CHAPTER 3

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3.1 Introduction

Fish has long been regarded as one of the basic sources of animal protein and were it not for the fragility of fish bones, archaeology would probably rank fish more highly in lists of foods from prehistoric times. The basic preservation technologies for fish were probably first drying and later salting, with or without smoking, as dictated by climate and local resources. Strong food flavours were a daily fact of life through the middle ages and although fresh fish was at times available, oxidation of fats was probably barely recognized as a problem in most fishery products.

But in recent times, with the advent of technology the most popular method of long term preservation of fish is by frozen storage. Prolonged frozen storage is known to lead to the deterioration in the quality of fish and shell fish items. Quality changes in fish include lipid oxidation, particularly in fatty fish and protein denaturation and textural changes due to interaction of proteins with oxidized lipids resulting in toughening of texture. For the long term storage of fish fillets, chilling alone has limitations in appreciable extension of shelf life and combination of chilling with other treatments incorporating permitted food additives have been applied to further augment the shelf life.

Antioxidants which include the phenolic compounds such as BHT (Butylated Hydroxy Toluene) and BHA (Butylated Hydroxy Anisole) have been used in the seafood industry. In recent years, there has been an increasing concern about the safety of synthetic food additives including the possible toxicity of the synthetic chemicals used as antioxidants. Naturally occurring compounds particularly spices and herbs have been chosen as a safe alternative to synthetic antioxidants. Much work has been focused on the antioxidant potential of spices on meat and meat products. The objective of the present study is to evaluate the efficacy of the use of naturally occurring spice compounds for maintaining the quality characteristics of fatty fish, mackerel, under chilled and frozen conditions.

3.2 Review of Literature

3.2.1. Muscle food as a substrate for lipid oxidation

Oxidation of lipids is a reaction of major significance in all living tissue and has both beneficial and detrimental consequence for the well being of living organism. (Sevanian and Hochstein, 1985). In muscle derived foods, the fine control mechanism that exist to control lipid oxidation
reactions in vivo are less effective and lipid oxidation proceeds in a comparatively uncontrolled manner (Sies, 1986).

After death the lipids in fish are subject to two major changes, lipolysis and autoxidation. Of the two processes, autoxidation is the most important, particularly in the deterioration of frozen fish products causing change in flavour (Banks, 1939), colour (Jones, 1962) and possibly textural changes of the lipid fraction in muscle foods (Sikorski et al., 1976). The polar phospholipids contain the highest proportion of unsaturated fatty acids and it has been established that this fraction as opposed to neutral lipid fraction, is primarily responsible for lipid oxidation in muscle foods.

### 3.2.2. Frozen storage of fish

Studies on mackerel (*Rastrelliger kanagurta*) of medium (4%) and high (11%) lipid contents, quick frozen individually (IQF) and as blocks (BF) and stored at -23°C showed that block frozen mackerel had higher frozen storage shelf-life than individually quick frozen samples based on sensory evaluation (Nair et al., 1976). Investigations carried out by Garg et al., (1982) on contact plate frozen stored (-18°C) ghol (*Pseudosciaena diacanthus*) in the fillets form indicated that ghol fillets remained in a highly acceptable condition up to 20 weeks and later acceptability steeply declined.

Chinnamma et al., (1995) observed that quick frozen mackerel (*Rastrelliger kanagurta*) stored at -10°C, -20°C and -30°C revealed that as the storage temperature was lowered a proportional extension of shelf life was obtained. Development of rancidity was found to be a limiting factor for acceptability when assessed by sensory and chemical parameters like free fatty acid and thiobarbituric acid value.

Frozen stored pink perch (*Nemipterus japonicus*) and oil sardine (*Sardinella longiciceps*) showed significant (p<0.05) increase in peroxide value (PV), thiobarbiturc acid (TBA) number and FFA. While sardine showed a greater rate of lipid oxidation, hydrolysis of lipids to FFA was more pronounced in pink perch. In sardine, oxidized and hydrolyzed products of lipids had an equally adverse impact on protein solubility. Hydrolysis of lipids and consequent accumulation of FFA and development of rancidity due to oxidation of the lipids were found to be the major problems during frozen storage of sardine (Gopakumar et al., 1978).
3.2.3 Factors affecting the rate of lipid oxidation

The influence of triglycerides on the development of rancidity was shown to depend upon the degree of unsaturation and the length of time in frozen storage. The relationship between oxidation of polyunsaturated fatty acid (PUFA) of the phospholipids and development of rancidity was confirmed by many workers (Watts, 1962 and Greene, 1969). Balogun and Talabi, (1984) have concluded that autoxidation of the triglycerides, principally in the adipose tissues, is responsible for the development of rancidity in raw frozen tuna.

In fish, oxidation is made even more complicated by the presence of protein (Sikorski et al., 1976; Fisher and Deng, 1977) especially heme proteins.

The most unsaturated lipids in all fish are the phospholipids, but they do not oxidise rapidly and this is caused by the physical disposition of the lipids making it difficult for them to participate in the oxidation chain reaction (Hardy et al., 1979). Exposed lipid surface of fillets and gutted fish will oxidise more rapidly than lipids embedded in tissue thorough which oxygen may diffuse only with difficulty (Bito and Kiriyama, 1973; Bligh and Regier, 1976). In fish oils, oxidation will proceed quite readily at ambient temperature (Smith et al., 1972). In wet fish between 0°C and ambient temperatures, oxidation does not appear to be a dominant spoilage factor (Smith et al., 1979). In sardine and mackerel rancid flavours have been reported to affect acceptability (Madhavan et al., 1972). Oxygen is mobilized by fish at these temperatures with a competition for it between microorganism, enzymes and lipids (Smith et al., 1972). Whole fish, when they are exposed and subjected to autoxidation, microorganism that are present which can interact with the oxidised lipid, can possibly affect the oxidation rate. This explains as to why certain fatty species such as trout and gutted mackerel, will oxidise at temperature above 0°C. (Madhavan et al., 1970; Hansen, 1972) where as others such as herring remain relatively unaffected.

Studies on conditions of cold storage have shown important consequences, if dehydration occurs then the rate of oxidation was increased, where as storage under condition of low water loss by packaging glazing or freezing in water had a protective influence (Tarr, 1948).

Lipid oxidation in fish is a free radical process, consisting of initiation and propagation steps. A number of components are normally present in fish tissues which serve as pro-oxidants or antioxidants to both the initiation and propagation steps (Hultin, 1988).
3.2.4. Effect of heme iron

Studies of Decker and Hultin, (1990) and Phillipy, (1984) showed that reducing compounds, such as ascorbate and glutathione decreased with post mortem storage of fish muscle. This decrease will affect the process of lipid oxidation in post mortem muscle. When the temperature of any membrane is lowered sufficiently, its phospholipids bilayer will pass from the liquid crystalline to gel state. Heme iron has been proposed as an initiator and promoter (Kanner and Hard, 1985; Rhee et al., 1987) of lipid oxidation in raw meats. In fish, low molecular weight iron is released from a precursor molecule, probably ferritin with time of storage. (Kanner et al., 1987; Decker and Hultin, 1990). The process is greatly accelerated if the muscle tissue has been frozen and thawed. When the muscle cells tend to be weak to maintain components in the reduced state, myoglobin and hemoglobin (Fe^{2+}) are oxidised to metmyoglobin and methemoglobin (Fe^{3+}). This in turn can react with hydrogen peroxide to produce (Fe^{3+}) which can initiate lipid oxidation (Kanner et al., 1987; John et al., 1989).

Tippleswamy et al., (2007) identified possible non controllable and controllable factors responsible for the occasional lack of haemoglobin (Hb) mediated lipid oxidation of washed cod mince. Among noncontrollable factors were initial peroxide value (PV), level of tocopherol and structure. Among controllable factors were washing, pH, moisture, Hb (haemoglobin) level and light during storage of food.

3.2.5 Effect of lipid oxidation products on protein structure

The myofibrillar protein particularly myosin of many species may be altered by the interaction with different types of lipids or lipid oxidation products during frozen storage (Saeed et al., 1999). This interaction caused considerable changes in some functional properties and in the texture of fish muscle (Howell, 1999).

Exposure of protein to peroxidising lipids of their secondary breakdown products can produce changes in proteins, including loss of enzyme activity, polymerization, insolubilisation, scission and formation of lipid protein complexes. (Sikorski et al., 1976; Sikorski et al., 1990).

Lipids, especially oxidized lipids, may affect the hydrogen bonds and hydrophobic interactions in the proteins of frozen fish. The fatty acid character of lipid molecules exerts a surfactant effect on
protein surfaces, leading to hydrophobic interaction and protein unfolding, thus exposing interior groups for reaction. Further more the carbonyl groups of oxidised lipids may participate in covalent bonding, leading to the formation of stable protein-lipid aggregates.

Jahrenback and Litjemark, (1975) shows the results of model experiments on protein lipid, interaction which gives circumstantial evidence for the effect of ordinary lipid on fish protein. Lipid protein interaction involves two basic mechanisms (Schaich and Karel, 1975). The first mechanism involves protein amino condensation reactions, involving lipid oxidation, break down products, such as malonaldehyde and amino groups. The second mechanism involves the reaction of proteins with lipid oxidation products, which results in the formation of protein centered free radicals (Saeed et al., 1999).

3.2.6. Lipid oxidation in cooked system

In cooked meats, work about the relative pro-oxidant rules of heme and non heme iron in lipid oxidation have been done (Sato et al., 1971). Love and Pearson (1974); Verma et al., (1985) and Apte and Morrissey, (1987) found that iron was released from heme pigments following cooking and proposed that the resultant increase in non heme iron was responsible for the rapid oxidation of stored cooked meats. Studies by Kristensen and Andersen, (1997) show that there was a negligible increase in free iron at the expense of heme iron in myoglobin solutions heated up to 90°C. They also showed an increase in pro-oxidant activity of myoglobin around its thermal denaturation temperature (60-90°C) and attributed this to exposure of the catalytic heme group to lipid hydroperoxides. At levels relevant to meat and meat products, the pro-oxidant activity of heme iron in cooked meats exceeds that of free iron. Other studies using model system have shown that ferritin, when heated may promote lipid oxidation. The other major iron-containing fraction in muscle is the insoluble hemosiderin fraction, which accounts for as much as 30% of total iron in beef and 60% in chicken (Hazell, 1982). In a study in which the relative contributions of different iron-containing fractions to lipid oxidation were found in beef, pork and fish. Apte and Morrissey, (1987) concluded that haemosiderin did not play a significant role in lipid oxidation. Rhee, (1987) has pointed out that the greater susceptibility of cooked meat towards lipid oxidation may also be due to the disruption of meat tissues by cooking; thus bringing the lipid substrate and catalyst in to closer contact.
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Similar studies by Tichivangana and Morrissey, (1985) revealed that only in raw fish metmyoglobin was a significant pro-oxidant, whereas significant increases in TBARS, were seen with pork, chicken, turkey and fish in the presence of ferrous iron.

The cooking process leads to thermal denaturation of antioxidant enzymes and enzyme inactivation is believed to contribute in part to lipid oxidation in cooked meats (Lee et al., 1996).

3.2.7. Factors influencing oxidation in Muscle Foods

3.2.7.1. Temperature

A number of extrinsic factors influence lipid oxidation in muscle foods, such as temperature, light etc. Hultin, (1994) showed that increased dissolved oxygen at lower temperatures may offset the effect of reduced temperature on the rate of lipid oxidation, and in fish, frozen thawed muscle may oxidise more than the non frozen tissue (Decker and Hultin, 1990). Lipid oxidation in raw meats in refrigerated storage can be greatly accelerated if the meat has been restructured (O’Grady et al., 1997). In the absence of particular condition that promote lipid oxidation in refrigerated raw meats, quality deterioration due to microbial growth may precede oxidative deterioration (Monahan et al., 1992). In frozen storage both microbial deterioration and oxidative deterioration are retarded in raw meats but lipid oxidation leading to rancidity occurs with increasing storage time (Bremner et al., 1976).

3.2.7.2. Phosphate

Polyphosphate improves texture, processing/cooking yield and storage stability of meat products (Molins, 1991). Sodium tripolyphosphate (STPP) had no antimicrobial effect in temperature abused; frozen raw ground beef and refrigerated raw pork containing salt (Chu et al., 1987).

3.2.7.3. Ascorbates

Meat products treated with ascorbic acid and sodium ascorbate have inhibited lipid oxidation and preserved desirable meat flavours (Rhee et al., 1997). Kanner et al., (1987) observed that ascorbic acid at low concentration (up to 10-3 M) reduced copper to its catalytically active form, stimulating lipid oxidation in a α-Carotene–linoleate model system.
3.2.8 Herbs and spices as sources of antioxidants

The anti oxidant activity of spices has been known for more than 50 years. (Chipault et al., 1952). They evaluated the anti oxidant properties of 72 kinds of spices, their petroleum ether extract and found 32 spices to retard the oxidation of lard. In their study, the anti oxidant activities of ground sage and rosemary were particularly strong, and oregano, thyme, turmeric, and nutmeg possessed relatively strong antioxidant activities.

The most active substances are produced from rosemary (Rosmarinus officinalis. L.) Chang et al., 1977) and from Sage (Salvia officianalis. L.) Both of them contain carnosol and carnosic acid as active constituents. Naturally occurring compounds in rosemary extracts have been reported to exhibit anti oxidant properties greater than BHA and equal to BHT (Wu et al., 1982). Addition of rosemary extract to simulated minced turkey meat has been shown to provide increased protection from oxidation during cooking. Three more antioxidant substances from rosemary, all phenol diterpene compounds, were also isolated and determined the structural formula for each. Houlihan et al., (1985) have isolated more compounds of rosemary possessing superior antioxidant property to BHA but less effective than BHT. Shelef et al., (1980) has studied the sensitivity of common food borne bacteria to the spices; sage, rosemary and all spice.

Bracco et al., (1981) has described the recovery of anti oxidants from spices and vegetable material. The effect of the essential oil of cinnamon and cloves and their primary constituents, cinnamic acid and eugenol respectively on mold growth and of aflatoxin production has also been studied (Gourama et al., 1995; Rajkumar and Berwal, 2003).

Spices are widely used in a variety of food products. Many spices including cloves, cinnamons, Black pepper turmeric ginger, garlic and onions exhibit anti oxidative activities in a variety of food system (Al – Jalay et al., 1987).

Several investigators in Japan reported that ginger and ginger extract added to lard or other food showed reasonably strong anti oxidant activity (Saito et al., 1976). Lee and Ahn, (1985) investigated the properties; of antioxidant substances in ginger rhizome and their effectiveness as a source of antioxidants in fresh, frozen or pre cooked patties. Ramanathan and Das, (1993) has studied the effectiveness of spices and other related natural products as anti oxidants when present in salted cooked fish.
Ahn et al., (2002) have investigated the comparative antioxidant activity of a grape seed extract and a pine bark extract in cooked ground beef. Yu et al., (2002) conducted studies to determine the potential benefits of water soluble rosemary extracts on stability and quality of cooked turkey products.

Sanchez et al., (2003) conducted studies to determine if natural antioxidant rosemary, oregano and borage alone or in combination with vitamin C, were effective in delaying lipid and myoglobin formation in pork meat, thereby stabilizing meat colour.

3.2.9. Nutritional and health benefits of synthetic antioxidants

Antioxidants are defined by the United states (U.S.) Food and Drug Administration (FDA) as substances used to preserve food by retarding deterioration rancidity or discolouration due to oxidation, (2) code of Federal Regulations (CFR) 170-3 (0) (3).

Stuckey, (1972) has attributed the inhibitory effect of these anti oxidants, to their donation of electrons or hydrogen to fat containing a free radical, and to the formation of a complex between the antioxidant and the fatty acid. Most of the above studies pertain to meat and meat products while, the effect of spices on the storage stability of fatty fish during frozen storage is very limited.

3.3 Materials and methods

3.3.1 Raw Materials

3.3.1.1 Fish

Fresh mackerel was procured from Munambam Harbour, Kochi. The size of fish was around 13-14 cm with 6 – 7 numbers/kg. The fish was washed, iced and transported to the freezing plant of Matsyafed, Govt. of Kerala, Kochi, where the initial treatment, freezing and frozen storage were carried out.

3.3.1.2 Spice oleoresins and synthetic antioxidant

Four different spice oleoresins viz, Rosemary, Ginger, Pepper and Clove were used for the study. These were obtained from M/s Synthite Chemicals, Kolenchery and Butylated Hydroxy Anisole (BHA) was used as the synthetic antioxidant. Solutions of oleoresins of the spices and BHA were prepared in two concentrations of 0.02% and 0.05%.
Plate 2.5 Oleoresin Samples of spices
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3.3.1.3 Polythene tubes and master cartons

Polythene tubes of 200 gauge were used to pack the treated samples meant for frozen storage studies. The fish samples after treatment were stored in 5 ply master cartons after proper labeling.

3.3.2 Sample Preparation

The procured fish was divided into three lots, depending on the method of study.

A. Whole fish for iced storage studies

B. Whole fish for frozen storage studies

C. Fish fillets for frozen storage studies.

A. Whole fish for iced storage studies

Fish was dip treated with four spice extracts and BHA of 0.02% concentration for 20 minutes. A control sample was also taken without any spice extract or synthetic antioxidant treatment. Throughout the preparation and dip treatment, the temperature was maintained at 5°C. The dipped samples were individually packed in polythene tubes and stored in layers of ice for study. There was no direct contact of the fish surface with ice. Each set of treated fish were kept in ice on separate trays. The trays were kept in a chill room at 5°C. From this sampling was done every alternate days for a period of 10 days.

B. Whole fish for Frozen Storage Study

Whole fish for frozen storage studies were categorized into two lots. One for dip treatment and the other for glaze treatment.

i) Dip treatment

Samples of fish were dip treated at two different concentrations, 0.02% and 0.05% for 20 minutes with each spice oleoresin as well as BHA along with two control samples. The samples were frozen in a tunnel freezer at -40°C for four hours. The frozen samples were packed in polythene tubes individually, and stored in master cartons. The product was stored in a cold store at temperature -20°C. Sampling was done immediately (0 month) and further at regular intervals of two months for a period of nine months.

For glaze treatment, the fish was subjected to freezing in a tunnel freezer, and on the subsequent day, the frozen samples were subjected to uniform application of glaze. For this the frozen samples were dipped in two different concentrations (0.02% and 0.05%) of treatment solutions. The samples dipped in 0.02% concentration were given two coatings of glaze for a period of 5 seconds each. The samples were frozen in between glazing. The samples dipped in 0.05% concentration were given a single dip for 2 seconds only.

C. Fish fillets for frozen storage studies

Fresh mackerel was filleted to skin on fillets. The fillets were subjected to dip treatment with 0.005% spice extracts, following the method of treatment as mentioned in the whole fish study, but the time of dip was 10 minutes. The controls along with the treated samples were frozen in a tunnel freezer at -40°C for four hours. The frozen fillets were wrapped and sealed in polythene tubes, packed in master cartons and stored in the cold store at -20°C. Samples were drawn at zero time and at regular intervals of two months for analyzing various parameters of lipid oxidation.

In all the above sampling methods triplicates were taken at every stage.

3.3.3 Analysis of Biochemical parameters

3.3.3.1 Proximate Composition

The proximate composition of the tissue viz: moisture, protein, fat and ash of mackerel was analysed according to AOAC, (1995).

3.3.3.2. pH

10g of sample after the treatment along with control was blended with 90ml of distilled water and the pH of the resultant suspension was measured (AOAC, 1995). A digital pH meter (Cyber Scan pH-500 MERCK) was calibrated with standard buffers of pH 4.0, pH 7.0 and pH 9.0 (SIGMA).

3.3.3.3 Peroxide Value

Peroxide value was determined by the method of AOCS, (1999). 10 g of treated fish muscle was ground well with anhydrous sodium sulphate to remove moisture. It was then transferred to an iodine flask, and extracted with small quantities of chloroform, filtered and made up to 100ml.
10ml of the extract was taken in a pre weighed petridish and evaporated to dryness. The weight of the petridish was taken again, to determine the weight of the residue in the petridish. Another 10ml of the extract was pipetted into a dry iodine flask. About 20ml of glacial acetic acid and 1ml of saturated potassium iodide solution was added. The mixture was kept in the dark for 15min. The liberated iodine was diluted and titrated against N/100 sodium thiosulphate solution, using 1% starch as indicator.

Calculation

\[
\text{Peroxide Value} = \frac{\text{Volume of } Na_2S_2O_3 \times \text{Normaility of } Na_2S_2O_3 \times 1000}{\text{(milleq/Kg of fat)}} \times \text{Weight of sample in 10ml extract.}
\]

3.3.3.4 Thio barbituric acid value (TBA)

Thio barbituric acid reactive substances (TBARS) was performed as described by Tarladigs et al., (1960). 10g of treated sample was mixed with 50 ml distilled water in a waring blender for 2 min. The mixture was transferred quantitatively into a Kjeldahl flask by washing with an additional 47.5 ml of distilled water. 2.5ml of HCl solution was added to maintain pH to 1.5. A few saddle stones were added to prevent bumping. A small amount of Dow antifoam A was placed on to the lower neck of the flask to prevent foaming. Assembled the apparatus and heated the flask at the highest obtainable heat, on the kjeldahl distillation apparatus. 50ml of distillate was collected, from the moment the boiling begins. The distillate was mixed, and 5ml was pipetted into a glass stoppered tube, with 5ml of TBA (Thio barbituric acid) reagent. The tubes were stoppered, the content were mixed and immersed in a boiling water bath for 35 minutes. A distilled water TBA reagent blank was also prepared and treated like the samples. After heating, the tubes were cooled in running tap water for 10min. A portion of it was transferred to the cuvette, and the optical density of the sample against the blank was read in a UV/VIS spectrophotometer (HITACHI, U 2800) at 578nm. The reading was multiplied by 7.8 to convert to mg of malonaldehyde per 1000g tissue.

3.3.3.5 Heme iron

Heme iron was determined using the method of Hornsey, (1956). 2g of the samples were transferred into a 50ml poly propylene tube and 9ml of acid acetone (90% acetone + 8% deionised water +
2% HCl) was added. The meat was macerated with a glass rod and allowed to stand for 1 hour in a dark cabinet at room temperature. The extract was filtered with Whatman filter paper No: 42 and the absorbance (A) was read at 640nm against the acid acetone blank.

Total pigments, as acid hematin, were calculated using the formula: Total pigment (ppm) = A x 680 and heme iron was calculated as follows (Clark et al., 1997).

\[
\text{Heme iron (ppm)} = \frac{\text{Total pigments (ppm)} \times 8.82}{100}
\]

**3.3.3.6 Met Myoglobin (MMb)**

Met myoglobin content was determined by the method of Lopez et al., (2003) 5 g of treated minced tissue was used to determine MMb concentration in each sample. Myoglobin was extracted with cold 0.04M phosphate buffer in the ratio of 1:10 (tissue: buffer). Samples were homogenized for 15 seconds in a Yorco Tissue Homogenizer, at 10,800 rpm. The homogenates were then centrifuged in a refrigerated centrifuge for 30min at 5°C (50,000g). The absorbance of the supernatant was read at 525, 572 and 730nm. Percentage of MMb was determined using the formula of Krzywicki (1979).

\[
\text{MMb (\%)} = \frac{1.395 - (A_{572} - A_{730})}{A_{525} - A_{730}} \times 100.
\]

Samples were kept in ice at all points of assay.

**3.3.4. Statistical Analysis**

The experimental design was a randomized block design of 5 rows for five treatments and 2 columns for the 2 storage periods. Analysis of variance (ANOVA) was carried out using the generalized linear model procedure. The difference of means between pairs was resolved by using the least significant difference. The level significance was set at p<0.01 and p< 0.05 (Snedecor and Cochran, 1989)

**3.4 Results**

The proximate composition of the tissue of mackerel on analysis gave the following values in percentage:

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>71.19 ± .2</td>
</tr>
<tr>
<td>Protein</td>
<td>21.21 ± .4</td>
</tr>
<tr>
<td>Fat</td>
<td>7.51 ± .1</td>
</tr>
<tr>
<td>Ash</td>
<td>1.33 ± .3</td>
</tr>
</tbody>
</table>
Table 3.1 shows the variation of pH for the frozen samples of mackerel fillets dip treated with 0.005% spice extracts and there was no significant difference between periods as well as between treatments (Appendix 3.1).

Table 3.1 pH of Mackerel fillets given 0.005% dip and stored at -18°C

<table>
<thead>
<tr>
<th>Period</th>
<th>1 month</th>
<th>4 months</th>
<th>5 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM</td>
<td>6.697</td>
<td>6.663</td>
<td>6.167</td>
<td>6.450</td>
</tr>
<tr>
<td>PEP</td>
<td>6.903</td>
<td>6.763</td>
<td>6.662</td>
<td>6.387</td>
</tr>
<tr>
<td>CLO</td>
<td>6.900</td>
<td>6.907</td>
<td>6.270</td>
<td>6.313</td>
</tr>
<tr>
<td>SYN</td>
<td>6.920</td>
<td>6.433</td>
<td>6.433</td>
<td>6.423</td>
</tr>
</tbody>
</table>

3.4.1 Peroxide value

Fig 3.1 shows the changes in peroxide value of treated and chill stored samples given dip treatment at 0.02% concentration. There is significant difference between treatments (p<.05) [Appendix 3.2(a)]. In the ANOVA for comparison between samples of control and rosemary, the difference is significant (p<.05) [Appendix 3.2(b)]. Rosemary and clove treated samples shows a lower peroxide value at end of 10 days storage in chilled condition. For samples in chilled storage there is significance between samples stored between 10 days and 8 days period. Significant difference is shown between treatments (p<.05) [Appendix 3.2(c)].
3.4.2 Thiobarbituric acid reactive substances (TBARS)

Fig 3.2 shows the variation obtained for glazed mackerel (0.02 %) stored at -18°C. ANOVA shows significant difference between treatments (p<.05) during the period of 11 months storage, clove and rosemary treated samples shows lower values [Appendix 3.3 (a)].

![Figure 3.2 TBARS in glazed whole mackerel at 0.02%](image)

TBA measured for glazed mackerel samples at 0.02% concentration and stored at -18°C shows significant difference between treatments (p<.05). Clove and Rosemary treated sample show significantly lower values for TBARS [Appendix 3.3(a)].
Fig 3.3 shows the variation of TBARS for the sample of glazed mackerel at 0.05% concentration and stored at −18°C, shows significant difference between treatments and storage time (p<.05). 11 months stored samples shows significantly higher values than 7 months. Control samples showed significantly high TBARS value compared to the treatment samples [Appendix 3.3 (b)].
Fig 3.4 shows the variation in TBARS obtained from dip treated mackerel (0.02%) stored in ice. There is significant difference between treatments (p<.05). The values of clove and rosemary are significantly lower than control [Appendix 3.3 (c)].

![Figure 3.4 TBARS of dip treated (0.02%) whole mackerel at chilled storage](image)

TBARS value for fish fillets dip at 0.005% concentration for 20 minutes (Fig 3.5) shows significant difference between storage period (p<.05), there is also significant difference between treatments (p<.05). Clove and rosemary showed significantly lower values and control showed significantly higher values [Appendix 3.3(d)]. 9 months samples shows significantly higher values than 8 months and results show that pepper is not an effective anti oxidant [Appendix 3.3 (e)]. Appendix 3.3 (f) shows the TBARS values for fish fillets given a dip treatment 0.005% concentration there is significance between 9 months and 8 months storage time. Control shows higher value than BHA treated samples.

![Figure 3.5 TBARS of frozen dip treated mackerel fillets(0.005%) stored at -18 °C](image)
Table 3.2 Heme Iron content of treated (0.02 % dip) and stored at -18°C

<table>
<thead>
<tr>
<th>Period</th>
<th>0</th>
<th>1 month</th>
<th>3 months</th>
<th>5 months</th>
<th>7 months</th>
<th>9 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT</td>
<td>6.830</td>
<td>4.900</td>
<td>3.927</td>
<td>2.850</td>
<td>2.490</td>
<td>2.669</td>
</tr>
<tr>
<td>RM</td>
<td>7.010</td>
<td>5.750</td>
<td>5.030</td>
<td>5.010</td>
<td>5.297</td>
<td>4.783</td>
</tr>
<tr>
<td>GIN</td>
<td>6.230</td>
<td>4.073</td>
<td>3.957</td>
<td>3.353</td>
<td>2.897</td>
<td>2.293</td>
</tr>
<tr>
<td>PEP</td>
<td>6.700</td>
<td>3.937</td>
<td>3.943</td>
<td>3.423</td>
<td>3.143</td>
<td>2.783</td>
</tr>
<tr>
<td>CLO</td>
<td>6.800</td>
<td>5.963</td>
<td>5.093</td>
<td>4.327</td>
<td>4.110</td>
<td>4.377</td>
</tr>
<tr>
<td>SYN</td>
<td>6.750</td>
<td>3.557</td>
<td>3.217</td>
<td>1.970</td>
<td>1.003</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Table 3.2 shows the variation of Heme iron for dip treated samples 0.02 % of whole mackerel stored at -18°C. For samples subjected to whole dip treatment of 0.02% concentration for 20 minutes there is significance difference between samples of 1 month and 9 month storage (p<.01) [Appendix 3.4(a)]. One month storage sample gives significantly higher value than 9 month storage sample and there is significant difference between treatments (p<.01). Clove and rosemary gives significantly higher value and BHA treatment gives significantly lower values. Control at one month storage gives significantly higher values than 9 months storage samples (p<.05) [Appendix 3.4(b)].

Table 3.3 Heme iron content of treated (0.02% glaze) and stored at -18°C

<table>
<thead>
<tr>
<th>Period</th>
<th>0</th>
<th>1 month</th>
<th>3 months</th>
<th>5 months</th>
<th>7 months</th>
<th>9 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIN</td>
<td>6.150</td>
<td>6.092</td>
<td>7.544</td>
<td>5.679</td>
<td>4.257</td>
<td>4.952</td>
</tr>
<tr>
<td>PEP</td>
<td>8.170</td>
<td>8.070</td>
<td>5.325</td>
<td>5.461</td>
<td>4.678</td>
<td>4.240</td>
</tr>
<tr>
<td>SYN</td>
<td>8.750</td>
<td>8.738</td>
<td>5.588</td>
<td>5.935</td>
<td>2.942</td>
<td>3.149</td>
</tr>
</tbody>
</table>

Table 3.3 shows the variance for heme iron content for glazed samples. For samples glazed with 0.02% concentration there is significant difference between treatments. Rosemary and clove show high average values while BHA shows significantly lower value. On comparison of control and rosemary treated samples, rosemary gives significantly higher value (p<.01) than control. Seven months storage showed significantly higher values than nine months storage [Appendix 3.5(a) and 3.5(b)].
3.4.4 Metmyoglobin

Table 3.4 Metmyoglobin content of glazed samples (0.05% dip) at -18°C

<table>
<thead>
<tr>
<th>Period</th>
<th>0</th>
<th>1 months</th>
<th>3 months</th>
<th>5 months</th>
<th>7 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT</td>
<td>0.723</td>
<td>0.905</td>
<td>0.929</td>
<td>0.921</td>
<td>0.985</td>
</tr>
<tr>
<td>RM</td>
<td>0.472</td>
<td>0.496</td>
<td>0.674</td>
<td>0.688</td>
<td>0.651</td>
</tr>
<tr>
<td>GIN</td>
<td>0.685</td>
<td>0.719</td>
<td>0.482</td>
<td>0.765</td>
<td>0.849</td>
</tr>
<tr>
<td>PEP</td>
<td>0.455</td>
<td>0.510</td>
<td>0.600</td>
<td>0.630</td>
<td>0.714</td>
</tr>
<tr>
<td>CLO</td>
<td>0.436</td>
<td>0.451</td>
<td>0.548</td>
<td>0.536</td>
<td>0.683</td>
</tr>
<tr>
<td>SYN</td>
<td>0.442</td>
<td>0.468</td>
<td>0.815</td>
<td>0.773</td>
<td>0.905</td>
</tr>
</tbody>
</table>

Table 3.4 shows the variation of metmyoglobin, in glaze treated samples stored at -18°C. For samples treated with 0.05% glaze, there is significant difference between five months and seven months storage (p<.05). Values of seven months are significantly higher than that in five months. There is also significant difference between treatments (p<.01). Clove and rosemary showed significantly lower values than BHA treated samples. In control versus ginger, control shows higher values than ginger (p<.05). On comparison, pepper treatments shows significantly lower value than control (p<.05) [Appendix 3.6]

3.4.5 Acid haematin pigment

![Figure 3.6 Acid hematin pigments of frozen mackerel(0.02% glaze) stored at -18°C](image_url)
Fig 3.6 shows the variation of total pigments as acid haematin in treated frozen samples. For samples glazed with 0.02% concentration there is significant difference in hematin pigment between storage periods. One month shows significantly higher values than nine months. There is significant difference between treatments ($p<.01$). Clove and rosemary show significantly higher values and synthetic gives very low value. In ANOVA on comparison of control and BHA there is significant difference between months. [Appendix 3.7]

3.5 Discussion

In the process of lipid deterioration, the fundamental reaction is generally accepted to be the process of autoxidation of the unsaturated fatty acids, which are abundant in fish lipids. The highly unsaturated fatty acids are very reactive. During frozen storage oxidative rancidity occurs which is caused by the reaction between oxygen and PUFA, e.g. Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), to form hydroperoxides that decompose to volatile aldehydes, ketones and acids responsible for rancid odour. The initial reaction between lipid and oxygen requires a catalyst. Once the reaction has started it becomes, self-propagating and difficult to control (Tall and Harris, 1995; Gopakumar, 2002).

Earlier studies indicated changes in moisture (%) of individually quick frozen (IQF) and block frozen (BF) mackerel (*Rastrelliger kanagurta*) of two batches containing fat of 4% and 11% respectively, stored at -23°C and attributed these changes to the difference in fat content (Nair et al., 1976). It was also observed that there was no significant change in moisture content during frozen storage, probably due to the effective protection of glaze and packing. Lipid hydrolysis is more when the fat content is less, and the production of FFA in mackerel is higher when it is lean and is lower when the fish has maximum lipid.

The peroxide values of samples treated with rosemary and clove show relatively lower value when compared to that of control. This shows that at concentration of 0.02% the spices play an active role as a strong antioxidant and between the two spices, rosemary gives better results when compared to that of control.
3.5.1 TBA values

Thiobarbituric acid reactive substances (TBARS) obtained from mackerel stored at -18°C shows that frozen samples glazed at 0.02% clove and rosemary have significantly lower values for TBARS when compared to control. For frozen whole fish samples glazed at 0.02%, clove and rosemary treated samples have given significantly lower values for TBA when compared to control. Early research (Chipault et al., 1956) has proven that rosemary had remarkable antioxidant effects in lard. In the present study clove showed extremely high antioxidant effect in the case of TBA while control shows significantly higher values compared to rest of treatments, confirming the well pronounced antioxidative effect of spices in treated fish. TBARS values for samples of chilled storage also showed remarkably low values for clove and rosemary. This again highlights the strong antioxidative properties of rosemary. A strong synergistic effect between rosemary extract (0.02 %) and tocopherol (0.05 %) in sardine oil at 30°C in frozen fish meat models has been reported (Wada and Fang, 1992). It has been established that molecule of carnosol and the radicals formed from them participate in the reactions of chain initiations and propagations to a much lower degree than the case with most natural and synthetic antioxidants (Marinova et al., 1991), thereby decreasing the rate of oxidation to a considerable extent. Houlihan et al., (1985) found rosmaridiphenol to be more active than BHA in lard and was equivalent to BHT. Rosemary oleoresin (RO) was superior to BHA and equivalent to BHT. The present result on TBARS values analysed for various chilled and frozen stored products of mackerel supports the view of the antioxidative principles of rosemary oleoresin. Carnosic acid and carnosol showed the ability to chelate the iron and were effective radical scavengers of peroxide radicals (Aruoma et al., 1992). Barbut et al., (1985) studied the effectiveness of rosemary oleoresin (RO) in turkey breakfast sausages. RO was effective as a combination of BHA and BHT with citric acid in suppressing oxidative activity.

Studies show that at the end of 5 months storage, the sample stored at -10°C had 15 times more TBARS value than that stored at -30°C. TBARS value started to increase from the initial stationary phase after 6 months storage in samples stored at -20°C and after 9 months in samples stored at -30°C (Chinnamma et al., 1995). Frozen mackerel stored at -30°C had a shelf life of more than 9 months provided the material is glazed and packed in moisture proof packaging material. Formation of ice glaze on the surface of a myosystem effectively limits the entrance of oxygen and thereby retards autoxidation. Antioxidants provide additional protection against lipid oxidation. Bremner
and Gerald, (1998) showed that oxidation occurs within one month in freshly caught pilchards (Sardinops neopitchardus) when stored at temperature of -20°C. In blue whiting fillets during frozen storage (-10°C and -30°C for 1, 3, 5, 7, 9 and 12 months), lipid damage increased showing high value of free fatty acids, peroxide value and conjugated dienes. TBARS index and fluorescence detection showed a good positive correlation with storage time (Aubourg, 1999). Thiobarbituric acid value (TBARS), which is a good index of fat oxidation, was also found to increase in a number of species during frozen storage. In Nile perch (Lates niloticus) fillets stored at -13°C, TBARS value and off odor and flavour increased with storage time. Flavour, texture and overall acceptability scores were lower in the samples and decreased with storage time.

Peroxide value shows a general increase during frozen storage. A high positive correlation between rancidity scores of a taste panel and peroxide values was found in frozen fish (Fennema et al., 1973). Awad et al., (1969) observed that the peroxide values increase to a maximum and subsequently decline to a minimum when frozen fish were stored near -10°C. This indicates that they are decomposed faster than they are being formed.

### 3.5.2 Heme iron

There is a great range in the concentration of haematin components in muscles from different species of fish. These components are present in relatively large concentration in the muscles of fatty fish especially in the lateral band of dark muscles. The characteristic brownish colour of the fish muscles is due to hemoglobin and myoglobin. Fish with highest haematin content are those that are most susceptible to oxidative rancidity. In the lipid oxidation hydroxyl free radicals can be produced by the reaction of transition metals with the reduction products of molecular oxygen like superoxide and hydrogen peroxide. Iron is probably of primary importance in fish tissue. Due to the low solubility of Fe³⁺ at physiological pH almost all cellular iron is complexed. Heme pigments can be activated by hydrogen peroxide to a compound able to initiate lipid oxidation.

The role of metmyoglobin (MetMb) and nonheme iron in accelerating lipid oxidation in cooked meat studied using a model system, containing water extracted muscle residue shows that non heme iron acts as prooxidant in cooked meat, while MetMb has little or no prooxidant activity. Heme compounds may act either as accelerator or inhibitors of lipid oxidation with their action depending on the ratio of the heme to unsaturated fatty acid. There are reports that haemoglobin
dissociation can promote lipid oxidation reactions in tilapia and that heme proteins, myoglobin and haemoglobin and various other derivates are responsible for development of off colours in canned tuna.

The rate and extent of lipid oxidation catalyzed by metmyoglobin in heat treatment was greatest in fish mainly due to level of poly unsaturated fatty acid present in the particular system (Mark et al., 2007). In the samples glazed (0.02 % concentration) there is significant difference between treatments. This probably may be due to the fact that in rosemary treated samples, the heme iron does not dissociate thereby preventing oxidation.

3.6 Conclusion

Freezing is one most effective ways of preservation of fish. The result of fat oxidation indices in the study shows that oxidation can be prevented to a considerable extent by the use of natural compounds. The use of rosemary oleoresin has shown to suppress the oxidative changes in fish muscles. This is well established by the results obtained from the TBARS values of frozen mackerel and products of mackerel stored at -18°C for a period of nine months. Clove also exhibits strong antioxidative effect. On the whole, from the results of various lipid oxidation parameters of samples given, different treatments with spice extracts in comparison with a synthetic antioxidant, BHA and control samples, the spices used could be graded as Rosemary > Clove > Ginger > Pepper, for their antioxidant properties. Among the various pretreatments given as glaze and dip of whole fish and frozen stored at -18°C, the glazed samples with 0.02% spice treatment is found to be most effective. Fillets dip treated with 0.005% concentration and frozen stored gave the optimum results. In this case also, the antioxidant properties was maximum for rosemary followed by clove, ginger, pepper and synthetic antioxidants as confirmed by the TBARS and Peroxide values.

The retention of heme iron, metmyoglobin and acid pigments were also maximum in rosemary treated samples followed by clove explaining the protective effect of the treatment in maintaining the colour and other textural profiles of the samples. The synthetic antioxidant treated samples on frozen storage gave low values of pigments compared to spice treated samples, showing a bleaching action and hence loss of sensory qualities. The fact that the active constituents of rosemary and clove can be made use of for effectively preventing oxidation, thereby maintaining the original characteristics of mackerel has been confirmed.