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Investigation on the Antioxidant Capabilities of Spice Extracts in Inhibition of Long Chain Polyunsaturated Fatty Acids Oxidation and Cholesterol

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INVESTIGATION ON THE ANTIOXIDANT CAPABILITIES OF SPICE EXTRACTS IN INHIBITION OF LONG CHAIN POLYUNSATURATED FATTY ACIDS OXIDATION AND CHOLESTEROL OXIDATION

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ABSTRACT

Antioxidant capabilities of the spice extract of ginger, oregano, rosemary and turmeric were evaluated by monitoring inhibition of DHA and EPA in a menhaden fish oil model and cholesterol oxidation model system. The spice extracts were mixed with the fish oil at 1, 2.5 and 5 % (w/w) and 1, 2.5 and 5mg (dw) were used in cholesterol study. The temperatures $150^{\circ}C$ and $175^{\circ}C$ were used for the oxidation study and a $60^{\circ}C$ was used for incubation storage. The methanol extracts of all four spices demonstrated significant capability in retaining the DHA and EPA, after subjected to oxidation when compared with the control. The oregano retained 66% and 41% of DHA for 5 % at 150 and 175 $^{\circ}C$ respectively. For the $60^{\circ}C$ storage study, at the end of the 6 day period, rosemary extract showed the highest retention of 84% DHA at 2.5%. For the cholesterol oxidation study, at $150^{\circ}C$, 2.5 mg extract retained 83, 81, 73 and 87 % cholesterol for ginger, oregano, rosemary and turmeric respectively. At $175^{\circ}C$, all the spice extracts were still significantly effective in retaining cholesterol as compared with the control and the antioxidant capabilities in decreasing order were oregano > rosemary > ginger > turmeric. For the antioxidant and total phenolic study, without heating, the antioxidant activity (TEAC) values ranged from 0.18 to 0.60 µmol of Trolox equivalent/gm of extract, with oregano the lowest and rosemary showing the highest activity. After heating, the antioxidant activity declined for all the spice tissues. During the $60^{\circ}C$ incubation storage, only rosemary demonstrated a 10 % increase in the antioxidant capability. The result for total phenolic content values in decreasing order were turmeric > rosemary > ginger > oregano. With heating at both 150 and $175^{\circ}C$, turmeric, rosemary and oregano showed no significant reduction in phenolics. The storage study showed a uniform decrease in the total phenolic content for all the spices and turmeric demonstrated highest

values. Thus the spice extracts of ginger, oregano, rosemary and turmeric demonstrated significant antioxidant capability by retaining DHA and EPA in menhaden fish oil and cholesterol during oxidative degradation.

CHAPTER 1. INTRODUCTION

Spices and herbs have been used in cooking since antiquity to enhance the palatability of foods; however, spices are also helpful in removing harmful microorganisms in food according to scientific research and they play an important role in preserving health (Luther 2006). **"**Spice" is a culinary term, not a botanical category and generally refers to dried parts of plants and "herbs" specifically relates to their fresh state. Herbs are known for their medicinal uses as well as for various food uses. Both spices and herbs have unique aroma and flavor characteristics that are derived from phytochemicals or secondary compounds in plants, often called their defense mechanisms against diseases, pathogens, fungi and parasites (Farrel 1999). The Food and Drug administration in the code of federal regulations for food labeling states that "spices are any aromatic vegetable substance in whole, broken or ground form whose major function is seasoning and from which no portion of any volatile oil or other flavoring principle has been removed"(Farrel 1999). Spices and herbs have been used in Mediterranean and Asian countries for centuries. Since ancient times, they have been known to impact flavor, preserve food, and recent research has established them as potent sources of natural antioxidants and antimicrobial agents. Parker et al (2003) reported that consumer awareness is an important factor in the food industry and consumers tend to buy food products that promote health benefits and disease prevention. Phenolic antioxidants derived from spices and herbs are abundant in flavanoids, polyphenolic compounds that are the result of the secondary metabolism in plants and are excellent sources of natural antioxidants in human diets.

Lipid oxidation in food can be beneficial in certain cases or can lead to detrimental effects in other cases. Ramarathnam et al (1995) reported that the peroxidation of lipids at

low levels are responsible for the desirable aroma of fried foods and some of the characteristic flavors, as in case of cooked meats, roasted nuts, and others. On the other hand, large scale degradation of lipids poses problems in the development of rancidity in processed foods, and can cause serious damage to the human body. The excess production of reactive oxygen species, particularly hydroxyl radicals, can easily initiate lipid oxidation in the cell membrane, resulting in the formation of lipid peroxides. Many researchers have stated that lipid peroxides are instrumental in the development of a variety of diseases, including cancer, heart related diseases and acceleration of aging (Yanishlieva and Marinova 2001, Ross and Smith 2005). Several secondary products of lipid oxidation, such as malondialdehyde and 4-hydroxynonenal, are very reactive; and they have been shown to react with biological components such as proteins, amino acids and DNA. Malondialdehyde has been shown to be formed both enzymatically and non-enzymatically, and has been implicated in health problems like mutagenesis and carcinogenesis (Madhavi et al 1996).

Lipid oxidation is the single most detrimental factor implicated in the degradation of lipids or oils in the food industry. Antioxidants can protect the lipids and oils in food against oxidative degradation. The antioxidants are defined as the substances that when present in food or in the body at low concentrations remarkably delay or inhibit the oxidation of the substrate. In foods, the antioxidants are endogenously present or may be added later to protect the product from oxidative degradation (Shahidi 1992, Ong and Packer 1992). Synthetic antioxidants like BHA, BHT and TBHQ are commonly used in the food industry to protect the foods from extensive lipid peroxidation. However, both consumer preference and toxicological investigations are focusing the interest on the research of natural plant antioxidants due to their excellent preservative, antioxidant and antimicrobial properties. Due

to the limitations of carcinogenicity of synthetic antioxidants, natural phenolic antioxidants are being widely explored and studied as alternatives (Chun et al 2004).

Cholesterol resembles a lipid; produced by liver and is useful in performing several biological functions of the body. Cholesterol is made up of a wax like substance that is also supplied by animal products such as meats, poultry, fish and dairy products. Cholesterol is used by the body to perform biological functions, to insulate nerves, make cell membranes and produce hormones, and it forms an important lipid in some membranes (plasma) (Ma 2006). The other lipids in the membranes are phospholipid and sphingolipid. Cholesterol in the food can undergo rapid autoxidation in the air to form several cholesterol oxidation products (COPs) (Li et al 1996). However, the other lipid molecules in food are the one who control the rate of cholesterol oxidation. The complex structured lipids, polyunsaturated fatty acids have been associated with an increase in the production of COP's (Li et al 1994, Nawar 1991). Just like PUFA's, the cholesterol in foods like fish oil is highly susceptible to form cholesterol oxidation products (COPs) when exposed to extreme conditions like heat, light and oxygen. COPs are as fatal as oxidized polyunsaturated fatty acids as the presence of COP's are responsible for various heart related disorders, certain types of cancers and other adverse health effects (Addis 1990, Jacobson 1987).

During the last 20 years, spices, as the most studied plants, have aroused great interest for the food industry. This fact is reflected from the number of publications on antioxidants and oxidative stress that have quadrupled in the past decade (1684 in 1993; 6510 in 2003) (Huang et al 2005). The interest in natural antioxidants from spices has arisen from their excellent antioxidant potential due to polyphenolic compounds. The natural antioxidant capabilities of these compounds are comparable to and in many cases exceed those of

synthetic antioxidants used in the food industry, particularly BHA (butylated hydroxyanisole) (Benkeblia 2005) and BHT (butylated hydroxytoluene) (Barlow, 1990).

The consensus of evidence over the last two decades suggests that the dietary intake of omega-3 (n-3) polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), is beneficial in various human disorders including cardiovascular diseases, inflammatory conditions and life threatening diseases (Shiota et al 1999). Fatty fish and fish oils are considered rich and valuable sources of polyunsaturated fatty acids like DHA and EPA (Yanishlieva and Marinova 2001). The importance of DHA is reflected in its efficiency in the normal neural development of the human fetus and premature infants. It is the reason why the DHA is incorporated in to infant foods as it contributes in the brain development, vision development (retina) and other health benfits. However, PUFAs in fish oil are very susceptible to peroxidation, even under mild ambient conditions. The PUFA content of fish oil has also been a factor in enhancing the production of COPs, as a continuous increase in the amount of cholesterol oxides is often accompanied by a remarkable decrease in PUFA residues (Kubow 1993). Spices and herbs rich in phenolic antioxidants can play a major role in reducing oxidative degeneration especially from lipid peroxidation and further development of off-flavor and rancidity in foods (Shahidi 1997, Lee and Shibamoto 2002, Yanishlieva and Marinova 2001). The phenolic antioxidant compounds that inhibit unsaturated fatty acid oxidation could reasonably also be expected to inhibit cholesterol oxidation. Natural antioxidants from spices and herbs include flavanoids, phenolic acids, and tocopherols that can effectively counter the oxidation of PUFAs in the fish oil and could eventually help in retarding cholesterol oxidation. This protection can be explained by the capacity of

antioxidants to scavenge free radicals that are mainly responsible for the oxidative damage of lipids, proteins and nucleic acids in food systems. Antioxidants from the spices have been proven to reduce the risk of various health disorders by lowering the oxidative stress caused by the free radicals (Shahidi 1997). Researchers, initially paid attention only to natural compounds, e.g. vitamin C, vitamin E and carotenoids, and only during recent years has the powerful antioxidant capacity of other plant phenolics aroused greater interests (Shahidi et al 1992). Thus, there is a current need to investigate the usefulness of phenolics rich natural food ingredients like spices as antioxidants that will also contribute to the general well being and improve the health-related functional value of food.

Various researchers have investigated the stabilizing effect of selected spices that play a key role during the autoxidation of lipids and breaking the reaction and further degradation of oils (Yanishlieva and Marinova 1997). Compounds like flavanoids, terpenoids, lignans and polyphenolics in these spices are responsible for their excellent antioxidant characteristics (Craig 1999). The preservation of plant parts, which are used as spices are often achieved by direct exposure to heat and often involves extended storage. The preservation process may also include sanitation that can also consist of several heating steps (Harnik et al 2004). It is therefore equally important to look at the effect of heat and storage on the changes in the phenolic antioxidants in the spices.

The phenolics from the spices can be explored for their potential to prevent lipid peroxidation in highly unstable fish oil. The effectiveness of antioxidants in retarding autoxidation in fish oils is a relatively less researched topic when compared with the wide range of reports on the properties of natural antioxidant such as spices in vegetable oils, lard and tallow. We therefore need to investigate thoroughly to clarify the antioxidative effects of

the spices in fish oil, which is highly susceptible to rapid degradation even at room temperature. The information on the use of phenolic antioxidants in spices can provide vital clues to tackle the problem of stability in fish oils. A few researchers in the past have investigated the use of spices from the *Labiatae* family (rosemary and oregano) and the *Gingerbacie* family (ginger and turmeric) in food systems. However, it is very hard to find research on the use of these particular spices, i.e., ginger, rosemary, turmeric and oregano, in a complex model like fish oil. A research study conducted by Sullivan et al (2005) investigated the use of naturally occurring antioxidants on fish oil systems extracted from livers of white pollack or cod. The study showed that the cod liver oil samples containing rosemary or tea catechins had lower levels of lipid oxidation. However, this and other studies were inconclusive regarding information on the oxidation of fish oil at higher temperatures resembling real time cooking or processing and storage conditions. Comprehensive research is needed to confirm the use of these spices in fish oil at accelerated heating and storage conditions to study their usefulness in maintaining the stability of fish oil. Although studies on the antioxidant phenolic constituents in numerous spices have been conducted, the composition data for the spices ginger, oregano, rosemary and turmeric is scarce and insufficient. Also, the wide variety of oxidation systems in the studies and ways to evaluate antioxidant activities make the studies inadequate and inconclusive. So we have selected the complex food system such as menhaden fish oil as a model for studying the antioxidant capability. The phenolics from spice extracts, if successful can help solve stability problems in highly unstable fish oil. Similarly we also used a cholesterol oxidation model to study the effect of phenolics in spices in retaining cholesterol during oxidation.

Frankel (2001) reported that it is necessary to understand the steps in oxidation including the free radical formation in order to simulate the mechanisms of lipid oxidation. The mechanism of protection of lipids from oxidation can be predicted and explained by free radical scavenging ability of the antioxidants. Previous scientists have recommended utilizing multidimensional methods in order to study antioxidant mechanism of spices in addition to the oxidation of a lipid substrate (Frankel et al 2001, Decker et al 2005). In our study, we have evaluated the antioxidant activity of phenolics in spices by the DPPH method (free radical scavenging) and total phenolic content by the Folin Ciocalteu method. This study may explain the mode and action of phenolic antioxidants in a lipid based medium. Further, spice plants, during the preservation are often subjected to several cooking and pre-processing steps involving high temperatures and long-term storage conditions. It is therefore important to evaluate the antioxidant activity of the polyphenolics in the spices under these conditions. In the past, the effects of heating and storage on antioxidant potential of the spices have not been explored. We have also attempted to study the changes in phenolic compound profile in the spices when subjected to heating and storage conditions.

In this research study, we have used the term spices for ginger, oregano, rosemary and turmeric, even though different terms such as herbs (for rosemary and oregano) and rhizomes (for ginger and turmeric) were also found in the literature about these plants.

CHAPTER 2. LITERATURE REVIEW

2.1 Spices and Herbs

Culinary herbs and spices have been grown and used for hundreds of years, and they are gaining popularity in the United States for their ability to enhance and complement the flavors of a wide variety of foods. According to Shan et al (2005) the term *herbs and spices* includes not only herbaceous plants but also bark; roots; leaves; seeds; flowers and fruit of trees, shrubs, and woody vines; and their extracts are valued for their savory and aromatic qualities. Spices used for cooking, or culinary spices, are commonly used as food adjuncts, used for flavoring, seasoning, coloring agents and even as preservatives. In most of the spices, flavor is provided by the aromatic ingredients present in their essential oils and oleoresins (Shan et al 2005). Spices have found extensive applications in fields like nutrition, beverages, repellents, fragrances, cosmetics, charms, smoking, and in other industrial uses (Zheng and Wang 2001). Since prehistoric times, spices were also used as traditional medicine in various Asian countries like China, India, Malaysia and Indonesia and their pharmacological effects have been extensively studied. Until the nineteenth century, spices formed an important constituent in all the traditional medicinal therapy. Even today, herbs have found their way in as much as 40% of prescription drugs (Hinneburg et al 2005). Widespread use of natural antioxidants from spices (i.e. tocopherols, ascorbic acid, and flavonoids) has captured the interest of scientists and researchers from medical and pharmaceutical industries because of their digestive stimulant property, and their anti-inflammatory and anti-carcinogenic activities (Berch et al, 2001). The phenolic compounds and flavonoids in spice plants are responsible for the different colors present in flowers, fruits, and leaves (Lee and Shibamoto 2002).
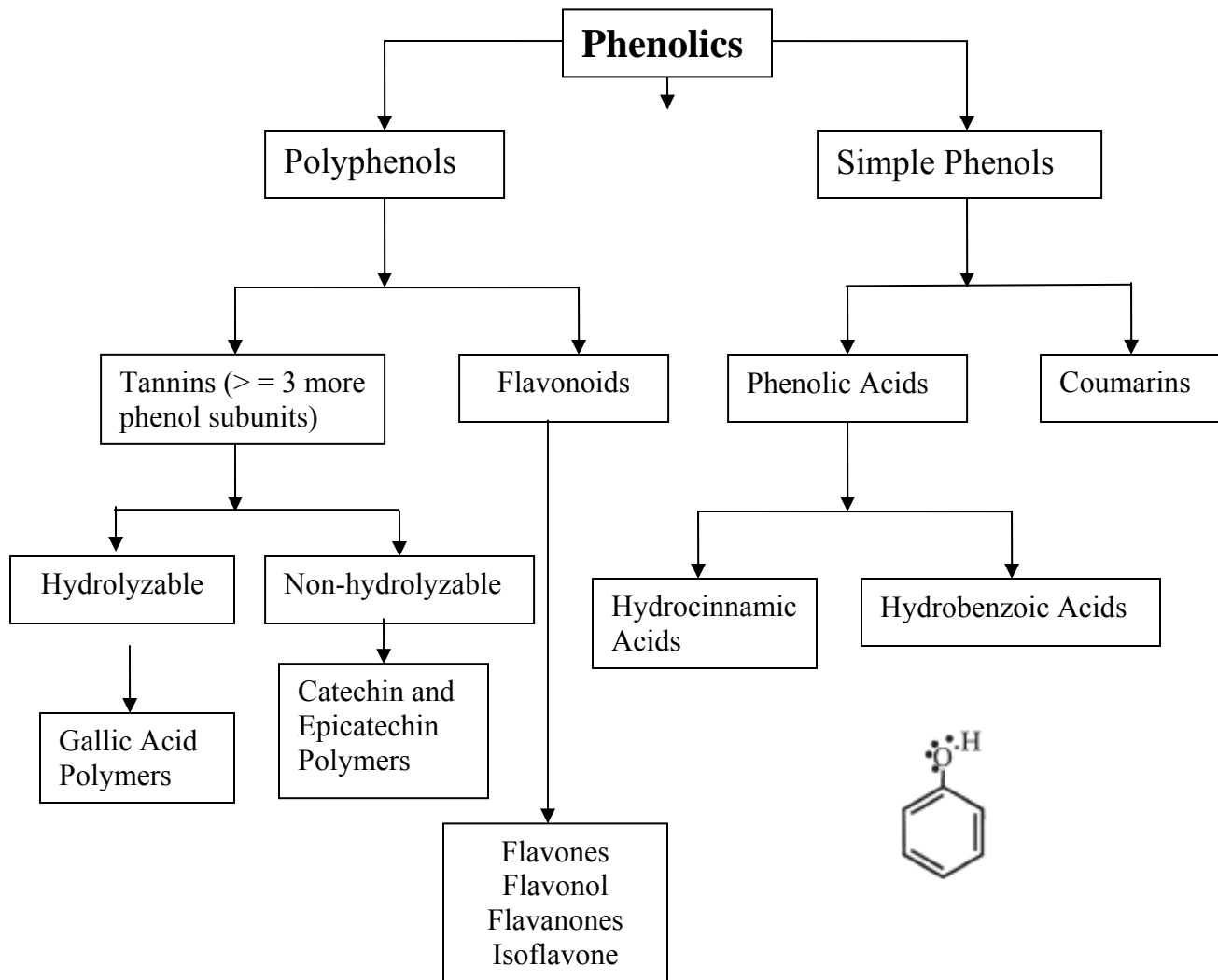
Figure 1: Classification of Phenolic Compounds (Luthria 2006)

Spices are an excellent source of phenolics that are responsible for the antioxidant activity, antibacterial and preservative property (Rice-Evans et al 1996; Zheng & Wang 2001). Kahkonen et al (2003) reported that phenolic compounds such as phenolic acids, stilbenes, tannins, lignans, and lignin, are commonly found in leaves, tissues, stems and barks of the plants. Plant phenols present in herbs and spices are broadly classified into vitamins, phenolic compounds (flavonoids and phenolic acids) and volatile compounds (Lee and Shibamoto 2002). The second classes of phenolics known for their antibacterial and preservative characteristics are flavonoids. Flavonoids are a sub-category of the plant

phenolics (figure 1), with widely found constituents represented by the structural element characterized by an aromatic ring bearing one or more hydroxyl groups; whereas, the phenolic acids family is usually comprised of cinnamic (C6-C3) and benzoic (C6-C1) acid derivatives (figure 1) that are characterized by the presence of a benzene ring substituted with one or more hydroxyl/methoxy groups and one carboxylic group. Flavanoids are compounds that are present in plants such as cinnamic acid derivatives such as chlorogenic acid, flavones, flavonols, flavanones, and isomers (figure 2) of flavones known as isoflavones (Liu 2004). Polyphenolics or flavonoids are universally present throughout all the plants, and in excess of 4000 of polyphenolic compounds have been identified (Harborne 1988). In the year 1994 alone, there were almost 1000 citations in scientific abstracts with the heading of flavonoids and polyphenols. Flavonoids and polyphenolics are a vital component in the plant defense system against infection and injuries and are also important in normal growth and in the development stages of plant life (Maria Cruz et al 2005). Many of these phenols and flavanoids have been found to be more powerful antioxidants than vitamins C, E, and *β*-carotene, and to possess radical scavenging activities, especially in protecting lipids as well as the lower density lipoproteins from oxidation (Vinson et al., 1998). The *in vivo* and *in vitro* mechanism for the protective effect of these phenol antioxidants has been recently reviewed and studied extensively (Vinson et al 1998). Spice vegetables, which are comprised of these polyphenolics, consumption is supposed to provide protection against oxidative stress, the mechanism mainly responsible for both carcinogenesis and atherosclerosis (Rababah et al 2004).

Herbs and spices have been used for a wide range of purposes in different types of foods due to their property of enhancement of organoleptic characteristics (Rababah et al

2004). Due to the combination of medicinal and nutritional (flavoring and seasoning) value of the spices (Shan et al 2005), they are gaining popularity as functional foods.
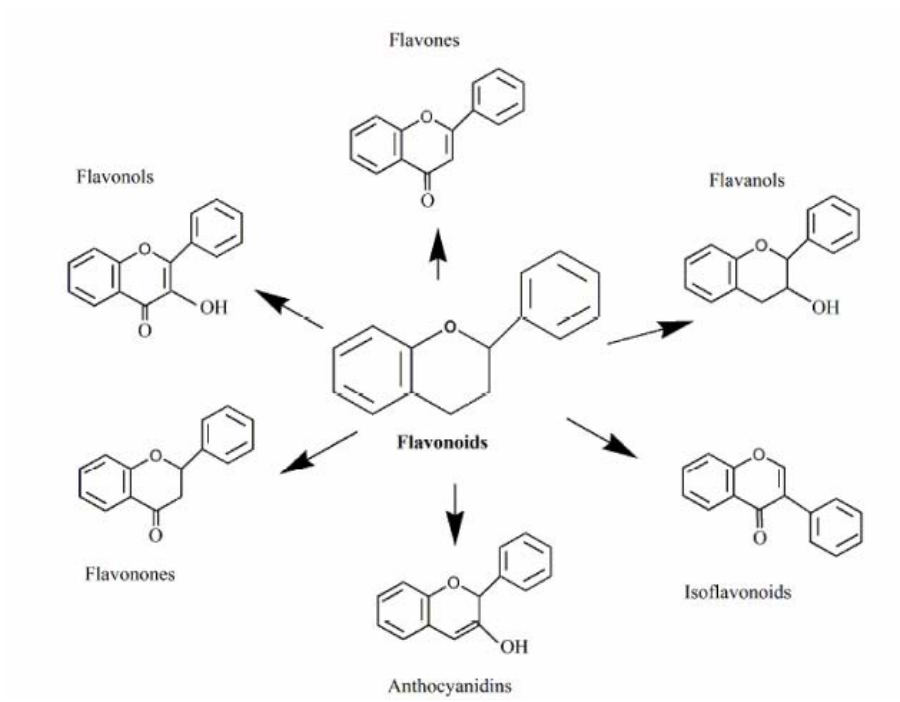
Figure 2: Structural Representation of Different Flavanoids (Liu 2004)

Several naturally extracted compounds from spice plants have demonstrated biological activities. Among these various kinds of natural substances, essential oils from aromatic and medicinal spice plants have received particular attention. The use of culinary spices (natural antioxidants) has considerably increased in foods as well as for uses in the medicines and is fast replacing the synthetic antioxidants (Hettiarachchy et al 1996).

A study by Kahnau (1976) confirmed that the average daily intake of flavonoids in the United States in 1971 was estimated to be 1 g/day of quercitrin, inclusive of all individual compounds found in foods. Phenolic compounds found in plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby may assist in retaining the quality and nutritional value of food. Kikuzaki

and Nakatani (1993) have noted that antioxidants are used as a means of reducing rancidity of fats and oils in various food systems. The antioxidant and antimicrobial benefits of spices and herbs are very useful for food preservation; apart from the fact that antioxidants also prevent damage caused from reactive oxygen species to tissues. The effectiveness and usefulness of natural antioxidants can be explained by their capacity to scavenge free radicals that are responsible for initiating the oxidation reaction in lipids, proteins, and nucleic acids (Odukoya et al 2005). Antioxidants possess the property of quenching free radicals especially those causing oxidation reactions in fatty-acid side-chains. Shan et al (2005) reported that the antioxidant effect of phenolic compounds is due to their redox properties and is the combined result of various possible mechanisms such as free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity. In addition, the natural antioxidants derived from plants possess other biological functions, like chelating, anti-inflammatory, antiallergic, antimicrobial, antiviral, anticarcinogenic and other preservative properties. The information on the total phenolics and antioxidant activities of plant extracts can be effectively used to study the inhibition of lipid oxidation in a variety of food products (Birch et al 2001 and Rababah et al 2004). Oxidative damage is a collaborative factor in causing cardiovascular diseases, cancers, neurological disorders, arthritis and other aging related denegenerative diseases. For this reason, there has been increasing interest in identifying plant phenolics and flavanoids and their use as antimicrobials, preservatives and antioxidants in foods, especially lipid-based food products (Rao 2003). Among plants, particularly spices are of special interest due to their spasmolytic, carminative, hepatoprotective, antiviral, and anticarcinogenic properties (Mimica-Dukic et al 2004).

The importance of the natural antioxidant phenolics extracted from plant materials are gaining importance as consumers are getting increasingly conscious of the use of synthetic antioxidants due to various health concerns. Although synthetic antioxidants, (e.g., BHA, BHT and TBHQ) are effective, they pose problems due to possible toxicity, carcinogenic effects and limitations like high volatility and instability at elevated temperature, and their property to impart an unpleasant flavor to the food in which it is used (Madhavi et al 1996, Dapkevicius et al 1998). The possibility of adverse effects by the use of synthetic antioxidants is increasing as it is with others synthetic food additives. Due to the health awareness among consumers about the possible ill effects of synthetic antioxidants, they are willing to pay high prices for antioxidants derived from natural products (Huang et al 2005). Consequently, increased consumer awareness stresses a strong need for effective antioxidants from natural sources as alternatives to prevent deterioration of foods and they may be safer to humans. Sources of natural antioxidants like spices and herbs, and such materials have been used throughout history not only for flavoring but also for preservative properties. Vegetable plants like spices not only have the extensive presence of flavonoids, phenolic acids, and tocopherols but the consumption of foodstuffs rich in these antioxidants can also provide effective remedy to prevent cancer and cardio- and cerebrovascular diseases (Vinson et al 2005, Shan et al 2005, Zheng and Wang 2001). Also, all the natural antioxidants like from spices are derived with resources that may not be depleted when grown in a minimal input environment. Spice plants like green tea extracts are comprised of phenolics, flavonoids such as catechin and epicatechin, which mainly contribute to the antioxidant activities (Velioglu et al 1998). The phenolic acids are classified as natural hydrophilic antioxidants that are prominently found in the plants such as vegetables, fruits, spices, and herbs (Maria Cruz et al

2005). They are of particular interest because of their potential biological properties, such as antioxidant, free radical scavenging and preservative properties. Due to the advantage of plant extracts over synthetic antioxidants and their effective role in delaying/retarding lipid oxidation, there has been a growing demand to elaborate the use of natural plant extracts as antioxidants. Various different sources of phenols and phenolic derivatives are available naturally and abundantly throughout the plant kingdom (Maria Cruz et al 2005). Previous reports have confirmed the efficiency of antioxidant spices or their constituents *in vivo*. Some researchers have reported the antioxidant capacity of the spices like ginger in relation to the LDL cholesterol oxidation (implicated in coronary heart disease) in a study conducted on apolipoprotein deficient mice (Lampe 2006). Only more recently, the scientists have begun to systematically study the health benefits from herbs and spices. The data from this present research study may lead to novel natural food preservatives and help to enhance the quality, safety, and nutritional value of food products.

2.2 Lipid Oxidation in Food

Lipids are fatty substances comprised of triacylglycerols, sterols and phospholipids and found in almost all food including plants and animals. The lipids are often added to increase the functional value of food. Lipids are responsible for a range of desirable attributes to the food like texture, structure, mouth feel, flavor and color (German 1999). Lipid oxidation is by far the single major factor causing food quality deterioration during the storage of lipids and lipid/fat containing foods (Martinez-Tome et al 2001). Lipid oxidation has been recognized as a leading cause of quality deterioration in muscle foods and is often the decisive factor in determining food product storage life (Frankel 1993, Ross and Smith 2001). The oxidation of lipids produces rancid odors and flavors and decreases the nutritional

quality by forming secondary products when subject to cooking and processing (Frankel 1996). Lipid oxidation is a very complex process where unsaturated fatty acids in lipid based foods react with molecular oxygen via a free radical mechanism (Asghar et al 1988). The primary products of the lipid oxidation reactions are hydroperoxides, that are relatively unstable and essentially odorless which further decompose into a wide range of secondary compounds, such as alkanes, alkenes, aldehydes, ketones, alcohols, esters, acids, and hydrocarbons. Of these compounds, aldehydes are considered the most important breakdown products due to their low threshold values and are the major contributors to the development of rancid off-flavors and odors (Ladikos and others 1988). Other problems associated with lipid oxidation or degradation include deterioration in flavor, color, and texture (Kanner 1994).

The fatty acid alkyl chain in a lipid is susceptible to oxidation both at double bonds and adjacent allylic carbons. Free radical and photooxidation at these carbons atoms are mostly responsible for deterioration of unsaturated oils and fats, resulting in rancid flavors and reduced nutritional quality of oils. Oxidation of double bonds is used in oleochemical production either to cleave the alkyl chain or to introduce additional functionality along the chain. Enzyme catalyzed oxidation is the initial step in the production of eicosanoids and jasmonates (biologically active metabolites in animals and plants respectively) (Ross and Smith 2001).

Initiation: (Radical formation)

$$RH + O_2 \rightarrow R\cdot + \cdot OH \dots\dots\dots\dots\dots\dots\dots 1 \text{ (reaction)}$$

Propagation: (Peroxy radical formation)

$$R\cdot + O_2 \rightarrow ROO\cdot \dots\dots\dots\dots\dots\dots\dots 2$$

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ROO· + RH → R· + ROOH ……………………3

ROOH → RO· + HO·…………………………….4

Termination: (Radicals combine)

R· + R· → RR …………………………….....5

R· + ROO· → ROOR…………………………….6

ROO· + ROO· → ROOR + O2…………………..7
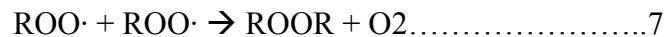
The start of the reaction is initiated by abstraction of allylic hydrogen to give a radical stabilized by delocalization over three or more carbons. This initiation of oxidation may probably be triggered by decomposition of hydroperoxides already present in the lipid. This decomposition may be thermal, however, may likely be promoted by traces of variable redox state metal ions by metal catalysis or by photooxidation through exposure to light. During the initiation step, the oxidation is characterized by an induction period during which the concentration of free radicals R· is increased (reaction 1), enough to start the propagation reaction. During the induction period, there is little increase in oxidation products. The first step of the propagation sequence is reaction of the allylic radical with molecular oxygen, producing a peroxy radical (ROO·) (reaction 2). This step is faster than the subsequent abstraction of hydrogen by the peroxy radical, producing both a hydroperoxide (ROOH) and new free radicals that continue the chain reaction (reaction 3 and 4). The abstraction of hydrogen is usually called the rate determining step and is therefore selective for the most readily abstracted hydrogen. Hydroperoxides, the initial products formed during oxidation are very unstable. They cause substrate degradation by forming several complex reactions to produce off-flavors (reaction 5 through 7). The free radicals cause shifting in the double bonds and form isomeric hydroperoxides with conjugated dienes. Methylene-interrupted

dienes and polyenes, where the free radical can be delocalized over five carbons, are oxidized faster than monoenes where the radical is delocalized over three carbons. The chain reaction is terminated by reactions that remove radicals that would otherwise produce more allylic radicals by hydrogen abstraction (Fenema 2001).

2.3   Protection of Lipid Oxidation by Antioxidants

Antioxidants are a class of compounds which prevent certain types of chemical damage caused by an excess of free radicals, charged molecules that are generated by a variety of sources including pesticides, smoking and exhaust fumes.

- Reaction of antioxidants with radicals:

    $R\cdot + A\cdot \rightarrow RA$……………………………….8 (from reaction 7 continued)

    $RO\cdot + A\cdot \rightarrow ROA$…………………………...9

    $ROO\cdot + A\cdot \rightarrow ROOA$………………………10

    Antioxidant + O_2 $\rightarrow$ Oxidized Antioxidant
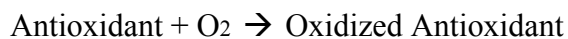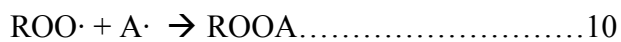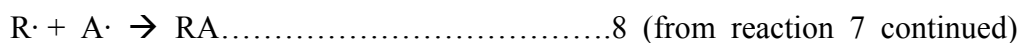
- Mechanism Involved: The hydrogen donation to free radicals is the main mechanism of action of antioxidants. The reaction proceeds further with the formation of a complex between the lipid radical and the antioxidant radical (free radical acceptor) (Yanishlieva and Marinova 2001). The antioxidant radicals $A\cdot$, formed in above reactions (reaction 8) have a very low reactivity and do not propagate the chain reaction by forming a complex lipid antioxidant radical (reaction 9 and 10), thereby inhibiting formation of extremely reactive peroxy radicals and hydroperoxides. Thus they terminate the free radical initiated reaction and protect the lipid from degradation.

Factors affecting the efficiency of antioxidants are activation energy of antioxidants, oxidation reduction potential, stability to pH and processing and the solubility of the antioxidants (Fenema 2001). Different antioxidants show substantially different antioxidant effectiveness in different fats and oils and food systems due to different molecular structures. The choice of antioxidants is dependent on various factors: safety, effectiveness of the antioxidant, imparting no off-odor and off-color, convenient to incorporate into foods, no carry-through effect, stable to pH and food processing, easily available, cost beneficial and non-absorbable (Madhavi et al 1996, Neilson 1998). Antioxidants, as is well known, are commonly used to retard or prevent lipid oxidation in foods (oils) by lowering the rate of oxidation of polyunsaturated fats, inhibiting the formation of off-flavors by preserving the overall food quality. Antioxidants are the compounds that can retard or inhibit the oxidation of lipids by inhibiting the initiation or propagation reactions that finally result in oxidation (Velioglu et al, 1998). According to the previous research by Shan et al (2001) free radicals produced during oxidation are mainly responsible for cardiovascular and other degenerative disorders. The destruction of these free radicals can assist in fighting cancer, heart disease and stroke. Fruits and vegetables are being considered as a rich source of antioxidants. The health officials have been encouraging consumers to eat more fruits and vegetables in order to gain the benefits of antioxidants (Wang et al 1998).The study conducted by Maria Cruz et al (2005) states that a substance is termed an antioxidant when present even at low concentrations and significantly delays or prevents oxidation of the substrates (proteins, lipids). According to the same study it was noted that the requirements to select the most effective antioxidant for a particular food depends upon the properties of the antioxidant, oils being oxidized, effect of other components on the antioxidant activity, and also actual

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relevance of a model.

There are two major types of antioxidants that are commonly used in the food industry: Natural antioxidants such as tocopherols (delta>gamma>beta>alpha), Vitamin C, caratenoids, Gossypol; and synthetic antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG), tertiary butyl hydroquinone (TBHQ) (Madhavi et al 1996).

2.4   Methods

2.4.1   Spice Extraction

The extraction procedure is usually based on the types of antioxidant compounds present in the spice to be extracted. The concentration of extracted antioxidants depends on the suitability of the extraction procedure and relative to the plant material. In the extraction of phytochemicals and polyphenolic antioxidants from plant materials, three major extraction techniques are used: extraction using solvents, solid-phase extraction and supercritical extraction (Suhaj 2006).

The phenolic antioxidants found in the vegetables plants like spice and herbs are very unstable and can be degraded by enzyme action in undried plant materials. Freeze drying preserves the medicinal and preservative qualities of plants and thus the phenolic antioxidant extraction is performed using dry, frozen or lyophilized samples (Abascal et al 2005). For different extraction techniques, various solvents with varying polarities are used such as toluene, acetone, ethanol, methanol, ethyl acetate, water and petroleum ether. Some researchers have also used supercritical (CO_2) extraction and short chain triacylglycerols as the carrier in extraction techniques (Schwartz et al 2001). A microwave assisted extraction technique utilizes a combination of microwave energy and traditional solvent extraction, with

advantages of short time, less solvent consumption and also high extraction efficiencies. Ultrasound assisted extraction uses solvents such as hexane and methanol for the extraction of plant materials whereas soxhlet extraction uses a combination of percolation and immersion techniques (Suhaj 2006). One of the simplest extraction methods is using edible oil, where natural antioxidants such as spices are mixed with oils, and the mixture is moderately heated and filtered to obtain the extract (Pokorny et al 2001).

The solvent methanol is universally used for extraction of polyphenols from different plants, fruits and vegetables. Aqueous methanol is also commonly used for extraction of phenolics from plants like spices. According to previous research, the usefulness of aqueous methanol is largely due to its polarity, which helps to extract polyphenols linked to polar fibrous matrices more effectively (Decker et al 2005, Frankel et al 1993). On the other hand water/acetone solvent is useful for extracting polyphenols from proteic matrices as they may degrade the polyphenol protein complexes in the plant material. Another advanced but lesser known extraction technique is pressurized liquid extraction; which has been recently introduced for phenolic compounds extraction. This technique uses high pressure and temperature to accelerate the process of extracting phenolic compounds (Suhaj 2006). While all of these methods have useful applications, the extraction of spices is generally performed with methanol or ethanol extraction techniques due to the extensive presence of polar matrices in the oil fraction of spices.

2.4.2   Lipid Oxidation

Lipid oxidation is also called autooxidation and is the result of free radical mechanism in the bulk fats and oils. The result of this reaction leads to the formation of hydroperoxides (initial or primary products of oxidation) that further undergo scission reactions to form aldehydes,

ketones and organic acids (final or secondary products of oxidation). Their measurements can be accomplished with either predictive tests or indicator tests. The predictive tests utilize the accelerated conditions to measure the fat stability in the finished food product. The oxidation tests are used to determine ingredient quality, measure preservative effectiveness, and also are used to estimate shelf life of the product.

➢ **Oxidative Stability Tests**

- **OSI (Oxidative Stability Index)**. A sample is held at constant temperature and air is passed through, then the sample is bubbled through a trap of deionized water. The results report the temperature as well as induction period time. The conductivity of water is increased as volatile acids produced during lipid oxidation are dissolved. The change in the conductivity is continuously monitored and the OSI value is defined as the hours required for rate of conductivity change to reach a predetermined value. The instrument commonly used for this method is the Rancimat (Brinkman Instruments, Inc.) and Oxidative Stability Instrument (Omnion, Inc). This process method can be utilized to analyze multiple samples simultaneously (Nielson 1999).

- **AOM (Active Oxygen Method)**. The AOM method is similar to the OSI method, except the induction time is determined by discontinuous measurements of peroxide values or sensory perception of the odor. This method measures the stability of a fat by bubbling air through a fat solution using different flow rate, temperature, and concentration. Peroxides and hydroperoxides are determined by this treatment at different time intervals by titration with iodine. The AOM value measures the number of hours required for the peroxide concentration to reach 100 meq/kg of fat. The method is very time consuming as a stable fat requires 48 hours or more to reach the

required peroxide concentration. The other disadvantage of this method is its more labor intensive. It has been coupled with faster automated techniques and is still used by many researchers (Nielson 1999).

- **Iodine Number**. The Iodine number measures the degree of unsaturation of fats or the potential of a fat to undergo oxidization. This method is based on the reaction of iodine with double bonds of unsaturated fatty acids. The more unsaturated the fat is, the greater are the number of double bonds; therefore, more iodine is absorbed. Iodine number does not provide an accurate measure, as fat stability can be altered by many other factors (Nielson 1999).

- **Oxygen Bomb Method.** The oxygen bomb test is used to measure the amount of oxygen uptake from the surrounding environment in a closed system. The oxygen consumption rate indicates the oxidative stability of the tested product. The test is usually used to estimate the stability of antioxidants in fats and finished food products. The major advantage of this method is that it measures the stability of the complete product prior to the extraction of the fat. The interference of other components like transition metals or chemical oxidants, however, can assist oxidation, and so the extracted fat may not indicate the true product stability (Nielson 1999).

- **Schaal Oven Storage Method.** Schaal oven storage method is used as an accelerated oxidative stability test. This test is performed by placing the fat or oil in a heated environment at a temperature of 60°C. The mechanism of oxidation at 60°C temperature is considered similar to oxidation at room temperature (Frankel 1993).

The results of oxidative stability determinations obtained at 60ºC by the Schaal test mimics actual shelf life determinations (Nielson 1999).

➢ **Measuring Present Oxidative Status**

- **Peroxide Value**. Peroxide value (PV) determines the transient products like peroxides and hydroperoxides formed during the initial stages of lipid oxidation. PV is expressed as milliequivalents of peroxide per kilogram of fat measured by titration with iodide ion. This is one of the most frequently used tests for oxidative rancidity. Low peroxide value usually indicates either the beginning of oxidation or oxidation at advanced stages, whereas high peroxide values are a definite indication of a rancid fat. However, moderate values may be the result of the depletion of peroxides after reaching high concentrations (Hahm and Min 1995, Nielson 1999).

- **TBA Test**. The TBA test measures the amount of secondary oxidation products of lipid oxidation. Saturated aldehydes, 2-enals, 2-dienals, or malonaldehyde produced during lipid oxidation, are detected during this thiobarbituric acid test. The reaction produces a colored compound that can be measured using a spectrophotometer. This test was originally developed to detect malonaldehyde, although TBA may react with other aldehydes, and also with other substances like phenols in smoke flavors. The reaction is not specific to malonaldehyde and is usually reported in terms of thiobarbituric acid reactive substances (TBARS). (Nielson 1999, Kulas Ackmen 2000, Rossel 1991, Decker et al 2005)

- **Anisidine Value and Trotox Value**. The anisidine values measures the amount of aldehydes produced during oxidation in fats and oils. The breakdown of hydroperoxides leads to the production of volatile aldehydes like hexanal; this

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reaction produces a non-volatile portion of the fatty acid attached to the glyceride molecule. This non-volatile portion reacts with n-anisidine to form a chromogen that is measured by a spectrophotometer. The totox value indicates the total oxidation using both anisidine values and peroxide values.

$$\text{Totox value} = \text{Anisidine value (AV)} + 2(\text{peroxide value})$$

The high AV values even if TBA and other aldehyde show low results, indicates that a fat has been oxidized, because volatile aldehydes may incidentally or intentionally be removed during the processing operation. The totox value increases continuously during the process of lipid oxidation (Nielson 1998, Kulas Ackmen 2000, Decker et al 2005).

- **Hexanal Value**. Hexanal is one of the most common aldehydes produced during the process of lipid oxidation. Hexanal is produced during the termination phase of lipid oxidation and can be measured by gas chromatography incorporated with direct headspace analysis. Usually the method used is direct headspace analysis, referred to the volume of vapors obtained from headspace above the sample in a closed container. The principle of this method is utilizing the headspace hexanal as an indicator of lipid oxidation. The obtained aliquots of vapors thus obtained are injected onto a GC column with a flame ionization detector to separate hexanal from other volatile components. Hexanal is one of the important factors in the offlavor formation and it is directly proportional to the sensory characteristics of lipid oxidation (Nielson 1999).

- **Free Fatty Acids (FFA) and Acid Value**. Free fatty acids in a fat (fat extracted) can be easily determined by a simple titration technique. The FFA value is then expressed

as the amount of fatty acids hydrolyzed from triacylglycerols. FFA is expressed as percentage by weight of a specific fatty acid. The values are expressed as % oleic acid for different oils. Acid value is defined as amount of KOH in milligram required to neutralize the free acids present in 1gm of oil. Along with FFA, acid phosphates and amino acids are also measured by the acid value. FFA may indicate hydrolytic rancidity; however, other lipid oxidation processes also produce acids (Neilson 1999).

- **Headspace Profile/Analysis**. The headspace profile is often used to measure total volatile profile of the headspace over a product using a technique similar to the one used for hexanal value. In lipid oxidation, a variety of volatile compounds are produced like hydrocarbons, aldehydes, enals, dienals, ketones, and organic acids. With the progress in oxidation, more and more volatiles are produced which are measured by injecting a portion of the headspace into a gas chromatograph. (Nielson 1999, Decker et al 2005).

➢ **Determining the lipid fractions**

- **Fatty Acid Composition by Fatty Acid Methyl Esters Test.** Fatty acid methyl esters test is used to determine the fatty acid composition or fatty acid profile of a food. The method measures the amount of fatty acids by extracting the lipids and analyzing them by capillary Gas Chromatography. The triacylglerols and phospholipids in the fat based foods are saponified to release the fatty acids, which are further esterified to form fatty acid methyl esters (FAME). The FAME is obtained by the reaction of extracted lipid with sodium hydroxide, methanol, boron triflouride and heptane. The FAME test is usually used in the industry to quantify the fats in

food labels: percent monounsaturated fatty acids, percent polyunsaturated fatty acids, and percent trans isomer fatty acids (Nielson 1999).

2.4.3 Antioxidant and Phenolics

Various methods have been developed in the past to measure the total antioxidant capacities of plants, vegetables and fruits. The antioxidant capacity methods are based on the ability of a compound to quench free radicals. These methods are called direct methods and are ORAC, DPPH and Total phenols.

- **ORAC Assay**

  The ORAC (Oxygen Radical Absorbance Capacity) assay is a relatively simple and sensitive method suitable for quantifying the antioxidant capacity of a number of products including whole fruits and vegetables, beverages such as fruit juices and wines, and other products. In the ORAC assay, antioxidant capacity is measured by the protection against the loss of PE (phycoerythrin) fluorescence in the ORAC assay against the radical species. The fluorescence of PE is highly sensitive to the conformation and chemical stability of the protein (Cao and Prior, 1999). The degree of radical damage is reflected by change in the fluorescence intensity. The original ORAC assay was primarily for water-soluble antioxidant activity against the peroxyl radicals. Results are expressed as micromoles of Trolox equivalents per gram or milliliter of the sample (Wang, 1996). An much improved ORAC assay, which uses FL (fluorescein) instead of PE, is common these days which measures the hydrophilic and lipophilic chain breaking antioxidant capability (Ou et al 2001).

  The ORAC assay has already been extensively used to measure the antioxidant capacity of a number of foods and nutritional products. Due to recent

media focus on the positive health benefits of antioxidants, the supplement and functional food market has introduced a plethora of antioxidant based products (Huang et al 2005, Decker et al 2005).

- **DPPH Activity by Trolox Equivalent Antioxidant Acivity**

2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay. DPPH is one of the stable and commercially available organic nitrogen radicals and has a UV absorption maximum of 515 nm. Upon reduction, the solution color fades; thus the reaction progress is conveniently monitored by a spectrophotometer. The DPPH assay is typically run by the following procedure: The DPPH solution (3.9 mL, 25 mg/L) in methanol is mixed with the sample solution (0.1 mL). The reaction progresses and absorbance of the mixture is monitored at 515 nm for 30 min or until the absorbance is stable. Upon reduction, the color of the solution fades. The percentage of the DPPH remaining is calculated as % DPPH and is proportional to the antioxidant concentrations, and the concentration that causes a decrease in the initial DPPH.
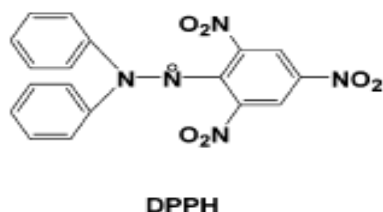
Figure 3 : DPPH Radical (Huang et al 2005)

Using standard curves for the reaction of Trolox with DPPH, the data is then converted to activity in terms of mmoles Trolox Equivalents/100 grams sample (TE) (Rababah et al 2004 and Kahkonen et al 1999).

DPPH is a nitrogen radical which has no similarity to the highly reactive and transient peroxyl radical that are mainly involved in lipid peroxidation. Many antioxidants that react faster with peroxyl radicals can react very slowly and may even be very inert to the DPPH solution (Huang et al 2005).

- **Total Phenols Content Assay by Folin-Ciocalteu Reagent**

Folin Ciocalteu Reagent was invented by scientists Folin and Ciocalteu in the year 1927 and was used for the analysis of proteins by using the reagent's characteristic activity toward protein's tyrosine (containing a phenol group) residue. The first use of the assay was for the analysis of total phenols in wine; after which the assay has found extensive applications in the food industry (Singleton et al 1999). The total phenolic activities for plant based extracts are usually obtained by the Folin Ciocalteu Reagent method using gallic acid as a standard. The Folin Ciocalteu Reagent is based on the reducing ability of phenols to quantify the amount of total phenolics (Waterman and Mole 1994). Various past publications have applied the total phenols assay by folin reagent and DPPH based antioxidant capacity assay and have found excellent linear correlations between the total phenolic profiles and the antioxidant activity (Vinson et al 1998).

The total phenolic content by Folin Ciocalteu reagent is very convenient, economical, simple and easily reproducible method. It is one of the frequently used assays in industry for studying the phenolic antioxidants in plant based materials.

2.5 Spices Ginger, Oregano, Rosemary and Turmeric

Spice plants are predominantly found in tropical and temperate areas and are comprised of botanical families such as *Lamiaceae*, *Lauraceae*, *Zingiberaceae* and several others. The Lamiaceae family includes rosemary, oregano, thyme and sage; Lauraceae family is

represented by plants like cinnamon and the *Zingiberaceae* family is represented by rhizomes like cumin, turmeric and ginger.

- **Ginger.**

Ginger (*Zingiber officinale Rosc.*) belongs to the family of Zingiberaceae and is widely used around the world as a spice or food additive and as a medicine. This plant is a perennial herb that consists of an underground stem or rhizome and bears erect leafy shoots. The pungent element of ginger is produced by the oleoresin-gingerols, shogaols and zingerone, which are credited with anti-nausea or antiemetic, abortifacient, anti-inflammatory,anticoagulant, antihypercholesterolemic, antihypertensive, antihyperglycaemic, anti-spasmodic, aperient, alexeteric, circulatory stimulant, counter irritant, sialagogue and vasodilator effects (Chandarana 2005). Ginger (*Zingiber officinale* L.), with its pleasant aroma and pungency, is used extensively for cooking purposes. It also possesses antimicrobial, antitumoral, and antiplatelet aggregation properties and is an excellent antioxidant (Murcia et al 2004).

In China, Japan, United States of America, and other countries, ginger is commonly used as either a food product or an herbal medicine. The essential oil, which that is one of the products from ginger, has been internationally commercialized as a flavoring agent and food additives and for use in pharmaceutical processing (Fan Gong et al 2004). The chemical components in the essential oil might affect the characteristic flavor and quality of ginger; on the other hand, there are substantial differences among chemical components of ginger oils from different sources. Chemical investigations carried out in the past showed that monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and nonterpenoid compounds are the main constituents in ginger oils.

Research shows that gingerol and shogaol (figure 4) are the main phenolic compounds found in ginger, especially shogaol as it provides the anti-emetic activity in ginger. Ginger also contains curcumin (like turmeric) in addition to the phenolic compounds, gingerols and diarylheptanoids that are high in antioxidants.

Ginger has gained a lot of attention as a novel source of natural antioxidant, because

Gingerol

Shogaol
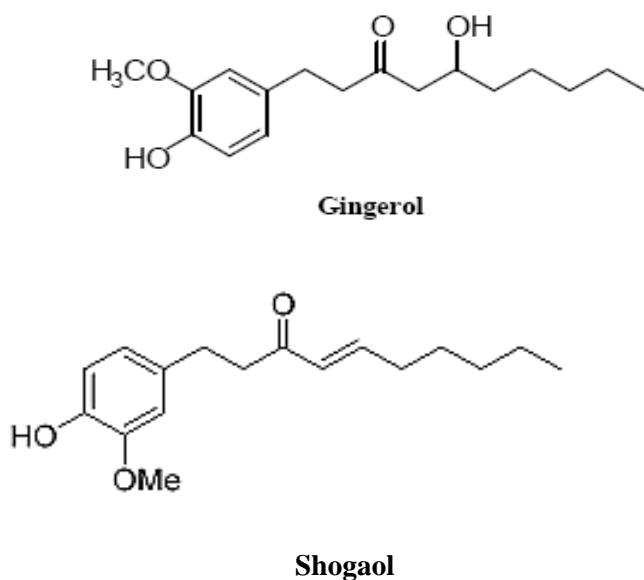
Figure 4: Main Antioxidant Phenolic Compound in Ginger

most ginger rhizomes are used for spices in tropical areas and spices are natural food additives established by the food culture. The rhizome of a popular ginger species, *Zingiber officinale,* is well known to possess potent antioxidant activity, and its antioxidant phenolics were isolated by Lee and Ahn (1985). In tropical countries, many kinds of gingers are cultivated and used not only for traditional medicines but also for spices (Jitoe et al 1992). However, little is known about the antioxidant activity and antioxidant compounds in tropical gingers. Interest in such gingers as an antioxidant source prompted us to investigate them.

- **Oregano.**

Researchers have studied many spices and it has been observed that rosemary, oregano and sage, from the *Lamiaceae* (*Labiateae*) family, possess strong antioxidant activity (Almeida and Regitano 2000). Oregano is one of the common spices in this family and is widely used in European and Mediterranean countries. The presence of naturally high phenolic content has made popular the use of herbal extracts from the *Lamiaceae* family suitable for antioxidant applications. The dried leaves as well as the volatile oil of this aromatic perennial herb have been used medicinally for centuries. The positive effects of oregano on human health have now been attributed to its antioxidant activity both in both the essential oil and soluble phenolic fractions (Chun et al, 2004). Kikuzaki and Nakatani (1996) reported the isolation of five different phenolic compounds from the methanol extract from the leaves of oregano, and claim that among these the rosmarinic acid was found to be present in highest concentrations. The compound rosmarinic acid is a caffeoyl ester and has gained importance due to its property as an antioxidant as well as an anti-inflammatory compound (Chun et al, 2004) Oregano is a genetically heterogeneous species as a result of natural cross pollination. This heterogeneity results in large variations in phenolic content that limits its use as an ingredient in functional foods.

As reported by Zheng and Wang (2001) the herb oregano has 3–20 times higher antioxidant activity than other herbs. The methods used in the study were ORAC and total phenolic content. In addition, Zheng and Wang (2001) have also reported that oregano possess 42 times more antioxidant activity (µg/g) than apples, 30 times more than potatoes, 12 times more than oranges and 4 times more than blueberries. Almeida and Regitano (2000) reported the presence of glucosides, phenolic acids fractions and terpene derivatives in oregano. The main phenolic compounds found in oregano are thymol and carvacrol (figure

5), which are reported to be primarily responsible for their antioxidant activity (Suhaj 2006, Aesbach et al 1994). Phenolic antioxidants donate the hydrogen to the free radicals, yielding low energy stable radicals. These antioxidants phenolic structure make them stable through resonance inside the ring. A study conducted by Zheng and Wang (2001) have documented from various sources that oregano extracts had high contents of rosmarinic acid (124.8-154.6 mg/100 g of fresh weight) and other hydroxycinnamic acid derivatives. Chromatographic and spectrophotometric analysis demonstrated the presence of the flavone apigenin, the flavanone, eriodictyol and the dihydroflavonols, dihydrokaempferol and dihydroquercetin.
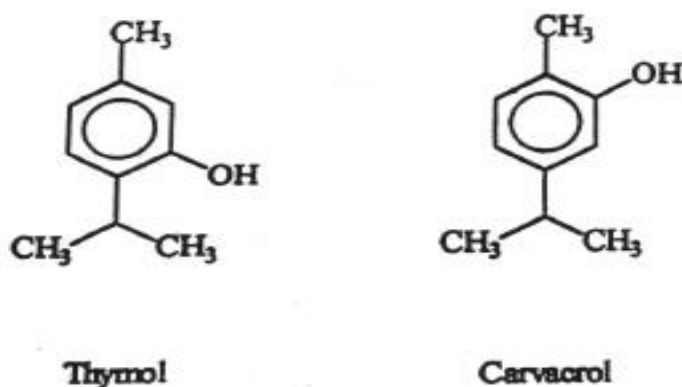
Figure 5: Main Antioxidant Phenolic Compounds in Oregano

Rosmarinic acid and hydroxycinnamic acid compounds have been demonstrated to possess strong antioxidant activity. The antioxidant activity of rosmarinic acid is much higher than that of α-tocopherol and BHT according to the experimental results of the Zheng and Wang (2001) study.

- **Rosemary.**

Rosemary (*Rosmarinus officinalis L.*) belongs to the *Lamiaceae* family and has historically being cultivated throughout the world, from ancient Egypt and Mesopotamia to China and India. Rosemary is a very important medicinal and aromatic plant; researchers and scientists

have found evidence in ancient literature of the use of rosemary herb for medicinal, culinary and cosmetic purposes in the ancient literature (Stefanovits-Banyai et al 2003).

Rosemary is one of the oldest recorded herbs in history. The origin of Rosemary can be traced back to the Mediterranean and Portugal, prior to its introduction to the Alps in the middle ages, and to the Americas by the early colonists and the first settlers in New England. Rosemary has been used to treat diseases like cancer, as well as tumor and cardiovascular disorders.

Today, Rosemary is the most commonly used aromatic and medicinal plant. The essential oil extracted from rosemary enhances the blood-circulation of the limbs and other medicinal properties.
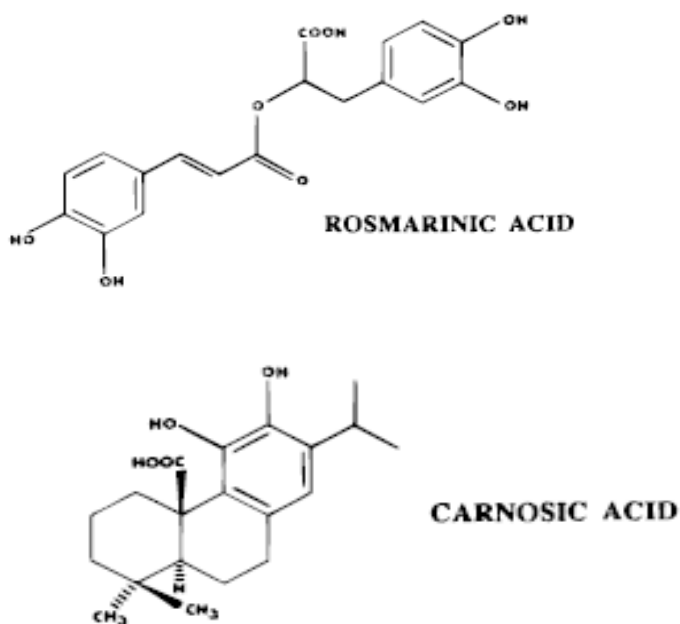
Figure 6: Main Antioxidant Phenolic Compounds in Rosemary

The preservative, antibacterial and antioxidant properties of rosemary have led to the applications of rosemary extracts in various foods such as sausages (Barbut et al 1985) and to preserve beef steaks (Stoick et al 1991).

Frankel et al (2003) reported the presence of a vast number of compounds in the extracts of rosemary, including carnosic acid and rosmarinic acid (figure 6), which contribute to the property of rosemary as a natural antioxidant and preservative. Carnosol and carnosic acid are the most important active antioxidant components in the rosemary extracts (Aeschbach et al 1990, Loliger 1991).

A study conducted by Kahkonen et al (1999) isolated and examined compounds from rosemary, including flavones, diterpenes, sterols, and triterpenes, and several other antioxidative diterpenes such as rosmanol, epirosmanol, and isorosmanol, rosmaridiphenol, and rosmariquinone, that have also been reported to contribute to the antioxidant activity of rosemary extracts (Nakatani and Inatani, 1984). In fresh rosemary leaves carnosic acid is the major phenolic diterpene. Carnosol constitutes approximately 10% of the carnosic acid (figure 6) content, and the other diterpenes are found in minor amounts.

- **Turmeric.**

Turmeric (*C. longa* L.) belongs to the family of *Zingiberaceae*, and its extract is an oleoresin consisting of a light (volatile oil) fraction and a heavy fraction of yellowish brown color. The turmeric spice is indigenous to South and Southwest Asia and is primarily cultivated in China, India, the Caribbean Islands, and some South American countries (Govindrajan 1980). This spice is used in various food preparations as it preserves the food's freshness and retains its nutritive values in addition to imparting flavor and color (Chandarana et al 2005). According to the history of turmeric, this spice spread to China some time in the 700's CE. The crop then spread to West Africa in the thirteenth century and to East Africa in the seventeenth century. It was introduced to Jamaica in the year 1783, then from the Caribbean, it eventually reached North America. Some of the medicinal benefits of turmeric, as reported

in previous literature, includes it usage as a stomachic and a blood purifier, as well as its usefulness in treating common colds, leprosy, intermittent fevers, afflictions of the liver, dropsy, purulent ophthalmia, indolent ulcers, pyogenic afflictions, wounds and inflammation. Turmeric can also be beneficial in treating a variety of ailments and metabolic disorders; it is also used as a remedy for hypercholesterolemia, arthritis, indigestion and liver problems (Chun et al 1999 and Srimal 1997). In addition to its medical and religious functions, Turmeric is commonly used in the culinary preparation of curry powder, chicken bouillon, sauces, gravies, dry seasonings and foods and in addition to this it's also used in medicine.
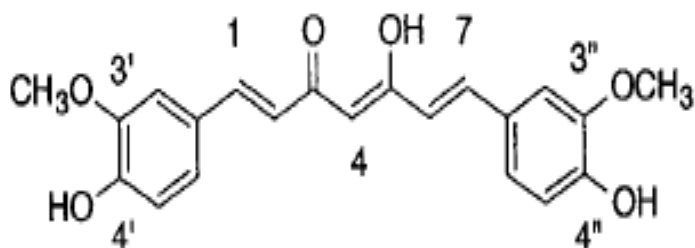
a. Curcumin
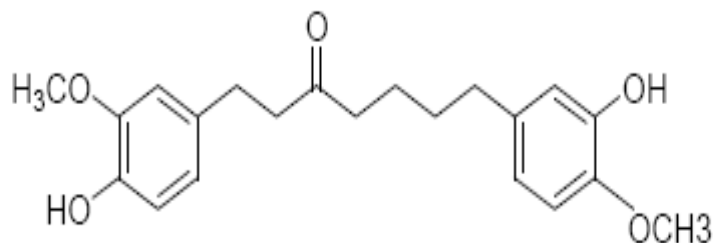
b. Tetra-hydrocurcumin

Figure 7: Main Antioxidant Phenolic Compounds in Turmeric

The rhizome of turmeric (*Curcuma longa L.*) has a rich history in South Asia as a spice, food preservative, and coloring agent and has been used for centuries in the Ayurvedic system of medicine (Guddadarangavvanahally et al 2003). Long before the time of cheaper synthetic food preservatives and coloring agents, turmeric played a key role as food

additives. Continuing research indicates that turmeric's active principle, curcumin, (figure 7a) has unique antioxidant, antimutagenic, antitumorigenic, and anticarcinogenic, anti-inflammatory, antiarthritic, antimicrobial, and hypocholesterolemic properties (Majeed et al 1995).

The compounds responsible for the yellow color are the curcumin (see figure 7 a) (1, 7-bis (4-hydroxy-3-methoxyfenil-1,6-eptadiene-3,5-dione) and curcuminoids, tetrahydrocurcumin (see figure 7 b), demethoxy-curcumin and bis-demethoxy- curcumin. The curcuminoids are very potent, naturally occurring antioxidants that have several pharmacological properties such as anti-inflammatory and anticancer action (Priyadarsini 1997).

The amount of oleoresin in the rhizomes varies from 3 to 6% and it is predominantly formed of sesquiterpenic ketones and 2-8% of curcuminoids. Curcuminoids have shown antimutagenic activities in different animals and cell cultures. One of the biochemical mechanisms attributed to the anticarcinogenic activity of curcumin is related to its carbonyl group (Mara Braga et al 2003).

The US Department of Agriculture (USDA) scientists have found that turmeric could be used as a natural antioxidant to keep dill pickles fresh after being packaged in plastic jars or pouches. Food technologist Roger McFeeters, and one another researcher with the USDA's agricultural research service, found that turmeric prevents the formation of aldehyde compounds that cause oxidative off-flavors in pickled cucumbers (Anonymous 2006).

2.6 Long Chain Polyunsaturated Fatty Acids in Fish Oil

Polyunsaturated fatty acids (PUFA) are an important group of fatty acids which are not synthesized by the human body and are supplied through the diet. Fish oil is an abundant

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source of PUFA's such as Docahexanoic acid (DHA) and eicosapentanoic acid (EPA) for human consumption. These PUFA's are very important to one's health as they have been reported to prevent coronary heart disease, hypertension, diabetes, arthritis and inflammation, autoimmune disorders, and some cancers, and are required for the normal growth and development of infants (Perez-Mateos et al 2004, Luther 2006). Masahiro et al (1998) confirmed PUFA's as the most prominent lipids found in fish oils. According to Frankel et al (2002) fish oils rich in long-chain ω-3 PUFA's have long been recognized for their nutritional value and are often incorporated into fortified foods.

The levels of palmitoleic acid (16:1), oleic acid (18:1), and eicosapentaenoic acid (20:5) in menhaden oil are 11.2-17.9, 10.7-23.4, and 10.2-14.1%, respectively (Stansby, 1981). Lipid oxidation, however, limits the utilization of these fish oils in processed foods and as supplements in fortified food. These ω-3 polyunsaturated fatty acids from fish oils have one disadvantage: the high concentration of PUFAs makes fish oil susceptible to oxidation. Despite these shortcomings, however, increased demands for PUFAs for their nutrition and health benefits have triggered the need for thorough investigation of methods in increasing the stability of fish oil against oxidation and reducing the formation of off-flavors. PUFAs are highly susceptible to oxidation, which results in simultaneous formation of adverse flavors and odors (Kulas and Ackman 2001) as they degrade into various carbonyl compounds or radicals such as $\cdot CHO$, $CH_3O\cdot$, $\cdot CO$, and $\cdot CH_2O$, and may cause a secondary reaction with amine compounds to produce a cooked flavor and aroma (Horuichi et al 1998). These secondary processes eventually reduce the shelf life of the product and limit their end use.

The literature on the oxidative stability of long-chain ω -3 PUFA is very limited and inconclusive. The problem may be attributed to the wide variation of fatty acids and triglyceride composition of fish oils and to the wide range of methods and lipid systems used in oxidative stability tests. The incorporation of EPA, DHA rich fish oil into foods is a challenging task, requires efficient antioxidants to protect the unsaturated lipids and to prevent the formation of any undesirable oxidative flavors. A related investigation was conducted on the use of fish oil with PUFA's (EPA and DHA) to make mayonnaise in hopes of improving oxidative protection in real food emulsions (Jacobsen et al, 2001). Another previous study conducted by Leonardis & Macciola (2003) investigated the effectiveness of caffeic acid and butylated hydroxyanisole (BHA) as antioxidants in cod liver oil. However the studies were not focused on the stability of PUFA during oxidation nor the mechanism behind the antioxidant activity. Thus, there is an urgent need for comprehensive research on lipid oxidation and the antioxidant mechanisms in complex food systems like fish oils.

Phenolics are responsible for antioxidant property and are secondary metabolites present typically in plants (Shahidi & Naczk, 1995; Chen & Ahn, 1998; Fukumoto & Mazza, 2000). A lot of research has been conducted on the use of spices like rosemary and oregano and their role in retarding oxidation in other edible oils. In the past, a few researchers have worked on the use of phenolic substances in plants to study their antioxidants potential in fish oil (Leonardis & Macciola (2003); however, no thorough and comprehensive investigation has been done. The vast spectrum of phenolic substances present in these spices like oregano, rosemary, turmeric and ginger still remains untapped. Phenolic antioxidants could potentially serve as preservative agents as they are already known to posses various medicinal properties. In the past, Sullivan et al (2005) investigated the use of naturally occurring

antioxidants on fish oil systems extracted from livers of white pollack. The study was conducted over a 16-day time period at a temperature of 30°C. The results showed the effectiveness of rosemary compared with other antioxidants in lowering the levels of lipid oxidation. However, this study and other related studies were insufficient and inconclusive regarding information on the oxidation of fish oil at higher temperatures that resemble real time cooking or processing and storage conditions. Thus, a comprehensive work on the spices ginger, oregano, rosemary and turmeric was conducted in this research study to examine the antioxidant phenolics in their role of retarding PUFA oxidation in menhaden oil during heating and accelerated storage conditions.

2.7   Cholesterol Oxidation in Food and its Protection by Antioxidants

The liver produces essential cholesterol enough to satisfy the requirement of the human body but cholesterol is also provided externally sources of foods like egg yolk, meat and whole milk products. Cholesterol is a vital component and is needed by the human body to digest fats, make hormones, and build cell walls and various other important functions. Lipoproteins are special molecules that circulate (carry or transport) cholesterol around the body. The blood flow carries the cholesterol in these particles (lipoproteins) and circulates it to the body tissues. The excess of this circulating cholesterol can cause damage to arteries, especially the coronary type that supply the blood to heart; it can also cause accumulation of cholesterol which is plaque in the vessel linings, which can lead to atherosclerosis (Anonymous 1999).

- Types of lipoproteins:

❖ Low-density lipoprotein (LDL): This cholesterol is the form in which is carried into the blood and is the main reason for harmful fatty buildup in the arteries. So it's also termed as bad cholesterol.

❖ High-density lipoprotein (HDL): HDL is termed as "good" cholesterol and it carries blood cholesterol back to the liver, from where it can be excreted. HDL helps in preventing the cholesterol buildup in the vessels.

❖ Very Low Density Lipoproteins (VLDL): VLDL, are the lipoproteins which carry cholesterol from the liver to the organs and tissues in the body. They are slightly heavier than the LDL's (low density lipoproteins), but are often associated with atherosclerosis and heart disease.
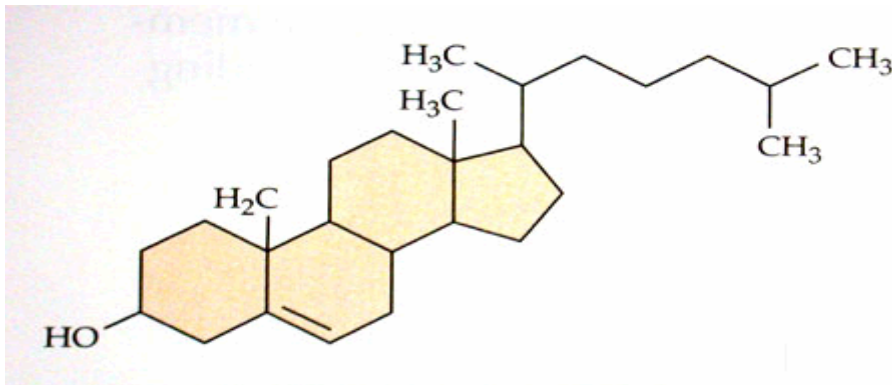
Figure 8: Structure of Cholesterol Molecule

LDL carries nearly about 60%-70% of the cholesterol in the bloodstream. A fairly high levels of LDLs can lead to heart disease or disorders which can be caused by buildup of plaque and blockage in the arteries. Whereas the high-density lipoprotein (HDL) may carry cholesterol away from the arteries to the liver and adrenal glands. Thus, the main focus should be to keep the LDL cholesterol low and the HDL cholesterol high (Anonymous 1999).

High cholesterol in the serum is the number one cause of heart diseases and strokes in the USA (Tabas 2002). This molecule is very unstable with an unsaturated bond at position 5-6 of the sterol nucleus (figure 8), making it susceptible to rapid oxidation (Maerker, 1987).

Cholesterol has the same structure as lipids, and as in lipids, the free radical is the main factor triggering the autoxidation of cholesterol; which further leads to the formation of hydroperoxides and other oxidation products that are termed oxysterols. The oxysterols are similar to cholesterol in structure except for the presence of extra oxygen.

In food, cholesterol stays in close proximity to the lipids and it is possible that oxidation of polyunsaturated fatty acids initiates the oxidation of cholesterol (Kubow, 1993). Interaction of lipid triacylglycerols with cholesterol molecules may accelerate the oxidation process of the sterol, and in turn cholesterol may also influence triglyceride oxidation (Kim & Nawar, 1991).

Cholesterol oxidation proceeds via a free radical mechanism, similar to polyunsaturated fatty acid oxidation (Kubow, 1993).

2.8   Hypothesis and Rationale

The natural preservatives like spices are getting very popular as they are safe due to their natural occurrence in foods and have been used for centuries. Spice and herbs contain very small organic biomolecules which are one of the reasons for their antioxidant characteristics. Consumers are attracted towards products which are minimally processed, and have natural preservatives, with an added functional value (Brul & Coote 1999).

The phenolic principles in spices ginger, oregano, rosemary and turmeric are considered to be responsible for the biological effects, one of the important being antioxidant potential. The phenolic compounds in these spices after extraction are hypothesized to

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exhibit the antioxidant properties through various possible mechanisms like free-radical scavenging activity by prevent degradation of foods especially lipid food (oils). One of such food is fish oil which is comprised of long chain unsaturated fatty acids (PUFA) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The presence of polyunsaturated fatty acids make these fish oils highly susceptible to undergo oxidation by producing rancid flavors volatiles when exposed to heat and also during storage.

➤ **Three Different Areas of the Research Work**

- Evaluate antioxidants capabilities of spice extracts from ginger, oregano, rosemary and turmeric in inhibiting oxidation of DHA and EPA in menhaden fish oil during heating and incubation conditions.

- Evaluate the effect of heating and incubation on the antioxidant activity (DPPH method) and total phenolic content (Folin-Ciolteau method) of the four spice extracts.

- Antioxidant capabilities of the spice extracts from ginger, oregano, rosemary and turmeric in inhibiting oxidation of cholesterol.

The literature review will be provided as a (Chapter 2), which will also include a review of methodologies in measuring oxidation in foods and detailed discussion of all four spices. Further the antioxidant capability of the four spice extracts with a menhaden fish oil model will be discussed (Chapter 3), and with a cholesterol model (Chapter 5). The fatty acid methyl esters method along with the Gas Chromatography will be used to determine the antioxidant (oxidation inhibition of PUFA) capability while High Performance Liquid Chromatography will be used to determine the retained cholesterol during heating. The overall hypothesis, as stated earlier is based on the antioxidant potential of the spices. As the spices are hypothesized to possess free radical scavenging activity, the DPPH method is used

to study the antioxidant capacity (Chapter 4). The spices are often subject to heat and storage during cooking and food uses, thus the effect of heating and storage on the retention of antioxidant activity and phenolic content of the spices will also be discussed (Chapter 4). The Chapter 4 will be concluded by identifying the main compounds in the above four spice extracts by Gas Chromatography method assisted spectral library software.

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CHAPTER 3. ANTIOXIDANT CAPABILITIES OF SPICE EXTRACTS IN INHIBITION OF DHA AND EPA OXIDATION IN MENHADEN FISH OIL

3.1 Introduction

The culinary herbs and spices have been used since antiquity, and are gaining popularity due to their ability to impart flavor characteristics to a wide variety of foods (Bin Shan et al 2005). According to Shan et al (2005) in an herbal medicine study, the herb or spice is used not only for savory and aromatic purposes but also medicinal qualities, such as but not limited to antioxidant activity, anti-inflammatory, antimicrobial and digestive stimulant action. The phenolics in the spice vegetable plants are mainly responsible for the biological effects such as antioxidant potential, preservative and antimicrobial properties (Kahkonen et al 2003). Shan et al (2005) have reported that the antioxidant effect of phenolic compounds is due to their redox properties and is the result of various possible mechanisms such as free-radical scavenging activity, transition-metal chelating activity, and/or singlet-oxygen quenching capacity. Plant phenolics are increasingly of interest to the food industry because they retard oxidative degradation of lipids and, thereby improve the quality and nutritional value of foods.

A number of previous research works have reported the use of antioxidants from plant sources to retard or prevent lipid oxidation in a variety of food products (Birch et al 2001; Rababah et al 2004). Plant phenolics and flavanoids have known to effectively minimize lipid oxidation in lipid based food products (Rababah et al 2004). Nakatani (2001) found that the spices ginger and turmeric from the *Zingiberaceae* family have greater antioxidant activity than α-tocopherol. Further, Shobana and Naidu (2000) also reported that the antioxidant activity of ginger extracts were retained even after boiling for 30 min at 100°C, strongly indicating that the constituents were resistant to thermal denaturation.

Frankel (1992) observed and stated that rosemary extracts contain a large number of compounds including carnosic acid, carnosol, and rosmarinic acid and provide a major source of natural antioxidants used commercially at present in foods (Loliger, 1983). Several studies have confirmed that leafy spices, especially those belonging to the *Labiatae* family such as rosemary, sage, oregano, and thyme, show a strong antioxidant activity, greater than synthetic antioxidants like BHA and BHT (Hirasa and Takemasa 1998).

Fish oil is comprised of abundant long chain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A considerable amount of research during the past three decades on fish oil containing ω-3 fatty acids EPA and DHA have concluded that they can reduce the risk factors associated with severe degenerative diseases including cancer, cardiovascular disorders and other inflammatory conditions (Perez-Mateos et al, 2004). Lipid oxidation, however, limits the utilization of these fish oils in processed foods and as supplements in fortified food. PUFA's are prone to oxidize rapidly and the oxidized polyunsaturated fatty acids have been reported to induce aging and carcinogenesis (Sasaki 1996; Miller 1995). Despite these shortcomings, increased demand of PUFA for better nutrition and health benefits has triggered a need for a thorough investigative effort on increasing the stability of fish oil against oxidation and formation of off-flavors.

A lot of research has been conducted on use of spices ginger, rosemary, turmeric and oregano and their role in retarding oxidation in oils (Martinez et al 2001; Sullivan et al 2005 and Murcia et al 2001). However except for a few studies, it is very hard to find research on the use of these spices in a system susceptible to oxidation, like fish oil. One such study conducted by Sullivan et al (2005) evaluated the use of naturally occurring antioxidants on

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fish oil systems, extracted from livers of white pollock or cod. The study was conducted over a 16-day time period at a temperature of 30°C. The results demonstrated that the cod liver oil and pollock liver oil samples containing rosemary or tea catechins (0.5 %) had lower levels of lipid oxidation in comparison to the samples containing other natural antioxidants. However this and other studies were inconclusive regarding information on the oxidation of fish oil at higher temperatures resembling real time cooking or processing conditions. So comprehensive research is needed to investigate and confirm the use of spices in fish oil at cooking conditions to study their usefulness in maintaining the stability of fish oil.

According to previous research on natural antioxidants, the efficiency of an antioxidant depends on the tested temperatures used for oxidation (Frankel 1993). It has been reported that rosemary, ginger and turmeric are useful as antioxidants with a thermal resistance stronger than BHA and BHT (Murcia et al 2004; Shobana and Naidu 2000; Yanishlieva and Marinova 2001); the synthetic antioxidants that degrade easily at higher temperatures (Yaakob et al 2000). However all these above referred studies were conducted on food grade oils and not specifically fish oils. And, it is equally important to conduct stability tests at temperatures closed to ambient due to the high tendency of fish oil to undergo rapid oxidation.

In the present study, antioxidant activity of ginger, turmeric, rosemary and oregano extracts in methanol were evaluated at oxidation during heating at 150°C and 175°C and also during a 6-day accelerated storage study at 60°C. The heating temperature (150°C and 175 °C) works as ideal scenario for cooking conditions with oxygen and air resembling deep frying conditions (Yanieshleiva and Marinova 2001). According to the literature on lipid based foods including oils, the accelerated storage at 60 C for 6 days is equivalent to 25° C

for 6 months (Osman 2004). The antioxidant activity of each extract was determined by evaluating the retention of polyunsaturated fatty acids, DHA and EPA, during oxidation of the menhaden fish oil.

3.2 Materials and Methods

- **Materials and Chemicals**

Ginger and turmeric were purchased from a local market. Rosemary and oregano plants were obtained from the Department of Horticulture, Louisiana State University, Baton Rouge, Louisiana. Menhaden fish oil (Lab use) was purchased from Sigma Aldrich Company. The solutions for fatty acid test; BCl_3-methanol and 2-2 dimethoxypropane were purchased from Supelco (Belafonte, PA), Heptadecanoic acid (C17:0) was purchased from the Sigma Aldrich (St. Louis, MO). Other chemicals and solvents used were HPLC grade.

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┌─────────────────────────────────────────┐
│          Freeze dried spices             │
│ Ginger, Oregano, Rosemary and Turmeric   │
└─────────────────────────────────────────┘
                    │
                    ▼
         ⬭ Extraction with methanol ⬭
                    │
                    ▼
        ┌─────────────────────────┐
        │     Mixed with fish oil  │
        └─────────────────────────┘
                    │
                    │   Heating at 150 and 175°C and
                    │   Storage at 60°C for 6 days
                    ▼
        ┌─────────────────────────┐
        │   Measuring DHA and EPA  │
        │        by GC-FID         │
        └─────────────────────────┘
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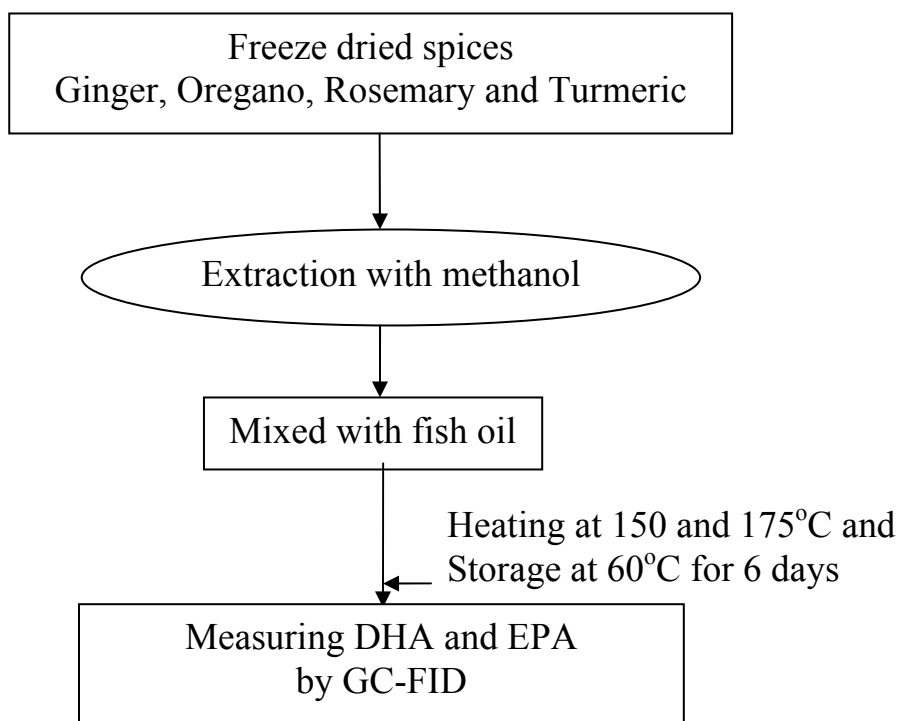
Figure 9: Flow Diagram Showing Menhaden Oil Oxidation Study

- **Extraction**

All spices were ground and freeze dried for 48 hours at -80°C before use. One gram of plant material was weighed and extracted with 100 mL of methanol for 2 hrs at 30°C. The methanol supernatant was separated by centrifuging at 1500 x g for 15 min. The residual was re-extracted with 100 mL of methanol. The supernatant was separated using the same centrifugation and combined with the previous. Each dried extract was obtained after all methanol solvent was evaporated by a centrifuge vacuum evaporator (CentriVap Mobile System; Labconco, Kansas City, MO).

- **Sample Preparation**

Each of the spice extract was prepared by re-dissolving 0.1 g of the dried extract in 10 mL of methanol solvent. Further all the extracts of ginger, oregano rosemary and turmeric were homogenized with two grams of menhaden oil in concentrations of 1 %, 2.5 % and 5 % (w/w). One gram of the mixture of each spice extract and fish oil at different concentrations was added to 15-mL test tubes. After evaporating the solvent, the oil samples with the extracts were heated at 150 and 175°C sand bath for 30 min and incubated at 60°C for 6 days. One gram of menhaden oil without adding any extracts was weighed in test tubes and used as a control group. For incubation at 60°C, test tubes with the mixture were taken on each day up to 6 days; the analysis was performed as above. The experimental study was conducted at each of three concentrations during heating and incubation storage condition, in triplicate.

- **Determination of DHA and EPA Oxidation after Heating and Incubation Storage**

One hundred μL of fish oil sample along with spice extracts was added to each test tube (13 ×100 mm). Heptadecanoic acid (C17:0) (1 mg/mL), as an internal standard for the DHA and

EPA analysis, was added to each tube. Except the control sample, the other fish oil samples with spice extracts were subject to heating and incubation conditions.

After adding 2 mL BCl_3-methanol and 1 mL 2, 2'-dimethoxypropane, all test tubes were capped and incubated in a 60°C water bath for 10 min to perform the derivatization of fatty acid methyl esters. Then, 1 mL hexane and 1 mL water were added to the tubes and vortexed for 30 seconds. The upper hexane layer was transferred to another tube, dried with anhydrous sodium sulfate and transferred to a GC vial.

A gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Palo Alto, CA) with a FID detector was used to determine DHA and EPA concentration. Helium was used as a carrier gas with a column flow rate of 1.2 mL/min. The injection volume was 5 μl and the split ratio was 1:100. The injector and detector temperature was 250 and 270°C, respectively. The oven temperature program was set to hold at 50°C for 3 min and then increased at 4.0°C/min to 250°C. The column was a Supelco SP2380 (30m × 0.25mm) (Bellefonte, PA). The concentrations of DHA and EPA were calculated using the C17:0 internal standard as a reference. The percentage of DHA and EPA that remained in each tube was obtained by comparing its final concentration to its original concentration.

3.3 Statistical Analysis

For each treatment group, the experiments were performed in triplicate of 3 at three different concentrations at each of the heating and incubation conditions. The experiments were conducted twice (2 sets). The data was analyzed by ANOVA and then Tukey pair wise comparison test was used to obtain the estimates of the values for different treatment with multiple comparisons to determine significant difference at $p < 0.05$.

3.4 Results and Discussion

- **Inhibition of Oxidation of DHA and EPA in Menhaden Fish Oil During Heating**

Inhibition capabilities of the four spice extracts in preventing DHA and EPA oxidation at heating temperatures of 150°C and 175°C with different concentrations are shown in Table 1 and 2. All the extracts demonstrated significant capabilities at each study condition. Without adding any extract, the DHA and EPA level in Menhaden fish oil were decreased respectively to 13.0 and 15.9 % of original concentration after heating at 150°C for 30 min. At 150°C, oregano extract had the strongest inhibition of oxidation as compared to the other spice extracts and retained 38.8, 57.6, and 65.9 % of DHA at concentrations of 1, 2.5, and 5%. The inhibition values for EPA at these concentrations were 44.6, 61.6 and 69 %. The oregano extracts demonstrated greater retention values for DHA and EPA at 2.5 % and 5 % concentrations when compared with ginger, turmeric and rosemary. Oregano methanol extract was found to be effective against lipid oxidation when used with lard and demonstrated higher activity than rosemary extract (Economou et al 1991), and the rate of the reaction decreased slightly with the increase in plant extract concentration, which was the opposite of what we observed in our study. The study conducted by Economou et al (1991) was different with respect to the model (lard) and also during storage at 75°C temperature.

In the rosemary extract treatment group, 20.8, 56.9, and 45.8 % of DHA were retained at concentrations 1, 2.5, and 5 %, respectively, at 150°C. Antioxidant capability of rosemary is mainly due to the chemical rosmarinic acid and also due to other chemicals like carnosic acid, carnosol, which are the major flavonoids contributing towards high antioxidation potential (Loliger 1983; Frankel 1995).

Table 1: DHA (%) Retained in Menhaden Fish Oil after Heating at Different Temperatures

Temp	Turmeric (% conc.)				Ginger (% conc.)				Rosemary (% conc.)				Oregano (% conc.)			
	0	1	2.5	5	0	1	2.5	5	0	1	2.5	5	0	1	2.5	5
150	13.0 ± 1.4^{G}	20.6 ± 1.4^{FG}	37 ± 0.5^{DE}	46 ± 2^{CD}	14.5 ± 1^{FG}	24 ± 3.0^{F}	34 ± 1.6^{E}	47.5 ± 0.6^{BC}	15 ± 0.6^{FG}	20.8 ± 1^{FG}	57 ± 5^{AB}	45.8 ± 8^{CD}	16 ± 0.6^{FG}	38.7 ± 2^{DE}	57.6 ± 2^{A}	66 ± 6^{A}
175	5.2 ± 0.5^{G}	14 ± 0.9^{FG}	27.7 ± 1^{DE}	36 ± 1.4^{CD}	4 ± 0.2^{FG}	17.1 ± 1.2^{F}	34 ± 0.6^{E}	18.6 ± 1.2^{BC}	2.6 ± 1.3^{FG}	19 ± 3^{FG}	39.6 ± 7^{AB}	37.5 ± 1.2^{CD}	6.5 ± 1^{FG}	16 ± 1^{DE}	27.5 ± 1.6^{A}	41.3 ± 2^{A}

*Significant difference ($P < 0.05$) is expressed by the different letters in each row

Table 2: EPA (%) Retained in Menhaden Fish oil after Heating at Different Temperatures

Temp	Turmeric (% conc.)				Ginger (% conc.)				Rosemary (% conc.)				Oregano (% conc.)			
	0	1	2.5	5	0	1	2.5	5	0	1	2.5	5	0	1	2.5	5
150	14.8 ± 1.4^{G}	22.4 ± 1.4^{FG}	38 ± 1^{DE}	47.2 ± 1.6^{CD}	16.6 ± 1^{FG}	28 ± 3^{F}	39 ± 1.6^{E}	55 ± 0.6^{BC}	13.1 ± 0.6^{FG}	25 ± 1^{FG}	58 ± 5^{AB}	46.8 ± 8^{CD}	18.4 ± 0.6^{FG}	44.6 ± 2.5^{DE}	61.6 ± 2^{A}	69 ± 6^{A}
175	7.0 ± 0.5^{G}	15.8 ± 1^{FG}	29 ± 1^{DE}	36.7 ± 1.4^{CD}	6.5 ± 0.2^{FG}	20.8 ± 1^{F}	40.6 ± 1^{E}	21.1 ± 0.6^{BC}	6.5 ± 1.3^{FG}	24 ± 2.7^{FG}	43 ± 7^{AB}	41 ± 0.6^{CD}	8.4 ± 0.6^{FG}	18.8 ± 1^{DE}	30 ± 1.7^{A}	44.2 ± 1^{A}

*Significant difference ($P < 0.05$) is expressed by the different letters in each row

In our present study, an interesting phenomenon was observed that the capability of rosemary extract did not increase with each higher concentration. At both 150°C and 175°C, the rosemary extract at 2.5 % concentration exhibited high retained values for DHA and EPA as compared with 5% concentration. This phenomenon may be due to the fact that the rosemary spice extract demonstrates marked variations in antioxidant capability in different lipid systems (Frankel 2000), and that the structure of rosemary may be more complicated when compared with extracts of oregano and turmeric. Other components in the rosemary extract could have pro-oxidation effect and that effect could suppress its antioxidant capability when their concentrations increase to a certain level. For example, carotenoids are antioxidant but not stable at relatively higher concentration in the presence of oxygen and degraded to hydroperoxide compounds due to their long conjugated polyene backbone (Subagio and Morita. 2001).

At 150°C, turmeric extract group retained 20.6, 37.0, and 46.0 % of DHA at concentrations of 1, 2.5, and 5%. Ginger extract was capable of inhibiting oxidation of DHA and the retention values were 23.9, 34 and 47.5 % and 28, 39 and 55% for EPA for the concentrations 1, 2.5 and 5 % respectively. The results showed decrease in inhibition by ginger with a decrease in activity when temperature increased to 175°C. They were consistent with regard to the effectiveness of ginger in retarding oxidation of fatty acids in different lipid systems (Hiroe and Nakatani 1993, Zia ur Rehman et al 2003).

The same study conducted by Zia ur Rehman et al (2003) has also confirmed the effectiveness of ginger comparable to synthetic antioxidants BHA and BHT. Tiwari et al (2006) also reported that turmeric has the capability to stabilize oil at the cooking conditions and showed higher values when compared with ginger extracts. Several

studies have also confirmed that leafy spices, especially those belonging to the Labiatae family such as rosemary, sage, oregano, and thyme, show a strong antioxidant activity (Hirasa and Takemasa, 1998; Martinez et al 2001).

At the heating temperature of $175^{o}C$, without adding any extract, the DHA in Menhaden fish oil was reduced to $2.6 - 6.5$ % of the original concentration and was approximately two to four times lower than heating at $150^{o}C$. Also, the retained DHA in each treatment group at $175^{o}C$ was lower than at $150^{o}C$. The pattern of the changes with increasing concentration in each group at $175^{o}C$ was similar to its pattern at $150^{o}C$. At 5 % concentration, oregano and turmeric extract had 41.3 and 35.8 % of retained DHA and 44.2 and 36.7% of EPA respectively.

The results for ginger extracts at $175^{o}C$ showed retention values of 17.1, 34.3, 18.6 % for DHA and 20.8, 40.6, 21.1 % for EPA over 1 to 5 % increasing concentrations. These results reveal that the trend was not similar to that observed at $150^{o}C$ for ginger in particular at the 5 % concentration. A study conducted by Lee et al (2003) on Vitamin C, has reported that the extract may exert a prooxidant activity under certain conditions, particularly in the presence of transition metal ions or alkali. The results of the inhibition of PUFA oxidation by ginger extracts were significantly different than the control samples. Previous studies have reported problems with physical properties of synthetic antioxidants like BHA and BHT due to their high volatility and instability at elevated temperatures (Dapkevicius et al 1998). Further, Yanishlieva and Marinova (2001) have reported that methanolic extracts of ginger showed high inhibition against oxidation of peanut oil and the process was superior to commercial antioxidants like BHT.

For all four extracts, the intensity of DHA and EPA oxidation was increased with a heating temperature increase from 150°C to 175°C. Also, the antioxidants in these extracts may not be stable and could have caused degradation at the higher temperature. Tiwari et al (2006) found that the stabilities of ginger and turmeric powder vary with the cooking conditions. They also stated that higher temperatures could be related to the denaturation of antioxidants in those samples, causing a decrease in antioxidant activity. Shobana and Naidu (2000) studied the stabilities of antioxidant activity of ginger, garlic, pepper and cinnamon and found that they were heat stable in the temperature range of 105°C–165°C. Mansour and Khalil (2000) have underlined the effectiveness of ginger in inhibiting lipid oxidation. However, the same study also reported that increasing the time of boiling resulted in a significant decrease in the antioxidant activity of the freeze-dried extracts; the study in discussion reported reduction in the antioxidant activity of ginger rhizome extract to about 28 % after 120 min heating at 100°C. Ginger extracts after subject to heating may affect the volatile oil content and this phenomenon may be responsible for the reduction in antioxidant activity.

The results obtained in our study are very significant due to the heating temperature (150°C and 175°C) used, which resemble actual cooking conditions (deep frying), to study the oxidation of menhaden fish oil. This study showed significant antioxidant capability for all spice extract groups at 150°C and 175°C and protected the PUFA's in menhaden fish oil from oxidative degradation. The reason for retention of antioxidant capability even during heating may be due to the fact that the polyphenols, when in an intermediate oxidation state are subject to heat induced oxidation and may exhibit higher radical scavenging efficiency than the non-oxidized ones (Kikugava et al

1990). In the past, effect of methanolic extract of spices has never been investigated in a fish oil model; however, a study with a methanol extract from grains of oats has demonstrated effectiveness in preventing DHA oxidation during heating (Sun et al 2006).

- **Stability of DHA and EPA in Menhaden Fish Oil during 60°C Incubation Storage**

Osman et al (2003) have reported that oxidation test conducted at (60°C-65°C) incubation in an oil based medium is normally equivalent to one month of storage time at room temperature; therefore we used 60°C incubation storage in this study for evaluating antioxidant capabilities of the four spice extracts in preventing Menhaden fish oil. The retained percentage of DHA and EPA in Menhaden fish oil without adding any extracts decreased approximately to 50% after 1 day of storage and to an undetectable level after 3 days of storage (Figure 11). Spice extract from rosemary and turmeric at three different concentrations exhibited significant capability in retaining DHA and EPA at the end of 6-day storage period when compared with other extracts.

However, the results were different from the heating study for oregano; here the retained DHA for oregano extract group was the lowest at each concentration after 6-day of storage. It only retained 18.8% of DHA while 46.2% of DHA was retained in turmeric extract at a concentration of 5%. Interestingly, rosemary extract showed significantly higher capability in preventing DHA oxidation during storage. Similar to the heating study, its capability was not enhanced with increasing concentration. The retained DHA and EPA (%) with 2.5 % rosemary extract were higher than either 1 or 5 % concentration. After the 6 th day of storage, 83.7 % of DHA and 84.4 % of EPA was retained in Menhaden fish oil at the 2.5 % concentration for rosemary extract and it was significantly higher than the ginger, turmeric and oregano extracts.

60
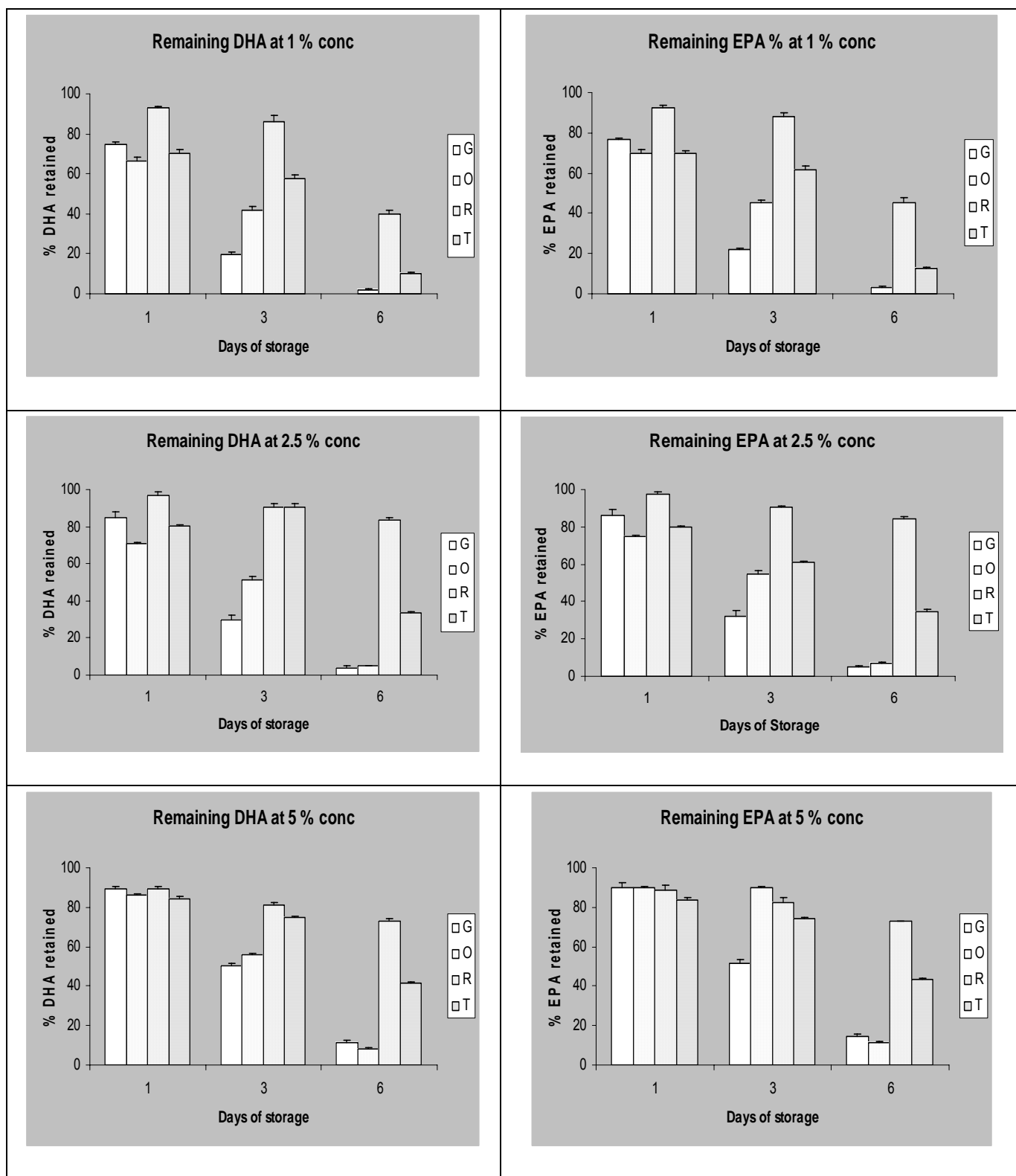
Figure 10: Docahexanoic acid and Eicosapentanoic Acid Retention by all Spice Extracts during 60 °C Storage at 1, 3 and 6 th day of storage, G-ginger extract, O-oregano extract, R-rosemary extract, T-turmeric extract.

61
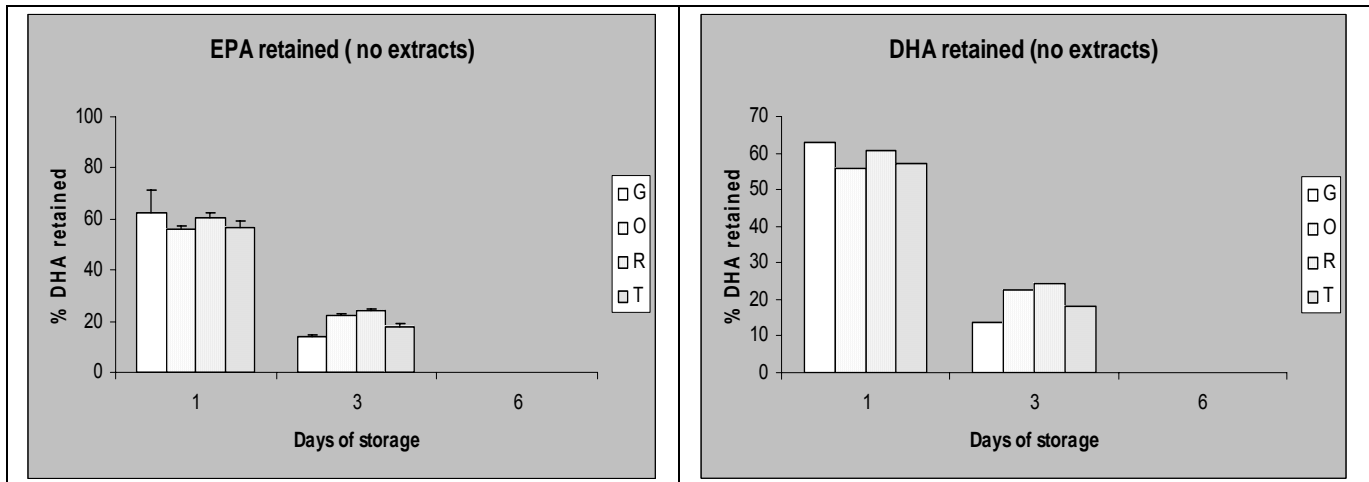
Figure 11: Heated Control Values During 60 ^{o}C (1, 3 and 6 th day of Storage) for 4 Set of Controls: G-ginger extract, O-oregano extract, R-rosemary extract, T-turmeric extract

In a study conducted by Ozcan (2003) with peanut oil, the rosemary methanol extract at 4 % (w/v) during a storage study demonstrated the highest antioxidant activity when compared with other spices like sage and sumac extracts. Another study used an oven storage at 63 ^{o}C over a 7 day period, and reported antioxidant efficiencies of rosemary in reducing oxidation of soybean oil, the activity was similar to the mixture of synthetic antioxidants of BHT +BHA (Almeida and Regitano 2000).

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CHAPTER 4. EVALUATION OF ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF SPICE EXTRACTS FROM GINGER, OREGANO, ROSEMARY AND TURMERIC

4.1 Introduction

The food industry has recently focused on the use of natural preservatives derived from fruits, vegetables, spices and herbs. Plant materials such as spices and herbs are attracting a lot of interest from food scientists due to the abundant presence of phenolic compounds or phytochemicals (Kahkonen et al 1999). The phenolic compounds in these spice plants are primarily responsible for the antioxidant potential, antibacterial and other preservative properties. The phenolic compounds are reported to act as biological response modifiers in plants, often referred to as secondary metabolites (Maria Cruz et al 2005, Berch et al 2001). Synthetic antioxidants are extensively used in foods; however, the use of synthetic antioxidants such as BHT, BHA, is governed by strict regulations due to toxicological limitations of and other safety concerns (Hettiarachchy et al 1996). The concerns and health hazards in relation to these synthetic antioxidants have encouraged the scientific community to explore natural substitutes. Past research has confirmed that the consumption of food enriched with phytochemicals from plant extracts can protect against major disorders including cancer and other cardiovascular diseases. Antioxidant research has generated a lot of interest in the past decade that is reflected in the four fold increase in number of articles published from 1684 in 1993 to 6510 during this decade (Huang et al 2005).

The antioxidant activity of phenolics and flavanoids in spice plants is due to their ability to scavenge free radicals, redox potential, transition metal chelating capacity, and singlet oxygen quenching capacity (Rababah et al 2004). The free radicals or the reactive

oxygen species produced during oxidation reactions are responsible in various degenerative diseases including cancer, atherosclerosis and arthritis (Thomas et al 1995). The phenols obtained from the plant extracts like spices have been found to be more powerful than other natural antioxidants like vitamins C, vitamin E, and β-carotene (Vinson et al 1995).

The study of individual phenolic contents is vital because the phenolic compounds are considered to be a major group of compounds that mainly contribute to the antioxidant activity of plant extracts including those of spices and herbs. It is confirmed from previous research that the information on the antioxidant activities of plant extracts and their individual phenolic compounds can be utilized as an important criteria to understand the prevention of lipid oxidation in a variety of food products (Rababah et al 2004).

A large number of phenolic compounds with strong antioxidant activity have been identified in various spice and herbs extracts (Nakatani 1997). Decker et al (2005) have reported that phenolic compounds are the source of major antioxidants in a crude plant extract, further it is also recommended to study the phenol content data of the extract along with the antioxidant activity in order to study and compare the antioxidant capacity of the samples. A direct correlation usually exists between phenolic content and the antioxidant activity of the plant extracts as reported in previous research and is considered an important factor taken in to consideration while characterizing spice extracts by different assays (Kaur and Kapoor 2002; Ivanova et al 2005; Hinneburg et al 2006).

Antioxidant activity assays consist of accelerating oxidation in a lipid system,

usually by heat, and then monitoring oxygen consumption, substrate loss, or product formation. The results from oxidation tests can vary based on several factors affecting oxidation; which includes temperature, oxygen pressure, metal catalysts, fat composition, and form of fat (Frankel et al 1993). The antioxidant activity may be reflected by radical scavenging ability of the phenolic compounds in the extracts. These tests usually resemble the basic mechanisms involved in lipid oxidation that measure the reduction of stable radicals or radicals generally represented by the use of a stable DPPH radical (Martinez Tome 2001). The radical scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is considered an easy and best way to evaluate scavenging activity of antioxidants; this radical compound is very stable and is not required to be generated like other radical scavenging assays (Suhaj et al 2006).

Despite the extensive data available on the antioxidant activity of phenolics in the spice extracts, research on the effect of heating on the phenolic content and the antioxidant activity of spices is rather rare and inconclusive. Previous studies by Tiwari et al (2001) have indicated an increase in antioxidant activity of turmeric when subject to heating at 120°C. The study conducted by Dewanto et al (2002) reported an increase in antioxidant activity of lycopene in tomato when subjected to thermal processing and a subsequent enhancement in the nutrional value. Another research work conducted by Shetty and Randhir (2004) reported that microwave heating produced acute heat stress in plant cells of fava beans causing an increase in the phenolics with a corresponding stimulation of the antioxidant activity. Jaramillo et al (2003) studied the effect of thermal treatment on the concentration, antioxidant activity and the phenolics of the carotenoids;

the results showed a positive effect of the thermal treatment on the carotenoids with a subsequent increase in their phenolic antioxidants.

Anwar and Nawar (1991) conducted a study to examine stability of phenolic antioxidant in different synthetic antioxidants such as BHT, PG, BHA and TBHQ. Studies on the effect of heating (150-175°C, simulating cooking conditions) and accelerated storage (60°C incubation) on the phenolics in spices have not been investigated in the past research studies. The effect of heating on the extraction yield, total phenolic content and antioxidant activity may provide valuable and useful information on the use of these spices as they are often subjected to thermal pre-processing and long term storage conditions during the preservation process.

The present study was conducted with 4 spices, two each from *Gingerbacie* and the other two from *Labiatae* families. These spices are extensively used as culinary herbs but little information is available on the phenolic antioxidants of these spice extracts. In the present study, we evaluated the antioxidant activity using 2,2-diphenyl-2-picrylhydrazyl (DPPH)-scavenging ability, and phenolic content as measured by the Folin-Ciocalteu reagent. The objectives of this study were;

1. Evaluate the antioxidant activity and total phenolic content of spice extracts from ginger, oregano, rosemary and turmeric;

2. Evaluate the effect of heating and storage conditions on the antioxidant activity, extraction yield and phenolic content of the spices;

3. Determine the effect on the changes in profile of major phenolic compounds in the four spice extracts after subject to heating and storage conditions by GC-MS.

4.2 Materials and Methods

- **Materials and Chemicals** Ginger and Turmeric were purchased from a local market. The rosemary and oregano plants were obtained from the Department of Horticulture, Louisiana State University, Baton Rouge, Louisiana. The spices were stored at -80 $^{\circ}$C before use. DPPH, 6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid (Trolox), Folin-Ciocalteu reagent, and catechin were purchased from the Sigma Aldrich (St. Louis, MO). Other chemicals and solvents used were HPLC grade.

- **Extraction** All plants were ground and freeze dried for 48 hours before extraction. One gram each of freeze-dried spices were weighed and extracted with 100 mL of methanol for 2 hrs at 30°C. The methanol supernatant was separated by centrifuging at 1500-x g for 15 min. The residual was re-extracted with 100 mL of methanol. The supernatant was separated using the same centrifugation and combined with the previous extract. The dried extract of each spice was obtained after all methanol solvent was evaporated using a centrifuge vacuum evaporator at 50°C for 4 hours (CentriVap Mobile System; Labconco, Kansas City, MO).

- **Heating Treatment** The freeze-dried spices as obtained from above were heated at 150°C and 175°C for 30 min and then allowed to cool at room temperature. They were then extracted in the same manner with methanol and the dried extracts were obtained as explained above. The spice extracts were evaluated for antioxidant activity and phenolic content. For the storage study, spices were stored at 60°C in an incubator and samples were drawn at the 1 [st], 3rd and 6th day of storage and dried extracts were obtained after extraction with the solvent methanol.

- **Antioxidant Activity by DPPH** The concentration of DPPH solution used in the study was 0.025 g in 1000 mL of methanol. The spice extract solution for the DPPH test was prepared by re-dissolving 0.10 g of each dried spice extract in 10 mL methanol. Two mL of the DPPH solution was mixed with 5, 10, 25 and 50 µL of the spice extract in methanol solution and transferred to a cuvette. The reaction solution was monitored at 515 nm for 30 min at room temperature using a UV-Visible SpectraMax Plus[384] spectrophotometer (Molecular Devices, Sunnyvale, CA). The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Inhibition%= $(Abs_{t=0\ min} - Abs_{t=30\ min})/\ Abs_{t=0\ min} \times 100$

Where $Abs_{t=0\ min}$ was the absorbance of DPPH at zero time and $Abs_{t=30\ min}$ was the absorbance of DPPH after 30 min of incubation.

The inhibition percentage of the absorbance of DPPH was plotted against each of the spice extract solutions to obtain a regression line. Trolox (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of spice extracts solutions to the Trolox equivalent antioxidant activity. The ratio between the slopes of the regression lines of spice extraction solution and the Trolox solution was defined as the Trolox equivalent antioxidant activity (TEAC).

- **Phenolic Content by Folin Ciolteau Reagent** The total phenolic content of spice extract was determined using Folin-Ciocalteu reagent (Velioglu et al 1998). Folin-Ciocalteu reagent was diluted 10 times with deionized water. Dried spice extract (0.1 g) were re-dissolved in 10 mL methanol and 0.1 mL of this spice extract solution was mixed with 0.75 ml diluted Folin-Ciocalteu reagent. The reaction solution was left at

room temperature for 5 min. Then 0.75 mL of sodium bicarbonate solution (60 g/L) was added. The mixture was incubated at room temperature for 90 min and filtered through a 0.45 μm syringe filter (Pall Corporation, Ann Arbor, MI). The absorbance of the solution was determined at 750 nm using a UV-Visible SpectraMax Plus[384] spectrophotometer (Molecular Devices, Sunnyvale, CA). Catechin was used as a standard to prepare a standard curve. The total phenolic compound content of spice extracts was calculated and expressed as μg catechin equivalent / gram of extract.

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┌─────────────────────────────────────────┐
│              Freeze dried                │
│ Ginger, Oregano, Rosemary, Turmeric      │
└─────────────────────────────────────────┘
                    │
                    │   Heating at 150° C and 175° C
                    │   and Storage at 60° C for 6 days
                    ▼
┌─────────────────────────────────────────┐
│          Extraction with methanol         │
└─────────────────────────────────────────┘
         │                          │
         ▼                          ▼
┌──────────────────┐    ┌──────────────────────┐
│ Antioxidant      │    │  Phenolic content by │
│ activity         │    │  Folin-Ciolteau      │
│ By DPPH (TEAC)   │    │  reagent             │
└──────────────────┘    └──────────────────────┘
```
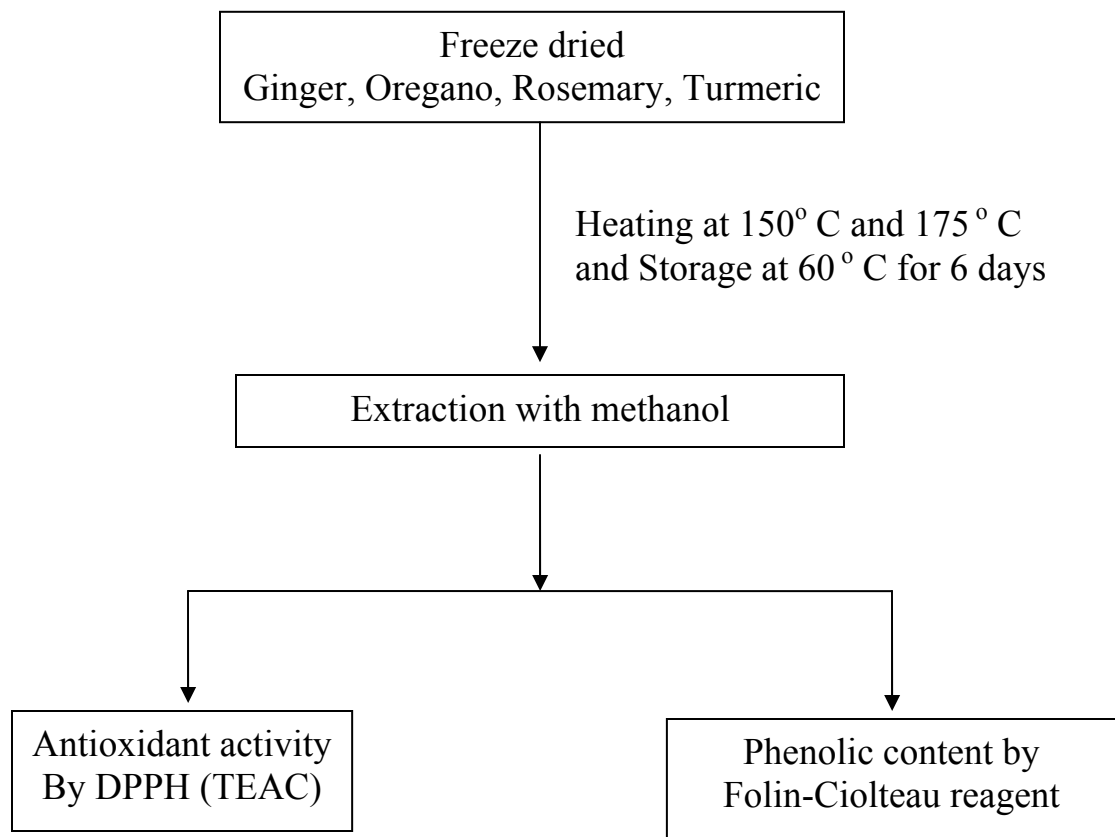
Figure 12. Flow Diagram of Evaluation of Antioxidant and Phenolics in the Spice extracts

- **Study of the Changes in Phenolics in the Spices during Heating and Storage by GC-MS**

All four spices after heating and storage were extracted with methanol and the dried extracts were obtained as described above. The phenolic compounds in the spice extracts were identified using NIST (National Institute of Standard and Technology) mass spectral search library software.

The dried extracts of each spice (0.5 g) were heated in a round bottom flask at 100 $^\circ$ C for 20 min. The flask was capped with a cork and a SPME fiber (Carboxen/DVD/PDMS) was inserted and exposed to the headspace to adsorb the volatiles 20 minutes . Once the volatiles from the spice extracts were trapped on the SPME fiber, the fiber was desorbed to a GC injection port for 6 min at 270 $^\circ$C. The oven temperature was held at 45°C for 5 min then ramped at 5 $^\circ$C/min to 220°C for a total run time of 40 min. Electron Ionization was used at 70 eV source with temperature at 150°C. The MS was operated in SIM mode at 40-400 *m/z* and was run in the selected ion monitoring mode.

4.3   Statistical Analysis

For each treatment group, the experiments were performed in triplicate and the experiments were conducted twice (2 sets) at two heating and one incubation storage conditions. The data were analyzed with ANOVA and studentized Tukey test was used to determine significant difference at $p < 0.05$.

4.4 Results and Discussions

- **Total Phenolic Content of the Non-Heated Freeze Dried Spices**

The total phenolic content of the spices were expressed per gram of freeze dried spices. The extraction yield (%) values for all four spice extracts were 8.4 % for oregano, 19.4 % for ginger, 27 % for rosemary and 31.7 % for turmeric. The phenolic compounds in the spices are one of the important factors responsible for the antioxidant property that is

represented by total phenolic content as determined by the Folin Ciocalteu method (Hinneburg et al 2006). The content of total phenols in the samples was expressed as catechin equivalent. The highest phenolic content for the non heated extracts was demonstrated by turmeric at 27 μg of catechin/ gm of freeze material (Table 3), while the lowest phenolic content was observed for oregano at 4.8 μg of catechin/ gm of freeze dried material. The overall total phenolic content in decreasing order was turmeric > rosemary > ginger > oregano.

Table 3: Total Phenolic Content of Spices During Heating Expressed as μg Catechin Equivalent/gm of Freeze Dried Material

Temp of heating	Ginger	Oregano	Rosemary	Turmeric
Non-heated	13.3 ± 0.42 [c]	4.8 ± 0.33 [f]	16.0 ± 0.35 [b]	27 ± 0.95 [a]
150 °C	17.2 ± 0.49 [b]	2.0 ± 0.23 [g]	7.22 ± 0.29 [e]	15.4 ± 0.69 [b]
175 °C	5.4 ± 0.15 [f]	2.4 ± 0.12 [g]	7.0 ± 0.18 [d]	14.8 ± 0.31 [b]

*Significant difference (P<0.05) is expressed by the different letters in all rows and columns.

In this study, ginger and oregano showed lower total phenolic content values as compared to rosemary and turmeric. Each spice contains different phenolic compounds; and the different phenolic compounds exhibit different responses by Folin-Ciocalteu method (Kahkonen et al 1999).

Antioxidant capacity of substance may not be solely interpreted by total phenolic content due to the presence of abundant specific phenolic components and their peculiar structural characteristics (Shahidi and Wanasundara 1992). The phenolic content of oregano was lowest among the four spices. The total phenolic content by the Folin-Ciocalteu procedure alone is not sufficient enough to give an exact measure of the quality of the phenolic constituents in case of the oregano extracts (Kahkonen et al 2004).

- **Phenolic Changes in the Freeze Dried Spices After Heating**

After heating at 150°C, the extraction yields showed a decreasing trend as compared with non heated spices and were 3.2 % for oregano, 7.3 % for ginger, 8.7 % for rosemary and 11.1 % for turmeric (see table in Appendix A). These yields were reduced approximately to 1/3 of the original value of the unheated samples. The gradual increase in the extracted phenolics for oregano and rosemary after heating may explain the upsurge in the antioxidant activity during the menhaden fish oil oxidation study discussed in previous Chapter 3, especially for oregano, which showed an increase in phenolics even more than the non heated extracts.

With an increase in the heating temperature from 150°C to 175°C a further decrease in extraction (%) yield was observed for ginger (4.9 %) and turmeric (8.6 %). However, the spices oregano and rosemary showed an increase in % yield with values 9.2 % and 9.8 % respectively. The rosemary has a tendency to form of diterpines and triterpenes during heating at elevated temperatures up to 200°C, and some of these thermal degradation products show antioxidant capabilities (Schwarz et al 1992).

At 150°C heating, there was a trend for increased phenolic content in ginger (13.3 µg/g to 17.2 µg/g). All the other spices, however, showed a decreasing trend in phenolics, with turmeric at 27.0 µg/g to 15.4 µg/g, rosemary at 16.0 µg/g to 7.22 µg/g and oregano at 4.8 µg/g to 2 µg/g. The carotenoids in cactus pears showed high extractability of pigments after thermal treatment and caused a decrease in the phenolic content with no such effect on the antioxidant activity (Jaramillo et al 2003).

With the heating of the spices to 175 °C there was a further decline in the total phenolics for all the spice tissues when compared with 150° C. The turmeric spice led the group with the highest phenolic content of 14.8 µg of catechin/ gm of freeze dried

material. The turmeric spice group showed significantly high phenolic content when compared with other spices at both the temperatures, except for ginger at 150°C. The natural antioxidants like lycopene and β-carotene are reported to be very heat stable even after intense heat treatments such as cooking (Elkins 1979, Yen and Hwang 1985).

Shobana and Naidu (2000) reported a higher heat stability for turmeric when compared with ginger, and found that both the ginger and turmeric were heat stable in the temperature range of 105–150°C. The study was conducted with powdered turmeric and ginger and their oils. Hinneburg et al (2006) have stated that some spices contain water soluble non phenolic material to a great extent; therefore their extracts have a high content of non Folin-Ciocalteu reactive substances.

The other three spices showed a decrease in phenolic content after heating at 175°C; oregano 50%, ginger 30% and rosemary showed 1% reduction when compared with 150 °C. Madsen and Bertelson (1995) have reported a reduction in the phenolic antioxidants of most of the spices with heating, as the steam sterilization reduces the volatile oil content which affects the antioxidant phenolics when extracted with different solvent interactions.

- **Phenolic Changes of the Freeze Dried Spices after Incubation Storage**

After the 60°C incubation storage, the extraction yields were reduced for all the spices at the end of 6 day period with 5.7 % for oregano, 13.2 % for ginger, 11.2 % for turmeric and 12.4 % for rosemary.

Table 4: Total Phenolic Content of Spices during Incubation Storage (60°C) Expressed as µg Catechin Equivalent/gm of Freeze Dried Material

Days of Storage	Ginger	Oregano	Rosemary	Turmeric
1	8.7 ± 0.25 [d]	4.4 ± 0.31 [e]	12.0 ± 0.45 [c]	17.9 ± 0.41 [a]
3	7.2 ± 0.22 [de]	3.9 ± 0.12 [e]	11.4 ± 0.43 [c]	16.3 ± 0.96 [b]

6	7.5 ± 0.17 [d]	1.7 ± 0.10 [dc]	10.7 ± 0.37 [d]	15.7 ± 0.40 [b]

*Significant difference ($P<0.05$) is expressed by the different letters in all rows and columns.

At the end of 6 day storage period, highest phenolic content was reported for turmeric; 15.7 µg of catechin/ gm of freeze dried material while the lowest phenolic content was observed for oregano, which was at 1.7 µg of catechin/ gm of freeze dried material. The oregano showed a decrease in the total phenolic content over the 6 day storage period. The compounds in oregano are reported to cause stearic hindrance that may alter the antioxidant phenolics profile (Yanishlieva et al 1999). The carotenoids in cactus pears showed a decrease in phenolic content with heat treatment and showed no similar effect on antioxidant activity (Jaramillo et al 2003). Rosemary has been reported to show increased antioxidant activity during a prolonged storage period and the activity was greater at the end of the storage period (Martinez et al 2001); however this study was conducted in a lipid system and performed at room temperature. In the present study, rosemary the values were not significantly different over a 6 day storage period. It has been reported by previous researchers that the prolonged storage times may enhance the progressive enzymatic/chemical oxidation of phenolic compounds in natural plant extracts like spices; the reactions rate may be different depending on some intrinsic food variables as well as on processing conditions (a_w, time, temperature, oxygen availability, etc.) (Nicoli et al 1999). The results found in our study are very significant as none of the other studies in the past evaluated changes in total phenolic content during incubation storage.

In the present study, no relation between extraction yields and phenolic content was observed for any of the spices. Similar to our study, Hinneburg et al (2006) conducted a study with hydrodistilled non heated spice extracts of basil, laurel, parsley,

juniper, aniseed, fennel, cumin, cardamom, and ginger and confirmed no significant association between extracted yield and phenolic content.

- **Total Phenolic Content of the Extracts Obtained After Heating and Incubation Storage of the Spices**

The phenolic content in this part are expressed per gram of extracts obtained after heating the spice sources. All the spice extracts showed an increase in phenolics after heating at 150°C.

Table 5: Total Phenolic Content of Spices during Heating Expressed as µg Catechin Equivalent/gm of extract

Temp of heating	Ginger	Oregano	Rosemary	Turmeric
Non-heated	68.7 ± 2.1 [e]	58.1 ± 4.0 [f]	16.0 ± 1.2 [f]	85.1 ± 3.0 [d]
150 °C	150.9 ± 6.6 [b]	62.2 ± 7.2 [f]	83 ± 3.3 [d]	139 ± 6.2 [b]
175 °C	111.3 ± 3.1 [c]	26.1 ± 1.3 [g]	71.4 ± 1.9 [e]	172 ± 3.6 [a]

*Significant difference (P<0.05) is expressed by the different letters in all rows and columns.

The non heated extracts showed lowest phenolic content at 58.1 µg of catechin /g for oregano and highest at 85.1 µg of catechin /gm of extract for turmeric.

After heating treatment, an increase in the phenolics for all spice extracts was observed. The highest increase was observed for ginger followed by turmeric, rosemary and oregano extracts. The heat treatment from 50 to 150°C showed an increase in the total phenolic concentration of Persimmon Peel (Kim et al 2006), although the study was conducted with ethanol and water extracts.

Table 6: Total Phenolic Content of Spices during Incubation Storage (60° C) Expressed as µg Catechin Equivalent/gm of Extract

Days of Storage	Ginger	Oregano	Rosemary	Turmeric
1	48.4 ± 1.3 [e]	56.3 ± 3.9 [d]	63.3 ± 2.3 [d]	112 ± 2.5 [b]
3	17.2 ± 1.3 [e]	56.2 ± 1.7 [d]	81.2 ± 3.1 [c]	119.7 ± 7.0 [b]
6	57.0 ± 1.3 [d]	30.4 ± 1.7 [g]	86.7 ± 3.0 [c]	139.9 ± 4.2 [a]

*Significant difference (P<0.05) is expressed by the different letters in all rows and columns.

The spices ginger, rosemary and turmeric showed an increase in the total phenolic content over the 6 day storage period On the other hand, oregano showed a decrease by 40% when compared with initial phenolic content. The highest increase at the end of storage period was observed for turmeric extract and was significantly different from all other spice extracts. The turmeric was followed by rosemary and then ginger in terms of increased values and also the total phenolic content values.

The difference in the total phenolic content results in case of the extracts and freeze dried materials can be explained by the fact that the decrease in the extraction yield of phenolics resulted in more concentration of phenolics which may have resulted an increase in the total phenolic content of the extracts. On the other hand, the total phenolic content expressed per gram of freeze dried material included the yield (%) value and thus reflected the phenolics in terms of freeze dried material.

- **Antioxidant Activities of Extracts Obtained From the Non-Heated Spices**

Free radical quenching can be directly related with oxidation in foods and biological systems. Scavenging of the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is considered a valid and easy assay to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays. The DPPH is the most widely used for all the antioxidant determinations (Chen and Ho (1995, Milan Suhaj 2006).

The results from this study are expressed as trolox equivalent antioxidant activity (TEAC). The results from our study showed antioxidant capability values ranging from 0.18 to 0.60 µmol of trolox equivalent per gram of extract.

Table 7: Antioxidant Activity of Freeze Dried Spices during Heating Expressed as μmol Trolox Equivalent/gm of Extract (TEAC)

Temp of heating	Ginger	Oregano	Rosemary	Turmeric
Non-heated	0.36 ± 0.01 [c]	0.18 ± 0.003 [d]	0.60 ± 0.003 [a]	0.57 ± 0.003 [a]
150 °C	0.36 ± 0.005 [c]	0.12 ± 0.005 [de]	0.55 ± 0.004 [a]	0.53 ± 0.005 [ab]
175 °C	0.33 ± 0.005 [c]	0.13 ± 0.005 [de]	0.40 ± 0.010 [a]	0.49 ± 0.016 [ab]

*Significant difference (P<0.05) is expressed by the different letters in all rows and columns.

For the non-heated samples, the lowest activity was found in the oregano (0.18 μmol/gm of extract) whereas rosemary showed the highest TEAC values. Among all the spice extracts, rosemary showed the greatest reduction in DPPH absorbance and reacted faster with the greatest TEAC values (0.60 μmol), followed by turmeric, ginger and oregano spice extracts in decreasing order of TEAC values. Kahkonen et al (1999) reported high antioxidant capability for rosemary extracts in methyl linoleate medium and showed antioxidant values of 37.8 mmol of trolox /100 g of extract (Shan et al 2005).

The study by Kahkonen et al (1999) found that the rosemary spice extracts were remarkably effective and showed high antioxidant activity in the ground form and as extracts.

As noted in the previous research studies the differences in the antioxidant activity in a particular assay are largely a function of the ratio of hydrophilic and hydrophobic nature of phenolics (Decker et al 2005, Frankel 1993). The DPPH assay has proven to be essentially measuring the antioxidant activity of the water-soluble phenolics and it is therefore, dependent on the amount of water-soluble phenolics available in the particular spices extracts. After rosemary, the turmeric extracts displayed greater antioxidant activity values of 0.57 μmol of trolox equivalent/gm of extract. Previous works conducted by Tiwari et al (2004) have reported high antioxidant activity for turmeric

when compared with ginger. In that study, Pyragallol solution (125 µg/ ml) was used as a reference corresponding to 100% radical scavenging activity. The ginger extracts in our study showed TEAC values (0.35 µmol) greater than oregano but lower than rosemary and turmeric. A study conducted by Murcia et al (2004) found that that ginger along with other spices had an effective radical scavenging activity as determined by the 3-ethyl benzthiazoline-6-sulphonic acid (ABTS) method expressed as TEAC equivalent, the activity was even higher than synthetic antioxidant BHT. Kaur and Kapoor (2002) reported in a study conducted with 32 vegetable extracts, that ginger showed more than 70% antioxidant activity; the study used ethanol as a solvent for extraction and stated that gingerol was a major component responsible for the antioxidant activity in ginger. In previous works by Chun et al (2004), the oregano extracted with ethanol showed excellent activity by DPPH; the results showed nearly 80% DPPH inhibition and also showed high scavenging activity for oregano. Zhang and Wang (2001) reported that the herb oregano had 3–20 times higher antioxidant activity than the other herbs when studied by the (ORAC) method.

- **Antioxidant Activities of the Extracts Obtained After Heating the Spices**

The capabilities of quenching of free DPPH radical were evaluated for the extracts obtained from the four spices that had been heated at 150°C and 175°C (table 7) and their antioxidant activities were compared with non-heated spice extracts. After heating at 150°C, rosemary still had the highest antioxidant capability among the four extracts and showed TEAC of 0.55 µmol of trolox/gm of extract. The heating treatment resulted in a 10% decrease in the antioxidant activity of rosemary when compared with the non heated extracts. The results obtained in this study were in agreement with the research conducted

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by Shobana and Naidu (2001), who reported that the antioxidant activity is dependent on the heating temperature and concentration of the extract.

The results from our study confirmed that the heating at 150^{o}C still retained 95 % of antioxidant capacity for turmeric extracts in methanol when compared with the non heated spices. Manzocco et al (1998) reported that the thermal treatment may enhance chain breaking activity and decrease oxygen uptake of the spice extracts.

After subject to heating at 150^{o}C the rosemary extracts showed antioxidant activity values of 0.55 μmol of trolox/gm of extract; these results in our study may have been influenced by the probable presence of polar components in rosemary extracts and the property of DPPH molecule to detect water soluble compounds in the extracts. The results from our DPPH study were similar to those from our previous menhaden oil oxidation study (see Chapter 3) that showed antioxidant capability of rosemary extracts was up to 50% retained for DHA and EPA in the fish oil. However, previous research work conducted by workers Kalucka et al (2005) have reported an increase in antioxidant activity of rosemary after being subjected to mild heating treatment (>100^{o}C). The antioxidant activity of ginger extract followed the rosemary in terms of the TEAC values and did not significantly change when compared with the non heated ginger extracts.

The turmeric showed greater values for antioxidant activity when compared with ginger. A study conducted by Tiwari et al (2001) found that the antioxidant activity of turmeric was higher than ginger when heated at 150^{o}C; in that study ginger extract showed a decrease in antioxidant activity when subjected to heating. Another study conducted by Shobana and Naidu (2000) studied the stabilities of antioxidant activity of ginger, garlic, pepper and cinnamon and found that they were heat stable in the

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temperature range of 105–150°C. In our study the oregano extracts were lowest in terms of antioxidant activity and showed very low values, in the range of 0.13-0.18 µmol of trolox / gm of extract. One of the major antioxidant compounds in oregano is thymol, predominantly present in the oil fractions of the extract (Zheng and Wang 2001) and that may have resulted in lower activity for oregano in our study. Interference in the oregano extract due to sugars and ascorbic acid components may also resulted in low antioxidant activity (Singleton and Rossi 1965).

With increased heating temperature from 150°C to 175°C, the antioxidant activities of all the spice extracts showed a decline (Table 7), except for oregano. The turmeric extracts had the highest value of 0.49 µmol of trolox/gm of extract, and showed lowest decline from 0.53 to 0.49 when compared with all other extracts. The heating resulted in the reduction of the antioxidant activity because steam sterilization may reduce the volatile oil content of the spices (major component responsible for the antioxidative activity) (Lee et al 1986, Jalay et al 1987).

Ginger extract retained antioxidant activity and exhibited a small reduction in TEAC next only to turmeric and was higher than rosemary and oregano. Marinova and Yanishlieva (2001) have reported loss of activity in ginger at higher temperatures above 150°C. In our study rosemary extract showed a steep decline (20%) in the antioxidant activity with heating from 150 to 175°C (table 7). The antioxidant capability of rosemary was reported to be contributed by the chemical rosmarinic acid, a flavonoid with high antioxidative activity and heat stability (Loliger, 1983). Frankel (1993) has confirmed the presence of a large number of compounds including carnosic acid, carnosol, in addition to rosmarinic acid in rosemary. The results from our study are in agreement harmony

with previous findings that have reported the possible formation of diterpines and triterpines at heated temperatures that may contribute to the antioxidant content of the rosemary extracts (Schwarz et al 1992). But this study and the other antioxidant studies were conducted in the lipid medium and did not confirmed any increase in antioxidant activity due to heating of the spices. In our study, during heating treatment turmeric spice extract showed significantly higher values when compared with all the other extracts.

- **Antioxidant Activity of the Extracts Obtained After Incubation Storage of the Spices**

For all the spice extracts there were no significant differences in the antioxidant activity at 1^{st}, 3^{rd} and 6^{th} day of storage. The antioxidant activities showed a decreasing trend in the activity for the extracts ginger, oregano and turmeric, however, only rosemary spice extract group showed an increase after the 6 day storage period (Table 8-next page). The antioxidant activity of rosemary extract showed a 10 % increase (0.57 to 0.63 μmol of trolox / gm of extract), whereas oregano extract was reduced by 29 % at the end of 6 day storage period.

Table 8: Antioxidant Activity of Spices during Incubation Storage (60 ^{o}C) Expressed as μmol Trolox Equivalent/gm of Extract (TEAC)

Days of Storage	Ginger	Oregano	Rosemary	Turmeric
1	0.39 ± 0.007^{c}	0.14 ± 0.008^{d}	0.57 ± 0.006^{a}	0.56 ± 0.008^{a}
3	0.36 ± 0.013^{c}	0.12 ± 0.008^{de}	0.60 ± 0.004^{a}	0.54 ± 0.003^{ab}
6	0.38 ± 0.011^{c}	0.10 ± 0.015^{de}	0.63 ± 0.009^{a}	0.53 ± 0.019^{ab}

*Significant difference (P<0.05) is expressed by the different letters

The increase in the antioxidant activity of rosemary during storage (0.57-0.63) was similar to previous results with menhaden fish oil study (Chapter 3) where rosemary had the highest retention of DHA at the end of 6 th day of the storage period. The ginger showed the least change (3 % decline) in the activity over the 6 day period. The

antioxidant activities of the four spice extracts in decreasing order were rosemary > turmeric > ginger > oregano. Aruoma et al (1996) reported similar observations during a 6 month storage study; the activity of the spices demonstrated a time-dependent increase in the extraction of active components from the spices.

The results obtained from our study are very significant as they provide important information on the ability of relatively less known spices turmeric and ginger as excellent antioxidants. Turmeric was found to have antimycotic as well as antioxidative property in butter cakes compared with synthetic antioxidants studied during 4 weeks of storage (Mansour and Khalil 1999).

In this study there was no correlation observed between total phenolic content and antioxidant activity of the four spice extracts. The spice extract groups of rosemary and turmeric showed significantly high antioxidant activities and total phenolic content when compared with oregano and ginger groups at both the heating and storage conditions. Similarly, the antioxidant activity of all the extracts at the end of 6 day incubation storage showed a decreasing trend except in the case of the rosemary, whereas the total phenolic content increased for all the extracts except for oregano. Similar to our results, previous studies conducted with natural extracts have found no correlation between antioxidant activity and total phenolic content, although these studies were not conducted with heating and storage treatments of the natural products (Sun et al 2006, Kahkonen et al 1999). Our study was very valuable regarding the information on the retention of phenolics in the spices during heat treatment and storage as often the spice plants are subjected to processing and storage conditions prior to preservation.

- **Changes in the Phenolic Profile of Spice Extracts During Heating and Storage Conditions**

The spices ginger, oregano, rosemary and turmeric are consumed after to cooking (heat treatment). Thus in this study we have attempted to study the phenolics changes in the spices during heating of the freeze dried spices (150 and 175 °C) and also during accelerated storage (60 °C incubation). After heating the spices were extracted with methanol and then after evaporating the solvent, the dried samples were subject to GC analysis. The National Institute of Standards and Technology mass spectral library in Gas Chromatography software was used to identify the phenolics. No studies conducted in the past have investigated the effect of heating and incubation on the phenolic content of these spices.

Table 9: Major Phenolic Compounds Identified in the Spice Extracts by the NIST Spectral Library by the GC-MC method

Ginger	Oregano	Rosemary	Turmeric
4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	Terpen-4-ol	-	Benzaldehyde-4-methyl
Geranial	p-Benzoquinone	Camphor	p-vinylguaiacol
Gamma-elemene	Thymine	α-terpineol	Longipinocarveol
Zingerberene	Pyrogallol	Isobornyl acetate	ar-curcumene
Beta-sesquiphellandrene	Carvacrol	Methyl-eugenol	dehydrocurcumene
Phorbol	Caryophyllene	Carophyllene oxide	1-4-diphenyl-pentanone
Ylangene	Spathulenol	Cadinene	beta-sesquiphellandrene
-	Nonylphenol	-	-

In case of ginger, the major compounds in the extracts tentatively identified were 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl, geranial, gamma-elemene, gingerberene, beta-sesquiphellandrene, Ylangene and phorbol. These compounds have also been reported in other research studies conducted with GC-MS on ginger oil (Wu et 1990; Chen and Ho 1988). The antioxidant activity may be due to the phenolic gingerberene which has been documented to exhibit major antioxidant characteristics. A new phenolic compound, 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl , was also detected, which has not been reported in the previous studies on ginger but may have contributed to the overall antioxidant capability. After subjected to heating treatment at 150°C, the abundance of all compounds were reduced by 50% of the original values except for the compounds pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl and only phorbol showed an increase in the peak values. After continuing the heating to 175°C, further decline in abundance values (m/z) of the compounds was observed.

However, in case of the storage study for ginger at 60°C, at the end of the storage period the compounds were similar to the control (no incubation) extract. The compound Ylangene was not detected in the non-heated ginger extract. The compounds detected from the GC-MS for both the heating and accelerated storage conditions did not contain shoagal or gingerol, which have been reported in the ginger extracts studies (Murcia et al 2004). Leal et al (2003) have observed and confirmed that temperature, and the use of solvents has a significant effect on the composition of ginger extracts.

In the case of oregano, the major phenolic compounds identified were carvacrol, terpin-4-ol, ethyllinalool, thymine, caryophyllene and spathulenol. These compounds have been identified in previous studies (Baranauskiene et al 2006, Baranska et al 2005)

with oregano and all of the studies have confirmed the presence of carvacrol, the main antioxidant compound present in oregano. A new antioxidant compound found in our study with oregano which has not been identified by previous studies was spathulenol (Kumar et al 2005). After increasing temperature from 150 ^{o}C to 175 ^{o}C, an increase in the peak abundance for the major compound carvacrol was observed during the heating study. The compounds terpen-4-ol and benzoquinone showed an increase in abundance with the heating treatment. This study showed an interesting phenomenon, the compounds thymine, caryophyllene and pyrogallol were detected after the heating treatment whereas they were not present in the nonheated and the incubation stored extracts.

The heating showed a decrease in all the phenolic compounds; however, during the incubation storage there was an increase in the main compound carvacrol (highest peak) at the end of the 6 day period. Martinez et al (2000) reported a high activity for oregano after being subjected to prolonged storage periods during an olive oil stability study. It has also been reported by other studies in the past that mild heating can improve phenolics in plants.

The main phenolic compounds identified in the rosemary spice extract used in our study were camphor, terpineol, methyleugenol, carophyllene oxide and cadinene. Previous studies by Boutekedjiret et al (2003) and Masatoshi Hori (1998) also reported these compounds in rosemary oil. Eucalyptol, camphor, carophyllene and verbenone were the main phenolic compounds detected in rosemary by using the multi-headspace solid phase microextraction and GC method (Carello and Tena 2006).

The heating of the rosemary spice extract at $150^{\circ}C$ and $175^{\circ}C$ showed a uniform decrease in phenolic peaks for all the compounds, except for 2, 4 decadienal and cadinene when compared with the control (non heated). In our study, an increase in the intensity of the peaks for rosemary extracts was observed for compounds carophyllene oxide and cadinene after 6-day storage at $60^{\circ}C$ when compared with the non-heated extract. Martinez et al (2001) conducted a storage study with rosemary (2, 4 and 6 months) and reported a time-dependent increase in the extraction of active components from the spices.

In the case of turmeric, the major compounds detected were methyl salicylate, 4-hydroxy-3-methylacetphenone, longipinocarveol and di-hydrocurcumene. Unlike the other three spice extracts, the phenolic compounds in turmeric showed an increase in the peak abundance after heating treatment ($150^{\circ}C$ and $175^{\circ}C$) and also during the incubation storage study. Shobana and Naidu (2001) reported an increase in the antioxidant phenolics in turmeric; the study was conducted with powdered ginger and turmeric oil and heated at $150^{\circ}C$. The antioxidant activity study and phenolic content studies also have indicated a similar increase in the activity for turmeric. However, the phenolic compounds profile in our study was not in agreement with previous characterization studies with turmeric. The studies in the past have confirmed the presence of the compounds, curcumin and tetrahydrocurcumin, which were not identified in our study; however these studies were mostly conducted with turmeric oil (Shobana and Naidu 2001). The methanol extraction played an important role and may have altered the phenolics in turmeric; further heating can also degrade the oil fraction in the turmeric that possesses the antioxidant compound fractions; especially curcumin.

For all four spices, ginger, oregano, rosemary and turmeric, changes in the phenolic profiles were observed after subjected to heating and storage conditions. In this study we investigated the changes in the phenolic compounds in the spices when subject to heating and storage, which could be beneficial during the post harvest processing and preservation of these spices. Moreover, all past research has confirmed that the composition of spice extracts is dependent on the solvent used for extraction (polarity) and the method of extraction (Murcia 2004, Suhaj 2006, Decker et al 2005).

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CHAPTER 5. ANTIOXIDANT CAPABILITIES OF SPICE EXTRACTS IN INHIBITION OF CHOLESTEROL OXIDATION

5.1 Introduction

Cholesterol is essential and required by the body to perform major biological functions including the cell membrane structure. The steroids along with phospholipids are formed in these cell membrane structures (Valenzuela et al 2003). The required cholesterol essential for the human body is supplied by the liver, although cholesterol is also supplied externally by food sources such as egg yolk, meat and whole milk products. High cholesterol in serum is the number one risk factor responsible for heart diseases and strokes in the USA (Tabas 2002). Serum cholesterol is a fat found in each cell of the body, it is carried through the blood by lipoproteins, lipoproteins are of 2 types, Low Density Lipoproteins (LDL) carry cholesterol (60-70%) from the liver to different parts of the body and are termed as bad cholesterol as it aids in lining of arteries which cause plaque buildup. The other type is High Density Lipoproteins (HDL) which carries cholesterol away from the arteries back to the liver from where it is discarded. HDL is considered good cholesterol and preferred over LDL as it can reduce the deposits on the inner lining of the blood vessels. Cholesterol is prone to oxidation due to the unsaturated structure of the molecule making it vulnerable at the C-7 position (Maerker 1987). Cholesterol is present in food, and is usually found along with lipids and other fatty acids. Like lipids, the oxidation of cholesterol may possibly be initiated by polyunsaturated fatty acids oxidation (Kubow 1993, Kim and Nawar 1991). Interaction of triglycerides with cholesterol can trigger the oxidation of cholesterol and may further cause rapid triglyceride oxidation (Kim & Nawar, 1991). The main cause of cholesterol oxidation are free radicals; the free-radical mechanism causes autooxidation of the cholesterol

molecule and further leads to the formation of hydroperoxides, and a number of oxidation products. The oxidation of cholesterol gives rise to a group of sterols similar in structure with cholesterol, but with an additional oxygen function such as a hydroxyl group, ketone group, or an epoxide group (Valenzuela et al 2003). These cholesterol oxidation products are often implicated in major health disorders such as cancers, atheroscelrosis, inflammation and heart related disorders (Addis 1986).

Scientists in the past have recommended that a regular intake of fruits and vegetables can help to lower the harmful effects of cholesterol (LDL) in the body. Djousse et al (2004) conducted a study on 4,466 subjects and found that the subjects who consumed more than four servings of fruits and vegetables a day had significantly lower levels of LDL (bad) cholesterol than who consumed less than four servings. Plant volatiles or polyphenols in spices are generally recognized as safe (GRAS) (Newberne et al 2000). Phenolics from plants like spices tend to possess the property of scavenging reactive oxygen and nitrogen free radical species, and also tend to decrease the molecular oxygen's oxidation potential and help in chelating metal ions. These free radicals are responsible for oxidative stress and cause large scale degradation of lipids and cholesterol in various foods. The phenolic antioxidants from plant based sources like spices have proven beneficial in limiting free radical damage from oxidizing low density lipoprotein (LDL) cholesterol and can further prevent the formation of cholesterol oxidation products (Siriwardhana and Shahidi 2002). Previous research studies have also found an increase in the antioxidant capacity of human blood plasma with ingestion of polyphenols with a resultant decrease in LDL cholesterol oxidation (Serafini et al 2000).

The past research on spices ginger, oregano, rosemary and turmeric implies the antioxidant phenolics are mainly responsible for medicinal capabilities and preservative and antioxidant capabilities (Murcia et al 2004, Martinez et al 2001, Frankel et al 1993). All these spices are comprised of phenols and secondary metabolites that are excellent source of various antioxidants. Ginger has gingerberene and gingerol, oregano has carvacrol and its derivatives, rosemary has rosmarinic acid, and turmeric has hydrocurcumin. They also contain many varying types of phenolic antioxidant compounds, such as eugenol, and other diterpines, triterpenes and quinones (Schwarz et al 1992). Owing to its high concentration of antioxidants, these spice extracts could serve as natural preservatives in preventing food oxidation during cooking and storage, especially for foods rich in cholesterol. The oxidation products generated from long-chain fatty acids are directly responsible for off-flavor and rancidity in foods causing deterioration of food quality. The oxidation of cholesterol and long chain fatty acids may be prevented by adding an extract of phenolic antioxidants to those foods (Shahidi 1997); however, the effect of natural antioxidants such as from spices on oxidative degradation of cholesterol has not been extensively studied and no information is available on the protective effect of spice extracts against oxidation reactions in food especially with the cholesterol molecule.

Previous studies on the use of antioxidants from plant sources in delaying cholesterol degradation have been conducted in *vivo*. Foods like meat, egg and the dairy products, butter and cheese are some of the principle external sources of cholesterol and these foods during heat processing have been reported to produce various toxic cholesterol oxidation products (Kumar and Singhal 1991). The consumption of these

deteriorated food products could lead to high levels of cholesterol oxidation products that could be fatal and can cause coronary heart disorders, cancers and inflammation (Addis 1990; Morel and Lin 1996). The plant based source materials of polyphenols such as spices are expected to exert a positive effect on the prevention of low-density lipoprotein (LDL) cholesterol oxidation which may be due to their free radical scavenging property (Siriwardhana and Shahidi 2002).

The information about the efficiency of phenolics in spices would be helpful in the development and utilization of these spices as food antioxidants or as an antioxidant nutritional supplement. Since heating is the most common process in food processing, it seems interesting to measure the oxidation products in heated foods.

In the present study, the antioxidant activity of the ginger, turmeric, rosemary and oregano extracts in methanol were evaluated during oxidation of cholesterol at temperature of 150 and 175 °C. The antioxidant activity of each extract was expressed as cholesterol (%) retained after being subject to oxidation.

5.2 Materials and Methods

- **Materials and Chemicals**

Ginger and Turmeric were purchased from a local market. The rosemary and oregano plants were obtained from the Department of Horticulture, Louisiana State University, Baton Rouge, Louisiana. Cholesterol (Lab use) was purchased from Sigma Aldrich Company. Other chemicals and solvents used were HPLC grade.

- **Extraction**

All plants were ground and freeze dried for 48 hours before extraction. One gram of each plant was weighed and extracted with 100 mL of methanol for 2 hrs at $30^{o}C$. The

methanol supernatant was separated by centrifuging at 1500 x g for 15 min. The residual was re-extracted with 50 mL of methanol. The supernatant was again separated using the same centrifugation and combined with the previous extract. Each dried extract was obtained after all methanol solvent was evaporated using a centrifuge vacuum evaporator (CentriVap Mobile System; Labconco, Kansas City, MO).

- **Determination of Cholesterol Retained after Heating**

The method used by Sun et al (2006) was used to determine the amount of cholesterol retained after subject to oxidation. One mL of cholesterol solution (0.1 mg/mL in hexane) was placed in test tubes (13 ×100 mm). The spice extract solution for the test was prepared by re-dissolving 0.10 g of each dried spice extract in 10 mL methanol. For each spice extract, 1, 5 and 10 mg of spice extract in methanol was added to the tubes containing cholesterol and vortexed for 30 sec. A tube without added spice extract solution was used as the control. Then, the solvent in the test tubes was evaporated using a vacuum centrifuge evaporator (CentriVap Mobile System; Labconco, Kansas City, MO). After evaporation, the tubes were immersed in a 175°C sand bath for 20 minutes. After the tubes were cooled down, 1 mL methanol was added and vortexed for 30 seconds. The methanol solution was transferred to an HPLC vial for analysis.

An HPLC system was used to determine the cholesterol concentration. It included a Waters 2690 system, a 960 PDA detector (Waters Corporation, Milford, MA), and a 25 cm × 4.6 mm diameter and 5- m C18 Discovery column (Supelco, Bellefonte, PA). The mobile phase was acetone: methanol (10:90) at a flow rate of 0.8 mL/min. The injection volume of sample was 100 μL. The HPLC was controlled using Waters Millennium chromatography software. The wavelength for quantifying cholesterol was

215 nm. The percentage of cholesterol that remained in each tube was obtained by comparing its final concentration to its original concentration.

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┌──────────────────────────────────────────────────┐
│              Freeze dried spices                   │
│   Ginger, Oregano, Rosemary and Turmeric          │
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                         ▼
          ╭──────────────────────────────╮
          │    Extraction with methanol   │
          ╰──────────────────────────────╯
                         │
                         ▼
          ┌──────────────────────────────┐
          │     Mixed with cholesterol    │
          │        (100ppm) solution      │
          └──────────────────────────────┘
                         │
                         │  ◄─── Heating at 150 and 175 C
                         ▼
          ┌──────────────────────────────┐
          │   Measuring the Cholesterol retained │
          │              by HPLC          │
          └──────────────────────────────┘
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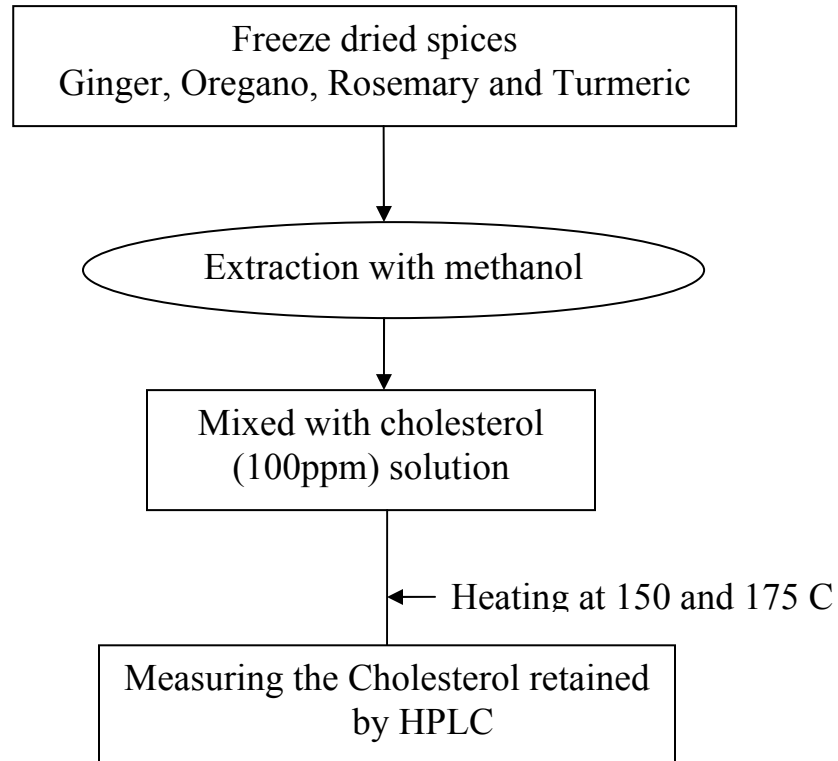
Figure 13. Flow Chart to Study Effect of the Spices on Cholesterol Oxidation

5.3   Statistical Analysis

For each treatment group, the experiments were performed in triplicates and the experiments were conducted twice (2 sets) at 3 different concentrations and at each of the two heating conditions. The data was analyzed using ANOVA and the studentized Tukey test to determine significant differences at $p < 0.05$.

5.4   Results and Discussions

- **Capabilities of the Spice Extracts in Retaining Cholesterol During Oxidation**

For the 150°C study, the control sample (cholesterol sample without any extract) had only 12.3 % of cholesterol remaining after 20 min of heating. All four spice extracts at three

different concentrations demonstrated significant capability in inhibition of cholesterol oxidation when compared with the control. In the case of ginger, 1 mg of extract retained the highest amount of cholesterol (78.7 %) while oregano, rosemary and turmeric retained 60.7, 61.9 and 55.1 %, respectively.

Table 10: Cholesterol (%) Retained by the Spice Extracts during Heating at 150°C

Spices (mg)	Ginger	Oregano	Rosemary	Turmeric
1	68.7 ± 1.42^{ab}	60.7 ± 2.4^{c}	61.9 ± 3.0^{c}	55.1 ± 2.2^{d}
2.5	83 ± 4.7^{a}	81.6 ± 8.5^{a}	73.7 ± 3.9^{b}	87.1 ± 6.4^{a}
5	73.2 ± 1.4^{b}	79.3 ± 2.2^{a}	65.9 ± 9.7^{c}	79.4 ± 3.2^{ab}

*Significant difference (P<0.05) is expressed by the different letters in all rows and columns
*Mean heated control values: 20 ± 3.4

However, with an increase in the amount of extract to 2.5 mg, antioxidant capability increased and retained 83, 81.6, 73.7 and 87.1 % cholesterol for ginger, oregano, rosemary and turmeric, respectively (Table 9). Among these four extracts, turmeric showed the highest retention with an increase from 1 mg to 2.5 mg. In a similar study, plant extracts from oats in methanol demonstrated inhibition of LDL cholesterol oxidation and the activity increased with an increase in concentration (Handelman et al 1999). The high activity of turmeric in our study was similar to previous studies, which reported that curcumin, the active ingredient of the spice turmeric, is a very strong antioxidant and was several times more potent than vitamin E as a free radical scavenger (Kermanshahi and Riasi 2006). The above discussed study conducted on hens demonstrated that the turmeric rhizome significantly decreased LDL cholesterol.

Valenzuela et al (2004) reported the effectiveness of rosemary antioxidants in preventing COP's formation. The further increase in the concentration of spice extracts (5mg) showed a uniform decrease in retained cholesterol for all spice extracts. The

increase in concentration of spices may have increased the non-phenolic components in the extracts including sugars and protein, which may have acted as prooxidants. Polyphenolics or flavonoids in the plants when used at high concentrations tend to produce reactive radicals through auto oxidation (Kessler et al 2003; Hodnick et al 1986). Although the prooxidant potential of these flavonoid radicals are reported to be lower than those of the other reactive species of radicals (Jorgenson et al 1998), they may play a role in reducing the antioxidant capability of the phenolics.

The 2.5 mg spice extracts concentration showed maximum retention capacity among all the extracts at $175^{\circ}C$; the activities of the spice extracts for preventing cholesterol oxidation in decreasing order at $175^{\circ}C$ were oregano > rosemary > ginger > turmeric. These results are similar to the order obtained for the antioxidant activity study.

After increasing the heating temperature from $150^{\circ}C$ to $175^{\circ}C$ the cholesterol retained in

Table 11: Cholesterol (%) retained by the spice extracts during heating at $175^{\circ}C$

Spices (mg)	Ginger	Oregano	Rosemary	Turmeric
1	70.7 ± 1.2 [ab]	56.1 ± 2.8 [c]	58.8 ± 2 [b]	33.1 ± 0.9 [f]
2.5	48.5 ± 3.1 [d]	76.3 ± 1.9 [a]	70.8 ± 6.7 [ab]	43.9 ± 6.7 [e]
5	38.0 ± 3 [ef]	64.8 ± 6.42 [b]	66.5 ± 1 [b]	49.2 ± 2.1 [a]

*Significant difference (P<0.05) is expressed by the different letters in all rows and columns
*Mean heated control values: 9.8 ± 1.7

the control decreased by 50 % (Table 10), but all the spice extracts were still significantly effective in retarding cholesterol oxidation as compared with the control (no extract) samples. The results from the ginger spice extract group for both temperatures showed higher values of cholesterol (%) retained; this phenomenon was also observed in the previous studies. Previous researchers have tested and reported the antioxidant capability of spice ginger conducted with *in-vivo* studies; ginger has been very effective in relation

to LDL cholesterol oxidation in a study conducted on apolipoprotein E–deficient mice (Fuhrman et al 2000).

In this study, for 150°C, turmeric extract at 2.5 mg concentration and at 175°C, oregano extract at 2.5 mg concentration yielded greater values for cholesterol (%) retained among all four spice extracts. The oregano spice extracts were able to scavenge the hydroxyl radicals and were more effective when compared with other synthetic antioxidants (Martinez et al 2001). Li et al (1996) found that natural antioxidants like tocopherols can effectively prevent formation of cholesterol oxidation products in commercial foods including oils. Valenzuela (2002b) found that the flavonoids quercetin, catechin, morin, and rutin were effective in preventing cholesterol oxidation during high temperature heating. Although the above studies used oils as substrate to study oxidation, the results were similar with our study in relation to the efficiency of natural antioxidant from spice extracts.

In our present study, there was a uniform decrease in cholesterol retained with the increase in concentration from 2.5 to 5 mg (Table 9 and 10). Previous research with the natural antioxidant tocopherol exerted a prooxidant effect at high concentrations and the antioxidant activity was temperature-dependent (Shahidi 1997).

The results from the present cholesterol study were different from the menhaden fish oil study (Chapter 3) where the increase in the concentration of the spice extracts increased the retention of EPA and DHA during oxidation. In the menhaden fish oil study, there are various ingredients and possible ingredient interactions involved between components of the fish oil and the spice extracts. The indigenous antioxidants present in fish oil may be reinforced (by synergism) with the addition of the spice extracts, whereas

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in the cholesterol model which uses pure cholesterol solution it is limited to interaction between plain cholesterol and the spice extract.

In a previous study by Gray et al (2002), they found that 50% ethanol extract from oats demonstrated significant antioxidant capability in protecting LDL from oxidation. This is one of the few studies which performed *in vitro* experiment to study cholesterol oxidation.

The spice extracts of rosemary, oregano, turmeric and ginger showed significant capabilities in retaining cholesterol after heating. However turmeric and ginger extracts showed a low activity at higher temperature (175^{o}C). Shobana and Naidu (2001) have found similar results with turmeric and ginger and have confirmed that the antioxidants in these extracts are not heat stable and may degrade at higher temperatures.

In this study the applicability of methanol solvent in extracting high antioxidant phenolics was confirmed. Thus, the compounds in all four spices ginger, oregano, rosemary and turmeric methanol extract played an important role in preventing cholesterol oxidation.

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CHAPTER 6. OVERALL CONCLUSIONS AND SUMMARY

The menhaden fish oil model system used in the present study was well suited for studying antioxidant capabilities of methanol extracts from ginger, oregano, rosemary and turmeric. The highly unstable polyunsaturated fatty acids, DHA and EPA, in Menhaden fish oil when mixed with the spice extracts significantly protected against oxidation during heating and incubation storage. The antioxidant capabilities in preventing the oxidation increased with an increase in the concentration for oregano and turmeric spice extracts. For the heating study, oregano showed greater capability in retaining PUFA's followed by rosemary, turmeric, and then ginger in a decreasing order. The accelerated storage study results showed rosemary retaining nearly 83 % DHA and 84 % EPA after 6 days of storage at 60°C at a concentration of 2.5 %. In the case of the storage study, a storage temperature of 60°C was used for 6 days as it simulates storage at 25°C for 6 months. The second best antioxidant capability during storage study was observed for turmeric followed by ginger and oregano in the decreasing order. These results are very significant considering the scarcity of information on these lesser known spices and the use menhaden fish oil in the oxidation studies. The basic mechanisms of action of these four different spice extracts in inhibiting polyunsaturated fatty acids (DHA and EPA) oxidation in the fish oil may help to provide insight into their role as antioxidants and their potential use to solve the stability issue in the fish oils. Thus this study provided the information of the use of these spice extracts in retaining the complex PUFA's in menhaden fish oil.

The spice extracts were equally effective in protecting cholesterol from oxidation at 150°C and showed (82-87 %) retained value at 2.5 mg concentration of extracts for

oregano, ginger and turmeric and 76 % for oregano. While the cholesterol retained values were 76 % for oregano and 71 % for rosemary in the case of heating temperature at 175^{o}C. The heating temperature (150^{o}C) study showed higher cholesterol retained values for ginger and turmeric but these values decreased with the increase in temperature to 175^{o}C at all the concentrations. The decrease in the inhibition of cholesterol oxidation was affected by the temperature and in case of ginger and turmeric the high temperatures may have reduced the volatile oil content which carries most of the antioxidant phenolics. But in both these models all four spices extracts were capable of retaining PUFA and also cholesterol when subject to oxidation by heat.

As seen in above study, spices are often subject to prolong cooking, processing and storage after adding to the food. It is thus very important to know the retention of antioxidants in these spices after subject to heating and storage conditions. In addition to that, the mechanism of action of the four spices extract in the menhaden fish oil oxidation study could also be associated with their scavenging ability of spices in quenching the free radicals. The results from the scavenging antioxidant activity study showed a high activity for rosemary followed by turmeric, ginger and then oregano extracts. These spices were then subject to heating (150^{o}C and 175^{o}C) and incubation storage (60^{o} C). After heat treatment, antioxidant activity was retained (80-90%) in all the spices; except ginger, which showed no significant reduction at both the heating temperatures of 150^{o}C and 175^{o}C. The results from the storage study showed a decline in the antioxidant activity for the spices of ginger, oregano and turmeric. However, rosemary showed a significant increase in the activity, from 0.63 to 0.66 µmol of Trolox equivalent/ gm of extract at the end of the 6 day storage period. The antioxidant activity of oregano and rosemary at

higher temperatures in the menhaden oil study could be explained by the possible release of bound phytochemicals during interaction with the lipid matrix. The antioxidant activity for rosemary was more prominent and showed a visible increase at mild heat (oven storage) storage conditions.

The phenolics are primarily responsible for the antioxidant activity of the extracts and the four spices exhibited phenolic content values at 4.8 to 27.0 µg catechin equivalent/gm of dry weight. The lowest value was observed for oregano and the highest phenolics were obtained for turmeric. After heat treatment (150^{o}C and 175^{o}C); phenolics were retained in case of turmeric (80%) and rosemary (82%) spices as compared to other two spices. During the incubation storage, turmeric and rosemary exhibited the highest total phenolic content, at 10.7 and 15.7 µg catechin equivalent/gm of dry weight respectively, showing minor (11-13 %) decline over the 6 day incubation storage.

The main volatile compounds responsible for antioxidant activity in rosemary were phenolic flavanoids. Our GC-MS study with the NIST spectral library search showed no evidence of rosmarinic acid and carnosol in the rosemary methanol extracts, however, other new compounds largely unknown in the past research were detected including methyl eugenol. While these results may suggest some interference in the MS chromatograph but may also be associated with the type of spice plants and their genetical structure and origin which was beyond the scope of this research. The GC-MS study did show main compounds in oregano as carvacrol, which may have been responsible for the antioxidant activity during heating in the menhaden oil study. The principal phenolic volatile compound detected by the GC-MS study in ginger was gingerberene and in turmeric it was hydrocurcumin.

In this study, the results from the antioxidant capacity and total phenolic content studies showed rosemary and turmeric had a high activity and the same was true for the menhaden fish oil oxidation study (rosemary was the highest) during the incubation storage. The oregano showed highest activity during heating study for menhaden fish oil and also for the cholesterol study while the antioxidant activity and phenolic content studies showed very low values. The results from our study provides vital information on the effect of heating and storage on the phenolic antioxidants in the spices ginger, oregano, rosemary and turmeric which haven't thoroughly been investigated in the past and are valuable for the end use of these spices. All the three studies in this research work confirmed excellent antioxidant capabilities for spices rosemary and turmeric. However, these methods to evaluate the antioxidant activity were based on different chemical and physical principles in monitoring oxidation and accordingly the activity of antioxidants in each spice extracts may have varied according to the assay's used. However from our study, it can be noted that extracts of rosemary, turmeric, ginger and oregano displayed high capability in preventing not only DHA and EPA oxidation but also cholesterol oxidation when compared with the control (no spice extracts added). This study showed that the extracts from ginger, oregano, rosemary and turmeric can be effectively used to maintain the stability of menhaden fish oil during cooking and incubation storage conditions. The extracts were able to retain the antioxidant phenolics even after heating and showed a significant change in phenolics during incubation storage at 60^{o}C. This study demonstrated antioxidant effectiveness of all the spice extracts in the following ways: (1) DHA and EPA were retained during oxidation in menhaden fish oil with heating and storage. (2) The antioxidant activity and total phenolic content were retained

even after the spices were subjected to heating and accelerated storage conditions (3) Cholesterol was retained when subjected to thermal oxidation. These results with more thorough analysis could help deal with the stability issue in the fish oil with the use of these spices, which are considered as functional foods due to their antioxidants as well as other benefits. The observations in this study can be helpful in exploring new frontiers in the use of these spices as natural antioxidants in replacing synthetic antioxidants, which have various limiting factors including toxicological problems, carcinogenesis etc.

Possible future work could be to evaluate the antioxidative effect of these spice extracts by using different types of lipid oxidation models. Other possible future work may be to look at the synergistic effects of these spices when used in combination. It will also be important to characterize the phenolic antioxidant compounds and study the interactive antioxidant effects during synergism.

APPENDIX A. EXTRACTION YIELD DURING HEATING AND INCUBATION STORAGE

Table 1: Extraction yield (%) of spices during incubation

Days of storage	Ginger(%)	Oregano(%)	Rosemary(%)	Turmeric(%)
1	18 ± 0.4^a	8.9 ± 0.3^c	19 ± 0.6^a	16 ± 0.4^a
3	18 ± 0.5^a	6.9 ± 0.4^d	14 ± 0.5^b	13.6 ± 0.4^b
6	13.2 ± 0.5^b	5.7 ± 0.3^e	12.4 ± 0.7^b	11.2 ± 0.5^c

*Significant difference (P<0.05) is expressed by the different letters in the same row.

Table 2: Extraction yield (%) of spices during heating

Temp	Ginger(%)	Oregano(%)	Rosemary(%)	Turmeric(%)
Non heated	19.4 ± 0.4^b	8.4 ± 0.5^d	27 ± 0.5^a	31.7 ± 0.7^a
150	7.3 ± 0.6^d	3.2 ± 0.6^e	8.7 ± 0.4^c	11.1 ± 0.6^c
175	4.9 ± 0.6^e	9.2 ± 0.5^c	9.8 ± 0.5^c	8.6 ± 0.6^d

*Significant difference (P<0.05) is expressed by the different letters

APPENDIX B. GINGER PHENOLIC PROFILE BY GC-MS

Phenolic Compounds	Non-heated				60^{o}C-6 days				150^{o}C				175^{o}C			
	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure
4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	14.04	0.3	43, 144	C6H8O4	14.0	5.08	43, 144	C6H8O4	13.9	2.39	43, 144	C6H8O4	-	-	-	-
Geranial	16.6	3.57	41,69	C10H16O	16.7	1.57	41,69	C10H16O	16.6	1.15	41,69	C10H16O	16.7	0.174	41,69	C10H16O
Gamma-elemene	19.9	1.98	41,69	C15H24	19.8	1.26	41,69	C15H24	19.8	1.1	41,69	C15H24	19.8	0.26	41,69	C15H24
Zingerberene	22.8	26.1	119, 91	C15H24	22.7	25.5	119, 91	C15H24	22.6	15.4	119, 91	C15H24	22.5	3.5	119, 91	C15H24
Beta-sesquiphellandrene	23.4	15.99	91,41	C15H24	23.3	18.1	91,41	C15H24	23.4	11.05	91	C15H24	23.4	3.7	91	C15H24
Phorbol	26.9	6.27	83, 119	C20H28O6	27	16	83, 119	C20H28O6	26.9	7.36	83, 119	C20H28O6	-	-	-	-
Ylangene	-	-	-	-	27.7	9.47	120, 39	C15H24	-	-	-	-	-	-	-	-

where Pk-peak height, RT- retention time, MC- MegaCounts, m/z- unit of abundance

Phenolic Compounds	Non-heated				60 °C -6 days				150°C				175°C			
	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure
Terpen-4-ol	13.7	0.6	43, 81	C10H18O	13.8	4.5	71, 41	C10H18O	13.8	1.2	1.2	C6H8O4	-	-	-	-
p-Benzoquinone	15.9	1.7	149, 91	C11H16O	15.9	4.8	93, 39	C10H12O2	16	5.2	93	C10H12O2	16.06	0.63	93	C10H12O2
Thymine	-	-	-	-	-	-	-	-	17.0	0.31	39	C5H6N2O2	-	-	-	-
Pyrogallol	-	-	-	-	-	-	-	-	-	-	-	-	16.9	1.01	97	C6H6O3
Carvacrol	18.6	30	135, 107	C10H14O	18.6	59	135, 91	C10H14O	18.5	0.5	135	C10H14O	18.6	1.7	135	C10H14O
Caryophyllene		-	-	-		-	-	-	23.3	2.5	91	C15H24	-	-	-	-
Spathulenol	24.8	6.8	43, 91	C15H24O	24.8	13.5	39, 91	C15H24O	24.8	1.2	91	C15H24O	24.6	0.7	91	C14H30O
Nonylphenol	28.6	2.8	120, 91	C15H24O	28.7	17	135, 107	C15H24O		15	135	C15H24O	28.6	7.1	135	C15H24O

where Pk-peak height, RT- retention time, MC- MegaCounts, m/z- unit of abundance

APPENDIX B2. ROSEMARY PHENOLIC PROFILE BY GC-MS

Phenolic Compounds	Non-heated				60 °C -6 days				150°C				175°C			
	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure
2,4 decadienal	7.68	0.22	39, 81	C7 H10O	7.55 3	0.15	39, 81	C7H 10O	7.64	0.65	39, 81	C7H 10O	7.77	0.8	39, 81	C7H 10O
Camphor	12.3 8	3.75	95, 152	C10 H16O	12.3 2	1.66	95, 39	C10 H16O	12.4 2	0.05	95, 152	C10 H16O	12.32	0.9	95, 126	C10 H16O
α-terpineol	14.5 8	3.22	43, 93	C10 H18O	14.4 6	0.13	43, 93	C10 H18O	14.3	0.9	43, 93	C9 H12O	14.58	0.8	43, 93	C13 H22O
Isobornyl acetate	-	-	-	-	16.7 8	2.33	95, 43	C10 H 20O2	16.8	1.77	95, 43	C10 H 20O2	16.83	1.95	95, 43	C10 H 20O2
Methyl-eugenol	20.5 1	9.55	178, 91	C11 H14O2	20.5 2	5.9	178, 91	C11H14 O2	20.5 3	4.3	178, 91	C11 H14O2	-	-	-	-
Carophyllene oxide	24.7	2.11	39, 79	C15 H24O		2.67	39, 79	C15 H24O	24.6 5	0.9	39, 79	C15 H24O	24.69	0.4	39, 79	C15 H24O
Cadinene	31.9 3	1.81	257, 105	C20 H32	31.9 2	3.13	257, 105	C20 H32	31.9 3	2.21	173, 255	C20 H32	31.93	1.96	257	C20 H32

where Pk-peak height, RT- retention time, MC- MegaCounts, m/z- unit of abundance

APPENDIX B3. TURMERIC PHENOLIC PROFILE BY GC-MS

Phenolic Compounds	Non-heated				60 °C -6 days				150 °C				175 °C			
	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure	RT	Pk MC	Ion (m/z)	structure
Benzaldehyde-4-methyl	-	-	-	-	14.3	0.75	120	C8H8O3	14.2	0.4	119, 91	C8H8O	14.3	0.51	119, 91	C8H8O
p-vinylguaiacol	-	-	-	-	18.4	0.59	135, 107	C9H10O2	18.37	2.53	135, 107	C9H10O2	18.3	2.79	135, 107	C9H10O2
Longipinocarveol	-	-	-	-	21.2	1.45	151, 91	C15H24O	21.2	2.45	151, 91	C15H24O	-	-	-	-
ar-curcumene	22.2	1.48	132, 39	C15H22	22.3	3.2	132, 39	C15H22	22.3	5.14	132, 39	C15H22	22.2	3.13	132, 39	C15H22
dehydrocurcumene	25.5	0.9	119, 91	C15H24	25.4	4.02	119, 91	C15H24	25.4	11.4	119, 91	C15H24	-	-	-	-
1-4-diphenyl-pentanone	27.7	15	120, 39	C17H18O	27.9	33.5	120, 39	C17H18O	27.9	40	120, 39	C17H18O	27.9	33.8	120, 39	C17H18O
beta-sesquiphellandrene	32.2	1.44	91	C20H34O2	32.1	2.44	91	C20H34O2	-	-	-	-	-	-	-	-

where Pk-peak height, RT- retention time, MC- MegaCounts, m/z- unit of abundance

VITA

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His primary expertise is in investigating the phenolic antioxidants in herbs and spices like ginger, oregano, rosemary and turmeric in a fish oil model. He received the Gamma Sigma Delta Student honor roll awarded for academic excellence in the year 2005. He was Food Science Club President for the year 2005-2006 at the department of Food Science. He is a certified trainee in hazard analysis and critical control point (HACCP) awarded by the Association of Food and Drug Officials (AFDO) and in retort operations, processing system operations, aseptic processing and packaging operations course prescribed by the U.S.F.D.A. at Louisiana State University A & M, Baton Rouge, Louisiana. He is an accredited member of the Institute of Food Technologists (IFT), the American Society of Agricultural and Biological Engineers.