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Antioxidant Distribution And Effectiveness In A Model Muscle System

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ANTIOXIDANT DISTRIBUTION AND EFFECTIVENESS IN A MODEL MUSCLE SYSTEM

A Dissertation Presented

by

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DEDICATION

To my marvelous mentor and extraordinary human being, Dr. Herbert. O. Hultin.

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ABSTRACT

ANTIOXIDANT DISTRIBUTION AND EFFECTIVENESS IN A MODEL MUSCLE SYSTEM

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Gallic acid esters (GAE) of varying alkyl chain length were used to determine how antioxidant physical location and partitioning influence hemoglobin-catalyzed lipid oxidation. Specific GAE used were propyl gallate (PG), octyl gallate (OG), and lauryl gallate (LG). GAE partitioning experiments were performed with either isolated cod muscle membranes or washed cod muscle, which primarily contain polar membrane lipids and myofibrillar proteins. Canola oil was used in some experiments to determine how neutral lipids impact partitioning behavior. GAE distribution was determined spectrophotometrically in the recovered membranes, aqueous phase, and oil layer after employing differential centrifugation. Oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides development.

When GAE were added to the membrane suspensions, significant differences ($p < 0.05$) in GAE partitioning were observed in the aqueous phase and membrane sediment, where increases in GAE alkyl chain length corresponded with a decrease in aqueous phase concentrations and increases in the membranes. GAE partitioning in the oil fraction did not show significant differences. Also, increases in GAE alkyl chain

correlated with increases in GAE membrane detection when GAE were added to the washed fish muscle ($p < 0.05$).

Adding GAE to the washed cod muscle before the canola oil was the most effective sequence of addition for extending the storage time before lipid oxidation was detected. Among the three GAE tested, PG showed the greatest inhibition against lipid oxidation. The effectiveness of the GAE in the washed cod-canola oil system follows the order, PG > OG > LG, which corresponds with decreasing hydrophobicity.

The conclusions of this study are twofold. First, GAE partitioning into the muscle membranes was not the primary factor for delaying the onset of lipid oxidation. Rather, solubility in the aqueous phase showed the greatest impact on extending storage time. Secondly, the order in which GAE and canola oil were added to the washed cod (WC) muscle system influenced hemoglobin-catalyzed lipid oxidation behavior. Adding GAE before the neutral oil may have allowed the GAE to partition more easily into the polar regions of the washed muscle, which in turn provided the most effective protection against oxidation.

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CHAPTER 1

INTRODUCTION

Lipid oxidation is a major quality challenge in meat processing. Oxidative damage in muscle food causes unpleasant quality changes such as off-odor and unpalatable flavor development, nutrient degradation, undesirable toughening, and color changes (Ke & Ackman, 1976; Ladikos & Lougovois, 1990). Although unprocessed animal products possess several native antioxidants and biological repair mechanisms for coping with oxidative stresses, these compounds and indigenous system can be removed, inactivated, or overwhelmed during food processing operations. Therefore, exogenous antioxidants are often added to foods during processing in order to extend product shelf-life. Antioxidants include a wide range of both natural and synthetic compounds capable of interfering with the various stages of oxidation. Generally speaking, antioxidants function to limit or delay the action of free radicals, prooxidants, and oxidative intermediates (Decker & McClements, 2007; Nawar, 1996).

Understanding the physicochemical properties of antioxidants, particularly how structure and physical location affects lipid oxidation, is important for explaining how antioxidants work in fish muscle. It is well-established that antioxidant structure impacts its ability to stabilize radicals which are formed during oxidation (Belin et al., 2003; Porter et al., 1989). The results obtained from this project will help fill knowledge gaps and contribute to the pool of empirical data pertaining to the evidence-supported (but often challenged) theory that suggests a direct relationship exists between concentrating antioxidants into the membranes of muscle tissue and the delay of lipid oxidation (Caltural et al., 2003; Ollila et al., 2002; Pazos et al., 2006ab; Raghavan,

2005; Raghavan & Hultin, 2005; Rawel et al., 2005; Tappel, 1955; Verstraeten et al., 2003). Also, many researchers have acknowledged the relevancy of determining antioxidant physical location and interaction with muscle membranes in order to provide a more complete explanation of antioxidant efficacy in muscle foods (Decker et al., 2005; Lee, C-H et al., 2006; Pazos et al., 2006b).

Our hypothesis is that a positive relationship exists between directing gallic acid esters (GAE) into the membranes of washed fish muscle and their ability to delay lipid oxidation. This hypothesis is based on several studies that suggest membrane phospholipids in fish muscle tissue are the primary substrates of lipid oxidation and that antioxidant-membrane interactions are important for antioxidant efficacy (Caturla et al, 2003; Kumazawa et al, 2004; Pazos et al, 2006b; Raghavan and Hultin, 2005ab). Data collected from the GAE distribution studies will be compared to the hemoglobin-mediated lipid oxidation studies in washed fish muscle. By understanding how GAE structure and physical location in washed fish muscle affects lipid oxidation, seafood product quality and shelf-life can be maximized.

Our long-term goal is to increase the understanding of how antioxidant structure and physical location within food systems affects its ability to delay lipid oxidation. The results and conclusions revealed by this project will provide the food industry with new information for improving the quality of muscle food products. Specifically, we plan to determine if GAE of varying alkyl chain lengths can be directed into the membranes of washed fish muscle and how this relates to the efficacy of this particular class of antioxidants in inhibiting hemoglobin-catalyzed lipid oxidation in washed fish muscle.

In order to fully attain the previously stated long-term goal, the following three key objectives must be achieved:

1.) Determine the distribution of GAE when added to prepared fish muscle membranes
2.) Determine the distribution of GAE in isolated membranes prepared from washed fish muscle with previously added GAE
3.) Monitor the development of lipid oxidation products in hemoglobin-treated washed fish muscle with added GAE

CHAPTER 2

LITERATURE REVIEW

2.1 Fish Muscle Tissue

2.1 1 Tissue organization and major constituents

Fish muscle is vastly different from land animal and avian tissue. Since fish are neutrally buoyant in their aquatic environments, their muscles do not require extensive connective tissue support to hold the muscles together. This decrease in collagen content coupled with the easy melting of fish collagen during cooking allows for fewer texture-related problems in fresh fish as compared with land animals. Since fish are required to swim, the arrangement of fish muscle is also different than in animal muscle. This muscle organization is markedly different compared to birds and mammals, which exhibits layers of increasingly slender fibers bound together by increasingly thinner connective tissue. Rather, fish muscles are arranged in W-shaped myotomes. Myotomes measure one cell in depth and are connected by thin layers of collagenous myosepta. Also, many desirable fish species live in colder waters and thus requires protein and lipid composition to be different from traditional warm-blooded animals. For example, the percentage of lipid classified as polyenoic is between 0 and 6% in beef compared to 48% in cod. Special proteinases produced by fish muscle enable these poikilotherms to restructure proteins as needed (Foegeding et al, 1996).

The major constituents of muscle tissue include sarcolemma, contractile fibers, sarcoplasm, and organelles. Soluble components of muscle cells consist of proteins such as myogen, myoglobin and various enzymes, as well as non-protein compounds like

amino acids, nucleotides, glycolysis intermediates, and inorganic ions. The insoluble fraction of muscle cells is mainly connective tissue, but also includes proteins like titin and desmin, various membrane components, glycogen particles, and fat droplets (Foegeding, et al., 1996).

2.1.2 Proximate Composition

Fish muscle composition varies greatly depending on species, life cycle stage, season, nutritive status, sex, and age (Foegeding et al, 1996). Table 2.1 shows typical, broad variations in fish muscle composition. It is important to note that the largest variations in composition are related to lipid percentages, which range between 0.2 and 25% (FAO, 1995). Fat storage mechanism, movement in the water, migratory patterns, sexual maturity, and feeding habits primarily account for the large variation in lipids. For example, many species display a seasonal curve, with the lowest lipid content around spawning (FAO, 1995). It is important to note that lean fish lipid content varies at a lesser extent compared to fatty fish (FAO, 1995; Kent et al, 1992). Although protein content fluctuates to a lesser extent as compared to lipids, these protein discrepancies can be partly attributed to spawning and migration patterns in species such as salmon. Also, when a fish is starved, it utilizes its muscle as energy and the muscle is replaced with water. According to Love (1980), the water content of a severely starved cod can raise to as much as 95%.

Table 2.1: Variations in fish muscle composition, expressed as percentages (FAO, 1995).

Constituent	Fish (fillet)		
	Minimum	Normal Variation	Maximum
Protein	6	16-21	28
Lipid	0.1	0.2-25	67
Carbohydrate		<0.5	
Water	28	66-81	96

2.1.3 Neutral Lipids

Neutral lipids in fish muscle primarily exist as triacylglycerols. These lipids are neutrally charged at physiological pH and are considered hydrophobic (Ettinger, 2000). Triacylglycerols belong to the glycerol family and contain three fatty acid chains which are attached to a glycerol molecule through esterification. Esterified lipids have reduced surface activity and lower cytotoxicity (McClements & Decker, 2007; Nawar, 1996). Since these lipids are both anhydrous and reduced, triacylglycerols are able to be stored in nearly anhydrous conditions and contain tremendous quantities of metabolic energy (Berg et al, 2002).These neutral lipids are sequestered in droplets within the dark muscle tissue. When needed, dark muscle metabolizes the stored lipids and converts them into energy.

2.1.4 Phospholipids

Phospholipids are triacylglycerols modified with a phosphate group in the third position and are derivatives of phosphatidic acid. Through an esterification process, phosphatidic acid is attached to a nitrogen-containing molecule, such as choline, ethanolamine, inositol, or serine. The general structure of a phospholipid molecule has a

saturated fatty acid at the C1 position of the glycerol molecule, a polyunsaturated fatty acid at the C2 position, and a nitrogenous base esterified to the phosphate at the C3 position (Berg et al, 2002). Phospholipid nomenclature is derived from both phosphatidic acid and the nitrogenous base. The major phospholipids in muscle are phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and acidic glycerol phosphatides (Foegeding et al, 1996).

Phospholipids are considered polar lipids because they carry a charge at physiological pH. The phosphate-containing head groups face outward into the extracellular environment and are able to form hydrogen bonds with water. Conversely, the hydrophobic tail regions form hydrophobic bonds with neutral lipids located in the center of the membranes. Phospholipids are considered amphiphiles because they are able to form an emulsion at the oil-water interface by aid to disperse lipids into the water. These polar lipids comprise more than 50% of the membrane lipid bilayer of the plasma membrane and 90% of lipids in the mitochondrial membrane. In general, phospholipid content in a variety of membranes comprises 60-90% of the total lipids (Alberts et al, 1989). A major function of the membrane is to provide a hydrophobic barrier capable of regulating the exchange of ions between the intra- and extracellular regions (Ettinger, 2000; Foegeding et al, 1996).

Lipids in lean muscle tissue contain more phospholipids than adipose tissue. Lean muscle contains 0.5-1% phospholipids, which are composed of more highly unsaturated fatty acids as compared to the more saturated triacylglycerols in the adipose tissue. Phospholipids in fish muscle are located the membrane structures such as the outer cell

membrane, organelle membranes, membranes surrounding lipid droplets, endoplasmic reticulum, and intracellular tubule systems (Foegeding et al, 1996).

2.2 Membranes

2.2.1 Fluid mosaic model

The generalized model for biological membranes is the fluid mosaic model and was first introduced by Singer and Nicolson (1972). As shown in Figure 2.1, the basic structure of membranes is a fluid lipid bilayer with a wide variety of amphiphilic molecules that traverse, attach to, or protrude from the bilayer. Phospholipids form a bilayer by orienting the polar head regions towards the aqueous environments while the hydrophobic tails are stacked closely together and create the nonpolar fraction of the membrane. This bilayer is responsible for establishing closed biomolecular sheets. These sheets are capable of encasing individual cells as well as the organelles within cells, which prohibit the flow of polar molecules. Compartmentalizing organelles within the cellular environment is necessary for the proper function and regulation of important processes.

Hydrophobic forces are responsible for the formation of these lipid bilayers, electrostatic and hydrogen-bonding attractions force the polar head groups of the phospholipids to associate with the aqueous environment, while the tight packing of the hydrophobic tails is due to van der Walls forces. Thus, a wide-array of reinforcing, non-covalent interactions actively provide stability to the membrane structure (Berg et al, 2002; Campbell et al, 1999; Singer & Nicolson, 1972).

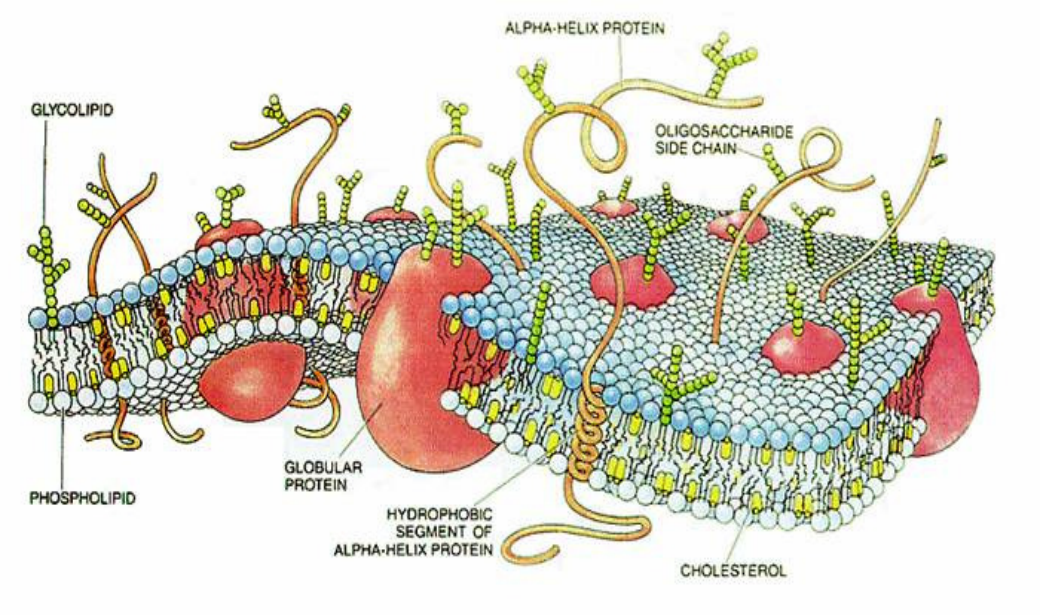

Figure 2.1: Fluid mosaic model of membranes illustrated by Dana Burns (http://commons.wikimedia.org/wiki/Image:CellMembraneDrawing.jpg) (Bretscher, 1985).

2.2.2.1 Additional membrane constituents and their function

2.2.2.1.1 Proteins

Membrane proteins vary in concentration and exist in various forms and composition depending on function and location. These proteins are essential to membrane function, such as acting as channels for solute exchange, signal transduction, and functioning as enzymes. There are two general types of membrane proteins: integral and peripheral. Integral proteins are located embedded in between the membrane phospholipids, with the hydrophobic portion inside the hydrophobic membrane interior while the hydrophilic regions project into the polar environment. The hydrophobic portion of the integral proteins contains at least one hydrophobic section of amino acids and primarily exists as α-helical or globular structures. Peripheral proteins, however, are

not rooted in the membrane structure but attach rather loosely to membrane surface structures. The majority of peripheral proteins are attracted to the membranes primarily through electrostatic and hydrogen-bonding, causing them to be easily displaced by adjusting salt concentration or pH. However, some peripheral proteins are covalently attached to membrane lipids by a hydrophobic chain (Berg et al, 2002; Campbell et al, 1999; Singer & Nicolson, 1972).

2.2.2.2.2 Cholesterol

Cholesterol modulates cell membrane fluidity and is a precursor to steroid hormones, vitamin D, and bile salts. All 27 carbons in the cholesterol molecules are derived from acetyl coenzyme A in a three-step process. Cholesterol molecules consist of four fused rings (three six-carbon rings and one five-carbon ring), a hydrocarbon tail, and a hydroxyl group. The hydroxyl group allows the cholesterol molecule to penetrate the polar phospholipid head groups of the membranes and burry the hydrophobic portion within the membrane. Cholesterol is able to decrease membrane fluidity by restricting the movement of phospholipids, hence losing their ability to pack closely together. This action reduces the temperature required for membrane solidification, thus limiting phase transitions (Berg et al, 2002; Campbell et al, 1999).

2.2.2.2.3 Carbohydrates

Carbohydrates are restricted to the polar surface of the membrane. These molecules exist as oligosaccharides and are branched and contain less than 15 subunits. Oligosaccharides play a key role in cell-to-cell recognition. Oligosaccharides covalently

bonded to proteins are termed glycoproteins, while those attached to lipids are called glycolipids (Berg et al, 2002; Campbell et al, 1999).

2.3 Lipid Oxidation

2.3.1 General theory

Lipid oxidation involves reactions between oxygen species and unsaturated fatty acids. Basically, atmospheric oxygen binds to fatty acids and forms compounds that are easily broken down to undesirable flavor and odor volatiles. The most common mechanism responsible for lipid oxidation involves a radical generating system involving three phases:

Initiation: $In\cdot + RH \rightarrow InH + R\cdot$

Propagation: $R\cdot + O_2 \rightarrow ROO\cdot$

$ROO\cdot + RH \rightarrow R\cdot + ROOH$

Termination: $2RO_2^{\cdot} \rightarrow O_2 + RO_2R$

$RO_2^{\cdot} + R\cdot \rightarrow RO_2R$

2.3.1.1 Initiation

Lipids most commonly associated with oxidation are triacylglycerols and phospholipids containing unsaturated fatty acid chains. Unsaturated fatty acids in either of these types of lipids are uniquely susceptible to oxidation due to the instability of the methyl groups adjacent to C=C double bonds. These methyl groups are particularly vulnerable to free radical attack by readily losing a hydrogen atom. The consequence of

this hydrogen loss is the formation of an alkyl free radical and sometimes a double bond shift. Alkyl free radicals are highly unstable and therefore are short-lived reaction intermediates (Laguerre et al, 2007). The process of forming alkyl free radicals is the initiation phase of oxidation.

2.3.1.2 Propagation

The second phase of lipid oxidation, propagation, involves first the marrying of atmospheric oxygen to the alkyl free radical. Atmospheric oxygen (or triplet state oxygen) is the oxygen species of choice due to its biradical nature. One radical readily forms a covalent bond with the alkyl free radical while the lingering radical remains unbound. This unbound oxygen free radical is the peroxyl radical. Peroxyl radicals are known to cause serious damage to fatty acids since they easily disrupt the covalent bonding between carbon and hydrogen atoms. When carbon-hydrogen bonds are broken in fatty acids by peroxyl radicals, not only are fatty acid hydroperoxides produced, but also new fatty acid alkyl radicals from other fatty acids are formed. These types of reactions may occur between 10 and 100 times before termination (Borg & Schaich, 1988; Erickson, 2002; Gutteridge & Halliwell, 1990).

2.3.1.3 Termination

Termination, the final stage of lipid oxidation, is achieved when the formed radical species form nonradicals. Two types of reactions are responsible for termination: radical-radical coupling and radical-radical disproportionation (Erickson, 2002).

2.3.2 Muscle tissue oxidation

Several studies have demonstrated that the primary substrate of lipid oxidation in muscle tissue is the phospholipids associated with membranes (Grandemer, 1999; Undeland et al, 2002; Younathan and Watts, 1959). Membrane phospholipids are exceptionally vulnerable to lipid oxidation not only because of their high degree of polyunsaturated fatty acids, but also their close association with cytoplasmic pro-oxidants such as heme and non-heme iron, high surface area, and their close proximity to the oxygen-activating systems found in most membranes (Hultin, 1995; Nawar, 1996).

2.3.2.1 Enzymic and nonenzymic non-heme iron systems

Both enzymic and nonenzymic non-heme iron systems participate in membrane lipid oxidation. One such system was described by Lin and Hultin (1976), which demonstrated the ability of chicken sarcoplasmic reticulum to enzymatically initiate lipid oxidation in the presence of iron and NADH as the electron source. Soyer and Hultin (2000) further explored lipid oxidation in fish sarcoplasmic reticulum by both enzymic systems and nonenzymic systems. Their studies suggest that the enzymic system is capable of initiating lipid oxidation by first attacking sulfhydryl groups of the proteins in the inner membrane. These protein oxidation products, in turn, initiate lipid oxidation and eventually produce lipid hydroperoxides. The nonenzymic systems of ferrous iron-catalyzed oxidation were also found to oxidize protein sulfhydryl groups, but this action primarily involved the surface proteins of the membranes and required preformed peroxides to be present (Huang et al., 1993; Soyer and Hultin, 2000).

Work by Luo and Hultin (1986) showed that an enzymic ferric iron-reducing system exists in the inner membrane of the mitochondria, which is nearly identical to that in sarcoplasmic reticulum. It has been well established that mitochondria produce superoxide, a major radical initiator in lipid oxidation, in complex I of the electron transport chain. The superoxide radicals produced here are converted by superoxide dismutase into hydrogen peroxide. Then, by action of catalase or glutathione peroxidase, hydrogen peroxide is converted into water and oxygen. Although the majority of superoxide is converted to harmless substances, this system is not completely efficient, leaving behind small quantities of highly reactive superoxide molecules that have been shown to directly damage proteins, DNA, and lipids. In the presence of ferrous ions, hydrogen peroxide is converted into highly reactive hydroxyl radicals by Fenton chemistry. These hydroxyl radicals are capable of initiating lipid peroxidation cascades in membranes by extracting hydrogen atoms from polyunsaturated fatty acid chains of membrane phospholipids, creating carbon-centered fatty acyl radicals that further interact with oxygen to form peroxyl radicals. Peroxyl radicals propagate lipid oxidation chain reactions (Brand et al., 2004).

2.3.2.2 Heme proteins

Another pro-oxidant source in muscle tissue is the heme proteins. Kanner and others (1987) determined that both the ferrous (Fe^{+2}) and ferric (Fe^{+3}) forms of hemoglobin can contribute to lipid oxidation. Oxyhemoglobin (Fe^{+2}) can be reduced to methemoglobin (Fe^{+3}) by spontaneous autoxidation, also producing the highly reactive

(and previously described) superoxide radical anion. Methemoglobin freely attacks peroxides, producing highly reactive hydroperoxyl free radical species as well as the further reduced ferryl (Fe^{+4}) form of hemoglobin. This ferryl form of hemoglobin is also able to attack polyunsaturated lipids by removing hydrogen atoms.

Richards and others (1998) demonstrated that as little as one minute of contact between dark mackerel muscle and blood resulted in a significant decrease in frozen storage stability. In an additional study, Richards and Hultin (2002) used a washed cod model system to show the influence of hemoglobin-catalyzed lipid oxidation. This washed cod model system contains very low concentrations of triacylglycerols, which renders it particularly useful in studying the effects of membrane phospholipid oxidation against a backdrop of muscle without the influence of native water-soluble pro- and antioxidant species. Richards and Hultin (2000) discovered a delicate relationship between muscle pH and the effects of lipid oxidation catalyzed by hemoglobin based on the development of thiobarbituric acid-reactive substances (TBARS) and changes in sensory scores. Their conclusion was that as pH decreases, heme-catalyzed lipid oxidation activity increases.

Due to the highly destructive potential of hemoglobin in the muscle tissue, this powerful pro-oxidant is contained and protected by red blood cells. However, red blood cells are easily disrupted by mechanical processing means, such as in the filleting of fish, as well as during various other processing techniques like freezing. When red blood cells lose their structural integrity, the once sequestered hemoglobin is allowed to freely enter the muscle tissue and release its oxygen constituent. The liberation of oxygen from hemoglobin is favored at low muscle pH and in the presence of ATP, yielding a variety of

reactive species (Kelleher et al., 1992). These molecules include oxygen with great reduction potential and highly reactive hemoglobin dimers and monomers which bind readily to lipids due to their strong hydrophobic nature (Everse and Hsia, 1997; Griffith and Kaltashov, 2003). In progressive oxidation of heme, iron may eventually be released from heme.

2.3.3 Consequences in muscle food

Lipid oxidation impacts muscle food flavor, aroma, texture, nutritive value, and appearance (Ladikos & Lougovois, 1990). The extent of lipid oxidation depends on the characteristics of the muscle tissue in question. For example, the concentration of native pro-oxidants and antioxidants as well as polyunsaturated fatty acid content will greatly affect the extent and rate of oxidation.

The development of the off-odors and flavors is due to the accumulation of volatile ketones and aldehydes, which are end-products of hydroperoxide decomposition via scission of the oxygen-oxygen bond followed by hemolytic cleavage of the resulting alkoxy group (Nawar, 1996). These aldehydes and ketones are also able to react with neighboring proteins to create even more unfavorable sensory compounds. For example, when fish muscle is stored, “cold-store” flavor develops and is attributed to the formation of cis-4-heptenal. Another common class of fishy cod-liver oil type odor compounds formed form the autoxidation of long-chain ω-3-unsaturated fatty acids include 2,4,7-decatrienal isomers (Lindsay, 1990; Lindsay, 1996 Lindsay, 2007).

When considering meats, a common phenomenon results when meats are cooked then stored under refrigeration conditions and then reheated. This characteristic is called warmed over flavor. Warmed over flavor is attributed to the disruption and dehydration of muscle membranes during the cooking process which are then oxidized by traces of metal ions released from heat-degraded heme proteins (Fisher & Scott, 1997; Foegeding, et al 1996).

Muscle food texture is negatively affected by lipid oxidation and manifests in toughening. Meat toughening is the results of oxidation products denaturing contractile proteins and is often associated with frozen storage of fish muscle. When contractile proteins denature, the proteins cross-link, thus decreasing water-holding capacity and increasing toughness (Foegeding, et al 1996).

Unfavorable yellowing in muscle foods can also be partly attributed to lipid oxidation. For example, Thanonkaew and others (2006) demonstrated that squid amine groups located on the phospholipid polar head groups were able to react with aldehydes formed from phospholipid oxidation and created yellow pigments by means of nonenzymatic browning-type reactions. Also, Lauritzen and Martinsen (1999) detected yellow and brown color changes in cod fish fillets during the salting process.

2.4 Antioxidants

2.4.1 General

Antioxidants are added to lipid-containing foods with the purpose of extending shelf-life and maintaining quality. Since a wide range of antioxidant compounds exist and

operate in various ways, it is difficult to develop an all-encompassing definition for all antioxidants. In many food systems, more than one antioxidant is used to increase efficacy against different mechanisms of oxidation. The majority of lipid soluble antioxidants used in the food industry are phenolic compounds with various substituted ring constituents (Decker and McClements 2007; Nawar, 1996).

Antioxidants intentionally added to foods can be either extracted from natural sources or chemically synthesized. Several examples of naturally occurring antioxidants include tocopherols, carotenoids, ascorbic acids, ascorbate salts, enzymes, amino acids, proteins, protein hydrolysates, as well as a wide variety of phenolic compounds and plant extracts. Commonly used synthetic antioxidants are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), gallic acid esters (GAE), and ethylenediamine tetraacetic acid (EDTA). Regardless of origin, antioxidants must be economical, nontoxic, effective at low concentrations, capable of retaining function through various processing procedures, and have low impact on product color, flavor, and odor. Antioxidants protect lipids by utilizing a wide range of mechanisms such as chelating metal ions, quenching singlet oxygen, detoxifying reactive oxygen species, as well as controlling various oxidation intermediates (Laguerre et al, 2007; McClements & Decker, 2007; Nawar, 1996; Reische et al, 2002).

2.4.2 Phenolic antioxidants

Phenolic antioxidants are one of the oldest and most frequently used antioxidants in the food industry (Cuvelier et al, 1992; Kawabata et al, 2002; Reische et al, 2002 Rice-

Evans et al; 1996). Phenolic compounds are products of secondary plant metabolism and are naturally and ubiquitously present in plants (Andjelkovic et al, 2006) or are chemically synthesized. Differences in the physical structure of phenolic antioxidants accounts for variations in antioxidant activity. Phenolic compounds containing alkyl groups act as extremely effective antioxidants and antimicrobials (Reische et al, 2002). The ability of phenolic compounds to act as antioxidants has been attributed to the number of phenolic hydroxyl groups present and their arrangement (Kawabata et al, 2002; Sroka & Cisowski, 2003). Studies by Rice-Evans and others (1996) and Joyeux and others (1995) suggest that *o*-hydroxyl substitution in the aromatic ring as being beneficial for antioxidant activity. A few examples of natural and synthetic phenolic antioxidants are located below in Figure 2.2 and include caffeic acid, quercetin, and catechin, BHT, TBHQ, and propyl gallate (Laguerre et al, 2007; McClements & Decker, 2007; Reische et al, 2002).

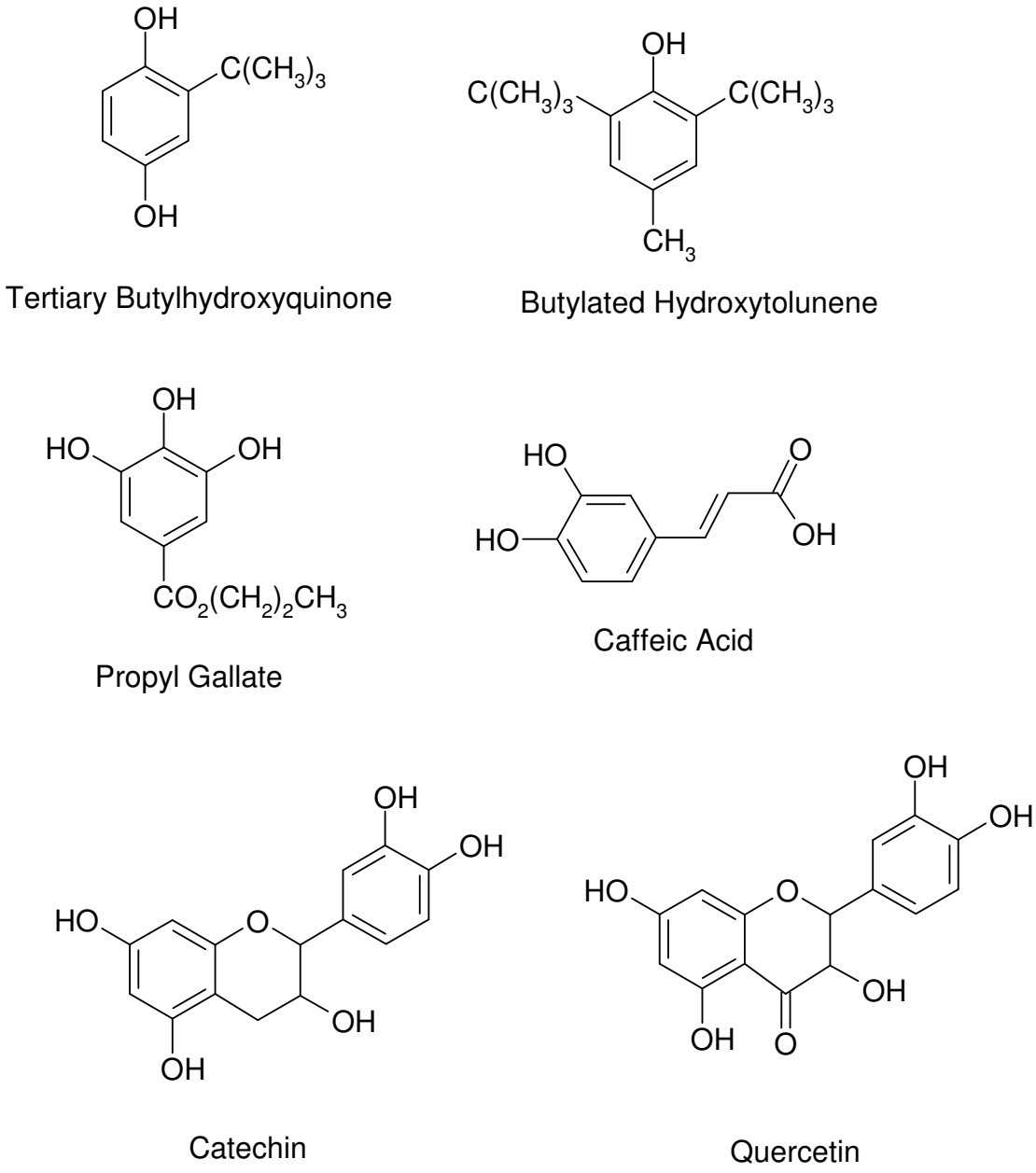

Figure 2.2: Structures of common phenolic compounds.

2.4.2.1 Gallic acid esters

Gallic acid esters (GAE) of varying alkyl chain length are the choice antioxidant compounds in the distribution and oxidation studies because they have the same reactive groups but vary in hydrophobicity degree. The hydrophobic nature of GAE increases as

alkyl chain length increases. GAE act as highly effective chain breaking antioxidants by transferring a hydrogen atom from a hydroxyl group to various lipid radical species. The resulting gallate semiquinone free radicals also effectively couple lipid radicals which create highly stable structures (Dwiecki et al, 2006; Terao at al, 1994). The specific GAE used in these studies include propyl gallate (PG), octyl gallate (OG), and lauryl gallate (LG). These GAE compounds are currently approved for use in food products (Aruoma et al, 1993; Kubo et al, 2002).

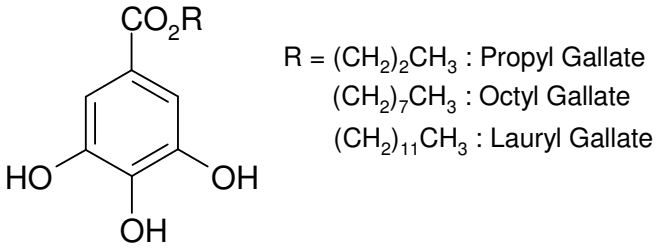

Figure 2.3: Structures of gallic acid esters.

2.5 Phenolic antioxidant mechanisms of action

2.5.1 Radical Scavenging

Phenolic antioxidants (ArOH) act as radical scavengers by donating hydrogen atoms and creating resonance-stabilized phenolic radicals, which has been shown by EPR spectroscopy and quantum calculation techniques (Amorati et al, 2006; Bors et al, 2002; Bors & Michael, 1999; Dwiecki et al, 2006; Lucarini at al, 2002; Kawabata et al, 2002). The formed phenolic radicals are low energy in nature, meaning they do not catalyze unsaturated lipids at rapid rates. EPR equilibration studies performed in *tert*-butyl alcohol by Lucarini and others (2002) showed that the propyl and octyl gallate radicals are

symmetric with the unpaired electron coupled with two each of equivalent ring and hydroxyl protons (Figure 2.4).

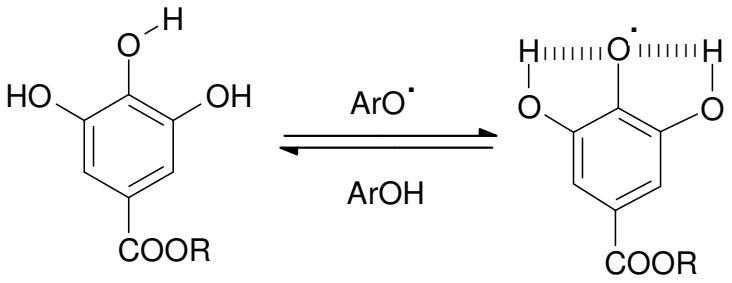

Figure 2.4 Phenolic radical formation, an adaptation from Lucarini and others (2002).

Hydrogen donation can occur as a straight forward, one-step reaction (Eq.1) or as a multi-step process involving electron and proton transferring (Eq. 2) (Ji et al, 2006; Zhang, 2005). Phenolic antioxidants in non-polar solvents favor radical scavenging by means of the single step method since charge separation is not required and characterized by the O-H bond dissociation enthalpy (BDE). When phenolic antioxidants are added into a polar solvent, a multi-step reaction scheme takes place due to charge separation processes and is governed by the ionization potential (IP) (Burton et al, 1985; de Heer et al 2000; Ji et al, 2006; Siquet et al, 2006).

(1) $RO\cdot + ArOH \rightarrow ROH + ArO\cdot$

(2) $RO\cdot + ArOH \rightarrow RO^{-} + ArOH\cdot^{+} \rightarrow ROH + ArO\cdot$

2.5.2 Chelating metal ions

Metal ions are capable of accelerating lipid oxidation by removing hydrogen atoms and destroying peroxides, which leads to free radical formation (Decker, 2002). Phenolic compounds are able to form stable metal complexes that inhibit participation in free radical generation reactions (Andjelkovic et al, 2006; Jovanovic et al, 1998; Leopoldini et al, 2006; Morel, et al 1994; Morgan et al, 1997; Pazos et al, 2006ab; Reische, 2002). Suggested structural properties that enable phenolic compounds to act as metal chelators include chatechol or galloyl groups, with galloyl groups showing the stronger chelating ability of the two (Andjelkovic et al, 2006). Metal chelation is able to occur at physiological pH; therefore it has a physiological significance (Khokhar & Apenten, 2003; Morgan et al., 1997)

2.5.3 Antioxidant partitioning

2.5.3.1 General

The ability of antioxidants to be effective at protecting lipids from the damaging consequences of lipid oxidation is highly dependent on their interactions with the vulnerable substrates. Lipid substrate characteristics such as type, degree of unsaturation, and structure, strongly shape individual antioxidant defense abilities (Becker et al, 2004; Frankel & Meyer, 2000). When added to a multiphase system, antioxidant solubility levels are critical for permitting interactions with the various components. Solubility impacts antioxidant phase distribution and to some extent determines both localization and orientation. Several studies suggest a “polar paradox” exists when evaluating antioxidant efficacy and is based on the assumption that lipid oxidation occurs at phase

interfaces where antioxidants are concentrated. This relationship was termed a paradox because polar antioxidants are more effective at protecting nonpolar bulk oils while nonpolar species perform better in polar membranes and emulsions (Becker et al, 1994; Porter, 1993; Porter et al, 1989). This reciprocal effect is highly pronounced when observing gallic acid and the gallate esters (Morris et al, 1947; Porter et al, 1989).

2.5.3.2 Antioxidant partitioning in cell membranes

Several studies suggest that antioxidants need to be in close proximity to membrane surfaces in order to provide defense against oxidation (Kikuzaki et al, 2002; Lee et al, 2006; Nakayama et al, 1998; Niki, 1990). Kikuzaki and others (2002) suggest that in addition to the radical scavenging activity of antioxidants, polarity and three-dimensional interactions with lipid bilayers also play a significant role in protecting liposomes. NMR evidence from studies using ferulic acid alkyl esters propose partitioning behavior may be related to the ability of the ester side chain to anchor into the phospholipid membrane while the exposed phenoxy group is in the aqueous region awaiting potential pro-oxidant species (Anselmi et al, 2004). In another study using evidence from spectral shift and fluorescence quenching experiments, Dwiecki and others (2006) demonstrated that propyl gallate associates with the hydrophilic portion of membranes and therefore may act as an aqueous phase antioxidant. An additional explanation of how antioxidants associate with the membranes is the phenolic groups may directly interact with the membrane proteins through hydrophobic interactions

(Almajano et al, 2007; Dwiecki et al, 2006; Lee et al, 2006; Oda et al, 1998; Pazos et al, 2006b; Rawel et al, 2005; Viljanen at al, 2005).

Studies using tea catechins have shown that hydroxyl group number, presence of the galloyl group, and stereochemistry determines their affinity towards lipid bilayers (Hashimoto et al, 1999; Kajiya, et al, 2001; Kumazawa et al, 2004). Kumazawa and others (2004) provided direct experimental evidence of epigallocatechin gallate (EGCG) interacting with a model membrane system of dimyristoylphosphatidylcholine using solid state ^{31}P and ^{2}H NMR techniques. In this study, EGCG was also shown to affect the phospholipid head-group motions and had one conformation that demonstrated rotational motion. Catechins have been shown to affect membrane structure and are mainly associated with membrane surfaces rather than the interior (Yoahioka et al, 2006). In addition to catechin structure, other factors that influence catechin membrane affinity include salt concentration, membrane electric charge, and the presence of other catechins (Kajiya et al, 2002).

2.5.4 Effects of exogenous antioxidants in membranes

2.5.4.1 Membrane fluidity

Several studies proposed that flavonols are able to localize in membrane interiors and protect the associated lipids from oxidation by altering the membrane fluidity (Arora et al, 2000; Calturla et al, 2003; Zhang et al, 2006). Zhang and others (2006) demonstrated a relationship exists between an increase in GAE alkyl chain length and an increase in membrane incorporation and retention. Tammel and others (2004) also concluded that there was an association between both the degree of hydroxylation and

molecular configuration with membrane affinity of flavonoids and alkyl gallates. Although longer alkyl side chains correspond to greater membrane associations, these longer side chains reduced the mobility of the antioxidants between and within the membranes and also caused decreases in membrane fluidity (Sunamoto et al, 1985; Yoshida et al, 2003). This change in fluidity could potentially halt free radical diffusion through the membrane due to steric hindrance, thus diminishing free radical reaction kinetics (Lee et al, 2006; Sunamoto, 1985). Lee and others (2006) suggest flavonols located in the lipid bilayer have a physical advantage for scavenging radicals because the lipid bilayer is the production site of alkoxyl and heme radicals.

2.5.4.2 Antioxidant mechanisms of action in membranes

The inhibitory properties of phenolic compounds are related to several physicochemical factors such as polarity, membrane association, reducing capacity, chelating activity, and protection against hemoglobin autoxidation (Pazos and others, 2006b). Pazos and others (2006b) suggested that phenolic compounds in microsomal systems were more active at delaying the induction period of oxidation rather than inhibiting the propagation period. Also, a high correlation of TBARS development inhibition existed between phenolic compound membrane association and their reducing and metal ion chelating abilities (Pazos et al, 2006b).

2.6 Phenolic antioxidants added to minced fish

Mincing fish incorporates air molecules into the muscle tissue. This processing technique exposes the lipids to oxygen and makes them more susceptible to oxidation (Lanier, 2000). This tendency towards rapid oxidation results in major quality problems during processing and storage (Suzuki, 1981). Like mincing, filleting fish also exposes the susceptible flesh to oxygen but to a lesser extent (Richards et al, 1998). To reduce the effects of postmortem exposure of fish muscle to air, freshly filleted fish can be dipped or sprayed with antioxidant solutions, powders, or extracts. In the case of minced muscle, antioxidants can be directly mixed into the minced tissue or be added in a carrier solvent. Carrier solvents function to deliver the antioxidants to the vulnerable muscle regions, namely the membrane phospholipids. The most common antioxidant carriers used in foods are oil, alcohol, and propylene glycol (Chipault, 1962; Chuang et al, 1974; Raghavan, 2005). Techniques such as forced injection, tumbling, soaking, marinating may be used to facilitate greater antioxidant penetration into the muscle (Chipault, 1962; Raghavan, 2005).

2.6.1 Natural phenolics

Several studies suggest phenolic compounds from various natural sources are able to delay lipid oxidation in various minced fish systems (Banerjee, 2006; Fagbenro & Jauncey, 1994; He & Shahidi, 1997; Lee et al., 2006; Medina et al, 2007; Pazos et al, 2005a; Ramanathan & Das, 1992; Vareltzis et al, 1997). For example, polyphenolic grape extracts have demonstrated lipid oxidation retardation in frozen minced fish muscle

(Pazos et al, 2005a). Two studies suggest that various green tea polyphenols show efficacy against lipid oxidation in minced mackerel tissue (Banerjee, 2006; He & Shahidi, 1997). Medina and others (2007) showed positive antioxidative effects of hydroxycinnamic acids and catechins on lipid oxidation in minced mackerel. In an earlier study, the polyphenols quercetin, myricetin, tannic acid, and ellagic acid all demonstrated potent antioxidant action when added to minced fish (Ramanathan & Das, 1992). Lee and others (2006) illustrated the antioxidant benefit of fractionated cranberry powder against hemoglobin-initiated oxidation in washed cod muscle. In addition, spice extracts from ginger and rosemary have also provided evidence for delaying lipid oxidation in minced fish muscle (Fagbenro & Jauncey, 1994; Vareltzis et al, 1997).

2.6.2 Synthetic phenolics

Synthetic phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) are currently approved for use in foods. He and Shahidi (1997) added BHT, BHA, and TBHQ to a minced mackerel fish model system and determined these phenolic compounds were effective in reducing TBARS development compared to the untreated samples. Raghavan (2005) added BHA, BHT, TBHQ, and PG to a washed haddock model systems with added canola oil and determined the most effective of the four synthetic phenolics at delaying the onset of TBARS development were TBHQ and PG.

2.7 Phenolic antioxidants as pro-oxidants

When the antioxidant-pro-oxidant balance is shifted toward pro-oxidant behavior, oxidative stress is induced. Consequences of oxidative stress in biological systems include a proliferation of radical production, decreases in antioxidant protection, and/or a marked increase in oxidative damage. A delicate balance exists between antioxidants and radicals, which is dependent on several variables such as reactive species concentration, environmental pH, oxygen tension, and associations with other antioxidants (Pezzuto & Park, 2002).

At high concentrations, phenolic compounds can act as pro-oxidants because of their high reactivity and participation in initiation reactions (Reische et al, 2002). Although phenolic compounds possess metal ion chelating abilities, sometimes they do so in such a fashion that either maintains or increases the catalytic activity of the metal. In addition, the metal reducing ability of phenolic compounds may increase their ability to create free radicals from peroxides (Pezzuto & Park, 2002). In an aqueous environment, flavonoids are capable of autoxidation (Sergediene et al, 1999). Specifically, catechins were shown to be capable of accelerating LDL cholesterol oxidation during the propagation phase (Yamanaka, 1997). Quercetin also exhibits pro-oxidant properties depending on concentration, number of radical species present, and cellular location (Metodiewa et al, 1999).

CHAPTER 3

PHYSICAL LOCATION DETERMINATION AND ANTIOXIDANT ACTIVITY OF GALLIC ACID ESTERS IN FISH MUSCLE

3.1 Introduction

Mincing fish incorporates air molecules into the muscle tissue and exposes lipids to oxygen which increases their susceptibility to oxidation (Lanier, 2000). This tendency towards rapid oxidation results in major quality problems during fish processing and storage (Suzuki, 1981). Adding antioxidants to minced fish is common practice in the food industry. However, many questions remain about how the structure of antioxidants influences their partitioning between lipid classes, such as neutral oil and the oxidatively liable cellular membranes, and how antioxidant partitioning behavior impacts the oxidative stability of muscle foods.

Since most food systems contain several physically different phases, antioxidants partition into the various phases based on their thermodynamic affinities to the different fractions of different polarity (Castle and Perkins, 1986; Pryor et al, 1988; Wedzicha, 1988). Many factors affect antioxidant partitioning behavior, such as antioxidant and lipid polarity, pH, temperature, surfactants, and the composition of the assorted phases (Barclay and Vinqvist, 1994; Cornell et al, 1970; Huang et al 1996a; Pryor et al, 1993; Schwartz et al, 1996). In addition to partitioning considerations, the milieu in which an antioxidant is located affects its hydrogen-donating ability, accessibility to free radicals and prooxidants, and diffusion rate (Castle and Perkins, 1986; Hopia et al, 1996; Huang

et al, 1996ab; Koga and Terao, 1994, 1995; Raghavan & Hultin, 2005b; Roginski, 1990; Yi et al, 1991).

Several studies have demonstrated that the primary substrate of lipid oxidation in muscle tissue is the phospholipids associated with cellular membranes (Grandemer, 1999; Undeland et al, 2002; Younathan and Watts, 1959). The membrane phospholipids are vulnerable to lipid oxidation not only because of their high degree of polyunsaturated fatty acids, but also due to their close association with cytoplasmic prooxidants and high surface area (Hultin, 1995; Nawar, 1996). Therefore, many researchers suggest that concentrating antioxidants into muscle cell membranes would increase the ability of the antioxidants to inhibit lipid oxidation (Caltural et al., 2003; Kikuzaki et al, 2002; Lee et al, 2006; Nakayama et al, 1998; Niki, 1990; Ollila et al., 2002; Pazos et al., 2006ab; Raghavan, 2005; Raghavan & Hultin, 2005; Rawel et al., 2005; Tappel, 1955; Verstraeten et al., 2003). Also, the order of antioxidant and oil addition can alter an antioxidant's ability to delay the onset of lipid oxidation. This phenomenon was shown by alternating the addition sequence of neutral oil and ethanolic tocopherol into a minced cod muscle system (Raghavan and Hultin, 2004).

The purpose of the work presented here was to illustrate how antioxidant structure (hydrophobicity) accounts for differences in the ability of compounds to inhibit lipid oxidation due to variations in phase partitioning. Specifically, gallic acid esters (GAE) were chosen due to their common use in the food industry and because they have the same reactive groups but various degrees of hydrophobicity (Arouma et al, 1993; Dwiecki et al, 2006; Joung et al, 2004; Terao at al, 1994). GAE are synthetic derivatives of gallic acid, a naturally occurring antioxidant whose hydrophobicity can be alter by esterification

with alkyl chains of various length. This antioxidant class acts as highly effective chain breaking antioxidants by transferring a hydrogen atom from its hydroxyl group to various lipid radical species. The resulting gallate semiquinone free radicals can then inactivate a second lipid radical via termination reactions forming a lipid-antioxidant conjugate (Dwiecki et al, 2006; Terao at al, 1994). Figure 3.1 depicts the three GAE selected for observation: propyl gallate (PG), octyl gallate (OG), and lauryl gallate (LG).

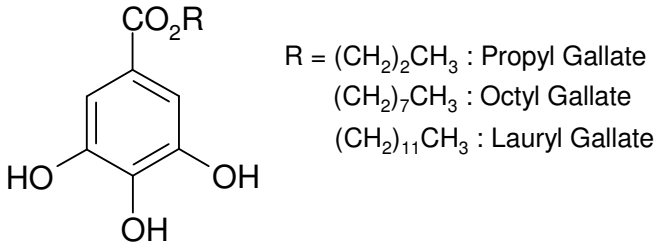

Figure 3.1: Chemical structures of gallic acid esters.

The objectives of this investigation were two fold. The first objective was to determine if gallic acid esters could be selectively incorporated into the membranes of fish muscle and evaluate how alkyl chain length on the gallic acid affected membrane partitioning. Selective incorporation refers to an increase in antioxidant uptake in the membrane lipids rather than a uniform distribution between triacylglycerols and membrane lipids (Raghavan & Hultin, 2004). The second objective was to determine how the order of GAE and canola oil addition affects antioxidant activity in a model system. Washed cod muscle with added hemoglobin was used as the model muscle oxidation system.

3.2 Materials and Methods

3.2.1 Materials

Fresh Atlantic cod (*Gadus morhua*) or haddock (*Melanogrammus aeglefinus*) fillets were purchased from a local fish company (J. B. Wright, Gloucester, MA) and kept on ice at all times. Gallic acid esters were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO) while all other dry chemicals and organic solvents were obtained from Fisher Scientific (Chicago, IL). Canola oil was purchased from a local supermarket.

3.2.2 Methods

3.2.2.1 GAE distribution when added to prepared fish membranes

3.2.2.1.1 Membrane isolation

White muscle from cod or haddock was separated from skin and dark muscle. The muscle was twice passed through a Kitchen Aid ultra power grinder (Model KSM90, St. Joseph, MI) fitted with a 5mm perforated plate. Membrane preparations were conducted using a method by Apgar and Hultin (1982) with slight modification. Four parts cold histidine buffer (5mmol histidine/0.12M KCl, pH 7.2) and one part minced fish were combined and homogenized two times at speed 4 for 40sec using a Polytron homogenizer (Model PT 10-35, Brinkmann Instruments, Westburg, NY). The pH of the homogenate was measured using an Orion pH meter (Model 420A) with an Orion Sure-Flow ROSS pH electrode (Orion Research Inc., Beverly, MA) and was adjusted to pH 7.2 with 2 N NaOH or 2 N HCl. The pH adjusted homogenate was then centrifuged under refrigerated conditions (8ºC) at 12,000*g* for 20 min using a Beckmann Ultracentrifuge with a Type 19

rotor (Model L5-65B, Beckman Instruments Inc., Palo Alto CA). The resulting supernatant was reserved and centrifuged again at 100,000*g* for 30 min using a Type 45 rotor. After this centrifugation step, the sediment and 0.6 M KCl buffer (6mL buffer/10g initial mince weight) were combined and homogenized in a Potter-Elvehjem tissue grinder. The resulting homogenate was centrifuged at 100,000*g* for 30 min. After this final centrifugation step, the membranous sediment was suspended in the histidine buffer (3mL/10g initial mince weight) using a Potter-Elvehjem tissue grinder and stored at 4°C for up to four days.

3.2.2.1.2 Washed fish muscle preparation

Washed fish muscle was prepared using a method by Vareltzis (2006) with slight alterations. White muscle from cod or haddock was separated from skin and dark muscle. The muscle was twice passed through a Kitchen Aid ultra power grinder (Model KSM 90, St. Joseph, MI) fitted with a 5 mm perforated plate. Cold water and minced fish were combined at a ratio of 1:5 (w/v) and stirred for 2 min with a plastic spatula. After a 10 min resting period on ice, the slurry was strained with a mesh screen. Two additional washing treatments were carried out except that a cold 100 mM NaCl solution was used rather than deionized water. After the final dewatering step (using a mesh screen as before) was completed, the pH of the washed fish (WF) was adjusted to 7.2 using either 2 N NaOH or 2 N HCl. The moisture content of WF was ~90%. WF was kept on ice throughout the procedures.

3.2.2.1.3 Isolation of membranes from washed fish muscle

Membranes were isolated from washed fish using the method by Raghavan and Hultin (2005a) with slight modification. Washed fish samples were combined with four volumes of 0.05 M phosphate buffer (pH 7.2) and homogenized two times at speed 4 for 40 sec using a Kinematica Polytron Homogenizer (Brinkmann Instruments, Westburg, NY). Homogenate pH was adjusted to pH 7.2 using 2 N NaOH or 2 N HCl and was then centrifuged at 12,000*g* for 20 min. The resulting supernatant was then divided equally among four clean ultracentrifuge tubes and centrifuged at 100,000*g* for 30 min to pellet the membrane fraction. The contents of two tubes were used for GAE quantification while the membranes in the remaining tubes were analyzed for protein, lipid, and phospholipid content.

3.2.2.1.4 Carrier solvent preparation

Gallic acid esters (GAE) were individually prepared in ethanol at 18.7 mmol/L to reflect a final concentration of 200 ppm (total lipid basis) of propyl gallate in the membrane suspension. GAE were prepared at equivalent molar concentrations to account for variations in alkyl chain length and thus molecular weight. Plain ethanol was used as the control. The carrier solvent was added to the membranes or washed fish at 0.1% of the total volume.

3.2.2.1.5 Sample preparation

Muscle membrane samples were prepared in a similar manner as described by Sigfusson and Hultin (2002) with a few modifications. For samples without added canola oil, ten milliliters of suspended membranes were combined with 0.1 mL GAE solution or ethanol in an ultracentrifuge tube and vortexed for 15 sec using a Fisher Scientific Touch Mixer (Model 232, Fisher Scientific, Pittsburg, PA). Canola oil, used to provide competition for GAE partitioning between neutral lipids and phospholipids, was added at 33% of the total membrane suspension volume. Therefore, 15mL suspended membranes and 4.5g (5 mL) oil were added to an ultracentrifuge tube and vortexed for 15 sec followed by the addition of 0.15 mL GAE solution or ethanol and subsequent vortexing for 15 sec. Regardless of oil addition or omission, 13.2 mL histidine buffer (5mmol histidine and 0.12M KCl) were added before phase separation. Phase separation was achieved by centrifuging the prepared samples under refrigerated conditions (8ºC) at 130,000*g* for 30 min and collecting the membrane sediment.

For washed muscle experiments, fifty grams of washed muscle and either ethanol or individually prepared GAE (0.5 mL) solutions were combined and then mixed using a mortar and pestle for 2.5min. Washed muscle samples were kept on ice throughout the preparation procedures.

3.2.2.1.6 Determining GAE content in the various phases

When oil was used in the sample preparation, the top oil phase was analyzed by carefully removing 100 uL and adding it into 4 mL ethanol. Absorbance values were read

against a blank at 280 nm using a Shimadzo UV-1201 UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan). GAE concentration in the oil phase was determined by subtracting the absorbance value of the control sample from the absorbance values of the treated samples and comparing this difference with a previously constructed standard curve of the appropriate GAE.

In studies with isolated membranes, the remaining oil layer (when present) was removed and the aqueous phase containing the membranes was analyzed for GAE content. The membrane fraction was isolated by centrifugation at 130,000*g* for 30 min. After the aqueous layer was removed, the interior walls of the ultracentrifuge tubes were carefully wiped to remove any remaining lipid or GAE. Histidine buffer (10mL) was then added to each tube and the membrane sediments were carefully loosened from the sides of the tubes using a thin metal spatula. The membrane samples were poured into a Potter-Elvehjem tissue grinder and homogenized using seven strokes (Raghavan and Hultin, 2005a). These membrane suspensions (MS) were transferred to clean ultracentrifuge tubes and 20 mL ethanol were added. The membrane/ethanol slurry was vortexed for 15 sec and then centrifuged under refrigerated conditions (8ºC) at 130,000*g* for 30 min. Absorbance values of the resulting ethanolic supernatants were determined at 280 nm. After correcting the absorbance values of the GAE-treated samples by subtracting the absorbance value of the control sample, the adjusted values were compared to the appropriate GAE standard curves to calculate concentrations.

For membranes isolated from washed fish, 4.0 mL ethanol were added to each tube containing the membrane pellet and the contents were homogenized with a Bio Homogenizer (Biospec Products Inc., Bartlesville, OK) for 1 min. The homogenized

contents of 2 tubes were combined and centrifuged under refrigerated conditions (8ºC) at 130,000*g* for 30 min. GAE concentrations in the ethanol fraction were measured as described above.

3.2.2.1.7 Measuring protein and lipids

Four milliliters of 0.05 M phosphate buffer were added to the pelleted membrane fractions. The membrane sediments from each tube were then carefully loosened using a thin metal spatula and poured into a Potter-Elvehjem tissue grinder and homogenized. These homogenates were then used to measure protein, total lipids and phopholipids.

Protein content was measured using a method by Markwell and others (1978). Protein concentrations were calculated from a bovine serum albumin standard curve. Total lipids were measured by combining 4.0 mL of membrane homogenates with 40 mL of chloroform/methanol (1:1 ratio) in a seperatory funnel. The contents were shaken and then 16 mL of 0.5% NaCl solution were added to enhance phase separation (Sigfusson & Hultin, 2002a). After vigorous shaking, the phases were allowed to separate for 3 hr and then the bottom phase (chloroform) was drained into a glass beaker. Five milliliters of the chloroform were transferred into a pre-weighed glass beaker which was placed onto a hot plate with the temperature set to 80ºC until the chloroform was completely evaporated. When the evaporation step was complete and beakers cooled, the beakers were weighed again. The difference between the initial and final beaker weights was calculated and total lipid content was determined by dividing the calculated difference by volume of membrane sample analyzed.

Total lipid content of washed fish was determined using a similar method to muscle membranes. In short, 10 g washed fish and 50 mL chloroform: methanol (1:1; v/v) were homogenized in a Waring blender (Waring Products Division, Dynamics Corporation of America, CT) at 50% speed for 60 s. The homogenate was filtered through a Whatman #4 filter paper into a separatory funnel and then the lipids in the chloroform layer were determined as described above.

A spectrophotometric method by Anderson and others (1982) was used to determine the phosphate content of the fish membrane lipids. One hundred μL aliquots of the chloroform from the total lipid analysis were transferred into screw cap test tubes. The tubes were placed into a Corning heating block (16.75mm well diameter, 12 wells per block, Corning Incorporated Science Product Division, Corning, NY) which was positioned onto a preheated (105ºC) hot plate to expedite chloroform evaporation. When the chloroform was completely evaporated, the tubes were removed and the temperature was increased to 155ºC. Meanwhile, 100 uL of concentrated sulfuric acid were added to each tube and vortexed for 15 sec. The tubes were returned to the hot plate for 10 min. The tubes were then cooled and 50 uL 6% hydrogen peroxide were added followed by vortexing. After vortexing, the tubes were once again returned to the heating block and hot plate for 40 min. The tubes were allowed to cool and 2 mL deionized water and 0.8 mL of a 1:1 mixture of 280 mM ascorbic acid and 10.1 mM ammonium molybdate were added and vortexed. The caps were screwed onto the tubes and they were placed into a boiling water bath (Model 181, Precision Scientific, Mumbai, India) for 7 min. After the samples were cooled, the absorbance values were measured at 797 nm. The absorbance values were compared to a previously constructed standard curve using sodium dibasic

phosphate. An average molecular weight of 750 for phosphatidylcholine was used to calculate phospholipid concentrations.

3.2.2.2 Oxidation Studies

3.2.2.2.1 Hemolysate preparation

Fresh cod frames were used as the hemolysate source. Hemolysate was prepared according to Fyhn and others (1979). Cod frames were severed at the tail and blood was collected from the vein using a glass pipette that was rinsed with heparin solution (30 units of heparin per mL and 150 mM NaCl) to prevent the red blood cells from bursting. Collected blood was transferred to glass test tubes containing the heparin solution. After blood collection was completed, Tris/NaCl (1 mM Tris and 290 mM NaCl) was added to each tube to achieve ¾ volume. Blood samples were then centrifuged (4ºC) at speed 5 for 10 min using a Sorvall RT6000 refrigerated centrifuge (DuPont, Wilmington, DE). The resulting plasma-rich supernatant was carefully removed and sediments were resuspended in the Tris/NaCl mixture and centrifuged again. This process was repeated until the supernatant becomes clear. The red blood cell pellets were then lysed by suspending them in 2 mL of 1mM Tris buffer (pH 8) and were transferred to an ultracentrifuge tube that was placed in ice for 1 hr. One-tenth volume 1 M NaCl was added to aid in stromal removal before centrifuging (8ºC) at 30,000*g* for 15 min in a Beckmann Ultracentrifuge (Model L5-65B, Beckman Instruments Inc., Palo Alto CA). The resulting supernatant was used as the hemolysate which was stored at -80ºC until needed.

The hemoglobin content of the hemolysate was determined using the method of Brown (1961). The hemolysate was thawed and poured into a large test tube covered with

aluminum foil (to block out light) and kept on ice at all times. In a test tube, 3 mL 50 mM Tris buffer (pH 8) and 30 µL hemolysate were combined. Three milliliters 50 mM Tris buffer served as the blank. To each test tube, 1.0 g sodium hydrosulfite was mixed into the samples followed by bubbling carbon monoxide into the solutions for 10 sec. Peak 420 nm) and valley (392 nm) absorbance values of the hemolysate were compared to that of the blank using a Hitachi U-3110 double beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA). The difference in absorbance values were compared to a standard curve prepared with bovine hemoglobin.

3.2.2.2.2 Sample preparation and oxidation storage studies

GAE and oil where added to the washed fish in different orders. GAE solution or ethanol (control) was added to 30 g washed fish at 1% volume by mixing with a mortar and pestle on ice for 2.5 min. Canola oil (5%, w/w), when used, was mixed in before or after the GAE for 30 sec. Streptomycin (200 ppm) was mixed in for 30 sec to delay microbial spoilage. Then, the hemolysate (6µmol hemoglobin/kg washed fish) was mixed in for 1 min. Time zero samples were immediately frozen at -80ºC for later analysis. The remaining samples were transferred to dark-tinted screw-capped containers and stored on ice at 4ºC.

GAE or ethanol (control) was mixed directly into the canola oil followed by addition of washed fish. GAE and canola oil (1% and 5%, respectively, based on 30 g washed fish) were mixed together for 1 min using a mortar and pestle. Thirty grams of washed fish were then added to the oil and ethanolic mixture and mixed for 3 min.

Streptomycin and hemolysate were then added and samples were stored at 4ºC as described above.

3.2.2.2.3 Measurement of lipid oxidation products

Thiobarbituric acid reactive substances (TBARS) concentrations in the treated samples were analyzed using a modified method of Lemon (1975). A trichloroacetic acid/antioxidant solution was prepared by mixing 7.5% trichloroacetic acid, 0.1% propyl gallate (first dissolved in 2mL ethanol) and 0.1% EDTA (first dissolved in 2 N NaOH) into distilled water. A thiobarbituric acid (TBA) solution (0.02 M) was prepared in deionized water and stored in a dark, glass bottle. Washed fish samples (1.0 g) were homogenized with 3.0 mL TCA solution for 1 min at high speed using a Bio Homogenizer (Model M133/1281-0, Biospec Products, Inc., Bartlesville, OK). The homogenate was then transferred to a disposable test tube and centrifuged (8°C) at speed 5 for 10 min using a Sorvall RT6000 refrigerated centrifuge. Two milliliters of the resulting supernatant were mixed with 2 mL of the TBA solution in a screw-capped test tube and then place into a boiling water bath for 40 min. After cooling at room temperature, absorbance was measured at 530 nm. TBARS (µmol TBARS per kg tissue) concentrations were calculated from a standard curve prepared with tetraethoxypropane.

Lipid hydroperoxides were measured using a method by Vareltzis (2006). One gram washed fish and 10 mL chloroform/methanol (2:1) containing 500 ppm BHT were homogenized for 20 sec at high speed using a Bio Homogenizer. The resulting homogenate was then transferred into a glass test tube and centrifuged (8ºC) at speed 5 for 10 min using a Sorvall RT6000 centrifuge. The chloroform layer was removed and

combined with 3 mL 0.5% NaCl in a clean test tube, vortexed for 15 sec and then centrifuged at speed 5 for 10 min using an IEC tabletop centrifuge. Two milliliters of the resulting bottom chloroform phase were collected and mixed with 8 mL chloroform/methanol (2:1) containing 500ppm BHT. The control sample was 10 mL chloroform/methanol (2:1). Ammonium thiocyanate and ferrous chloride solutions were prepared as described by Shantha and Decker (1994). To both the sample and control, 50 µL ammonium thiocyanate and 50 µL ferrous chloride were added followed by vortexing for 15 sec. Samples were then allowed to rest for 2 min. Absorbance values were determined at 500 nm compared to the blank. Lipid hydroperoxides (µmol hydroperoxide per kg tissue) were quantified using a standard curve prepared from cumene peroxide.

3.2.2.3 Statistical Analysis

GAE recovery in isolated muscle membranes: All experiments were performed twice. GAE in the aqueous, membrane, and triacylglycerols fractions was analyzed once within each experiment. Results from the two experimental replications were combined and analyzed. Analysis of variance was used to determine significant differences between samples using SAS 9.1 (SAS Institute, Cary, NC, U.S.A.). Least significant difference (LSD) was used to determine significance between samples.

GAE recovery in washed fish muscle: All experiments were performed three times. GAE in the membrane fractions was analyzed once within each experiment. Results from the three replications were combined and analyzed. Analysis of variance was used to determine significant differences between samples using SAS 9.1 (SAS

Institute, Cary, NC, U.S.A.). Least significant difference (LSD) was used to determine significance between samples.

Oxidation Studies: All oxidation experiments were performed twice. Analysis of variance was used to determine significant differences between the lag phase of the control and the individual treatments using SAS 9.1 (SAS Institute, Cary, NC, U.S.A.).

In all experiments, significance was established at $p < 0.05$ and results were expressed as mean ± standard error.

3.3 Results and Discussion

3.3.1 GAE distribution in muscle membranes

GAE were separately added at equivalent molar concentrations to prepared membrane suspensions with or without added canola oil and their partitioning into aqueous, membrane and oil phases were determined. The data in Table 3.1 provide the average protein, lipid, and phospholipid content of the membrane sediments. The protein, lipid, and phospholipid contents of the membrane sediments were not significantly different. However, the membranes treated with the canola oil did show slightly greater total lipid content. This difference could be due to some of the neural lipids from the added oil being incorporated into the membrane sediment in a manner in which the oil could not be completely separated during centrifugation.

No differences in the GAE concentrations were observed in either the oil phase (Table 3.2). However, the aqueous phase PG concentrations were significantly greater than OG and LG. Also, PG concentrations in the membrane sediments were significantly lower than in the OG or LG. The relative differences among antioxidant treatments did

not change in the presence of oil. In a 10% corn oil-water, over 90% of PG was detected in the aqueous phase after equilibrium was reached (Huang et al, 1997). Media and others (2007) reported approximately 50% of PG was detected into the aqueous phase of a 1:1 fish oil-water model system. On the other hand, more hydrophobic antioxidants, such as tocopherol, carnisol, and carnosic acid showed over 90% partitioning in the oil phase of a 1:1 corn oil-water system (Huang et al, 1997). In addition, 99.9% of the hydrophobic antioxidant BHT was recovered in the oil phase of a 1:1 fish oil-water model system (Medina et al, 2007).

Table 3.1: Membrane sediment protein, lipid, and phospholipid content from the recovered membranes of a fish membrane suspension-canola oil model system.

Gallic Acid Ester[1]	Protein (mg/mL)		Lipid (mg/mL)		Phospholipid (mg/mL)	
	Overall	Oil Only	Overall	Oil Only	Overall	Oil Only
PG	3.12 ± 0.14[a]	2.98 ± 0.41[a]	2.90 ± 0.55[a]	3.45 ± 1.45[a]	1.70 ± 0.05[a]	1.65 ± 0.45[a]
OG	2.99 ± 0.16[a]	2.83 ± 0.02[a]	2.60 ± 0.35[a]	2.95 ± 1.15[a]	1.65 ± 0.05[a]	1.60 ± 0.40[a]
LG	3.13 ± 0.09[a]	3.04 ± 0.42[a]	2.98 ± 0.28[a]	3.25 ± 1.25[a]	1.60 ± 0.10[a]	1.50 ± 0.40[a]

[1]Gallic acid ester = PG: propyl gallate; OG: octyl gallate; LG: lauryl gallate.
[a]Means with the same letter are not significantly different.

Table 3.2: Recovery of gallic acid esters (GAE, added at 18.7 mmol GAE/L) in the aqueous phase, membrane sediment, and neutral oil fractions of a fish membrane suspension-canola oil model system.

Gallic Acid Ester[1]	GAE Recovery				
	Aqueous Phase (µmol/L)		Membrane Sediment (mmol/kg lipid)		Oil (µmol/kg lipid)
	Overall	Oil Treated	Overall	Oil Treated	
PG	65.3 ± 5.6 [b]	70.9 ± 9.6[b]	21.5 ± 3.1[a]	18.4 ± 2.2 [a]	118 ± 14[a]
OG	8.4 ± 7.1[a]	15.5 ±6.0[a]	65.8 ± 0.9[b]	66.7 ± 11.5[b]	361 ± 29[a]
LG	10.6 ± 10.2[a]	20.8 ± 9.1[a]	57.5 ± 8.1[b]	65.5 ± 0.4[b]	170 ± 78[a]

[1]Gallic acid ester = PG: propyl gallate; OG: octyl gallate; LG: lauryl gallate.
[ab]Means with the same letter are not significantly different.

3.3.2 GAE recovery in membranes when added to washed fish muscle

In the next set of experiments, GAEs were added to washed fish muscle followed by isolation of the muscle membranes and analysis of the membrane fractions. Table 3.3 shows membrane sediment composition. There were no significant differences in the protein, lipid, or phospholipid contents among the GAEs. The protein content ranged from 2.83 ± 0.25mg/mL (LG) to 3.39 ± 0.26mg/mL (OG). Phospholipid content of the membrane sediments were determined to be between 1.20 ± 0.21mg/mL (OG) and 1.43 ± 0.03mg/mL (LG) while the total lipid content ranged between 1.70 ± 0.35mg/mL (OG) and 1.90 ± 0.23mg/mL (PG).

Table 3.4 shows that the GAE concentrations in the membrane sediments isolated from the washed fish muscle were significantly different. LG (17.5 ± 2.0 mmol/kg lipid) had the highest concentrations in the membrane sediment while PG (1.93 ± 0.81 mmol/kg lipid) was the lowest. Similar GAE partitioning trends have also been reported in other systems. Zhang and others (2006) found that as GAE alkyl chain length increased, the concentration of GAE in unilamellar vesicles increased. Tammela and others (2004) also

demonstrated that alkyl chain length governs membrane interaction of gallates and showed that OG was rapidly and almost completely incorporated (96%) into Caco-2 cell monolayers compared to the shorter-chained methyl gallate (54%) and PG (51%). A study by Nakayama and others (1998) showed similar GAE distribution trends when using a model liposomal system by showing 10% of added PG was incorporated into the model lipid bilayers while LG demonstrated 84% uptake. Pazos and others (2006b) reported 52% PG recovery in membranes when PG was added to isolated hake white muscle. In addition to GAE, tocopherol and ubiquinone homologs of varying hydrocarbon chain lengths also showed an increase in membrane incorporation as chain length increased (Kagan et al, 1988; Kagan et al, 1990; Landi et al, 1984).

It is important to mention that GAE recovery in the membranes when GAEs were added to the washed fish was not as high as when the GAE were added to the membrane suspensions. GAE was probably discarded in the aqueous phase while preparing the membranes from the washed muscle. Another possibility is that GAEs may have interacted with myofibrillar or other insoluble proteins by way of hydrophobic interactions or hydrogen bonding (Raghavan, 2005; Seufert et al, 1970).

Table 3.3: Membrane sediment protein, lipid, and phospholipid content from the recovered membranes of a washed fish model system.

Gallic Acid Ester[1]	Protein (mg/mL)	Lipid (mg/mL)	Phospholipid (mg/mL)
PG	3.36 ± 0.19[a]	1.90 ± 0.23[a]	1.27 ± 0.17[a]
OG	3.39 ± 0.26[a]	1.70 ± 0.35[a]	1.20 ± 0.21[a]
LG	2.83 ± 0.25[a]	1.73 ±0.33[a]	1.43 ± 0.03[a]

[1]Gallic acid ester = PG: propyl gallate; OG: octyl gallate; LG: lauryl gallate.

Table 3.4: Recovery of gallic acid esters in the membrane sediment of a washed fish model system.

Gallic Acid Ester[1]	GAE (ppm)/ Total lipid (g)[2]	Carrier Solvent[3] (mmol GAE/L)	GAE Recovery	
			Membrane Sediment (mmol/kg lipid)	Membrane Sediment (% Recovery)
PG	200	4.5	1.9 ± 0.81[a]	2.4 ± 1.1[a]
OG	266	4.5	12.6 ± 3.0[b]	13.1 ± 1.1[b]
LG	319	4.5	17.5 ± 2.0[b]	18.9 ± 0.58[b]

[1]Gallic acid esters = PG: propyl gallate; OG: octyl gallate; LG: lauryl gallate.
[2]GAE addition was based on washed cod lipids present + theoretical addition of 5% (w/w) canola oil.
[3]Carrier solvent as ethanol with dissolved GAE.

3.3.3 Effect of antioxidant addition on the oxidative stability of a washed cod-canola oil system

The ability of the GAEs to inhibit lipid oxidation in washed cod was determined by adding the antioxidant with and without added oil in different orders of addition. The treatments included: 1) direct GAE in ethanol addition without added oil; 2) oil first then GAE in ethanol; 3) GAE in ethanol first followed by oil and 4) GAE dissolved in oil and then added to washed cod. When the GAEs in ethanol were added directly to the WC system (treatment 1) in the absence of oil, they we not able to significantly inhibit

TBARS formation (Figure 3.2). In general, when the GAEs were added to washed cod to which canola oil had been added, inhibition of TBARS was observed. For example, when PG, OG and LG in ethanol were added after addition of canola oil (treatment 2, Figure 3.3), the lag phase of oxidation significantly increased 22-32 hr. When PG and OG in ethanol were added prior to oil addition (treatment 3, Figure 3.4), the lag phase increased 53 and 41 hr, respectively. In these studies, LG increased the lag phase, but not to the same extent as PG and OG. In the system where PG, OG and LG were dissolved in oil and then added to washed cod (treatment 4, Figure 3.5), all three GAE increased the lag phase. However, PG and OG increased the lag phase by 50 and 40 hr, respectively, while LG increased the lag phase by 16 hr. In all of the treatments studied, none of the GAEs where more effective at inhibiting TBARS formation than the others.

Lipid hydroperoxides were also measured to confirm if the GAEs could inhibit lipid oxidation in the washed cod system. In the treatments where GAE were added first followed by oil (treatment 3, Figure 3.6) and GAEs were dissolved in oil then added to the washed cod (treatment 4, Figure 3.7), all three GAE esters inhibited lipid hydroperoxide formation.

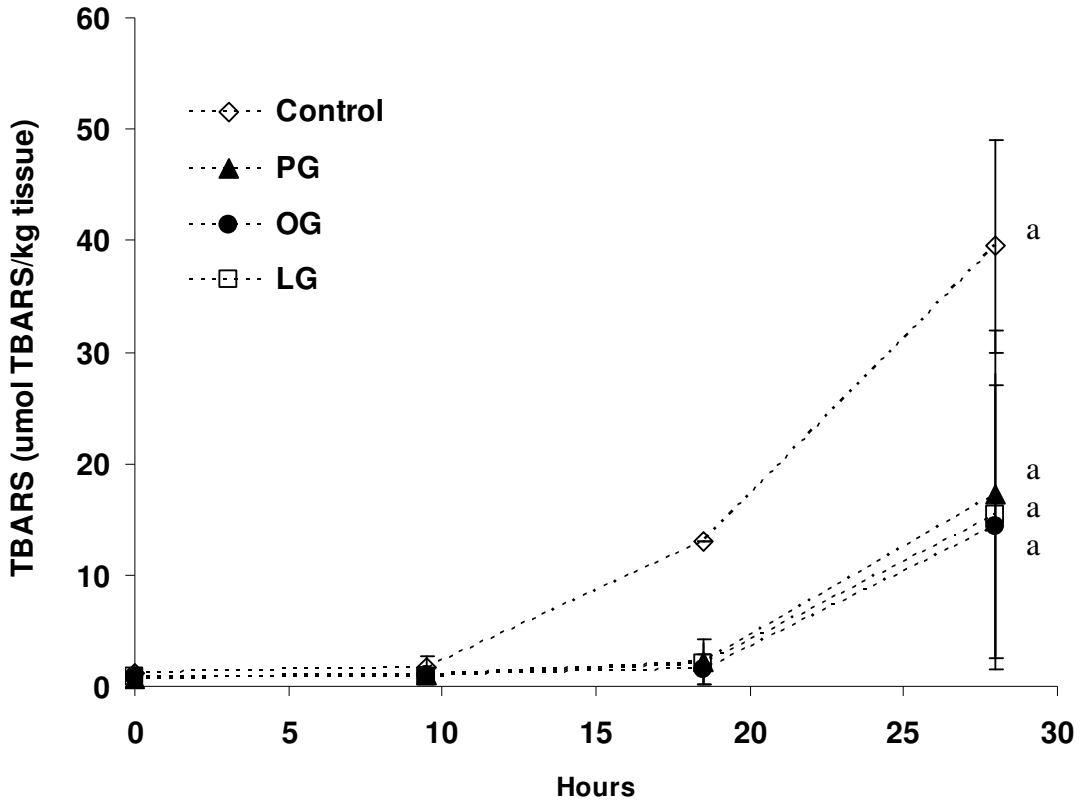

Figure 3.2. Effect of omitting canola oil but adding gallic acid esters or ethanol on the development of TBARS in washed cod. The final pH of washed cod was 6.6; the amount of hemolysate added was 6 µmol hemoglobin/kg tissue. PG, OG, and LG were added at 236µmol GAE/kg. Control = carrier solvent ethanol; PG = propyl gallate; OG = octyl gallate; LG = lauryl gallate. Experiments were performed in duplicate.

Due to wide variation in the lag phase and duration of the experiments between the two replications, statistical analysis was performed without taking into account the data during the lag phase of the control (WC) sample. As 19 hours in the figure above is assigned the time that the lag phase of the control ends. Error bars represent standard error within each experiment.

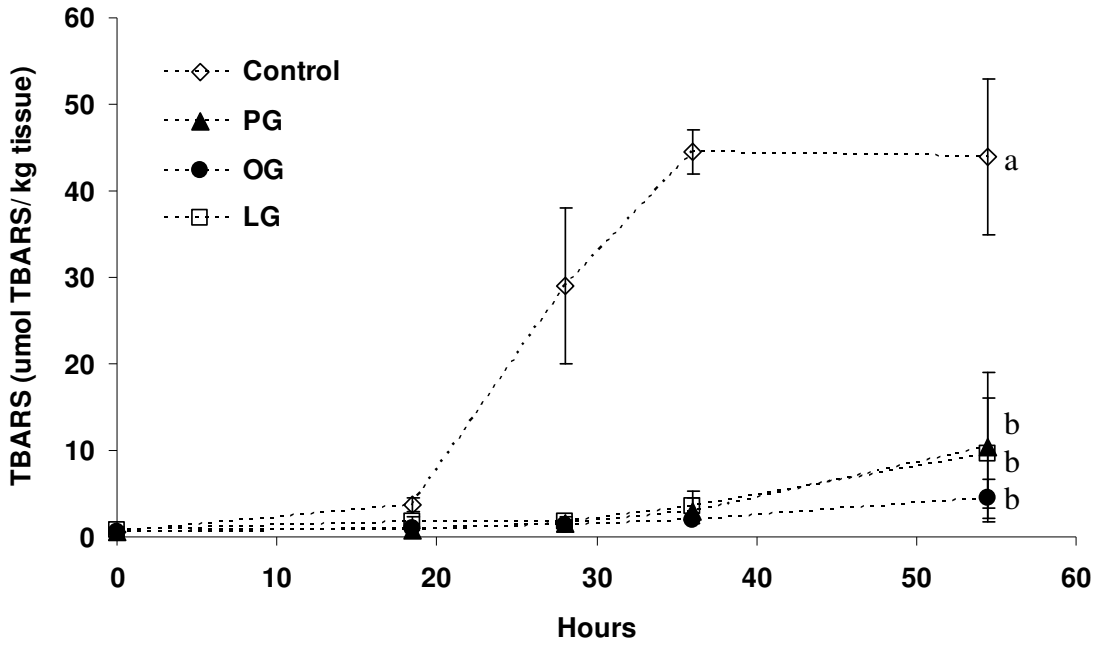

Figure 3.3. Effect of adding canola oil first followed by gallic acid esters or ethanol on the development of TBARS in washed cod. The final pH of washed cod was 6.6; the amount of hemolysate added was 6 µmol hemoglobin/kg tissue. PG, OG, and LG were added at 236µmol GAE/kg lipid. Control = carrier solvent ethanol; PG = propyl gallate; OG = octyl gallate; LG = lauryl gallate. Experiments were performed in duplicate.

Due to wide variation in the lag phase and duration of the experiments between the two replications, statistical analysis was performed without taking into account the data during the lag phase of the control (WC) sample. As 9.5 hours in the figure above is assigned the time that the lag phase of the control ends. Error bars represent standard error within each experiment.

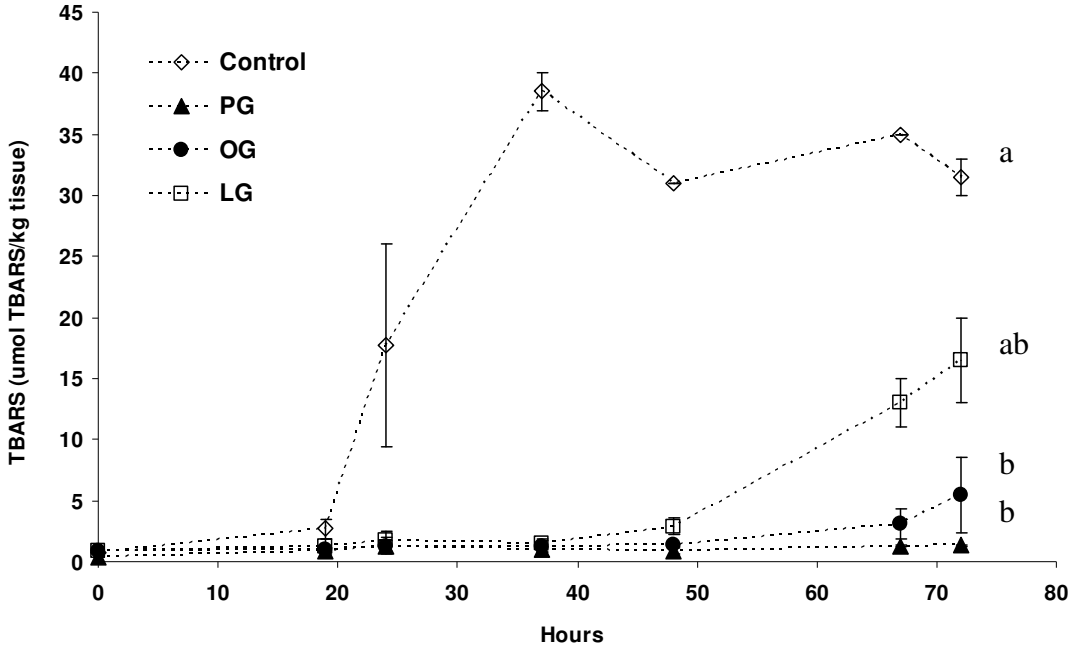

Figure 3.4. Effect of adding gallic acid esters or ethanol first followed by canola oil on the development of TBARS in washed cod. The final pH of washed cod was 6.6; the amount of hemolysate added was 6 µmol hemoglobin/kg tissue. PG, OG, and LG were added at 236µmol GAE/kg lipid. Control = carrier solvent ethanol; PG = propyl gallate; OG = octyl gallate; LG = lauryl gallate. Experiments were performed in duplicate.

Due to wide variation in the lag phase and duration of the experiments between the two replications, statistical analysis was performed without taking into account the data during the lag phase of the control (WC) sample. As 19 hours in the figure above is assigned the time that the lag phase of the control ends. Error bars represent standard error within each experiment.

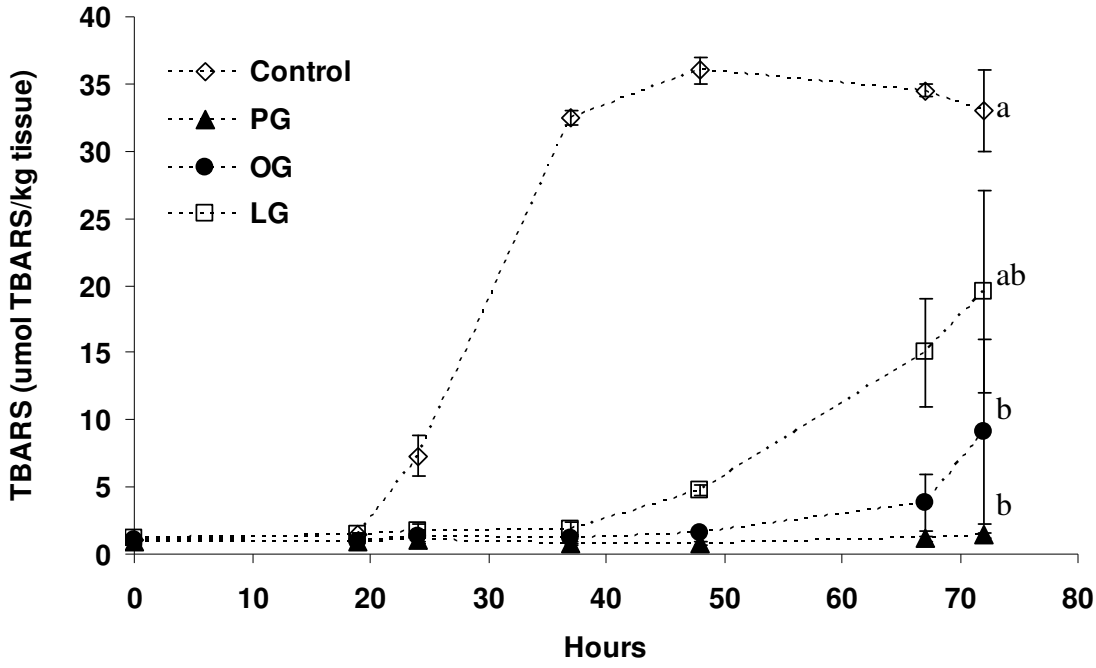

Figure 3.5. Effect of adding gallic acid esters or ethanol directly to canola oil on the development of TBARS in washed cod. The final pH of washed cod was 6.6; the amount of hemolysate added was 6 µmol hemoglobin/kg tissue. PG, OG, and LG were added at 236µmol GAE/kg lipid. Control = carrier solvent ethanol; PG = propyl gallate; OG = octyl gallate; LG = lauryl gallate. Experiments were performed in duplicate.

Due to wide variation in the lag phase and duration of the experiments between the two replications, statistical analysis was performed without taking into account the data during the lag phase of the control (WC) sample. As 19 hours in the figure above is assigned the time that the lag phase of the control ends. Error bars represent standard error within each experiment.

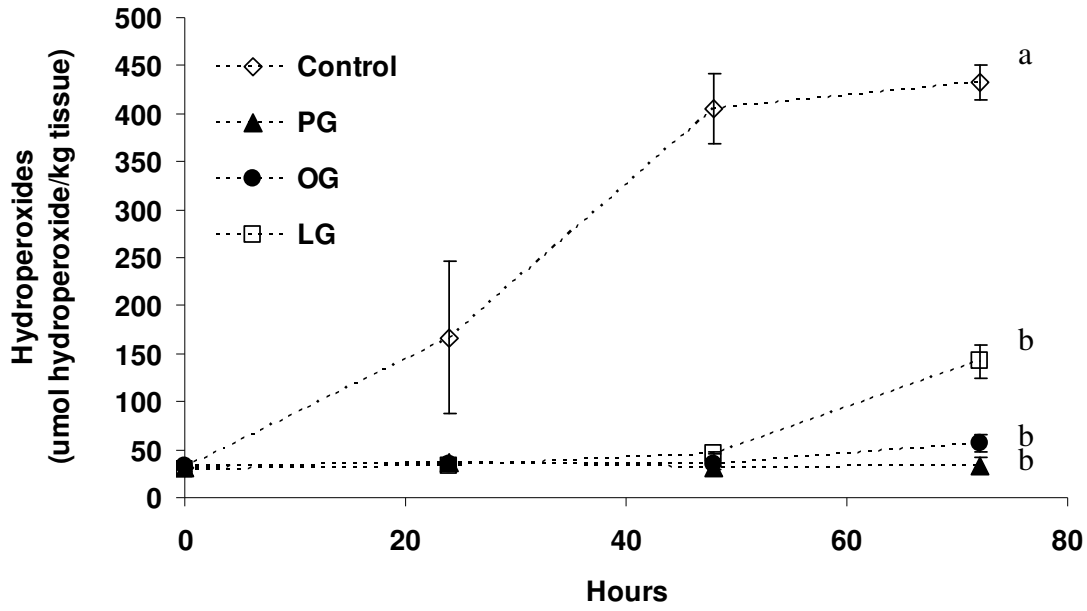

Figure 3.6. Effect of adding gallic acid esters or ethanol first followed by canola oil on the development of hydroperoxides in washed cod. The final pH of washed cod was 6.6; the amount of hemolysate added was 6 µmol hemoglobin/kg tissue. PG, OG, and LG were added at 236µmol GAE/kg lipid. Control = carrier solvent ethanol; PG = propyl gallate; OG = octyl gallate; LG = lauryl gallate. Experiments were performed in duplicate. Error bars represent standard error within each experiment.

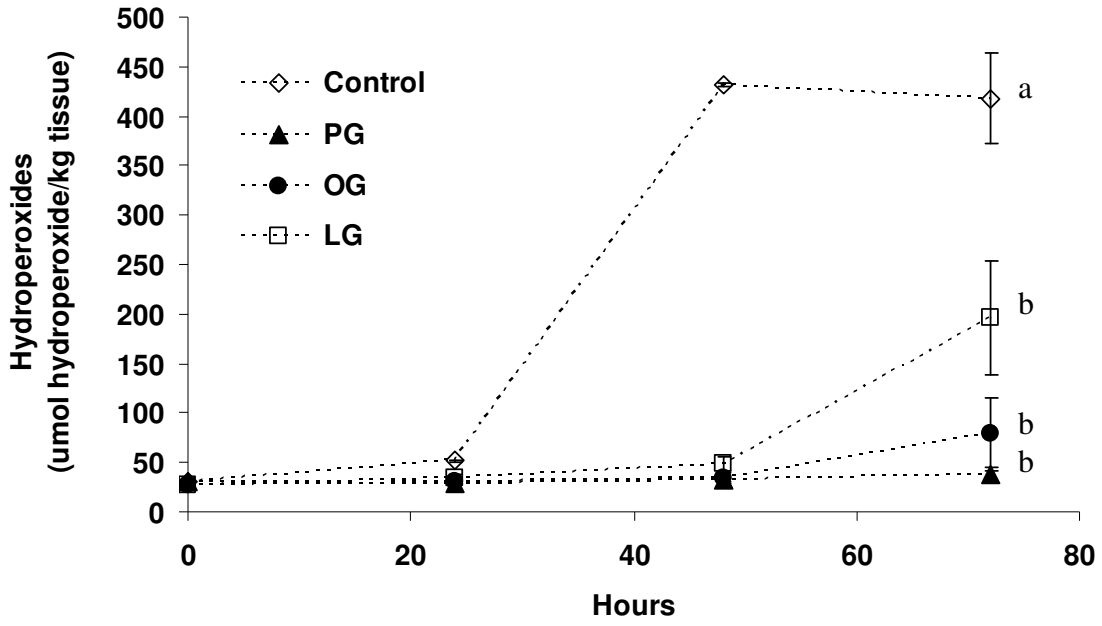

Figure 3.7. Effect of adding gallic acid esters or ethanol directly to canola oil on the development of hydroperoxides in washed cod. The final pH of washed cod was 6.6; the amount of hemolysate added was 6 µmol hemoglobin/kg tissue. PG, OG, and LG were added at 236µmol GAE/kg lipid. Control = carrier solvent ethanol; PG = propyl gallate; OG = octyl gallate; LG = lauryl gallate. Experiments were performed in duplicate.

Due to wide variation in the lag phase and duration of the experiments between the two replications, statistical analysis was performed without taking into account the data during the lag phase of the control (WC) sample. As 24 hours in the figure above is assigned the time that the lag phase of the control ends. Error bars represent standard error within each experiment

3.3.4 Discussion

The antioxidant partitioning studies showed that PG partitions more into the aqueous phase in the isolated muscle membranes system (Table 3.2) and less in the membrane phase of the membranes isolated from washed cod (Table 3.4). Lipid oxidation of muscle foods has been postulated to first occur in the cell membranes. This would suggest that increasing the concentration of the antioxidants in the membrane fraction would decrease lipid oxidation rates. Even though the partitioning studies indicate that PG would have the lowest muscle membrane concentration, it was just as effective as the more hydrophobic and less water soluble OG and LG.

One consistency in the oxidation studies was that use of the GAEs and canola oil in combination increased the ability of the GAEs to inhibit TBARS formation. The ability of canola oil to increase the oxidative stability of washed cod has also been shown by Raghavan and Hultin (2005b). Increased inhibition of TBARS formation in the treatments with added oil is likely due to the ability of antioxidants from the canola oil to inhibit lipid oxidation. The antioxidants from the canola oil and the GAEs could then act in combination to inhibit lipid oxidation more effectively than the GAEs alone.

The observation that PG has the highest water solubility and lowest partitioning into the membrane fraction while still being able to inhibit lipid oxidation could be due to several factors. Dwiecki and others (2006) demonstrated with spectroscopic spectral shift and fluorescence quenching experiments that propyl gallate can associate with the hydrophilic portion of phospholipid membranes potentially allowing PG to scavenge free radicals at the membrane surface. In addition, PG in the aqueous phase could also be regenerating oxidized antioxidants such as α-tocopherol in the cell membranes

making the α-tocopherol more effective. PG could also be inhibiting lipid oxidation by chelating metals in the aqueous phase of the washed cod muscle system.

3.4 Conclusions

The GAE partitioning results obtained from the two fish model systems indicate that PG has the lowest association with phospholipid membranes. Even though PG had the lowest concentration in the membrane fraction, it was still able to inhibit lipid oxidation possibly by acting as a metal chelator or interacting with antioxidants in the cell membranes.

CHAPTER 4

FUTURE WORK

In this research work, the distribution of gallic acid esters of varying hydrophobicities was determined in the phases of various washed cod-canola oil systems as well as their impact on lipid oxidation. Although many of the distribution and oxidation results did not show significant differences, perhaps other antioxidant classes would provide more insight into the relationships between antioxidant distribution and lipid oxidation delay in model muscle systems. Two possible antioxidants to study would be protochatechuic acid and its ethyl ester with hydroxyls at the 3 and 4 positions rather than positions 3, 4 and 5 of gallic acid. This way, differences could possibly be determined regarding the effect of three hydroxyls on a ring versus two.

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