Changes in Lipid Composition of Gulf Coast Marine Species as Influenced by Processing and Storage Treatments.

Rafida Idris

Louisiana State University and Agricultural & Mechanical College

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Changes in lipid composition of Gulf Coast marine species as influenced by processing and storage treatments

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The Louisiana State University and Agricultural and Mechanical Col., 1989
Changes in Lipid Composition of Gulf Coast Marine Species as Influenced by Processing and Storage Treatments

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Food Science

by

Rafida Idris

B.S., M.S., Biochemistry, Dhaka University
M.P.H., Nutrition, University of Hawaii at Manoa

May 1989
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ABSTRACT

The lipid composition, and changes in lipid components of several marine finfish due to frozen storage, canning and use of various physical and chemical treatments during storage were evaluated. In Phase I, the lipid content of mullet (*Mugil cephalus*), croaker (*Micropogon undulatus*), redfish (*Sciaenops ocellata*) and red snapper (*Lutiganus campechanus*) fillets obtained from a local supermarket, was approximately 5% with phospholipids representing < 5% of the total lipid. Vitamin A was not detectable but vitamin E was found in mullet. Mullet had a higher degree of oxidation (8.5 mg/1000 g) compared to other species. In Phase II, fresh mullet were wrapped in oxygen impermeable freezer paper or vacuum packaged and frozen at -20°C, or were thermally processed. After 90 days of frozen storage, a second batch of mullet was thermally processed. Total lipid, phospholipid, vitamins A and E and oxidation were determined at 0, 90, 180 and 360 days of storage. TBA number increased by 9.42mg/1000 g after 360 days of frozen storage. Oxidation did not occur in canned fish. Fish canned at 90 days evidenced greater oxidation after canning but further oxidation during storage did not occur. Vacuum packaging inhibited oxidation to a significant degree. Phospholipid decreased in frozen fish to the greatest
degree, followed by vacuum packaged samples. Loss of phospholipid in freshly canned mullet was small. In frozen canned mullet, the change was greater. Vitamin E level decreased during frozen storage from 0.69mg/100 g to a trace amount and the loss was 38% as a result of canning.

In Phase III a comparative effect of prefreezing dip treatment, use of antioxidants in the dip and oxygen limitation by vacuum packaging on the lipid composition of frozen stored blackdrum (*Pogonius cromis*) was studied. Analyses were made at 2 week interval during a 12 week storage period. Total lipid averaged 4% in all treatment samples and phospholipid was < 5% of total lipids. The effectiveness for the inhibition of oxidation in frozen blackdrum was 0.025% TBHQ (tert-butylhydroquinone) > 1% and 0.5% \( \alpha \)-tocopherol > water dip > vacuum pack > no dip treatment. Vitamin A was quantifiable but not Vitamin E. No significant change in Vitamin A content was observed in any treatment group.
CHAPTER I

INTRODUCTION

Consumer surveys and market trends reflect an increasing interest among consumers in high quality seafoods and a willingness to pay for quality. Medical research suggests that an increase in seafood consumption may have health benefits. Many public and private organizations have also promoted fish consumption for this reason.

The fishing industry has expanded the scope of available products, thereby increasing the number of market forms available to consumers. Underutilized or nonutilized resources are of interest because popular species are in short supply. Quality control is becoming an increasingly important factor, particularly at the processor level. The use of underutilized fish species is possible depending on the development of processing procedures compatible with current commercial and technological conditions in the seafood industry.

Fresh fish held on ice during transportation and marketing has traditionally been the form of choice. The quality of chilled seafood reaching consumers is greatly dependent on the care taken in icing and handling on the vessel. On average, the shelflife of fish stored in ice is usually less than one week. For long term storage, fish
must be frozen. The maintenance of temperature close to freezing, from the time of catch through all phases of processing and distribution, is important to maintain quality and wholesomeness. Deterioration of texture and flavor is a frequent problem for frozen fish.

Lipid oxidation in muscle foods is one of the main occurrences responsible for losses in quality. Marine lipids have a characteristic feature of having a high content of monenoic and polyenoic C\textsubscript{20} and C\textsubscript{22} fatty acids. These lipids are highly susceptible to oxidation when exposed to air. Autoxidation in lipids is the most serious quality problem encountered in frozen fatty fish (45, 103, 104, 195). Autoxidation leads to formation of short-chain aldehydes, ketones and fatty acids, as well as the development of certain polymers (65, 107, 135), all of which are believed to contribute to oxidized flavors in meat, poultry and fish (157, 229). The resulting off-flavor is generally referred to as rancidity.

Concern for the relative toxicity of oxidized compounds in flesh foods has not been expressed until recently (163). With increasing emphasis on the role of diet in human diseases such as coronary heart disease, cancer and stroke (186, 187), questions have been raised as to the relative role of lipids and their breakdown products in the diet.

Considering the interest and need for the use of non-
traditional fish resources, many fish species in the Gulf of Mexico are still underutilized. Mullet and blackdrum are two such underutilized species, which could have significant nutritional and commercial value because of their high protein content, variety of uses and their abundance along the southern coastal waters. Currently, mullet is used mainly for roe production. Because of the short distance between markets and fishing grounds, fishermen can readily supply processing plants with good quality fish. Lipids in these fish are highly unsaturated and oxidize quite readily. Both mullet and blackdrum have been marketed fresh or frozen as whole fish, headed and gutted, and as fillets, but generally with little success. Oxidative rancidity development has been one of the principle constrains for marketing them in these forms.

Canning perhaps offers the greatest potential of utilizing mullet, particularly during the peak production season. In Louisiana, shrimpers operate for 6 to 9 months and the plants are free for the rest of the year. Because of the seasonal nature of this relationship, it might also be necessary to freeze fresh mullet caught when shrimp processing is active for use during periods when shrimp processing is slack. Blackdrum offers a potential alternative for red drum which is in great demand. The present study was developed to appraise the influence of storage procedures and suggest treatments that would
maintain maximum quality in the fish under consideration. The objectives were to (1) determine the significance of oxidative rancidity as a spoilage mechanism in frozen and canned fish during storage and the role of the natural antioxidants, vitamins A and E, as a part of the cellular antioxidant system, (2) evaluate the effect of frozen storage and/or canning on the lipid components, and (3) study the effectiveness of prefreezing dip treatments with antioxidants, and vacuum packaging on rancidity development in fish during frozen storage.
CHAPTER II

LITERATURE REVIEW

2.1 An Overview of Underutilized Marine Species

The supply of marine resources is greatly influenced by the natural environment, which means that the fishing industry must contend with a higher degree of uncertainty than most other industries. In an effort to narrow the gap between supply and demand, the fishing industries are putting considerable effort into the development of new resources, modernization of fishing technology and upgrading of ports and distribution facilities.

Food from the sea has been an important renewable source of protein for man since ancient times. In modern history the need for protein is growing at a rate greater than that of protein production. Lands for livestock production are limited and pressure is increasing to obtain protein from the sea to feed a rapidly expanding world population. Underutilized finfish could help meet this demand, and pressure on major fisheries. The underutilized species program has, therefore, been developed in the U.S., mostly in the South, with the hope that some fishermen would transfer their efforts to underutilized species and
develop new gear.

An underutilized marine species, as classified by the Department of Wildlife and Fisheries of Louisiana, is "any species that does not have fishing restrictions" (4). It is estimated that in the coastal and inland waters of the United States there are 2,268 species of finfish. Only about 500 species are currently being harvested and out of those only 12 to 25 species are generally accepted by the American consumer as food (7). Currently, several varieties of fish classified as underutilized are gaining interest as targeted fisheries. A targeted fishery is one that has not been previously utilized, but with changing economic climates may have potential value.

Some common underutilized species of the Gulf of Mexico include croaker, mullet, sea catfish, shark and squid. These species have in common the designation as 'trash fish' due to the fact that they are by-catch. The Louisiana landing figures for the species mentioned above are given in Fig.1 for the years 1975-84.

Development of a mullet fishery seems to offer the most promising future. The National Marine Fisheries Service indicates that the landing of mullet could increase by 30 million pounds annually and the total economic impact of harvesting mullet for commercial marketing could result in total sales of up to 45 million dollars with a projected net income of 17.8 million dollars (4). These figures
Figure 1. Louisiana landings of underutilized marine species (1975-1984 period). (Ref. 4).
indicate a significant market potential. The markets for processed mullet are predominantly in the Southeast (65%), especially Florida. Another species which has been landed is black drum. Unlike Mullet, significant catches have been made with both purse seines and monofilament webbing. Relatively minor catches have been reported for white trout, croaker and mackerel.

2.2. Lipid Composition of Marine Species

The total lipid content (in weight percent) varies widely among species, from as low as 0.7% for pike, northern (Exox lucius) to 21.5% for eel (53,217). A difference in lipid content has also been observed between spring and fall fish, with the latter generally having higher fat content (54). Most of this variation in lipid content is reflected in the differences in triglyceride content, as phospholipid show much less variation. The range of phospholipid concentrations reported in the literature, expressed as a percentage of muscle tissue, runs from 0.19% in rock bass to 0.87% in rainbow trout (21). Red muscle contains a higher concentration of total lipids and phospholipids than white muscle (138). The additional phospholipids in red muscle is presumably present as a membrane component of the additional mitochondria.
The lipids in marine fish contain fatty acids usually ranging in chain length from 14 to 22 carbon atoms (216). A high percentage (60-75%) of these fatty acids are unsaturated, having up to six double bonds. The degree of saturation of fatty acid is highly variable between species and depends on environmental factors (192). The limited data available on fish muscle phospholipid shows that phosphotidylcholine (PC) and phosphotidylethanolamine (PE) are the prominent classes of phospholipids present. A small portion of the lipid fraction consists of unsaponifiable matter which is predominantly sterols with lesser amounts of hydrocarbons, fat soluble vitamins, carotenoid pigments and wax ester (221). Also, lipids are not distributed uniformly throughout the body of the fish (195). Generally, the anterior sections of fillets are much richer in lipids than sections obtained from the posterior tail region and the ventral portion, especially in the belly flap area, which is much richer in lipids than the dorsal region.

2.3. Lipid Oxidation

Oxygen, which is ubiquitous in food systems during fresh-state storage, cooking, processing, and post-processing storage, may lead to deterioration in the quality of food (62). The appearance, texture, flavor, and
odor may be altered to a lesser degree. Some changes, such as rancidity of fats, are quite unpleasant and unacceptable. Many of these changes in foods result from autoxidation process (34,91). Oxygen mediated oxidation in many synthetic and natural systems, including foods, is commonly referred to as autoxidation. Autoxidation usually proceeds through free radical reactions, comprising initiation, chain propagation, and termination steps (72,168). Understanding the free radical process in autoxidation is, therefore, critical for avoiding or minimizing the deleterious effects of autoxidation.

 Compared to sugars and proteins, lipids are extremely sensitive to autoxidation. This undesirable property of lipids is a consequence of the much lower bond energy of allylic C-H bonds in unsaturated fatty acids, and double-allylic C-H bonds in polyunsaturated fatty acids (PUFA).

 The decrease in bond energies leads to facile initiation of PUFA radicals and chain peroxidation (deoxygenation) reactions. Fatty tissues in meats have different proportions of polyunsaturated and saturated fatty acids. The membranes in meats have different compositions. They incorporate phospholipids, sphingolipids, and glycolipids, which contain large proportions of PUFA. Mechanistic studies of autoxidation of these unsaturated fatty acids and of decomposition of resulting oxygenated products are basic to an understanding of quality and flavor changes in
meat and fish.

2.3.1. Mechanism of Lipid Oxidation

The classical mechanism of lipid oxidation is via free radical attack and the most important substrate for oxidation is unsaturated lipids. The free radical chain mechanism has been generally accepted as the only process involved in autoxidation. The following simplified scheme shows the various steps in autoxidation:

Initiation
- RH
- RH + O₂

Propagation
- R⁺ + O₂
- ROO⁻ + RH

Termination
- ROO⁻ + AH
- ROO⁻ (or R⁻) + AH
- ROOR + O₂
- ROO⁻ (or R⁻) + AH
- ROOR (RH) + A.

Free radicals in biological systems can be formed through abstraction of a hydrogen atom from an unsaturated fatty acid (RH) by a free radical initiator to form a free radical (R⁺). This is followed by the rearrangement of the
double bond and acceptance of oxygen by the free radical to produce a fatty acid peroxyl radical (ROO·). The peroxyl radical then reacts with another molecule of unsaturated fatty acid to form a semi-stable unsaturated hydroxide (ROOH), which also regenerates a molecule of free radical (R·). These reactions may propagate unless all of the free radical is scavenged by an antioxidant (AH) or by self quenching. Upon homolytic fission of the hydroperoxide, a free radical chain reaction may again be initiated. The origin of the initial free radical or hydroperoxide to initiate the peroxidation reaction under cellular conditions is not entirely clear. Hydroperoxide formation as a result of the reaction of oxygen with lipids requires a change in the total electron spin, since the hydroperoxide and lipids are in the single state, while oxygen is in the triplet ground state.

Conversion of triplet oxygen to singlet oxygen is normally achieved by sensitizers that are present in plant and animal tissue; these are photosensitive compounds such as chlorophyll, pheophytin and myoglobin. Hydroperoxides have been isolated from photosensitized oxidation in unsaturated fatty acid esters (215). Dihydroperoxide groups of all isomers were attached to the carbon atoms that originally existed at both sides of a double bond and the double bond shifted to the adjacent position. Photosensitized oxidation was not inhibited by
butylhydroxytoluene (BHT), a free radical stopper, but was inhibited by β-carotenoid, a singlet oxygen quencher. These results indicate that singlet oxygen oxidation differed from oxidation in air (215).

2.3.1.1. Formation of Malonaldehyde

Malonaldehyde is a three carbon dialdehyde that is produced during autoxidation of polyunsaturated fatty acids containing three or more double bonds (115,156,161). Different mechanisms for the formation of malonaldehyde have been proposed. Polyunsaturated fatty acid containing trienoic systems may undergo autoxidation forming a peroxide radical with unsaturation β-γ to the carbon bearing the peroxide group (Fig. 2) (43). This radical (compound 1) can cyclize to form a five membered cyclic peroxide radical (compound 2), which can abstract a hydrogen from the alkyl group to form compound 3, or undergo peroxidation and abstraction of hydrogen to form compound 4. Compounds 3 and 4, when exposed to heat or acid, form malonaldehyde.

Fatty acids containing less than three double bonds can also produce malonaldehyde. The proposed mechanism stated above, was criticized on the basis that it did not
Figure 2. Formation of malonaldehyde from a triene system (Ref. 43).
adequately explain why dienes produce very little malonaldehyde compared to trienes. Another group of workers proposed (168) a modified mechanism to more adequately explain the formation of malonaldehyde from polyunsaturated fatty acids (Fig. 3). Compound 2, whose formation is shown in Figure 2, undergoes ring closure to produce the cyclic endoperoxide radical (compound 10), which subsequently undergoes peroxidation and abstraction of an alkyl hydrogen to produce compounds 11 and 12. When either of these compounds is subject to heat or acid, malonaldehyde is formed. In a diene system, compound 6 would produce endoperoxide radical 9 (Fig. 4). This radical is about 10 Kcal less stable than the allylic radical 10 (Fig. 3). Thus it is expected that less endoperoxide would be formed from radical 9 than from radical 10, and consequently less malonaldehyde would be formed from the diene system. This latter mechanism seems to more adequately explain the reason PUFAS produce more malonaldehyde than fatty acids with one or two double bonds. This mechanism is similar to the one proposed for the synthesis of prostaglandins in which malonaldehyde is a side product.

2.3.2 Lipid Oxidation Catalysts

Catalysts of lipid oxidation are well known. Because
Figure 3. Prostaglandin-like endoperoxide mechanism for the formation of malonaldehyde in a triene system. (Ref. 168).
Figure 4. Formation of malonaldehyde precursors in a diene system (Ref. 43)
Lipid oxidation is a major problem in muscle foods, much effort has been devoted to identifying the catalysts of lipid oxidation in meat. It has been generally accepted that iron in some form promotes the oxidation of lipids in muscle foods. Muscle tissue contains a considerable amount of iron bound to proteins. Myoglobin is the major oxygen-storage protein in muscle cells and resembles hemoglobin. Muscle tissue also may contain hemoglobin residues from residual blood. Hemoglobin and myoglobin contain the prosthetic group heme, which has an iron atom at its center. The heme group promotes autoxidation of fats (131, 228); others have suggested that iron derived from the heme molecule may be responsible for the oxidation reaction (92). Myoglobin and hemoglobin are thus the primary catalysts of lipid oxidation in meat (32, 92, 182, 210, 239). Lipid oxidation in meat has been considered to be nonenzymatic; however, recent evidence indicates that muscle microsomes contain enzymatic lipid-oxidizing systems (127, 165, 175). Enzymatic as well as nonenzymatic mechanisms may be involved in promoting lipid oxidation in meat. Further insight into these mechanisms would facilitate the development of effective means of controlling lipid oxidation in meat and fish.

Metals can promote lipid oxidation by facilitating initiation or promoting hydroperoxide decomposition. Although the effects of metals in promoting lipid oxidation
are often ascribed to initiation, decomposition of pre-formed lipid hydroperoxides may actually be occurring since the commercially available polyunsaturated fatty acids used in model system studies contain hydroperoxides (72). In most in vitro studies of lipid oxidation, transition metal salts or chelates, usually iron chelate, are added to the lipid, which may be in whole cells or tissue homogenates. Possible mechanisms for iron-promoted hydroperoxide decomposition reactions follow:

\[
\begin{align*}
(1) & \quad \text{Fe}^{2+} + \text{LOOH} \rightarrow \text{Fe}^{3+} + \text{LO}^\cdot + \text{OH}^- \\
(2) & \quad \text{Fe}^{3+} + \text{LOOH} \rightarrow \text{Fe}^{2+} + \text{LOO}^\cdot + \text{H}^+ \\
\end{align*}
\]

Reaction two involving Fe^{3+}, is relatively slow, and the rate of this reaction can be greatly affected by chelation (11). Chelation can enhance, suppress or not affect the rate of this reaction, depending on the nature of the metal, chelator and complex that is formed. In general, reaction one is much more rapid than reaction two (11,233).

Many metal complexes, including simple complexes of iron with phosphate ion or phosphate ester, which might decompose lipid hydroperoxides, are present in vivo (5,72). Muscles contain other iron containing compounds such as peroxides, cytocrome p-450, other cytochromes and nonheme iron proteins that also may be effective catalysts.
The ability of transferrin and ferritin to stimulate hydroperoxide decomposition appears to depend on their degree of iron-loading (11).

Reports on the decomposition of lipid hydroperoxides induced by metal ions and hemes indicate that hematin and heme proteins were more effective than Fe2+, Fe3+, or Cu2+ (131). Hydroperoxide decomposition was ten times faster with Fe2+ than with Fe3+. With Fe3+, cysteine and ascorbic acid stimulated the reaction, presumably by keeping iron in the ferrous state, thus favoring radical formation. EDTA and ADP ferric chelates showed very little catalytic activity in lipid hydroperoxide-dependent lipid oxidation, while EDTA-Fe2+ significantly enhanced lipid oxidation. It is possible that copper, as well as iron, might play a role in lipid oxidation of fish (109). Copper catalysis of fish lipid oxidation has been reported in lipids prepared from mackerel skin and frozen fish muscle (137).

Attempts to assess the relative importance of heme and nonheme iron as catalysts of lipid oxidation in homogenates of a variety of tissues incubated in air demonstrated that both heme and nonheme iron were capable of promoting lipid oxidation, with nonheme iron catalysis being more important at acid pH values, whereas pH had less effect on catalysis due to heme iron (233). A comparative study of the effect of an iron:EDTA complex and purified myoglobin showed that ferrous iron was more active at the lower end of the pH
range investigated, and metmyoglobin accelerated oxygen uptake in the lipid emulsions over the pH range 5.6 to 7.8, with catalysis tending to, increase with increasing pH (129,130). Chelating agents inhibited catalysis by the nonheme iron complex, but did not affect myoglobin catalysis. Evidence has been presented favoring nonheme iron rather than myoglobin as being responsible for the rapid lipid oxidation noted in cooked meat (132,182). When FeCl$_2$ or FeCl$_3$ was added to meat, which had been extracted with water to remove all but traces of myoglobin, lipid oxidation was enhanced. Added myoglobin did not affect the development of off-flavors or TBA-reactive material in the extracted meat. Subsequent work also indicated that nonheme iron plays a role in the lipid oxidation in muscle foods (91,137). These researchers found that treating a pigment extract from beef with EDTA decreased the prooxidant activity of the extract. Adding the filtrate from a heated meat extract to the cooked beef residue caused TBA values in the residue to increase; the effect of the filtrate was nearly as great as that of the unfiltered extract. When the filtrate was treated with EDTA, the prooxidant effect was decreased.

The significance of enzymatic lipid oxidizing systems recently shown to exist in muscle microsomes is not clear (127,173,176). It has been reported that a muscle microsomal fraction with lipid oxidizing activity could
cause lipid oxidation in an emulsion prepared using lipids extracted from herring muscle. Recent reports of lipoxygenase activity in the skin tissue of fresh water trout have suggested that this in fact may be a significant source of initiating radicals leading to oxidation in muscle tissue of fish (68). The formation of carbonyls and alcohols in volatile aroma compounds of fresh fish have been reported to be inhibited by inhibitors of fatty acid cyclooxygenase and lipoxygenase enzymes (97). It has been concluded that these compounds were formed from the unsaturated fatty acids through a hydroperoxide forming mechanism. These hydroperoxides could accelerate the rate of oxidized flavor formation during frozen storage. The oxidized flavors in frozen white fish was suppressed by vacuum-packaging (96). Studies indicate that in flounder muscle microsomes treated with phospholipase A, production of malonaldehyde was drastically reduced (91). When the free fatty acids produced by the phospholipase treatment were removed from the membrane, about 60% of the original peroxidative activity was restored. The relevance of these findings to meat is not clear but there is considerable evidence that lipase activity affects lipid oxidation in meat and fish. Lipase treatment was shown to increase the rate of lipid and pigment oxidation in ground beef, but that phospholipase A reduced lipid and pigment oxidation (72). A protective effect of free fatty acids against
lipid oxidation has been observed in fish muscle and fish muscle homogenates (46,140), and in flounder muscle microsomes (193). The mechanisms for these protective effects have not yet been conclusively demonstrated.

2.3.2.1. Promotion of singlet oxygen by heme pigments

Catalysis of lipid oxidation by heme pigments was an accepted mechanism until the report that demonstrated no evidence of lipid oxidation when myoglobin and hemoglobin were added back to a muscle tissue from which heme pigments were removed by dialysis (181). Other reports confirmed this finding (92,132). The oxidative reactions that the native heme pigments, hemoglobin and myoglobin, accelerate are those that produce activated oxygen forms, superoxide anion and singlet oxygen. Hemoglobin catalyzes the oxidation of ascorbate, in which activity is postulated to be due to an electron transfer from the heme iron, forming an activated oxygen, superoxyferriheme (125). While oxygen activation may play a role in the initial production of lipid peroxides, such activation by native protein heme pigment does not appear to be part of the heme catalysis process (132). Another reason to believe that native pigments are not involved is that the heme is sterically hindered. Heme or hemin is the most reactive catalyst, has the lowest lipid to heme ratio for maximal activity of all the heme compounds studied, and has never shown a lag
period in any of the systems in which it has been studied. This observation further strengthen the conclusion that heme pigments are not catalysts of lipid oxidation but rather promoters of singlet oxygen formation.

Iron is necessary for the activity of heme, since protoporphyrin was found to be completely inactive while hematin showed an extremely high activity (101). Smaller heme compounds exhibited higher activity possibly due to greater diffusional mobility of these catalysts and/or better accessibility of the active site.

In almost all systems in which the pigments have been studied, hematin has been found to be the most active catalytic form of heme iron. Attempts to elucidate the roles of heme and free iron using model systems established the dependence of free iron and heme catalysis on various factors such as chelating agents, reductants and pH (130,131). For example, ascorbate did not affect the heme catalysis at pH 5.6, but inhibited at pH 7.8; in contrast, ascorbate accelerated the iron catalyzed reaction at all pH values. The qualitative and quantitative aspects of the effect of these conditions or additives were then compared with their effect on lipid oxidation in meats. The results indicated catalysis by both iron and heme. In conclusion, lipid oxidation in muscle foods is probably catalyzed by both heme and free iron, and possibly by other compounds not yet identified.
2.3.3. **Cellular Antioxidation Defense System**

In view of the potential damage that may be caused by free radicals and hydroperoxides and the possible direct effect of various oxidants on cellular components, it is important that cells contain an antioxidant defense system. The major antioxidation defense system appears to include several factors. Scavenging of free radicals and singlet oxygen in conjunction with vitamin E and superoxide dismutase, reduction of hydroperoxides by the activities of catalase and glutathione (GSH) peroxidase as well as dietary protein, lipids, carbohydrate, minerals and vitamins, all are involved in the overall cellular antioxidant defense. Thus the key cellular antioxidant defense mechanism appears to act through scavenging of free radicals and reduction or detoxification of hydroperoxides (Fig.5).

The enzyme superoxide dismutase is widely distributed in cells with high oxidative metabolism and has been proposed to protect such cells against deleterious effects of superoxide anion formed from gaseous oxidants as well as lipid hydroperoxides. The hydrogen peroxide that is formed can be reduced in the cells by the activity of catalase or
Figure 5. Cellular antioxidant defense systems. RH, polyunsaturated fatty acids; ROOH, fatty acid hydroxy fatty acid; GSH, reduced glutathione; Se, selenium; G-6-P, glucose-6-phosphate and 6-PG, 6-phosphogluconate.
glutathione (GSH) peroxidase as follows:

\[ 20_2 + 2H^+ \rightarrow H_2O_2 + O_2 \]

The activities of GSH peroxidase, GSH reductase and G-6-p dehydrogenase have been shown to increase in certain tissue after animals were fed lipid hydroperoxides for prolonged periods (171) or made vitamin E deficient (39). Dietary selenium is an integral part of GSH peroxidase enzymes and is necessary for their activity.

Vitamin E, as an integral part of the membrane, is visualized as a biological antioxidant, which by sequestering free radical functions to terminate the propagation of an autoxidation process involving lipid peroxidation. Vitamin E was recognized in 1922 by Evans and Bishop as a dietary factor from plants that was essential for normal reproduction in rats. It was not until 1933 that it was identified as a group of substances known as tocopherols. Of the four different tocopherols that have been identified: Alpha (\(\alpha\)), beta (\(\beta\)), gamma (\(\gamma\)) and delta (\(\delta\)), the d-alpha tocopherol is most biologically active.

The quenching of a free radical by Vitamin E results in the formation of a tocopherol semiquinone radical that rapidly degrades to other products. Tocopherol acts as a free radical scavenger by donating a phenolic hydrogen atom.
to a free radical, thereby resolving the unpaired electron of the radical and oxidizing the tocopherol to its quinone form (Fig.6). \( \alpha \)-tocopherol has the added advantage of being nontoxic at required levels.

The fundamental biological role of vitamin E has been discussed recently and it seems to depend on its reactivity towards peroxy radicals. Microsomal vitamin E concentration has been shown to have an important bearing on the resistance of microsomes to lipid peroxidation (27). The efficiency of exogenous vitamin E has been found to be 50 fold lower than that exerted by the naturally occurring vitamin E in microsomal membranes. The lower efficiency of exogenous vitamin E might imply that the added vitamin could not be properly incorporated into specific sites where it could develop its activity. Another research publication provided evidence of the dependence of the lag phase of lipid peroxidation on vitamin E content of microsomes (85). The micronutrient selenium was studied also, because it has antioxidant effects \textit{in vivo}. This study suggested the existence and importance of two defenses against microsomal lipid peroxidation: the GSH dependent protein, which is responsible for the existence of the lag, and \( \alpha \)-tocopherol, which affects the length of lag. They suggested that these defenses function separately
Figure 6 Oxidation of $\alpha$-tocopherol to its quinone form in the process of quenching of a free radical.
to prevent peroxidation of membrane polyunsaturated fatty acids. It appears that they protect one another. It is conceivable that GSH dependent protein protrudes from the membrane and scavenges radicals at some distance from the lipid water interphase. This would make them complementary. The possibility that the GSH dependent microsomal protein is linked to \( \alpha \)-tocopherol function is an attractive one because vitamin E is the only vitamin for which no enzymatic role is known. The regeneration of d-\( \alpha \)-tocopherol by an enzyme using the reducing equivalents of GSH would go a long way towards explaining the in vivo antioxidant effect of this form of vitamin E. Selenium appears to affect microsomal \( \alpha \)-tocopherol content but has no effect on the microsomal lipid peroxidation system.

As vitamin E is considered nature's best lipid-soluble antioxidant, it is also known to have an effect in stabilizing Vitamin A (14). The nutritional influence of vitamin A on cellular redox status have been known for years. While high levels of Vitamin A has been shown to decrease the vitamin E concentration of plasma and tissues of several species (40), plasma selenium level as measured by GSH peroxide activity was increased. It has been suggested that a high level of dietary vitamin A may promote the enteric absorption of selenium, but interfere with the absorption of vitamin E (14). Vitamin A may also enhance the oxidation of vitamin E in the gut (73) or
influence the permeability of the intestinal mucosa to vitamin E and selenium (178). Vitamin E appears to be the first line of defense against peroxidation of cellular and subcellular membrane phospholipids. The antioxidant potential of tocopherol is effective at high oxygen concentrations, and thus it is not surprising that vitamin E tends to be concentrated in those lipid regions that are exposed to the highest partial pressures of oxygen, such as the erythrocyte membranes. However, even in the presence of adequate vitamin E, some peroxides are formed. GSH peroxidase provides a second line of defense to destroy the peroxides before they cause damage to the membranes. Thus the biochemical action of vitamin E and selenium seems to be prevention of peroxidative damage to cellular and subcellular elements, which thereby preserves the organelles necessary to cope with physical and chemical environmental insults and other stresses. Selenium spares vitamin E or reduces the vitamin E requirements for the maintenance of membrane integrity. In some unknown way, selenium aids in the retention of tocopherol in lipoproteins. Likewise, vitamin E appears to reduce the selenium requirement. Its specific role in selenium metabolism is incompletely understood, but by preventing autoxidation of membrane lipids from within, vitamin E reduces the amount of GSH peroxidase needed to destroy peroxides formed in the cell.
L-ascorbic acid, as a reducing compound and an important water soluble vitamin for humans and animals, functions to maintain sulfhydryl compounds, including GSH in a reduced state, and participate in many redox reactions (41). It has also been shown to have a synergistic effect with vitamin E and selenium (146). However, excessive amounts of L-ascorbic acid may promote oxidation in some biological systems (36). This may be due to the ability of ascorbic acid to maintain iron or other metal ions in a reduced state, which in turn allows free radical lipid peroxidation to proceed by catalyzing homolytic fission of hydroperoxides.

The antioxidant or prooxidant effect of various ascorbic acid concentrations in separated ground mullet light and dark colored muscle, as well as mixed flesh, was studied (47) to predict possible concentrations of ascorbic acid that would optimize its antioxidant effect in practical applications. The authors predicted the concentrations of ascorbic acid that would act as a prooxidant or shift from an antioxidant to prooxidant to be 70 ppm, 0.10-0.15 ppm and 2-425 ppm in light, dark and mixed mullet flesh, respectively. Use of a higher amount than the critical range of concentrations would assure an antioxidant effect.

The synergistic interaction of vitamin E and C was verified in a study where the oxidation of methyl linoleate
in solutions initiated with azo compounds in the absence and presence of vitamin E and C was conducted (151). Both vitamin E and vitamin C acted as chain breaking antioxidants. They also suppressed oxidation and produced an induction period. Vitamin E remained nearly unchanged and only vitamin C was consumed at the initial stage; vitamin E was consumed after vitamin C was exhausted. Vitamin E could trap two peroxy radicals where as vitamin C could trap only one under the reaction conditions employed. It was concluded that Vitamin E trapped the peroxy radicals and the resulting cromanoxy radical reacted with vitamin C to regenerate vitamin E.

Since vitamin E is oil soluble and vitamin C is water soluble, and in biological systems these two vitamins must be located in separate phases, a more biologically related study (17) was conducted where the interaction of vitamin E and vitamin C in liposome systems was investigated. Ascorbate was found to be an effective inhibitor of peroxidation initiated in aqueous phase, but a poor inhibitor of peroxidation initiated in the lipid phase. Peroxidation initiated in the lipid-phase in the presence of tocopherol showed ascorbate to be an excellent synergist with the phenolic antioxidant. Synergism of ascorbate with \( \alpha \)-tocopherol indicate that, tocopheroxyl radicals are more accessible to ascorbate than are membrane peroxyl radicals. This is consistent with the known stability of
tocopheroxyl (α-T) radical and the likelihood that the polar α-T head group is close to the membrane surface. This study made a good attempt to differentiate the effect of peroxidation initiated in the lipid phase from that in the aqueous phase. It also attempted to explain some recent findings on the in vivo protective effect of vitamin C and tocopherol against lipid preoxidation (207).

Since the antioxidant properties of vitamin C and vitamin E are thought to be coupled, the vitamin E status of membranes may determine whether a particular vitamin C concentration has pro or antioxidant properties. An investigation relevant to this idea reported the effect of vitamin E status of membrane on the balance between pro and antioxidant activity of ascorbic acid in microsomes for rat heart, kidney and liver (151). Within the Vitamin E range of the membrane preparations, the balance between the prooxidant and antioxidant capacities of ascorbic acid seemed mainly to be dependent on ascorbic acid concentration and not on vitamin E concentration. In contrast, vitamin E concentration influenced whether or not the prooxidant activity of vitamin C was expressed. Vitamin E proved to be the most important parameter determining sensitivity of lipid peroxidation. This study also emphasizes that vitamin E depletion does not always seem to make oxidative stress more detectable.
2.4. **Lipid Oxidation in Marine Species**

Of the various components that affect edible quality attributes, the lipids of fish are most important. The purchase of fish, either raw or in precooked state has increased recently as people have become more concerned with the consumption of fat and polyunsaturated fatty acids. However, one critical aspect of heated seafood products has been storage life, whether in a refrigerator or freezer (133; 238). The susceptibility of a particular fish flesh to oxidative rancidity is related to intrinsic factors such as the lipid content and degree of unsaturation, season, feed, fishing ground, stage of spawning cycle, stage of maturity, content of prooxidants and antioxidants, and to extrinsic factors such as a storage temperature and partial pressure of oxygen (2,30,118). Lean fish (<1% lipid), although not as prone to rancidity compared with fatty fish (>5% lipid), still develop this condition because of the highly unsaturated nature of their lipids. In lean fish, phospholipids constitute the major portion of total lipids. While fatty fish, in addition to phospholipids, contain deposits of depot fat usually located just beneath the skin and among the lateral line. Red and dark muscles located in the lateral line are rich in hematin compounds (hemoglobin, myoglobin, cytochromes). This class of compounds are known
to be potent catalysts of lipid oxidation (26,126). Semi-fatty fish are intermediate between lean and fatty fish in susceptibility to oxidative spoilage. Control of rancidity might improve sensory acceptability of marine foods, like mullet and blackdrum, because the chemical makeup of these fish contributes to the poor stability of the flesh during storage. This in turn will cause problems in the development of marketable products.

Traditionally oxidation of raw fresh fish muscle has not been considered a matter of great importance since the deterioration of muscle tissues has generally been attributed to microbial spoilage (157). One of the first reports on the incidence and rates of oxidation in the muscle tissue of several fish species from the Gulf included mullet, mackerel, red snapper, red drum and sea trout (227, 228). Significant increases in the thiobarbituric acid (TBA) numbers of the muscle tissues were observed during refrigerated storage following a heat treatment of 60-80°C. Changes in TBA numbers were attributed to the cooking process, which provided additional energy to the oxidation reaction. This conclusion is in agreement with the review of factors influencing the rate and course of lipid oxidation reactions (122). Very limited data are available, however, describing the extent of oxidation occurring in the muscle tissue of many of the Gulf species during harvest,
processing and storage, prior to cooking and consumption. Models to predict the shelf-life of frozen fish have been developed, based mostly on autoxidation of extracted fish lipid (106,113). These models could be considered as references to provide an estimation of the TBA number based on shelf life of frozen fish.

Frozen mullet in the round and mullet fillets with longer periods of iced storage produced higher amounts of free fatty acids than frozen mullet with shorter period of iced storage (15). In practice it would be ideal to freeze mullet in the round as early as possible during storage.

Fresh water prawns stored at -18°C have been reported to contain 23% saturated, 46% monounsaturated and 31% polyunsaturated fatty acids (172). Fatty acids, especially unsaturated ones, decreased during frozen storage for 6 months at 18°C, regardless of packaging procedures employed. No objectionable rancid flavor was detected during 6 months of frozen storage.

Oxidative rancidity leads to off-odors and flavors in cooked meats. Both freshly cooked meats or those stored in the freezer for several months are susceptible (28). Intramuscular phospholipids have been shown to be the most rapidly oxidized components in cooked meat (23). Oxidation proceeds at a rapid rate following tissue heating because heme proteins are denatured and there is a general disorientation and destruction of cellular structure,
permitting intimate mixing of cellular constituents. The reaction probably is catalyzed by both heme and nonheme iron (92,176).

Oxidative rancidity in the skin and muscle lipids of oil sardine showed that total skin lipid content increased by 10% after 4 weeks of storage at -18°C and remained at this level up to 37 weeks (148). There was a significant increase in free fatty acids in muscle lipids due to the breakdown of phospholipids. No change in free fatty acid of skin lipids occurred because the phospholipid level was low. Changes in the levels of fatty acids and polyene indices in the muscle lipids during storage showed an increase in proportion of saturated acids and a decrease in polyunsaturated acids. The fall in polyene indices was significant in the skin lipids in the initial stages indicating a higher rate of rancidity. After 22 weeks, the rate of decrease in polyene indices of muscle lipids was faster than in the initial stage. Both peroxide value and TBA values increased in the skin, to levels 5 to 9 time higher, respectively, than that of the muscle lipids. This indicated that skin lipids of oil sardines are less prone to hydrolytic attack but more prone to autoxidation than the muscle lipids.

The catalytic activity of heme and nonheme iron in lipid oxidation was studied using a linoleate emulsion model system made from mullet dark flesh homogenate (63).
The addition of ascorbic acid, EDTA, and cyanide, at different pH levels, indicated that cyanide was the strongest inhibitor of lipid oxidation. Cyanide selectively inhibited lipid peroxidation by blocking the reaction of hydroperoxide with the iron in the heme molecules. Cyanide has been found to be the most effective inhibitor of this type. Based on the criteria of other researchers, heme iron is the major catalyst of lipid oxidation in mullet.

2.5. Effect of Antioxidants in Food Systems

Cooperative studies on the effect of various antioxidants on fish oils, fish tissue homogenates and fish meat have been investigated. The rate of oxygen absorption in a mixture of mackerel oil and egg albumin containing added hemin was reported to decrease as the concentration of \(\alpha\)-tocopherol increased (241). The oxygen absorption in the hemin mixture was characteristically accelerated above that of the induction period. Although EDTA showed little effect on the action of \(Cu^{2+}\) and hemin under conditions of investigation, \(\alpha\)-tocopherol and BHA acted synergistically. The effect of lipid antioxidants on the stability of meat during storage was investigated (17) using a 0.005% level of five additives individually derived from natural sources: tocopherol, ascorbic acid, L-ascorbyl stearate,
citric acid and ascorbic acid plus sodium bicarbonate. Meat samples were treated with antioxidants and stored at -1.1°C. Ascorbic acid exhibited a strong prooxidant action, where as the other additives showed antioxidant activity. Free fatty acid production decreased when mullet fillets were pre-immersed in the antioxidant solution (0.025%) monotertiarybutyl hydroquinone (TBHQ) + 2% ascorbic acid (46). The effect of antioxidants was interpreted as due to lowering of pH of the environment. In addition to a shorter period of iced storage and/or immediate antioxidant treatment, treatments like vacuum packaging was suggested as an important measure to retard rancidity development. A similar study reported the effect of a combination of vacuum packaging and antioxidants to extend the storage stability of mullet during the spawning season (45). Mullet in the round that were kept frozen at -29°C for one month were used because most mullet harvested during peak spawning season are frozen before shipping or further processing (47). Peroxide values and TBA number indicated that ascorbic acid alone or in combination with TBHQ or Na₂-EDTA was more effective than other antioxidant treatments. Vacuum packaging in combination with antioxidants improved rancidity control over the antioxidant treatment alone.
Antioxidative potency of TBHQ and other antioxidants used to reduce oxidation of mackerel skin lipids indicated the order of effectiveness to be TBHQ > α-tocopherol > tempeh oil > BHA > BHT, at concentrations of 0.02% for all synthetic compounds and 0.1% and 5% for α-tocopherol and tempeh oil respectively (8).

Riboflavin tertbutylate, a fat-soluble derivative of riboflavin is a legal food additive in Japan, although not in the United States. It is used to raise the Vitamin B₂ level of fats and oily foods. Ingested riboflavin tertbulylate is hydrolyzed in the tissue in vivo and quickly absorbed (139,218). When absorbed, riboflavin was claimed to limit lipid peroxidation in the tissues; the effect was increased by racemic DL-α-tocopherol (218). A study was conducted to examine the in vitro antioxidant effects of riboflavin tertbulytlate and the synergistic effect of tocopherol in a system that resembled emulsions used in food systems (139). Riboflavin tertbutylate had an antioxidant effect in the dark, but an oxidative effect when irradiated, perhaps because the activated oxygen and hydrogen peroxide that was produced induced lipid peroxidation. The effect of racemic α-tocopherol was synergistic in the dark. This study indicated that peroxidation could be prevented satisfactorily if the emulsion could be shielded from light.
The rate of oxidative rancidity in frozen fatty fish has been retarded by glazing. Glazing is the most common method of increasing the storage life of frozen fish. Oil sardine glazed with water could be stored frozen for 2-5 months at -20°C. Pomfret could be stored at -28°C with a water glaze for 3 months. Herring, anchovies and Baltic sprats could be stored only for short periods of time at -20°C (205).

2.6. Effect of Processing and Storage on Marine Species

Lipids may undergo several deteriorative reactions during processing and storage, e.g. hydrolysis and oxidation. These can adversely affect flavor, odor, color and texture. Earlier work on the effects of processing (134) including freezing (53,133) have been thoroughly reviewed. Little emphasis has been placed on this research area since these reviews were published. The holding time prior to freezing appears to have an effect on lipolysis in the frozen state. Holding of silver hake for 5 days in refrigerated sea water prior to freezing led to decreased lipolysis in frozen storage compared to those held on ice (86). Holding mullet on ice for 7 days prior to freezing led to a decrease in lipolysis upon subsequent frozen storage in the presence of the antioxidants TBHQ and
ascorbic acid, but an increase in lipolysis was observed in their absence (47).

Heat treatment halts fish muscle lipolysis (120,158). Microwave heating of herring inactivated constituent phospholipase more rapidly than lipase (23). Although these studies observed no appreciable accumulation of fatty acids during this cooking process, higher levels of fatty acids in cooked versus uncooked samples of mackerel muscle were observed in a later study (102).

Accumulation of free fatty acid upon frozen storage of fish has been found to be associated with quality deterioration. This relationship has led to the suggestion that free fatty acid concentration could be used as a quality index in frozen fish (24). Although free fatty acids have not been implicated directly in quality defects, indirect effects of textural changes by promotion of protein denaturation and flavor deterioration by enhancing lipid oxidation have been suggested. Other studies contradict these suggestions, however. The fatty acids of cod, despite considerable hydrolysis were noted to be surprisingly resistant to lipid oxidation (52). An inverse relationship between the production of free fatty acids and an increase in oxidation was noted in frozen cod (31), frozen mullet (124) and minced cod (226).

The possibility of direct interference of fatty acids in the oxidation process has been suggested (194), despite
an earlier rejection of this possibility (53). Free fatty acids may function as inhibitors of enzymatic lipid oxidation in membranes or may disrupt membrane structure or interfere with the propagation of both enzymatic and nonenzymatic oxidation. The role of lipolysis in fish quality deterioration remains somewhat uncertain despite a general correlation between free fatty acid production and quality deterioration. A growing body of evidence suggests that triglyceride hydrolysis stimulates lipid oxidation, whereas phospholipid hydrolysis retards lipid oxidation (167, 194).

Lipids and Protein Interaction

The interaction between lipid and protein during frozen storage was studied by investigating the effect of polar and nonpolar lipids on rainbow trout myofibrillar protein (6). Salt soluble proteins rapidly decreased during frozen storage. The study indicated that the digestibility of the cold acetone dehydrated precipitate was higher in the nonpolar and polar lipid systems than in the untreated system. This suggested to the authors that the water surrounding the site attacked by the digestive enzyme is held by lipid and therefore freeze dehydration is controlled. This shows the importance of interaction among protein, water and lipid.
Biochemical changes in proteins exposed to peroxidizing lipids are similar to those induced by ionizing radiation and include: loss of enzyme activity; destruction of individual amino acids, particularly methionine, lysine, histidine, cystine and tryptophan; and polymerization or cross-linking by products of scission. The degree of change will depend on the water activity of the system and the nature of the protein. Such characteristics have led to the proposal that reactions of free radicals arising during lipid oxidation are as important as those of peroxide breakdown products, such as malonaldehyde. Using an electron spin resonance (ESR) technique, free-radical reactions of peroxidizing lipid with amino acids and proteins were studied (184). Free-radicals transferred from oxidizing methyl linoleate to amino acids and proteins was detectable. It was shown that ESR signals in lipid protein systems required the presence of an oxidizing lipid and arose from non-lipid components of the system. These findings substantiate the fact that free radicals produced in the lipid oxidation chain react with proteins.

Reactions that contribute to quality changes in fish muscle lipids and protein are related to the interaction between oxidized lipid material and myosin (48). Compounds with fluorescence activity were isolated from an autoxidizing system consisting of sodium linoleate, coho
salmon myosin and buffer (25). Similar compounds were also present in extracts from freeze-dried salmon steaks and salmon kept frozen at -20°C for one year. Amino acid analysis of the myosin from the autoxidizing system, compared with the non-oxidized myosin-linoleate system, indicated a significant decrease in the amount of histidine, lysine and methionine following oxidation.

Color/Flavor:

The discoloration of fish muscle is partially caused by Maillard reactions of ribose with amino groups. Reactions of lipid oxidation products are believed to be directly involved in the browning process. Experimental evidence suggests that the pigments in browning reactions are probably formed by ionic condensation of primary amino groups of protein with conjugated unsaturated aldehydes or similarly active lipid oxidation products (166,167). Formation of brown pigment may be prevented by addition of an antioxidant (57).

The use of mechanical flesh separators to increase the utilization of the fish catch has received considerable attention. The major problems encountered were flavor and color changes that occurred in deboned flesh. Mechanical deboning of mullet can increase the hemoglobin and nonheme iron contents of the flesh (196). Studies have shown that
hemoprotein (32), as well as iron (123) can accelerate oxidative rancidity in fish, and the hemoproteins extracted from the dark muscle of mullet can increase the oxygen uptake of linoleic acid (63). The change in the amounts of myoglobin in the deboned fish flesh is not influenced by mechanical deboning. The large variations in the dark muscle content of mullets appears to have a greater influence on the hemoprotein concentrations of deboned fish flesh than the method of deboning.

2.7. Nutritional Effects of Lipid Oxidation

Long chain polyunsaturated fatty acids (PUFA) have received attention for their beneficial effect on the cardiovascular system (70,121). Among these fatty acids, the role of N-3, or omega-3 fatty acids, largely present in fish or fish oils were demonstrated to be more efficient in lowering plasma triglyceride and cholesterol levels than the PUFA from vegetable oils (51,55,114). Fish consumption results in decreased very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol, modified prostaglandin formation and altered hepatic lipoprotein synthesis (70,89,232). Fish oils affect lipoprotein metabolism by reducing hepatic secretion of VLDL (80,150) or by accelerating VLDL clearance (79), although the last point has been debated. Consumption of marine fish has
been shown to decrease intestinal cholesterol absorption, reduce total fatty acids recovery in thoracic lymph and increase the lymphatic eicosapentaenoic acid (EPA)/arachidonic acid (AA) ratio (37). This affirms the cardioprotective role suggested for dietary fish consumption. The n-3 PUFAs present in fish oil also have beneficial effect by reducing high aggregability of platelets, viscosity of blood and possibly hypertension (55,83,153).

Fish oil consumption could be of concern due to its oxidative potential. Although protective mechanisms that control lipid peroxidation exist in vivo, they may be hampered when fatty acid unsaturation is too high. Increased levels of long-chain n-3 fatty acids in the diet led to a marked incorporation of these fatty acids into membrane phospholipids (95). This increased their unsaturation and their sensitivity to peroxidation (141). Increased production of hydroperoxides may be deleterious to membrane integrity and may also result in accumulation of degradative products of peroxidized lipids (76). Some degeneration and inflammation of fat cells as well as some lipofuscin accumulation in Kupffer cells in liver of young growing pigs fed mackerel oil has been observed (179). These change were attributed to vitamin E deficiency induced by the high unsaturation of fatty acids in the diet (144). Such a pathological accumulation was also observed
in the heart of rats fed marine oils (159). The presence of cetoleic acid (22:1n-11) and erucic acid (22:1n-9) in fish oils, if they accumulate in heart, can lead to myocardial lipid storage. The exchange of heart sarcolemma n-6 PUFA by dietary n-3 PUFAs alters the double bond index of cardiac membranes and may thereby affect membrane fluidity, receptor sites for intrinsic enzymes or ion transport properties (16,89). It is also feasible that prostanoid synthesis is affected (84).

A low serum vitamin E level has been reported in rats fed fish diet (207). Although the diet was supplemented with an increasing amount of vitamin E, the serum vitamin E concentration did not increase as it did with other types of dietary fat. A significantly low level of serum vitamin E was observed in rats fed salmon, even after supplementing the diet with the vitamin (149). A rise in n-3 PUFA in serum lipids and most of the cellular membranes observed, indicated membrane peroxidation. A higher level of activity of defense system is probably required to prevent peroxidation. As a consequence a lower vitamin E status was observed, despite supplements of vitamin E. This may indicate that the defense system was somewhat impaired. Also it has been observed that in case of vitamin E deficiency, vitamin A is inadequately absorbed and more rapidly mobilized by the liver, resulting in a lower circulating vitamin A level (202).
The promising future of fish in the diet depends on the assurance that consumption of fish oils does not result in detrimental effects. The time course of changes in the risk factors after daily intake of fish oil is largely unknown. In many studies, the quantity of consumed fish and, therefore, the resulting n-3 PUFA intake have been in excess of what could reasonably be consumed by the public at large (84,212). The effect of fish fat, with particular consideration given to the n-6/n-3 dietary ratio, in all vital organs and functions should be investigated further, before being widely recommended for human consumption. Recent studies showing that a moderate amount of dietary fish oil did not alter pig heart performance (80) and reduced ischemic damage in rat heart (88) are interesting nutritional approaches to this concern.

2.8. METHODS OF ANALYSIS

2.8.1. Methods for Assessing Lipid Oxidation

One of the commonly used methods for assessing stability or rancidity of fats or fatty foods has been the thiobarbituric acid (TBA) test. The nature and mechanism of formation of the thiobarbituric acid (TBA)-reactive material produced in the autoxidation of polyunsaturated fatty acids (PUFA) or their esters have been studied. On
the basis of chemical studies and spectroscopic evidence, it is concluded that the TBA test detects malonaldehyde that arises at least in part from the acid catalyzed or thermal decomposition of endoperoxides (2,3-dioxanobornane compounds). It is difficult to achieve optimum reaction conditions necessary for this test because of a number of factors that could possibly influence the test results. Since lipids are exposed to heat, light, oxygen, and possibly trace metals during the test, it is reasonable to assume that the test conditions themselves will contribute, in varying degrees, to the results that are obtained (169). The presence of cupric ion or simply exposure to air can induce 2-enals to generate absorbance at 532 nm, as well as at 450 nm (161, 162). Thus, for example, the presence of copper or iron proteinates, such as blood, muscle tissue, etc. could be expected to influence test results. Other classes of lipid oxidation products, e.g., hydroperoxides, also are involved. It has been demonstrated that malonaldehyde, a three carbon compound, gives the characteristic absorbance at 532 nm in the test (197). The pigment so formed is the result of a 2:1 reaction between TBA and malonaldehyde. While compounds other than malonaldehyde may yield the essential three carbon structure during pigment formation, there is no evidence yet that the pigment absorbing at 532 nm in the TBA test may have a variety of structures. Thus the test appears to
be measuring malonaldehyde and structures capable of yielding a closely related three carbon derivative during the test reaction.

The TBA test has proven to be highly sensitive and useful as a method of monitoring lipid oxidation in many systems and under a wide variety of conditions. In light of the complex factors leading to pigment production in the TBA reaction, test results need to be considered with caution and should be compared with organoleptic evaluation or with findings by other suitable chemical tests.

Malonaldehyde is produced during the autoxidation of PUFA and is a highly reactive dicarbonyl. Because of its reactivity, most of the malonaldehyde present in fatty foods exists bound to other food constituents and very little of it is in the free form. The TBA test has been performed in various ways. The TBA test can be performed on: (1) the whole food followed by extraction of the red pigment formed (197), (2) a distillate of the food (214), and (3) an extract of the food (225,233). The distillation method is most widely used because of numerous advantages. Malonaldehyde is obtained in a clear aqueous solution, can be distilled rapidly, contains only steam volatile constituents that reduces the possibility of interfering compounds reacting with the TBA reagent and the TBA reagent is diluted by half when mixed with the distillate, again
lessening the possibility of side reactions occurring during the color-forming step.

When compared with the extraction method, TBA values are higher by the distillation procedure. The higher TBA values by the distillation procedure may be due to the thermal decomposition of malonaldehyde precursors to malonaldehyde and also to the liberation by heat of malonaldehyde from its bound state with proteins (117) during distillation. The extraction procedure does not involve heating but both procedures include an acid treatment of the tissue sample.

Propylgallate (PG) and EDTA have been used in the distillation and extraction TBA test of fish (mackerel) samples (224), presumably to minimize further oxidation of fish lipids during the test. Addition of PG and EDTA in the blending process has been recommended in the distillation TBA test of meat and particularly of fish to minimize further lipid oxidation (173).

Although the distillation procedure is preferable to the extraction method for many food products, there still remains the possibility that the TBA reagent will react with a variety of components, other than malonaldehyde, present in oxidized foods. Also impurities in the reagents themselves could lead to interference with the results. To avoid these risks, a high performance liquid chromatographic (HPLC) technique for the direct
quantitation of malonaldehyde in food products has been developed (87, 99).

2.8.2. Methods for Assessing Vitamins

The nutritional and biochemical role of Vitamin E as an antioxidant, free-radical scavenger and membrane lipid stabilizer has generated a need for the accurate determination of vitamin E in food. In the past, numerous analytical methods have been employed for the separation and quantitation of the various isomers and related compounds.

The oldest and most widely employed method is the Emmerie-Engle reaction based on the reduction of Fe$^{3+}$ (from FeCl$_3$) to Fe$^{2+}$ by tocopherol, forming a red-colored complex with dipyridin. This complex is measured colorimetrically at 520 nm. The difficulties and limitations inherent in this method include the fact that carotenoid, cholesterol and vitamin A, along with other nonspecific reducing compounds interfere with the colorimetric reactions (49). A modified Emmerie-Engle procedure employing bathophenanthroline, which forms a more stable chromophore with Fe$^{2+}$, increasing the sensitivity of the colorimetric reaction 2 to 3 fold has been developed (220), but does not eliminate the problem of interference.
Spectrofluorometry is also a sensitive method for assaying free and esterified tocopherols. The original method involved oxidation of tocopherol with nitric acid that fluoresces at excitation of 295 nm and emission at 340 nm. These methods are preferred over colorimetric procedures due to speed, simplicity, sensitivity and the absence of interference from the nonspecific reducing compounds mentioned previously (49).

The most useful chromatographic techniques employed for the separation and quantitation of various tocopherols have been thin layer chromatography (TLC) (38), gas chromatography (GC) (142) and liquid chromatography (119,223). In the past, TLC and GC methods have required lengthy analysis and allowed the possibility of oxidative loss of the compounds during sample preparation (29). With the advent of sensitive densitometric methods (154,219), however, the in situ separation of a variety of compounds has been made possible, reducing analysis time and opportunity for sample loss, and improving the sensitivity and reproducibility of the analyses.

A number of investigators have likewise demonstrated the advantages of HPLC methods for tocopherol analysis using normal phase (111) and reversed-phase systems (119). The advantages of these systems include the separation of \( \alpha -, \beta -, \gamma \) and \( \delta - \) tocopherols, high specificity and sensitivity often into nanogram range, good reproducibility
and sample recovery and speed and ease of sample application. Advantages of HPLC over spectrophotometry, fluorometry or gas chromatography include its relative freedom from interfering impurities, non destructive conditions and simplified methodology applicable to very small samples. The feasibility of HPLC for analyzing Vitamin A and Vitamin E and other fat soluble vitamins separately (1,44) as well as as simultaneously (13,20) has been described and has proven to be very useful for clinical studies and nutrition surveys.
CHAPTER III

MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN:

The research project was conducted in three phases. In Phase I a preliminary survey of the lipid composition was made to appraise the quality of selected varieties of fresh Gulf Coast finfish sold in local supermarkets. Oxidative status, lipid composition and lipid soluble vitamins A and E were determined. In Phase II, oxidative changes that occurred over time were analyzed in fish that were stored either frozen or canned. In Phase III, prefreezing dip treatments, antioxidants, and vacuum packaging were evaluated as processing variables for their effect upon lipid composition and oxidative stability. In Phase II, three replicates (one fish per replicate) were blocked and treatments were assigned in a 4x4 factorial arrangement. Processing and storage time were the treatment factors used. In Phase III, a completely randomized design was used with six prefreezing treatments and seven storage periods in a factorial arrangement.
3.2. EXPERIMENTAL TECHNIQUES:

3.2.1. Preparation and Processing of Fish

Fish samples were obtained from three different commercial sources in Louisiana. In Phase I, fillets of mullet, croaker, redfish and red snapper were bought from local markets. Mullet obtained from a commercial roe cutting plant was used in Phase II and blackdrum fillets from a commercial filleting plant were used for Phase III. The fish were packed in ice and transported to the laboratory. For the Phase II study, half of the mullet were wrapped in oxygen impermeable freezer paper and one fourth were vacuum packaged in polythene bags and stored in a blast freezer (-20°C). The remaining fish were washed, filleted and held in ice at 4°C until processed for canning. After 90 days of frozen storage in freezer paper, a second fourth were canned. For the Phase III study, blackdrum samples were given prefreezing treatments immediately upon arrival in the laboratory and stored for prescribed storage times.

The canning procedure in Phase II was accomplished by first chunking fillets and blanching in boiling water for 3 minutes. After blanching, meat was placed for 30 seconds in three different types of dips: 2% brine, 15% Brifisol 512 + 2% brine and 15% Lemophos + 2% Brine. Brifisol 512
is a food grade tripolyphosphate produced by BK - Ladenburg Corporation (Cress Kill, New Jersey). Lemophos (Stauffer Chemicals) is a mixture of tripolyphosphate (Brifisol 512), citric acid, erythrobate and lemon powder. Following the dip treatment, 182 g of fish was placed into 307 X 113 cans and topped off with a 1% hot (85-90°C) brine fill solution to a final weight of 205 g. Cans were then sealed using a Dixie Universal vacuum closing machine (Dixie Canners, Athens, GA) and placed into a Dixie Model RDSW still retort. The retort was set at 121°C (15 psi of steam). The cold spot of several cans was monitored using thermocouples (O.F. Ecklund Co., Coral Gables, FL). A chunk of mullet was put on the end of the thermocouple to ensure proper temperature determination. The can with the lowest temperature was monitored closely and the retort was turned off when the total lethality in the coldest can reached a value of 12. The temperature of the retort was quickly lowered by venting the steam and adding water. When sufficient steam had been released, the retort was opened and the cans placed in a chlorinated ice slush. The internal temperatures of the cans were monitored until the temperature reached 32°C. The cans were removed from the ice slush, dried and stored at room temperature (21°C).

In Phase III of the study, fresh blackdrum was chunked and weighed into six equal portions and assigned to individual treatments. Treatments included vacuum packaged
in polyethylene bags; water dip, by dipping the fish chunks in ice cold demineralized/distilled water for 1 minute and then wrapped in saran wrap; dipped in 0.025% TBHQ solution; dipped with 1% or 0.5% Vitamin E solutions and a control without any treatment. Weight of fish before and after dipping was recorded.

3.2.2. Chemical Tests

3.2.2.1. 2-Thiobarbituric Acid (TBA) Value Determination

TBA value was determined by a modified distillation method (213,214). Fifty ml distilled water was added to 10 g of fish sample and blended for 2 minutes in an Osterizer Galaxie Cycle Blend blender. The blend was quantitatively transferred to a flat bottom flask (500 ml) using 47.5 ml distilled water and 2.5 ml 4N hydrochloric acid was added to a final volume of 100 ml. To prevent foaming during distillation, five drops of Dow Antifoam A were added to each flask. The flask was attached to a distillation unit and 50 ml of distillate was collected. Two 5 ml aliquots from each distillate were used for the TBA assay.

A set of standards was run preceding every set of test sample in the following manner. A stock solution of 0.02M 1,1,3,3,-Tetraethoxypropane (TEP) (Sigma Chemical Co., St. Louis, MO) was prepared by dissolving 59.9 ul of TEP in 25
ml of distilled water. From this stock solution, $1 \times 10^{-8}$, $2 \times 10^{-8}$, $3 \times 10^{-8}$, $4 \times 10^{-8}$ and $5 \times 10^{-8}$ dilutions of TEP were made as follows. First, a $1 \times 10^{-5}$M TEP solution was prepared by diluting 0.1 ml of 0.02M TEP solution to 100 ml with distilled water. To five test tubes, increments of 1 ml of working TEP solution was pipetted. Distilled water was pipetted into each test tube to obtain a total volume of 5 ml per test tube. A blank was prepared with distilled water.

The TBA assay was accomplished by adding 5 ml of 0.02M TBA solution to the blank, standard solutions and fish sample solutions. The TBA solution was prepared by dissolving 0.288 g of TBA in 100 ml distilled water. The test tubes were immersed in a boiling water bath for 30 minutes. The tubes were then cooled to room temperature and the absorbance was measured at 532 nm with a Response UV-visible spectrophotometer. The standard curve was obtained by plotting absorbance vs moles of TEP. Regression analysis was used to calculate the slope and intercept of the line. The concentration of sample distillate was determined from the regression equation. TBA value (or number) was expressed as mg of malonaldehyde (MA) per 1,000 g of sample and was calculated by multiplying the distillate concentration by a constant K. All analyses were done in duplicate and TBA values were expressed as an average of the two readings. The K value was obtained as
follows:

\[ K = \frac{5 \text{ ml distillate} \times 10}{\text{sample wt.}} \times 1000 \text{ g} \times \frac{\text{M.W. malonaldehyde} \times 100}{\text{recovery}} \]

TBA number = \( K \times \text{concentration of distillate} \).

Percent recovery was determined as follows. To 1, 2, 3, 4 and 5 ml of \( 10^{-4}\text{M TEP stock solutions}, 2.5 \text{ ml 4N HCl solution was added and made up to 50 ml volume with distilled water. These solutions were distilled as previously described and 5 ml of each distillate was used in the TBA assay. A ratio was calculated for the absorbance values of distilled and corresponding undistilled standards. The percent recovery was calculated from an average of the five quotients multiplied by one hundred.}

3.2.2.2. Determination of Total Lipids:

Total lipids were determined by a modification of the method described by Kinsella et al. (110). Twenty grams of fish from each treatment group was weighed and blended with 60 ml methanol and 30 ml chloroform for 2 minutes. Another 30 ml chloroform was added and homogenized for 30 seconds. Thirty ml of distilled water was added and the mixture was stirred with a glass rod and filtered with suction through a Whatman No.1 filter paper placed in a buchner funnel.
The filtrate was transferred to a separatory funnel where it separated into an upper aqueous layer and a lower organic layer. The lower layer was collected in a 250 ml round bottomed flask and the contents were concentrated to between 1 and 2 ml with a rotary evaporator (Buchi Rotavapor-R, Buchi Laboratoriums - Technik Ag Ch-9230, Flawil/Schweiz, Switzerland) at 40°C. The concentrated lipid extract was then quantitatively transferred and adjusted to a final volume of 25 ml with chloroform. Aliquots (0.2 ml) were transferred to tared test tubes and evaporated under nitrogen to a constant weight. The difference between the final weight of the test tube and the initial weight of the tared tube at room temperature was the amount of lipid. All analyses were performed in duplicate and the results expressed as a percentage of total wet weight.

3.2.2.3. Determination of total phospholipids

Phospholipids were determined by the colorimetric method of Raheja et al. (170). A chromogenic solution was prepared by dissolving 16 g of ammonium molybdate (Mallinckordt, Inc., St. Louis, MO.) in 120 ml of distilled water. To an 80 ml aliquot of this solution, 40 ml concentrated HCl and 10 ml mercury (Fisher Scientific Co., Fair Lawon, NJ) were added. The combined solution
was mixed for 30 minutes on a Vortex mixer (Type 37600, Mode M 37615, Thermolyne Maxi Mix II, Thermolyne Corp., Dubuque, IA) and filtered. To the remainder of the original solution, 200 ml concentrated sulfuric acid (H$_2$SO$_4$) was added, followed by the addition of the second solution. The chromogenic solution was ultimately prepared by mixing 25 ml of the final solution with 45 ml methanol, 5 ml chloroform and 20 ml water. Chromogenic solution was stable for three months at refrigerated temperature.

To prepare the standard curve, a stock solution of phosphotidylcholine (PC) (Sigma Chemical Co., St.Louis, MO) was prepared by dissolving 500 ml of PC in 50 ml chloroform. Aliquots containing 0, 8, 16, 24, 32 and 40 micrograms of phosphorus were transferred into test tubes. The solutions were evaporated to dryness under nitrogen at room temperature. To the residual lipid material in test tubes, 0.4 ml chloroform and 0.1 ml chromogenic reagent were added. The test tubes were then placed in a boiling water bath for 1 to 1.5 minutes, then cooled to room temperature. Five ml of chloroform was added to the test tubes and gently shaken. Blue color appeared in the lower chloroform layer as the tubes were allowed to stand for 30 minutes. This blue layer was pipetted into quartz cuvettes and absorbance was measured at 724 nm against a blank using a Response UV-VIS spectrophotometer. The blank was prepared like the standards except distilled water replaced
PC. Absorbance was plotted against phosphorus content and the slope and intercept of the curve was calculated using regression analyses.

The test tubes containing the lipid obtained in total lipid analysis were used subsequently for determining the phospholipid content. The samples were analyzed in the same way as the standards. All analyses were done in duplicate and reported as mg of phospholipid per gram of sample.

3.2.2.4. Determination of Vitamin E (\(\alpha\)-tocopherol) and Vitamin A (retinol) by High Performance Liquid Chromatography (HPLC).

A 20 g sample of fish was saponified by mixing 20 ml of water, 15 ml potassium hydroxide (KOH) solution (50% w/w in water), 0.3 g ascorbic acid and 50 ml ethanol. After refluxing for 30 minutes, the mixture was filtered. The water-ethanol phase was extracted three times with 50 ml of n-hexane and the hexane phase was washed twice with an equal volume of a saturated NaCl solution and once with water. This solution was evaporated to dryness in a rotary evaporator. The residue was dissolved in 3 ml of methanol (HPLC grade) and filtered through a 0.45 um Millipore filter before injection into the HPLC.

A rapid microprocedure was used for the simultaneous
The determination of Vitamin A and Vitamin E in fish samples using HPLC (201). The HPLC instrumentation was from Waters Associates (Millipore R, Maple Street, Milford, MA 01757), consisting of a Model M-45 solvent delivery system, a model U6K universal liquid chromatograph injector and a model 481 variable wavelength UV-VIS spectrophotometer. Quantitation was accomplished with an HP-3390A recording integrator in the external standard mode. The column was stainless steel (3.9 mm X 15 cm) packed with Waters NOVA Pak C\textsubscript{18}. A guard column, u Bonda Pak C\textsubscript{18}, was attached in front of the primary column. The solvent was reagent grade methanol: water (96:4), filtered and degassed by a HPLC solvent filter - degasser (Model FG-256), using 0.5 um type FM Millipore filter. Samples were injected manually with a 25 \textmu l - syringe (Hamilton Co., Reno Nevada, parts No.2933087). The flow rate was maintained at 0.5 ml/min.

Standard compounds were all-trans retinol (Sigma Chemical Company, St. Louis, MO) and d-\textalpha-tocopherol (Sigma Chemical Company, St. Louis, MO). Stock solutions of individual standards were prepared with a concentration of 0.25 mg/ml. A mixture of standards was prepared by adjusting the volume of the individual standards in the mixture to reflect the optimum concentration needed for HPLC detection of the individual peaks. Reproducibility of the standards were checked for retention time and peak areas.
To assess linearity, different dilutions of the standards or a mixture of standards were injected and peak areas were plotted against the quantity injected. Regression analysis of all sets of readings established a linear relationship between peak height/area and quantity of standard injected. Finally, successively lower dilutions of each of the standards were injected until it was no longer detectable at the appropriate attenuation. The amount of standards from the next higher dilution was considered the minimum detectable level. Sample solution was manually injected each time and the resulting peak area obtained from the chromatogram was compared with peak area found for vitamin E standard and peak height for vitamin A standard, under identical chromatographic conditions, and the concentrations calculated by comparison with known concentrations.

3.3. STATISTICAL ANALYSIS:

Analysis of variance (200) was computed by a general linear model procedure, using programs in the Statistical Analysis System Software package from the SAS Institute, (SAS, Cary, NY). Differences between the means were determined by the Least Significant Differences (LSD).
method. Linear regression and correlation analyses (206) were used to test linear trends and linear relationship of selected variables.
4.1. PHASE I

4.1.1. Lipid Analyses of Gulf Coast Marine Species

Lipid composition and oxidative rancidity of mullet, croaker, redfish and red snapper fillets obtained from a local supermarket are summarized in Table 1. The total lipid content of all fish species was found to be within the range of 4 to 6 percent. Total lipid content of mullet was the highest (5.3%) followed by red snapper (5.1%), croaker (4.6%), and redfish (4.5%). This would categorize these species as having intermediate fatness. Phospholipids represented < 6.5% of the total lipid. Phospholipid content of mullet, croaker, redfish and red snapper were 0.17% of tissue, 0.15% of tissue, 0.1% of tissue and 0.13% of tissue, respectively. Fat is the most variable component of flesh for any given species,
Table 1. Lipid analysis of selected marine species

<table>
<thead>
<tr>
<th>Fish group</th>
<th>Total Lipid (mg/g)</th>
<th>Phospholipid (mg/g)</th>
<th>Vitamin E (mg/100g)</th>
<th>Vitamin A (mg/100g)</th>
<th>TBA Number (mg/1000g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mullet</td>
<td>53.19</td>
<td>1.74</td>
<td>2.45</td>
<td>ND</td>
<td>8.54</td>
</tr>
<tr>
<td></td>
<td>+ 2.9</td>
<td>+0.3</td>
<td></td>
<td></td>
<td>+1.3</td>
</tr>
<tr>
<td>Croaker</td>
<td>45.77</td>
<td>1.47</td>
<td>ND</td>
<td>ND</td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td>+ 2.14</td>
<td>+0.01</td>
<td></td>
<td></td>
<td>+0.3</td>
</tr>
<tr>
<td>Redfish</td>
<td>44.68</td>
<td>1.02</td>
<td>ND</td>
<td>ND</td>
<td>2.71</td>
</tr>
<tr>
<td></td>
<td>+ 2.14</td>
<td>+0.3</td>
<td></td>
<td></td>
<td>+0.1</td>
</tr>
<tr>
<td>Redsnapper</td>
<td>51.86</td>
<td>1.31</td>
<td>ND</td>
<td>ND</td>
<td>2.71</td>
</tr>
<tr>
<td></td>
<td>+1.84</td>
<td>+0.1</td>
<td></td>
<td></td>
<td>+0.8</td>
</tr>
</tbody>
</table>

1 Mean values (n = 3) (+ SD).
2 Not detectable.
especially fish. It varies widely with species (60), within individual species (204), with season (217), due to physiological status and spawning (204), location in the muscle or the body (203, 204), diet of fish (12) and geographic location (12, 203, 204). Therefore the variation in the lipid and phospholipid content among the groups of finfish studied could be due to differences in any of the above factors. Although the contribution of these variables cannot be identified in the present study because the history of the samples is unknown, the data provides an indication of the relative lipid composition.

TBA numbers for mullet, croaker, redfish and red snapper ranged from 8.5 to 2.7 mg/1000 g tissue. Mullet had a relatively high degree of oxidation as did croaker, but very little oxidation was evident in redfish. Reports related to possible significance of malonaldehyde in human health have increased concern for the quality of fresh supermarket products including meats, fish, vegetable oil, etc.(10, 19, 115, 190).

The incidence and rate of lipid oxidation in muscle tissues of several fish species from the Gulf were reported earlier (122, 198, 228). Limited information is available on the extent of oxidation occurring in the muscle tissue of many of these Gulf species during harvest, processing and storage. A number of factors could be responsible for the oxidative status of the species investigated. These
may include the chemical composition of the muscle, season of harvest, handling procedure and condition of storage. Variation may be due to migration patterns that influence the diet of fish and change the lipid composition, specifically the relative proportion of unsaturated fat. TBA number tends to increase during the spawning period when fish are in full roe, especially for mullet. Fish samples were obtained in late October, when the usual spawning period for mullet starts.

Rancidity in meat is caused by the oxidative decomposition of unsaturated fatty acids. Fish, because of its higher content of PUFA, has been shown to be more susceptible to lipid oxidation than other muscle foods. Heme pigments are present in fish, though mainly in the lateral band. It is well established that heme or nonheme iron of myoglobin are potent catalysts of lipid oxidation (32, 94, 102). Consequently it is reasonable to expect oxidative reactions in the tissues of the lateral band. Mullet is known to possess a characteristically large lateral band, which has been suggested as the reason that mullet is highly susceptible to oxidation (63, 106, 157). Croaker also has a lateral band, but to a lesser extent than mullet. Red snapper or redfish fillets did not have lateral dark meat. Hematin catalysis might partially have contributed to the higher level of oxidation evidenced in mullet and croaker compared to redfish and red snapper.
Evidence favoring hematin catalysis of autoxidation as the dominant mechanism of lipid peroxidation in tissues has long been reported (210). Rapid initiation and propagation reactions are characteristics of hematin catalysts.

Inorganic ferrous iron has also been demonstrated to be a catalyst of unsaturated lipid peroxidation in mitochondria (160) and microsomes (177), as well as in pure unsaturated lipids (234). Both types of catalysts, hemoprotein and inorganic iron, could be differentiated by their relative activities at different pH and in the presence of chelating agents such as ascorbic acid and thiol compounds (234). Both heme and nonheme iron containing enzymes present in microsomal fractions could catalyze lipid peroxidation if the iron were made accessible through some change in tertiary structure.

Another factor influencing oxidative rancidity is pH. An inverse relationship between the pH of meat samples and the TBA number has been found, which would be expected if nonheme iron is playing an active role (58, 108). On the other hand, this effect could result from an increased effect of reducing enzymes in raw tissues at higher pH (173). Oxygen can be removed from the tissues and hematin compounds, like metmyoglobin, may be reduced to myoglobin by the reducing enzymes. Research indicates that hematin catalysts would be of great physiological importance as a mechanism of lipid peroxidation, along with autoxidation,
in the process of pathological and deteriorative reactions (2, 116, 131). The condition of high PUFA and dark muscle content, therefore, make preservation of mullet and other finfish difficult.

Handling and packaging methods practiced at the wholesale and retail level influences the oxidative status of fish also (97). During peak season of harvest, surplus fish supplies are usually frozen either in the round or fellated without specific handling or packaging considerations. The chilling process may also effect lipid oxidation (96). The freshness of fish samples in this study is uncertain.

Prefreezing abuse may also impact the quality of frozen fish. Reports have stated that microsomes from both light and dark muscle of lean and fatty fish contain enzymes that catalyze lipid peroxidation, and these enzymes may also contribute to the oxidative deterioration of frozen fish (96, 97, 199). Lipid deterioration in fish muscle may be initiated by myeloperoxidases from phagocytic leukocytes (177).

Lipid soluble vitamins A and E analyses indicated a small quantity of vitamin E in mullet, but not in other fish. Vitamin A could not be quantified in any of the fish species. Probably, the levels of vitamins E and A of these species were low and already affected by oxidation by the time they were purchased and analyzed. Fatty fish like
mullet have been reported to have high PUFA and a low level of Vitamin E (71). The role of tocopherols as inhibitors and synergists in hemoglobin-catalyzed lipid oxidation has been discussed (210). Since vitamin A and vitamin E could not be measured in any of the fish species studied, it was not possible to speculate as to their role in the oxidative status of these samples.

The tocopherol in tissues is in contact with many constituents that could act synergistically in inhibiting lipid peroxidation. A mixture of tocopherol with ascorbic acid and citric acid was found to inhibit oxidation more effectively than ascorbate alone or in combination with citric acid in tissue fats of fish (17). Therefore, consideration of such influences seems appropriate in developing handling and packaging practices for maintaining marine products for extended periods with only limited losses in freshness. In the subsequent experiments Phase II and Phase III, processing conditions necessary to optimize the product quality and storage stability in relation to oxidative changes were investigated.
4.2. PHASE II

4.2.1. Lipid Changes in Mullet: Effect of Storage Condition and Time

The total lipid content of frozen, canned and vacuum packaged mullet samples as influenced by period of storage is presented in Fig. 7. Average total lipid values obtained from all fish samples were within the range of 5 to 5.8 g fat per 100 g tissue. A similar observation was made earlier for mullet (46). Most mullet are harvested during the spawning season (222) and are then frozen before shipping or further processing (33). Prior to spawning, fish feed voraciously and increase their body fat content substantially. After spawning, their fat content is very low and the water content of muscle is high. Such changes in composition make the quality of the flesh inferior. Lipid content during the post-harvest spawning period of February to July averages between 1 and 2%. Post-harvest changes in the lipid of mullet caught during spawning season have not been investigated.

Fish samples that were canned tended to have higher values compared to the frozen and vacuum packaged samples (Table 2), indicating that processing treatment and temperature had an effect on the levels of total extractable lipid of mullet. The canning process possibly
Figure 7. Lipid content of mullet as influenced by frozen storage and canning. Days indicate the period of storage until analyzed for all treatment groups. Each block represents the mean of three observations.
Table 2. Mean values of the total lipid content of mullet as influenced by canning.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Storage (day)</th>
<th>Lipid (g/100g)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen</td>
<td>0</td>
<td>5.22</td>
<td>0.19</td>
</tr>
<tr>
<td>Canned</td>
<td>0</td>
<td>5.35</td>
<td>0.08</td>
</tr>
</tbody>
</table>

1 Total lipid content.

a,b Means (n=3) with different superscripts are different (p<0.01), as tested by least significant difference (LSD) method.
caused lipid components that were bound in tissue membranes to become more extractable. Also, dehydration resulting from the cooking and canning process may have had a concentration effect. This possibility has been observed in cooked meat (183). ANOVA indicated that total lipid content did not change (P > 0.05) with time of storage. This observation is in agreement with a previous finding where the effect of storage method, temperature and time on lipid changes in fish was investigated (46).

Figure 8 presents the phospholipid content of frozen, canned and vacuum packaged mullet samples. The initial phospholipid values were within the range of 2.00 ± 0.1 mg/g to 1.81 ± 0.29 mg/g of tissue. The phospholipid content of mullet fillets that were canned, both fresh as well as after 90 days of frozen storage, were higher compared to fish fillets frozen and vacuum packaged on day 0 of storage. The levels of total extractable phospholipids were found to be influenced by the length of storage period and exhibited considerable change with time (P ≤0.01). The phospholipid values for freshly frozen mullet decreased approximately 4% from an initial value after 90 days, approximately 22% after 180 days and finally by 62% after 360 days of storage. This change was not as pronounced for mullet samples canned on day 0. The reduction in phospholipids levels in freshly canned samples was only 3% between day 0 and day 360 of storage. Mullet
Figure 8. Phospholipid content of mullet as influenced by frozen storage and canning. Each block represents the mean of three observations. Days indicate the period of storage until analyzed for all treatment groups.
canned after 90 days of frozen storage, showed a large decrease in phospholipid content between day 180 and day 360. Vacuum packaged mullet samples had a pattern of phospholipids losses, but to a lesser degree.

Highly unsaturated fatty acids are widely distributed in small quantities as constituents of the fatty acid moiety of phospholipids. The predominant polyunsaturated fatty acids in fish are the omega-3 fatty acids. Their presence makes the lipids more susceptible to oxidation. Marine lipid, rich in the n-3 fatty acids eicosapentanoic acid (EPA 20: 5) and docosapentanoic acid (DHA 22:6) greatly affect the degree of unsaturation of various membrane phospholipids (3, 59, 121).

Mullet will readily oxidize by free radical chain mechanisms to give peroxides (45). Enzymatic phospholipid hydrolysis in frozen fish also has been recognized as a major cause of quality deterioration (194). It is possible that enzymatic lipid peroxidation activates phospholipase A₂, to initiate phospholipid hydrolysis in frozen fish muscle, because the microsomal lipid peroxidation enzymic system is active at temperatures below the freezing point of fish tissue (9). Experiments using fish muscle microsomes suggested that lipid peroxidation expressed as TBRS per unit weight of total post mitochondrial lipid was inversely correlated with the recovery of both microsomes and lipids. This suggested a relationship between
peroxidation and destruction of lipid by hydrolysis (105). Changes in the phospholipid pattern similar to the observations made in this study were reported with fish frozen at 
-18°C over an extended period of storage (69).

Oxidative changes in mullet during storage are depicted in Fig.9. The TBA values initially were very low and similar in all treatments analyzed on day 0. TBA number ranged from 0.2 to 0.6 mg/1000 g tissue. Canned samples had relatively higher TBA numbers 0.58 ± 0.07 mg/1000 g compared to the freshly frozen fish 0.16 ± 0.03 mg/1000 g.

TBA number of frozen fish stored at -20°C increased sharply (P ≤ 0.01) and steadily with time from 0.16 ± 0.03 mg/1000 g on day 0 to 9.58 ± 0.18 mg/1000 g on day 360. The quick onset of oxidative rancidity of mullet flesh lipid during storage has been a major problem affecting the flavor, nutritional and textural quality of the fish (63, 242). The heme pigment in the lateral band acts as a catalyst for the oxidation of highly unsaturated fatty acids (209). This rapid development of oxidative rancidity during storage has been reported earlier (63, 233), which supports the present observation. Evidence for the occurrence of enzymatically derived hydroperoxides in fresh fish (18) suggests that these compounds also could play a role in the rate of autoxidation of polyunsaturated fatty acids and thus affect the rate of oxidative deterioration.
Figure 9. Lipid oxidation in mullet as influenced by frozen storage and canning. Thiobarbituric acid reactive substance is TBRS, frozen storage in a vacuum package is Froz-Vac, canned after 90 days of frozen storage is canned-90. Each point is the mean of three observations.
Seasonal variation could possibly explain part of the rapid increase in TBA values of frozen stored mullet samples, which were caught during spawning season. Chill processing immediately after catch has been found to have little effect on the rate of oxidative spoilage. Although several million pounds of mullet are harvested each year, the value per pound is one of the lowest among fish from the Gulf. Therefore, compared to other commercial species from the Gulf, mullet must be given special consideration since it has unique spoilage properties.

Studies to control oxidative rancidity in frozen mullet fillets have been reported. Canning has been shown to be the most effective means of storage to arrest increases in TBA numbers. The canned mullet in this study stored from day 0 to 360 days showed little change in TBA numbers, which was statistically insignificant ($P > 0.05$). Vacuum packaged fish had TBA values that were similar to canned samples in which TBA numbers increased from $0.16 \pm 0.04$ to $1.27 \pm 0.20$ mg/1000 g from 180 to 360 days.

Air was greatly reduced in the cans and vacumm packages. This was possibly responsible for the reduction in oxidation reactions of these samples. Also, use of brine in combination with polyphosphates as dips may have contributed to the effectiveness of preventing rancidity development in canned samples. Such evidence has been
reported in the literature (17, 45, 243). Antioxidants alone or in combination with curing salts have been used to inhibit oxidation in cooked mullet (241). In addition, heat treatments that were involved in the canning process of mullet might possibly have resulted in the production of Maillard reaction products (MRP). These MRPs could have exerted antioxidant activity or TBA retarding activity. Conclusive evidence supporting the influence of MRP as strong antioxidants has been reported on several occasions (57, 182, 236,). Suggestions have been made concerning the possible usefulness of MRP in preserving desirable flavor in cooked meat during frozen storage.

Canning after a short period of frozen storage was not as effective in controlling oxidation. The length of frozen storage before canning is crucial because in frozen fish the most important factor is the control of the induction period before bimolecular oxidation propagates. So far, no effective method to prevent the development of rancidity in the propagation period has been developed. It was apparent that, oxidative degradation had already occurred when mullet samples were canned after 90 days of frozen storage; however, oxidation was substantially retarded after canning. Perhaps this was due to the factors discussed earlier in relation to thermal processing. Canning after a period of frozen storage therefore, may not be as effective as canning of fresh
Vacuum packaging slowed the rise of TBA numbers and enhanced the retention of quality in frozen fish. This observation agrees with earlier findings where rancidity in fish was retarded by vacuum packaging (118, 128, 180). Vacuum packaging alone and in combination with antioxidants has been used effectively to retard oxidation in frozen fish (124, 180).

Finally, a significant correlation between TBA values and phospholipids was noted (234). Phospholipids have been found to contribute about 90% of the malonaldehyde production in meat fat (164). The observations of the present study indicate a direct relationship between the rate of oxidation and changes in phospholipid content. Freshly canned samples showed minimal changes in phospholipid content where TBA values were also the lowest compared to frozen stored samples. The correlation coefficients between TBA values and phospholipids was highly significant (p > 0.001) and negatively correlated (Table 3). With the increase of TBA values, there was decrease in phospholipid content during frozen storage, possibly as a consequence of fatty acid degradation in the phospholipids by oxidation. These results are in agreement with earlier findings (77, 148, 224). The correlation coefficients between the means of TBA values and of total
Table 3. Correlation coefficients between the means of TBA values and of total lipid, phospholipids and vitamin E.

<table>
<thead>
<tr>
<th>Components correlated with TBA</th>
<th>Correlation coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Total lipid</td>
<td>-0.09</td>
</tr>
<tr>
<td>2 Phospholipids</td>
<td>-0.41</td>
</tr>
<tr>
<td>3 Vitamin E</td>
<td>-0.64</td>
</tr>
</tbody>
</table>

1 % Lipid content.
2 mg Phospholipids per gram of sample.
3 mg α-Tocopherol per 100g of sample.

a Nonsignificant.
b \( P < 0.01 \) for HO: \( P = 0 \).
c \( P < 0.001 \) for HO: \( P = 0 \).
n = 48
lipids was not significant ($p > 0.05$) (Table 3).

4.2.2. **Changes in Lipid Soluble Vitamin A and E of Mullet Upon Storage as Determined by HPLC**

A typical HPLC elution profile of the standard solutions mixture of all trans retinol and DL-\(\alpha\)-tocopherol in HPLC grade methanol is depicted in Fig. 10. A detection wavelength of 280nm was chosen because this was a wavelength intermediate between the optimum wavelength of the two vitamins. The sensitivity obtained was satisfactory for the samples analyzed. Many different columns could have been used (189, 231). For this experimental protocol, C-18 Nova Pak was found to give good resolution of retinol and \(\alpha\)-tocopherol. With 96% methanol-4% water as the eluent, a chromatogram could be completed in about 25 minutes (Fig. 10). The reproducibility of peak heights, areas and retention times is given in Table 4. Peak height was used to calculate vitamin A content, whereas peak area was used for calculation of vitamin E content. The peaks of both vitamins were highly reproducible in terms of either peak heights or peak areas, and retention times. The recovery was approximately 80% for either of the vitamins. Linear analysis was done for retinol and \(\alpha\)-tocopherol standards with a correlation coefficient of 0.999 for concentration vs peak area with \(\alpha\)-tocopherol and 0.987 for
Figure 10. Typical HPLC chromatogram of the standards of retinol and DL-tocopherol. Standard solution concentration, 0.25mg per ml; 5ul per injection; C-18 NovaPak column, 5um; mobile phase, 96% methanol - 4% water; flow rate, 0.5ml per min; detection at 280nm.
Table 4. Reproducibility of peak heights/areas and retention times for retinol and DL-α-tocopherol.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Retinol peak height (mm)</th>
<th>$t_R$ (min)</th>
<th>DL-α-tocopherol peak area</th>
<th>$t_R$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>5.34</td>
<td>378230</td>
<td>21.59</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>5.35</td>
<td>383610</td>
<td>21.47</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>5.35</td>
<td>375910</td>
<td>21.53</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>5.34</td>
<td>385442</td>
<td>21.57</td>
</tr>
<tr>
<td>5</td>
<td>67</td>
<td>5.34</td>
<td>370378</td>
<td>21.53</td>
</tr>
</tbody>
</table>

1 Standard solution mixture of retinol and DL-α-tocopherol. Concentration, 0.025mg per ml; sample injection, 5 ul each.

$n = 5$.

$t_R$ = Retention time.
concentration vs peak height for retinol (Figs. A.1 and A.2).

The main nutritional implications of fat oxidation may be that nutritional value of polyunsaturated fatty acids is reduced and fat soluble vitamins, especially vitamins E and A are lost. Though the function of vitamin E is to prevent fat oxidation, in the process it becomes oxidized itself. Therefore the need for vitamin E is greater when there is high levels of PUFA in the system. Also, vitamin A content of most fish is very low, particularly in the muscle.

Figures 11-14 are chromatograms of Vitamin E (frozen, canned and vacuum packaged) in samples analyzed at different storage intervals. No Vitamin A was identifiable using the experimental conditions. On the other hand, α-tocopherol was present in detectable amounts in all samples. Figure 15 is a quantitative summary of changes in α-tocopherol content of mullet during storage of frozen, canned and vacuum packaged samples. The α-tocopherol content in frozen samples gradually decreased over the period of storage from 0.69 ± 0.03 mg/100 g to a trace amount at day 360. In samples canned on day 0 the change was significantly less (P < 0.05). Mullet canned after 90 days of frozen storage, had less vitamin E in comparison to frozen fish. There was a 54% reduction in vitamin E content from day 90 to 360 days of storage in samples canned after 90 days. However, canning did serve to
Figure 11. Typical HPLC chromatograms of fresh frozen mullet showing changes in \( \alpha \)-tocopherol as influenced by frozen storage over a period of 0, 90, 180 and 360 days.
Figure 12. Typical HPLC chromatograms of freshly canned mullet showing changes in tocopherol content as influenced by canning and storage over a period of 0, 90, 180 and 360 days.
Figure 13. Typical HPLC chromatograms of mullet canned after 90 days of frozen storage showing changes in α-tocopherol from day 90 through day 180 and 360.
Figure 14. Typical HPLC chromatograms of mullet freshly frozen in vacuum package showing changes in tocopherol content as influenced by storage over a period of 0, 90, 180 and 360 days.
Figure 15. Tocopherol content of mullet as influenced by frozen storage and canning. A, represents mullet frozen fresh; B, canned fresh; C, canned after 90 days of frozen storage and D, frozen fresh in vacuum package. Each point depicts the mean of three observations.
maintain vitamin E during subsequent storage, compared to frozen fish. For vacuum packaged fish, the change in \( \alpha \)-tocopherol concentration during storage was similar to fish frozen without vacuum packaging.

The presence of very low levels of vitamin E, along with fairly substantial losses of the vitamin during the period of storage in frozen fish samples suggests a possible correlation of \( \alpha \)-tocopherol with lipid peroxidation in mullet tissue. The correlation coefficients between the means of the TBA values and of \( \alpha \)-tocopherol content was found to be significant \((p \leq 0.01)\), with \( \alpha \)-tocopherol content decreasing as TBA values increased during frozen storage (Table 3). Vitamin E is a natural antioxidant and is known to play an important role in lipid peroxidation reaction. A decrease in concentration of \( \alpha \)-tocopherol as TBA number increased, may indicate that the level of vitamin E required to maintain a low basal level of lipid peroxidation was not present in mullet muscle. Therefore, it may not have exerted a significant antioxidant effect and was degraded as peroxidation proceeded with time. The results suggest that increased TBA values may have resulted, not so much from an increase in the initiation of lipid peroxidation, but from a decrease in vitamin E catalyzed termination of free-radical propagated lipid peroxidation chain reactions.

Vitamin E is believed to have metabolic importance in
the maintenance of the functional integrity of subcellular membranes. An increase in lipid peroxidation produces a significant impact on the vitamin E requirements for inhibition of lipid peroxidation. Vitamin E may be the only protective agent naturally present within cellular membranes. Reducing compounds like cysteine and glutathione may serve to regenerated vitamin E, if present in the system. A cooperative inhibition of oxidation by α-tocopherol and ascorbic acid was observed (50), and it was suggested that vitamin C and vitamin E function as water soluble and lipid soluble chain breaking antioxidants, respectively. A more detailed understanding of the quantitative relationship between lipid peroxidation and tissue requirements of vitamin E would therefore require additional investigation.

Mullet represents a substantial portion of by-catch in the Gulf coast. The results of this phase of study suggests the potential of producing a stable form of mullet through canning. This may provide a new product to the fish industry. The potential use of frozen mullet for canning would require proper protection against oxidation during frozen storage. Because of the unstable nature of this fish, pretreatment with vitamin E prior to canning, or addition of this antioxidant in the can, may improve the quality, stability and nutritional value of the product.
Therefore, mullet may provide a new mullet product for shrimp processors during the off season.
4.3. PHASE III

The low levels of vitamins A and E along with the high levels of lipid oxidation observed in the first phase of this study suggested the possibility of supplementing frozen fish with antioxidants through the use of prefreezing treatments. These included water dip, dips containing vitamin E or TBHQ and vacuum packaging. A comparative study of their effectiveness during frozen storage in blackdrum samples was done.

4.3.1. Lipid Changes in Blackdrum: Effects of Physical and Chemical Treatments Upon Frozen Storage.

Blackdrum contained approximately 4% total lipid and there was no variation due to treatment (Fig 16). Extractable phospholipids for all treatment groups ranged between 1.19 - 1.24 mg/g (Fig 17). Blackdrum would fall into the category of fish with intermediate fat content but appeared to be less fatty than mullet and also had proportionally less dark lateral band. Therefore oxidation was expected to be lower compared to mullet.

The relative effectiveness of the physical and chemical treatments in the prevention of lipid oxidation during frozen storage is depicted in Table 5. TBA number of blackdrum fillets with no treatment increased more than 14 fold during 12 weeks of storage. This group served as
Figure 16. Lipid content of blackdrum as influenced by physical and chemical treatments. Treatment 1 is no dip; 2 is water dip; 3 is 1% vitamin E dip; 4 is 0.5% vitamin E dip; 5 is 0.025% TBHQ dip and 6 is no dip-vacuum packaged. Each bar represents the mean value of three observations.
Figure 17. Phospholipid content of blackdrum as influenced by physical and chemical treatments. Treatment 1 is no dip; 2 is water dip; 3 is 1% vitamin E dip; 4 is 0.5% vitamin E dip; 5 is 0.025% TBHQ dip and 6 is no dip-vacuum packaged. Each bar represents the mean of three observations.
Table 5. Mean values of TBA of blackdrum as influenced by physical and chemical treatments during frozen storage

<table>
<thead>
<tr>
<th>Storage (days)</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1 2 3 4 5 6</td>
</tr>
<tr>
<td>15</td>
<td>H , I H , I 0.24 0.24 0.23 0.24 0.24 0.24</td>
</tr>
<tr>
<td></td>
<td>+0.05 +0.04 +0.05 +0.04 +0.04 +0.04</td>
</tr>
<tr>
<td>30</td>
<td>0.53 0.30 H , I H , I 0.28 0.34 0.28 0.36</td>
</tr>
<tr>
<td></td>
<td>+0.08 +0.01 +0.01 +0.01 +0.02 +0.02</td>
</tr>
<tr>
<td>45</td>
<td>0.80 0.38 G , H , I 0.31 H , I 0.42 G , H 0.38 G , H</td>
</tr>
<tr>
<td></td>
<td>+0.12 +0.01 +0.01 +0.02 +0.02 +0.03</td>
</tr>
<tr>
<td>60</td>
<td>1.38 0.45 G , H H , I 0.41 0.51 G , H , I H , G</td>
</tr>
<tr>
<td></td>
<td>+0.22 +0.01 +0.01 +0.02 +0.01 +0.10</td>
</tr>
<tr>
<td>75</td>
<td>1.57 0.47 G , H G , H 0.46 G , F G , H 0.86 E , F</td>
</tr>
<tr>
<td></td>
<td>+0.12 +0.01 +0.01 +0.85 +0.40 +0.04</td>
</tr>
<tr>
<td>90</td>
<td>1.78 0.48 G , H G , H 1.02 E Q , H E</td>
</tr>
<tr>
<td></td>
<td>+0.16 +0.01 +0.01 +0.13 +0.02 +0.03</td>
</tr>
</tbody>
</table>

1 means (N=3) (+SD). Means followed by the same letter are not significantly different (P>0.05). Treatment 1 is no dip; 2 is water dip; 3 is 1% -tocopherol dip; 4 is 0.5% -tocopherol dip; 5 is 0.025% TBHQ dip and 6 is no dip-vacuum packaged. TBA values are expressed as TBRS per 1000g sample. TBRS is thiobarbituric acid reactive substance.
the control group and was found to be most severely oxidized. The no dip-vacuum packaged group had a six fold increase in TBA values over the 12 week period of storage. The vacuum packaged group, however, was much lower \((p \leq 0.05)\) than the control group in TBRS production. Vacuum packaging slowed the rise in TBA numbers to an extent comparable to the 0.5% \(\alpha\)-tocopherol dipped group. Similar observations were made in previous a study on storage stability \((191)\). A combination of vacuum packaging and antioxidant treatments in a dip might greatly improve the effectiveness against oxidative changes. Such combinations were found to be effective in earlier studies \((45, 46)\). Water dip offered better protection compared to no dip-vacuum packaging treatment. This was unexpected. The water dip may have formed a continuous film, that protected against both dehydration and accessibility of oxygen. As long as water coating remains in contact with fish, only limited amount of oxygen could penetrate and reduced oxidation would occur \((64, 205)\).

TBHQ provided the greatest protection against autoxidation of marine lipids. The effectiveness of TBHQ in the highly unsaturated blackdrum muscle lipids is noteworthy as a way to improve oxidative stability of marine fatty fish. This observation confirms earlier findings that TBHQ is the most effective antioxidant for unsaturated lipids \((105, 188, 218)\). The finding with
regard to \(-\)tocopherol agrees with earlier investigations and demonstrates advantages of careful application of this vitamin to marine species because it is a natural nutrient.

The overall effectiveness of the various storage treatments investigated for inhibiting oxidation in blackdrum fillets were in the order of TBHQ > 1% \(\alpha\)-tocopherol > water dip > 0.5% \(\alpha\)-tocopherol > vacuum pack > no dip. The trend in recent years towards the inclusion of more highly unsaturated lipids in the diet has invoked greater consumption of fatty fish. Should this trend continue, TBHQ and \(\alpha\)-tocopherol would fulfill the need for quality retention by preventing oxidative deterioration in processed marine oils and fillets of fatty fish as well as newer varieties of processed fishery products based on minced and comminuted fish (22).

4.3.2. Changes in Lipid Soluble Vitamins A and E in Frozen Blackdrum: Effects of Physical and Chemical Treatments.

Preliminary analysis established the presence of a small peak with the approximate retention time of retinol. In order to verify the presence of retinol, typical blackdrum samples were spiked with standard all trans retinol. Figure 18 represents the response at two wavelengths for retinol in the spiking study,
Figure 18. HPLC chromatograms of frozen black-drum samples without dip treatment, spiked with vitamin A; A, detection is at 280nm; B, detection is at 340nm.
verifying the presence of retinol in blackdrum samples. Retinol was quantifiable in all samples but tocopherol was absent, with the possible exception of tocopherol dip treatments (Figure 19-24).

Changes in the retinol content in blackdrum samples over the storage period of 12 weeks is presented in Table 6. Control samples showed the greatest change in which retinol content decreased about 44% by the final week of storage. The 1.0% tocopherol dip was most effective in maintaining retinol levels, followed by the 0.5% tocopherol dip. Water dipping was nearly as effective as tocopherol dipping to prevent the loss of retinol during frozen storage. The magnitude of retinol loss during 90 days of frozen storage was 20%, 24% and 27.5% for the 1.0%, 0.5% \( \alpha \) -tocopherol and water dipped groups, respectively. The TBHQ dipped group had a 27.7% decrease in retinol content over 90 days of frozen storage. Therefore the order of effectiveness of the prefreezing treatment groups for the retention of vitamin A was 1.0% > \( \alpha \) -tocopherol > 0.5% \( \alpha \) -tocopherol > water dip > TBHQ dip > no dip-vacuum package > no dip. Correlation coefficients between TBA values and retinol content of blackdrum is given in Table 7. A significant negative correlation \( (p < 0.01) \) was obtained. This suggests that retinol retention was probably directly related to oxidation during storage.
Figure 19. Typical HPLC chromatograms of frozen blackdrum samples frozen stored with no treatment, showing changes in retinol content over a period of 90 days of storage. A represents chromatogram at day 0 and B at day 90 storage.
Figure 20. Typical HPLC chromatograms of frozen blackdrum samples dipped in 1% - tocopherol, showing changes in retinol content over a 90 days period of storage. A represents chromatogram at day 0 and B at day 90 storage.
Figure 21. Typical HPLC chromatograms of frozen blackdrum samples dipped in 0.5% tocopherol, showing changes in retinol content over a 90 days period of storage. A represents chromatogram at day 0 and B at day 90 storage.
Figure 22. Typical HPLC chromatograms of frozen blackdrum samples dipped in 0.025% TBHQ showing changes in retinol content over a 90 days period storage. A represents chromatogram at day 0 and B at day 90 storage.
Figure 23. Typical HPLC chromatograms of frozen blackdrum samples dipped in water, showing changes in retinol content over a 90 days period of storage. A represents chromatogram at day 0 and B at day 90 storage.
Figure 24. Typical HPLC chromatograms of frozen blackdrum samples stored in vacuum package without dip treatment, showing changes in retinol content over a 90 days period of storage. A represents chromatogram at day 0 and B at day 90 storage.
Table 6. Mean values of vitamin A content of blackdrum as influenced by physical and chemical treatments during frozen storage

<table>
<thead>
<tr>
<th>Storage (day)</th>
<th>Treatments 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>11.72</td>
</tr>
<tr>
<td></td>
<td>+0.04</td>
</tr>
<tr>
<td>15</td>
<td>10.63</td>
</tr>
<tr>
<td></td>
<td>+0.55</td>
</tr>
<tr>
<td>30</td>
<td>10.23</td>
</tr>
<tr>
<td></td>
<td>+0.07</td>
</tr>
<tr>
<td></td>
<td>+0.05</td>
</tr>
<tr>
<td>60</td>
<td>9.10</td>
</tr>
<tr>
<td></td>
<td>+0.04</td>
</tr>
<tr>
<td>75</td>
<td>8.15</td>
</tr>
<tr>
<td></td>
<td>+0.05</td>
</tr>
<tr>
<td>90</td>
<td>6.52</td>
</tr>
<tr>
<td></td>
<td>+0.05</td>
</tr>
</tbody>
</table>

1 means (n=3) (+ SD). Means followed by the same letter are not significantly different (P > 0.05). Treatment 1 is no dip; 2 is water dip; 3 is 1% tocopherol dip; 4 is 0.5% tocopherol dip; 5 is 0.025% TBHQ dip and 6 is no dip-vacuum packaged. Retinol content is expressed as ug per 100g.
Table 7. Correlation coefficients between the means of the TBA values and of vitamin A.

<table>
<thead>
<tr>
<th>Components correlated with TBA</th>
<th>Correlation coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Vitamin A</td>
<td>a -0.66</td>
</tr>
</tbody>
</table>

1 mg Retinol per 100g of sample.

P < 0.001 for H0 : P = 0.

n = 126.
TBHQ, which effectively arrested oxidation, was not as effective in preventing the loss of retinol in the tissue during storage. \( \alpha \)-tocopherol appeared to offer better oxidative protection and prevent loss of retinol. Tocopherols are well known inhibitors of hemoglobin catalyzed linoleic acid oxidation in animal fats (139). Retinol also has some antioxidative properties when it exists as \( \beta \)-carotene. \( \beta \)-carotene is an unusual type of lipid antioxidant that may play an important role in reducing the concentration of chain carrying peroxyl radicals in tissues and organelles at low partial pressures of oxygen. Although \( \beta \)-carotene also has some capacity to quench singlet oxygen. Retinol and \( \alpha \)-tocopherol may be complimentary in their action in a sense, because retinoids quench singlet oxygen while \( \alpha \)-tocopherol inactivate free radicals, thereby controlling lipid peroxidation. The presence of carotinoids in fat tissue is very limited. In fish muscle this fat soluble vitamin normally exists as retinol, and also at a very low concentration. This was observed in the present research. Moreover, its level present in this fish may not be high enough to exert an antioxidant effect.

The loss of retinol observed in the different treatment groups, indicates that the type of prefreezing treatment makes the greatest difference in the extent to which this vitamin was protected from oxidation. Both vitamins A and
E are known to be involved in the lipid oxidation process but their interrelated role in the overall process is still obscure. The incorporation of certain forms of provitamin A may provide potential antioxidant activity, but this possibility in fish has not been investigated. Tocopherols, because of their close co-existence with unsaturated fat in the tissues, also would offer a promising biological tool to achieve oxidative stability in finfish. Use of antioxidants in the form of dips or glazes appears to offer an effective method to control lipid oxidation in fish foods during frozen storage.

In recent years, there has been a trend to include more fish in the diet. This trend may have developed as a result of increased awareness toward health issues and concerns. This is leading to consumption of more polyunsaturated marine lipids. The seafood industry is, therefore, flourishing and at the same time facing an increased demand for a newer supply of fish products. Red drum, for example, has become a popular menu item in the form of blackened redfish. A large demand for this fish has increased the commercial fishery beyond the likely supply. Blackdrum, which has characteristics similar to reddrum, may provide an alternative, and is somewhat underutilized and has ample supply. The fishing industry could possibly utilize this resource by following proper handling and storage practices. A large supply of
blackdrum could be frozen, using glazes impregnated with antioxidants like \( \kappa \)-tocopherol and TBHQ, and/or sealed in vacuum packages. Thus, blackdrum could be a complementary new variety of seafood for restaurant fare.
CHAPTER V

SUMMARY AND CONCLUSIONS

Lipid composition of selected Gulf coast marine species as influenced by processing and storage treatments was evaluated. In the first phase of this study, a survey was made of the lipid composition of a variety of fresh Gulf coast marine fish obtained from a local supermarket. Fillets of mullet, croaker, redfish and red snapper were analyzed for total lipid, phospholipid, Vitamins A and E and oxidation. Total lipid content was determined gravimetrically and phospholipids colorimetrically after cold solvent extraction. Vitamin A and E were determined simultaneously by HPLC. Oxidation was measured as malonaldehyde.

Lipid content for all fish species was found to be about 5% and phospholipids represented < 5% of the total lipid. Mullet was found to have a small amount of Vitamin E but Vitamin A was not detected. Malonaldehyde levels indicated that mullet had a relatively high degree of oxidation as did croaker but very little oxidation was evident in redfish. High levels of lipid oxidation suggest instability of flesh during storage. Freezing technology has provided consumers with products that have a reasonably good acceptance. However, frozen mullet fillets have been
of low quality and rancid flavor has been considered a major factor responsible for quality loss. The control of lipid oxidation in mullet may be the key to expanded utilization of this potentially valuable fishery.

In the second phase, oxidative changes in tissue lipids and lipid soluble vitamins of frozen and canned mullet during storage were determined. Also, the influence of canning, both fresh and after frozen storage was evaluated with regard to oxidative and nutritional properties. Fresh mullet was obtained from a commercial source on the Louisiana Gulf coast. Fish were eviscerated and half were wrapped in freezer paper, one fourth were vacuum packed and stored at -20°C. The other one fourth were canned and thermally processed. After 90 days of frozen storage, a second fourth were canned and thermally processed. Total lipid, phospholipid, Vitamins A and E and lipid oxidation were determined at 0, 90, 180 and 360 days of storage.

Lipid content remained unchanged throughout the storage period and was about 5% in all treatments. Phospholipid comprised <5% of total lipid and showed a gradual decrease in concentration over the period of storage, mainly in frozen and vacuum packed samples. This change was more pronounced in mullet canned after 90 days of frozen storage, compared to the freshly canned fish. No Vitamin A could be measured in any of the samples.
Concentration of Vitamin E decreased during storage, with higher losses in frozen and vacuum packaged samples. Canned fish had slightly higher Vitamin E levels, possibly due to a concentration effect, which changed only slightly during storage. Vitamin E levels of fish canned after 90 days of frozen storage were lower than the uncanned frozen samples at 90 days, but did not decline as rapidly in subsequent storage.

Malonaldehyde concentration, expressed as TBA number, indicated that oxidation was initially higher in the canned samples compared to the frozen samples, possibly due to the catalytic effect of thermal processing. However, the rate of oxidation was arrested in the canned fish during storage as compared with the frozen samples. Fish canned after 90 days of frozen storage had higher levels of oxidation after canning but further oxidation during storage did not occur. Mullet that were vacuum packaged fresh and stored frozen had less oxidation compared to the no vacuum treatment.

It appears that canning fresh would assure improved oxidative stability and longer shelf life for mullet, compared to other methods of storage. The presence of dark lateral meat in mullet, possibly contributed to the high oxidation state of the frozen fish tissues. Low levels of vitamin E, along with substantial losses during frozen storage suggests that long term frozen storage of mullet would affect its nutritional quality.
In the third phase of study, effectiveness of various prefreezing chemical and physical treatments such as use of dips, antioxidants and oxygen limitation in the reduction of oxidative changes during frozen storage was investigated. Blackdrum, another underutilized but potentially important fishery, was used in this study. Fillets of freshly caught blackdrum were obtained from a commercial source and assigned to treatment groups of no treatment (control), water dip, vacuum packaged, 0.025% TBHQ dip, 1% α-tocopherol and 0.5% α-tocopherol dips. After treatment, samples were stored at -20°C for 12 weeks. Total lipid and phospholipid content was determined initially. Samples were then analyzed at biweekly intervals to monitor changes in Vitamin A and E and malonaldehyde content.

Lipid content of black drum was within a range of 4-4.5%, which places it into the category of intermediate fatty fish. Phospholipid concentration was approximately 5% of total lipid. Effectiveness to control oxidation, as measured by TBA values was TBHQ > 1.0% α-tocopherol > water dip > 0.5% α-tocopherol > vacuum pack > no dip. α-tocopherol has an added advantage of being a natural nutrient. As an antioxidant, α-tocopherol has been well documented for its crucial role in biological oxidation. Vacuum packaging was not as effective preservation by dip.
contained Vitamin A, but not E in measurable quantities. Retinol was retained best in the \(-\)tocopherol dipped fish. TBHQ dip was not as effective in this respect. Water dip however was unexpectedly more efficient compared to vacuum packaging in retaining the vitamin.

Use of dips impregnated with antioxidants was proven to be an effective way to reduce oxidation during frozen storage of blackdrum fillets. TBHQ, which is permitted at a level of 0.02% under U.S. regulations, was found to be the most effective control of oxidative rancidity. \(\alpha\)-tocopherol, a natural nutrient and a widely used antioxidant, was demonstrated to have advantages compared to treatments other than TBHQ in reducing oxidation. TBHQ, although more effective in controlling oxidation, was not as effective in protecting retinol loss from the fish. The relationship of retinol and \(\alpha\)-tocopherol in the oxidative defense system might explain this situation.
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Table A.1. Analysis of variance for total lipids (Phase II)

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Note: T = Treatment; S = Storage days; R = Replication.
Table A.2. Analysis of variance of phospholipids (Phase II)

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Note: T = Treatment; S = Storage days; R = Replication.
### Table A.3. Analysis of variance for TBA values (Phase II)

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Note: T = Treatment; S = Storage days; R = Replication.
Table A.4. Analysis of variance for vitamin E (Phase II)

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Note: T = Treatment; S = Storage days; R = Replication.
Table A.5. Analysis of variance for TBA values (Phase III)

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Note: T = Treatment; S = Storage days; R = Replication.
Table A.6. Analysis of variance for vitamin A (Phase III)

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Note: T = Treatment; S = Storage days; R = Replication.
Figure A.1. Linear regression line of standard DL-\(\alpha\)-tocopherol.
Figure A.2. The linear regression line of standard all trans retinol.
VITA

The author was born and raised in Dhaka, Bangladesh. She received her Bachelors and Masters degrees in Biochemistry from the University of Dhaka, Bangladesh. In 1979, the author earned a Masters in Public Health, with Nutrition as major, from the University of Hawaii at Manoa, Honolulu, Hawaii. This program was sponsored by the U. S. Agency for International Development. Earlier, she attended an applied nutrition program in the National Institute of Nutrition, Hyderabad, India. She was engaged in teaching and research in the Institute of Nutrition and Food Science of Dhaka University before coming to the Louisiana State University, in 1984.

The author is at present pursuing her doctoral program in Food Science at Louisiana State University, Baton Rouge, Louisiana, under the guidance of Dr. J. Samuel Godber. Her doctoral program is being sponsored by the Louisiana Methodist World Hunger Scholarship.

The author is associated with the Institute of Food Technologists, American Public Health Association, LSU-Food Science Club, Bangladesh Food Science and Nutrition Association and Bangladesh Biochemical Society. She perceives herself as an academician in areas of Food Science and Nutrition.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Rafida Idris

Major Field: Food Science

Title of Dissertation: Changes in Lipid Composition of Gulf Coast Marine Species as Influenced by Processing and Storage Treatments

Approved:

[Signatures]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

November 23, 1988