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# Impact of Chemical and Physical Properties on the Ability of Antioxidants to Inhibit Lipid Oxidation in Foods

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**IMPACT OF CHEMICAL AND PHYSICAL PROPERTIES ON THE ABILITY  
OF ANTIOXIDANTS TO INHIBIT LIPID OXIDATION IN FOODS**

A Thesis Presented

by

**JEAN ALAMED**

Submitted to the Graduate School of the  
University of Massachusetts Amherst in fulfillment  
of the requirements for the degree of

**MASTER OF SCIENCE**

September 2008

Food Science

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## ABSTRACT

### IMPACT OF CHEMICAL AND PHYSICAL PROPERTIES ON THE ABILITY OF ANTIOXIDANTS TO INHIBIT LIPID OXIDATION IN FOODS

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Lipid oxidation is a major problem in foods resulting in alteration of texture, appearance, off flavors, aroma and decreased nutritional quality. The ability of compounds to inhibit lipid oxidation in foods is dependent on both physical and chemical properties. The effects of heating (50-90°C), ethylenediaminetetraacetic acid (EDTA), and calcium on the oxidative and physical stability of salmon oil-in-water emulsions were investigated in the first study. Oil-in-water emulsions were prepared with 2% salmon oil, stabilized by 0.2% Brij 35 at pH 7. Above 2.5  $\mu\text{M}$ , EDTA dramatically decreased lipid oxidation in all samples. Addition of calcium to emulsions containing 7.5  $\mu\text{M}$  EDTA significantly increased both thiobarbituric acid reactive substances (TBARS) and hydroperoxide formation when calcium concentrations were 2-fold greater than EDTA concentrations. These results indicate that heat processed salmon oil-in-water emulsions with high physical and oxidative stability could be produced in the presence of EDTA. The objective of the second study was to compare how the free radical scavenging activity of various compounds relates to their ability to inhibit lipid oxidation in cooked ground beef and oil-in-water emulsion. The order of

free radical scavenging activity of the polar compounds was: ferulic acid > coumaric acid > propyl gallate > gallic acid > ascorbic acid as determined by oxygen radical absorbance capacity (ORAC). The free radical scavenging activity of the nonpolar compounds was rosmarinic acid > BHT  $\geq$  TBHQ >  $\alpha$ -tocopherol as determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH•). Of these compounds only propyl gallate and TBHQ were found to inhibit the formation of TBARS in cooked ground beef while propyl gallate, TBHQ, gallic acid and rosmarinic acid were able to decrease lipid hydroperoxides and hexanal in the oil-in-water emulsion. These data indicate that a compound's free radical scavenging activity did not directly correlate with their ability to inhibit lipid oxidation in cooked ground beef and emulsion suggesting that free radical scavenging assays have limited value in predicting the ability of a compound to act as an antioxidant in complex foods.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS .....	iv
ABSTRACT.....	v
LIST OF TABLES .....	x
LIST OF FIGURES.....	xi
CHAPTER	
1. INTRODUCTION.....	1
2. LITERATURE REVIEW.....	6
2.1 Lipid oxidation.....	6
2.1.1 Mechanisms of lipid oxidation .....	7
2.1.2 Lipid oxidation decomposition products .....	8
2.1.3 Factors influencing oxidative stability.....	8
2.1.3.1 Fatty acid composition.....	9
2.1.3.2 Oxygen concentration .....	9
2.1.3.3 Temperature .....	9
2.1.3.4 Surface area.....	10
2.1.3.5 Transition metals.....	10
2.1.3.6 Enzymes and singlet oxygen.....	12
2.2 Antioxidants.....	12
2.2.1 Free radical scavengers and chain breaking antioxidants .....	13
2.2.2 Metal inactivators and chelators .....	17
2.2.3 Singlet oxygen quenchers .....	18
2.2.4 Multiple antioxidant functions.....	19
2.3 Measurement of antioxidant capacity .....	19
2.3.1 HAT methods.....	21
2.3.1.1 ORAC .....	22
2.3.1.2 TRAP .....	23
2.3.2 ET methods .....	25



2.3.2.1 TEAC .....	25
2.3.2.2 FRAP.....	27
2.3.2.3 DPPH•.....	28
2.4 Measurement of lipid oxidation .....	29
2.4.1 Lipid hydroperoxides .....	30
2.4.2 Thiobarbituric acid reactive substances .....	31
2.4.3 Chromatographic method.....	31
2.5 Omega-3 fatty acids .....	32
2.6 Lipid oxidation in food systems.....	34
2.6.1 Emulsions.....	34
2.6.2 Edible (bulk) oils.....	38
2.6.3 Muscle foods .....	40
<b>3. INFLUENCE OF HEAT PROCESSING AND CALCIUM IONS ON THE ABILITY OF EDTA TO INHIBIT LIPID OXIDATION IN OIL-IN-WATER EMULSIONS CONTAINING OMEGA-3 FATTY ACIDS..</b>	<b>43</b>
3.1 Abstract.....	43
3.2 Introduction.....	44
3.3 Materials and Methods.....	46
3.3.1 Materials .....	46
3.3.2 Methods.....	47
3.3.2.1 Preparation of salmon oil .....	47
3.3.2.2 Preparation of emulsion .....	47
3.3.2.3 Preparation of EDTA containing samples .....	48
3.3.2.4 Preparation of calcium components.....	48
3.3.2.5 Lipid oxidation measurements .....	48
3.3.2.6 Statistics .....	49
3.4 Results and Discussion .....	50
3.4.1 Heat processing and holding time.....	50
3.4.2 Influence of EDTA.....	51
3.4.3 Influence of adding EDTA before or after heating .....	54
3.4.4 Influence of calcium on antioxidant effects of EDTA.....	56
3.5 Conclusions.....	58

4.	THE RELATIONSHIP BETWEEN A COMOUNDS FREE RADICAL SCAVENGING ACTIVITY AND ITS ABILITY TO INHIBIT LIPID OXIDATION IN FOODS .....	59
	4.1 Abstract .....	59
	4.2 Introduction.....	60
	4.3 Materials and Methods.....	63
	4.3.1 Materials .....	63
	4.3.2 Methods.....	63
	4.3.2.1 Free Radical Scavenging Assays.....	63
	4.3.2.2 Lipid Oxidation in Cooked Ground Beef.....	68
	4.3.2.3 Lipid Oxidation in Oil-in-Water Emulsion.....	69
	4.3.2.4 Statistics .....	71
	4.4 Results.....	71
	4.4.1 Free Radical Scavenging Capacity Assays .....	71
	4.4.2 Inhibition of Lipid Oxidation in Foods .....	75
	4.5 Discussion.....	82
	4.6 Conclusions.....	87
5.	OVERALL CONCLUSIONS .....	89
6.	FUTURE WORK.....	92
	BIBLIOGRAPHY .....	95

## LIST OF TABLES

Table		Page
4.1.	The oxygen radical absorbance capacity (ORAC) values of selected compounds expressed as $\mu\text{mol}$ of trolox equivalents (TE)/mL. A higher ORAC value represents greater free radical scavenging capacity .....	72
4.2.	Free radical scavenging activity of non-polar antioxidants as determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) assay .....	74

## LIST OF FIGURES

Figure	Page	
2.1.	Schematic of the potential pathways that impact the oxidative deterioration of foods, where $Mn^{+n}$ and $Mn^{+n+1}$ are transition metals in their reduced and oxidized states; RH, ROOH and AOH are unsaturated fatty acid, lipid hydroperoxide and chain breaking antioxidant; and $R\bullet$ , $RO\bullet$ , $ROO\bullet$ are alkyl, alkoxy and peroxy radicals; and $^1O_2$ and LOX are singlet oxygen and lipoxygenase, respectively (Modified from Decker et al., 2002).....	11
2.2.	Partitioning of polar and non-polar free radical scavengers (FRS) in bulk oil and oil-in-water emulsions as defined by the polar paradox (Modified from Frankel 2005 and Decker 1998b).....	15
2.3.	A schematic of the physical environments of an oil-in-water emulsion including the possible physical location of reactants in the transition metal-promoted decomposition of lipid hydroperoxide. $Mn^{+n}$ and $Mn^{+n+1}$ are transition metals in their reduced and oxidized states, respectively. LOOH is a lipid hydroperoxide and $LO\bullet$ is an alkoxy radical. The components and phases are not drawn to scale (Modified from Decker et al., 2002).....	37
3.1.	TBARS concentration of salmon oil-in-water emulsions exposed to heat treatments of 50°C, 70°C, 90°C and one control (not heated) stored at 20°C and measured over 8 days.....	51
3.2.	TBARS concentration (a) of salmon oil-in-water emulsion stored at 20°C and measured over 8 days. Samples contain varying amounts of EDTA as well as one control with no EDTA added. TBAR values comparison of EDTA concentration and it's effect on lipid oxidation at day 7 (b).....	53
3.3.	TBARS concentration (a) and hydroperoxide concentration (b) of salmon oil-in-water emulsions heat processed at 90°C, stored at 20°C and measured over 8 days. EDTA (10 $\mu$ M) was added to samples before (bh) or after (ah) heat processing .....	55
3.4.	TBARS concentrations (a) and hydroperoxide concentration (b) of non-heated salmon oil-in-water emulsions stored at 20°C and measured over 8 days. Samples contain 7.5 $\mu$ M EDTA and varying amounts of CaCl, added after homogenization, as well as a control with no EDTA and no CaCl added .....	57

4.1.	Structures of polar test compounds.....	65
4.2.	Structures of non-polar test compounds .....	67
4.3.	Changes in the relative fluorescence intensity of 45 nM fluorescein ( $\lambda$ EM 493 nm, $\lambda$ EX 515 nm) in the presence of 20 mM 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) and 5 $\mu$ M ferulic acid (FA), propyl gallate (PG), gallic acid (GA), coumaric acid (CA) or ascorbic acid (AA) at 37°C .....	72
4.4.	The ability of butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), rosmarinic acid, or $\alpha$ -tocopherol to inactivate the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) .....	74
4.5.	The formation of thiobarbituric acid reactive substance (TBARS) in cooked ground beef containing ferulic acid (FA), propyl gallate (PG), gallic acid (GA), coumaric acid (CA) or ascorbic acid (AA) (50 $\mu$ M) during storage at 4°C for 96 hrs .....	77
4.6.	The formation of thiobarbituric acid reactive substance (TBARS) in cooked ground beef containing 50 $\mu$ M butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), rosmarinic acid, or $\alpha$ -tocopherol during storage at 4°C for 96 hrs .....	77
4.7.	Formation of lipid hydroperoxide concentration (a) and hexanal (b) in corn oil-in-water emulsion containing 50 $\mu$ M ferulic acid (FA), propyl gallate (PG), gallic acid (GA), coumaric acid (CA) or ascorbic acid (AA) during storage at 55°C in the dark for 24 days.....	79
4.8.	Formation of lipid hydroperoxide concentration (a) and hexanal (b) in corn oil-in-water emulsion containing 50 $\mu$ M butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), rosmarinic acid, or $\alpha$ -tocopherol during storage at 55°C in the dark for 24 days.....	81
4.9.	Comparison of free radical scavenging assay and lipid oxidation results of polar (a) and non-polar antioxidants (b) in oil-in-water emulsion (day 14) and cooked ground beef (day 3). Zero value was set to equal the no additive control. Negative values represent antioxidant activity while positive values represent prooxidant activity .....	83

## CHAPTER 1

### INTRODUCTION

Lipid oxidation is a serious problem in the food industry, not only because it produces rancid odors, off flavors, decreases shelf life and can alter the texture and appearance of foods, but also because it can decrease the nutritional quality and safety of foods. Foods contain a variety of fatty acids that differ in chemical properties, physical properties and also their susceptibility to oxidation (Nawar, 1996). Food chemists have been studying the development of rancidity in foods for many decades. There are numerous aspects that can influence the oxidative stability of foods, including heat, processing, oxygen concentration, trace metals and degree of unsaturation (Frankel, 1996; Vercellotti et al., 1992). Progress has been made in controlling lipid oxidation by altering various factors, including improving processing and packaging techniques and the use of metal chelators and antioxidants (Frankel, 2007).

There are many studied and proven health benefits of dietary  $\omega$ -3 fatty acids, including protection against cardiovascular disease, cancer, hypertension, diabetes and rheumatoid arthritis, improved brain and retinal function in infants and reduced susceptibility to tumors (Tong et al., 2000; Simopoulos, 1991; Mori and Beilin, 2001; Patil and Gislerod, 2006). Although  $\omega$ -3 fatty acids have many health benefits, they are subject to rapid and/or extensive oxidation and other chemical changes by means of exposure to air, light, trace metals or heat during processing (Lytle et al., 1992). It is of great interest to food manufacturers to use  $\omega$ -3 fatty acids as functional ingredients to improve the nutritional profile of food products. However, lipid oxidation of  $\omega$ -3 fatty

acids can result in alteration of the quality of the food (Nuchi et al., 2001), making their inclusion in food a challenge.

Food antioxidants are substances, synthetic or naturally occurring, that can delay the onset or slow the rate of oxidation of biomolecules (Nawar, 1996). Antioxidants have the ability to inhibit lipid oxidation by various mechanisms, such as free radical scavenging which inhibits free-radical oxidation reactions at the initiation and/or propagation phase and metal chelating which converts metal prooxidants into stable products (Pokorny, 2007).

Free radical scavengers (FRS) have the ability to slow or inhibit oxidation by interfering with either chain initiation and/or propagation. Peroxyl radicals are found in the greatest concentration of all radicals in the system and have a lower energy than other radicals; therefore peroxyl radicals preferentially react with the low energy hydrogens of the free radical scavengers rather than the unsaturated fatty acids, resulting in the formation of a free radical scavenger radical (FRS•). The resulting low energy FRS• will be less likely to catalyze the oxidation of unsaturated fatty acids. The inactivation of the FRS• will come about during a termination reaction with another FRS• or lipid radical (Decker, 2005; Buettner, 1993; Frankel, 2005). Free radical scavengers can be classified into two groups: 1. polar (water loving) and 2. non-polar (oil loving). The difference in their behavior in food systems is referred to as the *polar paradox*. This theory is based on the observation that, in emulsified oils, non-polar FRS are more effective than polar FRS, while polar FRS are more effective than non-polar FRS in bulk oils (Frankel, 2005; Chaiyasit et al., 2005; Porter, 1993; Decker, 1998a). The key to this occurrence is the ability of the FRS to concentrate where lipid oxidation

is most prevalent. Polar FRS, such as ascorbic acid, concentrate at oil-air or oil-water interfaces in bulk oils, where the majority of oxidation occurs due to high concentrations of oxygen and prooxidants. In emulsions, non-polar FRS, for instance  $\alpha$ -tocopherol, accumulate in the lipid phase and at the oil-water interface, where interactions between hydroperoxides at the droplet surface and prooxidants in the aqueous phase occur (Decker, 1998b; Chaiyasit et al., 2005; Decker, 2005).

Metal chelators are compounds that can inhibit lipid oxidation by mechanisms that do not involve the inactivation of free radicals (Frankel, 2005; Pokorny, 2007). Chelators inhibit metal-catalyzed reactions by prevention of metal redox cycling, formation of insoluble metal complexes and/or occupation of all metal coordination sites (Decker, 1998a). Most chelators accumulate in the aqueous phase of foods, however, in order to inactivate lipid-soluble metals some chelators must also partition into the lipid phase (Decker, 2005). Ethylenediamine tetraacetic acid (EDTA), one of the most effective metal chelators, can inactivate metals by forming stable coordination complexes with prooxidant metals, thus effectively inhibiting both metal-catalyzed initiation and decomposition of hydroperoxides (Frankel, 2005).

There is increasing interest in the use and measurement of antioxidant capacity in the food, pharmaceutical, and cosmetic industries. Much of this interest is derived from the increasing evidence of the importance of reactive oxygen/nitrogen species (ROS/RON) in aging and pathogenesis (Brand-Williams et al., 1995). For foods the ideal antioxidant evaluation method should be conducted under the chemical, physical, and environmental conditions expected in food systems in order to accurately evaluate antioxidant potential. However, in food products, these conditions vary widely so



individual evaluation methods are needed (Decker et al., 2005). Many simplistic one-dimensional methods that use a broad range of conditions, oxidants and methods to measure end points of oxidation have been developed to measure the free radical scavenging or “antiradical” ability of antioxidants (Frankel, 2005; Brand-Williams et al., 1995). Antioxidant capacity assays can generally be classified into two types: hydrogen atom transfer (HAT) reactions or electron transfer (ET) assays. HAT assays, such as oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP), apply a competitive reaction scheme where antioxidant and substrate compete for thermally generated peroxy radicals through the decomposition of azo compounds. ET assays involve two components in the reaction mixture: the antioxidant and the oxidant (which is the probe). The probe will abstract an electron from the antioxidant causing a color change of the probe. The color change is used to monitor the reaction and works as an indicator of the reaction endpoint. Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) are examples of ET assays (Huang et al., 2005; Frankel, 2007; Sanchez-Moreno, 2002).

The rate of lipid oxidation in food systems is dependent on a range of factors and can vary between food systems, such as emulsions, bulk oils and muscle foods. The composition of foods is complex and contains a variety of fatty acids that differ in chemical and physical properties and susceptibility to oxidation. Numerous reactions, such as heating, metal interactions and oxidation, can lead to the development of rancidity in foods. The overall goal of this research is to investigate how the chemical and physical properties of antioxidants can impact their ability to inhibit lipid oxidation

in foods. In the first experiment we will study the interactions between EDTA and calcium ions on their ability to inhibit lipid oxidation in emulsions. In the following study we will look at how the ability of antioxidants to scavenge free radicals is related to their ability to inhibit lipid oxidation in various food systems.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Lipid oxidation

In the food industry lipid oxidation is a serious problem, not only because it produces rancid odors and flavors, decreases shelf life and can alter texture and appearance of foods, but also because it can decrease the nutritional quality and safety of foods. In the presence of an initiator, polyunsaturated lipids can become oxidized and form alkyl radicals. When exposed to oxygen, the alkyl radicals rapidly form peroxy radicals and these radicals can react with more lipids to produce hydroperoxides (Frankel, 1996). As a lipid hydroperoxide decomposes, it forms numerous volatile compounds such as hexanal and propanal, which impact the sensory quality of foods. By measuring the ability of a compound to decrease the formation of hydroperoxides and volatile oxidation products, the potential of the compound to act as an antioxidant can be determined (Adegoke et al., 1998). Antioxidants work to inhibit free radical oxidation by either reacting with peroxy radicals to stop chain propagation or with alkoxy radicals to inhibit the decomposition of hydroperoxides and thus the formation of aldehydes (Frankel, 1996). Secondary antioxidants, such as chelators, reduce the activity of prooxidant metals and inhibit lipid oxidation by mechanisms that do not involve the deactivation of free radical chains (Decker, 1998a; Frankel, 2005).

### 2.1.1 Mechanisms of lipid oxidation.

When molecular oxygen reacts with organic compounds under mild conditions it is referred to as autoxidation. Oxygen, in the ground state, behaves as a biradical due to its two unpaired electrons ( $\bullet\text{O}-\text{O}\bullet$ ). The oxidation of lipids occurs by a free radical chain reaction involving three processes: (1) initiation – the formation of free radicals; (2) propagation – the free radical chain reactions; and (3) termination – the formation of non-radical products (Frankel, 2005; Nawar, 1996). Below is a schematic of this process (Frankel, 2005; Erickson, 2002; Ingold, 1962).



In the initiation step a lipid free radical known as the alkyl radical ( $\text{L}\bullet$ ) is formed [1]. The alkyl radical contains an unpaired electron that reacts rapidly with the oxygen biradical to form peroxy radicals ( $\text{LOO}\bullet$ ) [2] in the propagation step. The following hydrogen transfer reaction that occurs with unsaturated lipids to convert the peroxy radical to a hydroperoxide ( $\text{LOOH}$ ) happens slower than the previous step [3]. Termination is the last step of autoxidation. In the termination stage [4-6], peroxy

radicals accumulate and react with each other forming non-radical products (Frankel, 2005; Erickson, 2002; Nawar, 1996; Ingold, 1962).

### **2.1.2 Decomposition products.**

Fatty acid hydroperoxides produced during propagation do not have a direct adverse effect on the flavor and aroma of foods. However, lipid hydroperoxide decomposition produces alkoxy radicals (LO•) which, in turn, can cause the decomposition of the fatty acid.  $\beta$ -scission reactions occur after hydroperoxides decompose into alkoxy radicals. These highly energetic alkoxy radicals have the ability to abstract an electron from the carbon-carbon bond on either side of the oxygen radical in order to cleave the fatty acid chain. The  $\beta$ -scission reaction is important because it causes fatty acids to decompose into low molecular weight, volatile, compounds (Decker, 2005; Chaiyasit et al., 2007). The aldehydes and ketones produced from the  $\beta$ -scission reaction are the source of the characteristic rancid flavors and aromas in foods (Coleman and Williams, 2007).

### **2.1.3 Factors influencing oxidative stability.**

Foods contain a variety of fatty acids that differ in chemical and physical properties and their susceptibility to oxidation (Nawar, 1996). There are many factors that can influence the oxidative stability of foods, some of which include temperature, surface area, oxygen concentration, trace metals and degree of unsaturation (Frankel, 1996; Vercellotti et al., 1992).

#### **2.1.3.1 Fatty acid composition.**

The rate of lipid oxidation is affected by the number, position and geometry of the double bonds (Nawar, 1996). Hydrogen atoms adjacent to double bonds are most susceptible to abstraction during the propagation step in lipid oxidation. The greater the number of double bonds a fatty acid contains, the more rapidly it will oxidize (Coleman and Williams, 2007). In addition, fatty acids in the *cis* form oxidize more readily than their *trans* isomers (Nawar, 1996).

#### **2.1.3.2 Oxygen concentration.**

When oxygen is present at low levels the rate of oxidation is approximately proportional to the oxygen concentration. If abundant levels of oxygen are present, the rate of oxidation is independent of the oxygen concentration (Nawar, 1996; McClements and Decker, 2000). Since the addition of oxygen to the alkyl radical is a diffusion limited reaction the majority of oxygen must be removed from the system in order to inhibit lipid oxidation. Vacuum conditions are often needed to reduce oxidation since the removal of oxygen can be difficult due to its solubility being higher in oil than water (Decker, 2005).

#### **2.1.3.3 Temperature.**

The general rule is that as temperature increases, the rate of oxidation increases. At room temperature, autooxidation of saturated fatty acids is slow. At high temperatures, saturated fatty acids can undergo oxidation at substantial rates (Nawar, 1996; Decker, 2005). However, in bulk oils, increasing temperature can decrease

oxygen solubility which, consequently, has the ability to slow oxidation rates (Decker, 2005).

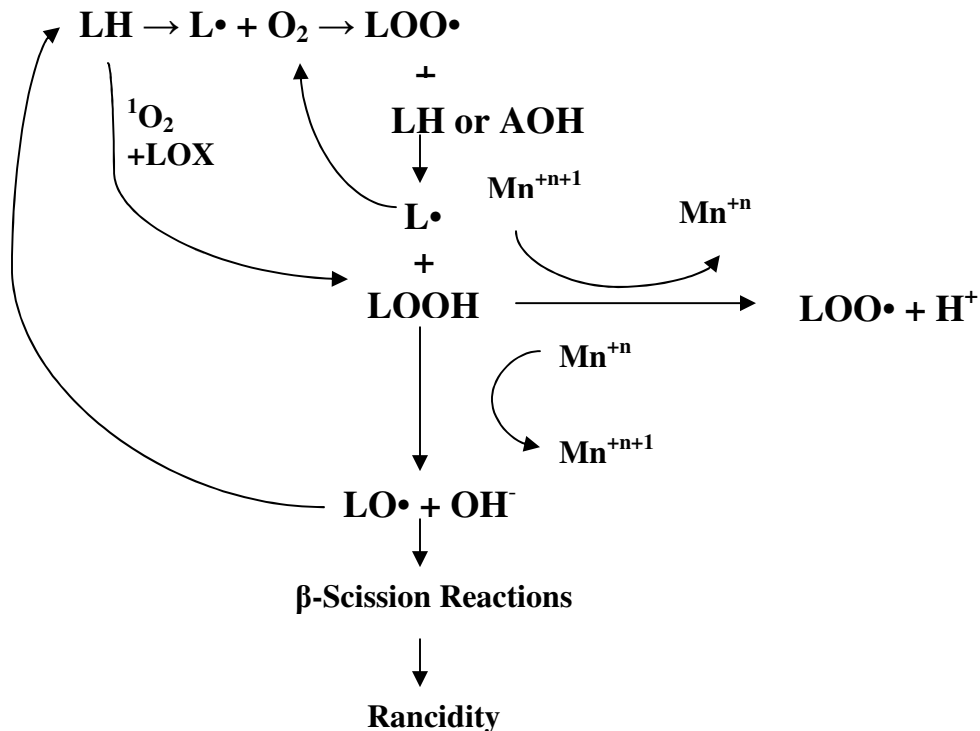
#### **2.1.3.4 Surface area.**

The rate of lipid oxidation will increase in direct proportion to the surface area of the lipid which is exposed to oxygen and prooxidants, as can be seen in bulk oil systems (Nawar, 1996; Decker, 2005) and in muscle tissue (Erickson, 2002). In oil-in-water emulsions, surface area does not seem to impact lipid oxidation rates presumably because the surface area is already very large (McClements and Decker, 2000).

#### **2.1.3.5 Transition metals.**

Trace amounts of heavy metals, such as iron and copper, are commonly present in edible oils and muscle foods. These metals originate from animals, plants, soil, dust, and the metallic equipment used in processing and storage. The concentration at which these trace metals occur in natural lipids is a major factor determining the rate of oxidative deterioration of lipids (Watts, 1962; Nawar, 1996). Even when present at concentrations as low as 0.1 ppm, transition metals can decrease the induction period and increase the rate of oxidation (Nawar, 1996). Transition metals aid in the formation of free radicals by hydrogen abstraction and hydroperoxide decomposition, which accelerate lipid oxidation reactions, therefore, decreasing the quality of foods. Hydrogen abstraction from an unsaturated fatty acid results in the formation of a single alkyl radical. Proceeding hydrogen abstraction, oxygen adds to the alkyl radical forming a peroxy radical. The subsequent abstraction of a hydrogen from another fatty acid or

antioxidant leads to the formation of lipid hydroperoxide (Figure 2.1). By themselves these reactions do not result in increased free radical numbers and if these reactions were the only steps in lipid oxidation reactions rapid exponential increases in oxidation, which are commonly observed in lipids, would not occur. Transition metal-promoted decomposition of lipid hydroperoxides is a result of the formation of additional radicals, such as alkoxy and peroxy radicals. These additional radicals start to attack other unsaturated fatty acids, which leads to an exponential increase in oxidation rates (Decker et al., 2002; Chaiyasit et al., 2007; Kanner, 1992).



**Figure 2.1.** Schematic of the potential pathways that impact the oxidative deterioration of foods, where  $\text{Mn}^{n+}$  and  $\text{Mn}^{n+1}$  are transition metals in their reduced and oxidized states; RH, ROOH and AOH are unsaturated fatty acid, lipid hydroperoxide and chain breaking antioxidant; and  $\text{R}\cdot$ ,  $\text{RO}\cdot$ ,  $\text{ROO}\cdot$  are alkyl, alkoxy and peroxy radicals; and  $^1\text{O}_2$  and LOX are singlet oxygen and lipoxygenase, respectively (Modified from Decker et al., 2002).



### **2.1.3.6 Enzymes and Singlet Oxygen.**

Lipoxygenases are found in plants and some animal tissues (Decker, 1998a). Lipoxygenases promote lipid oxidation by catalyzing the formation of lipid hydroperoxides. Singlet oxygen is formed in the presence of light and a photosensitizer, such as chlorophyll or riboflavin. Singlet oxygen directly interacts with unsaturated fatty acids to form lipid hydroperoxides (Frankel, 2005).

## **2.2 Antioxidants**

Antioxidants are substances, synthetic or naturally occurring, that can delay the onset or slow the rate of oxidation of autoxidizable materials. However, their use in food products is limited by certain requirements, including sufficient proof of safety (Nawar, 1996). The activity of antioxidants is strongly influenced by numerous factors, thus, compounds that are effective antioxidants in one system may be unsuitable in other systems. Some factors that influence antioxidant activity are the nature of the lipid substrate, the hydrophilic-lipophilic balance of the antioxidant, physical and chemical environments and interfacial interactions (Chang et al., 2003; Porter, 1993).

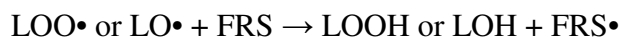
According to their mechanism of action, antioxidants can be classified as primary or secondary antioxidants. Primary antioxidants are chain breaking antioxidants and can inhibit lipid oxidation by interfering at the propagation or initiation phase or in  $\beta$ -scission reactions by accepting free radicals to form stable free radicals. Secondary antioxidants are considered preventative antioxidants, such as chelators, oxygen scavengers and singlet oxygen quenchers. These antioxidants decrease the rate

of oxidation through numerous mechanisms; however, they do not convert free radicals into more stable products (Chaiyasit et al., 2007; Frankel, 2005; Reische et al., 2002).

Antioxidants can inhibit lipid oxidation by numerous mechanisms, some of the major mechanisms include: 1) free radical scavengers which are inhibitors of free-radical oxidation reactions that can stop oxidation at the initiation phase and chain breaking antioxidants that interrupt the propagation phase, 2) metal chelators that can convert metal prooxidants into stable products singlet oxygen quenchers, 3) singlet oxygen quenchers and 4) synergists that, when in the presence of the proper combination of antioxidants, can increase the activity of chain-breaking antioxidants in a mixture (Pokorny, 2007).

### **2.2.1 Free radical scavengers and chain breaking antioxidants.**

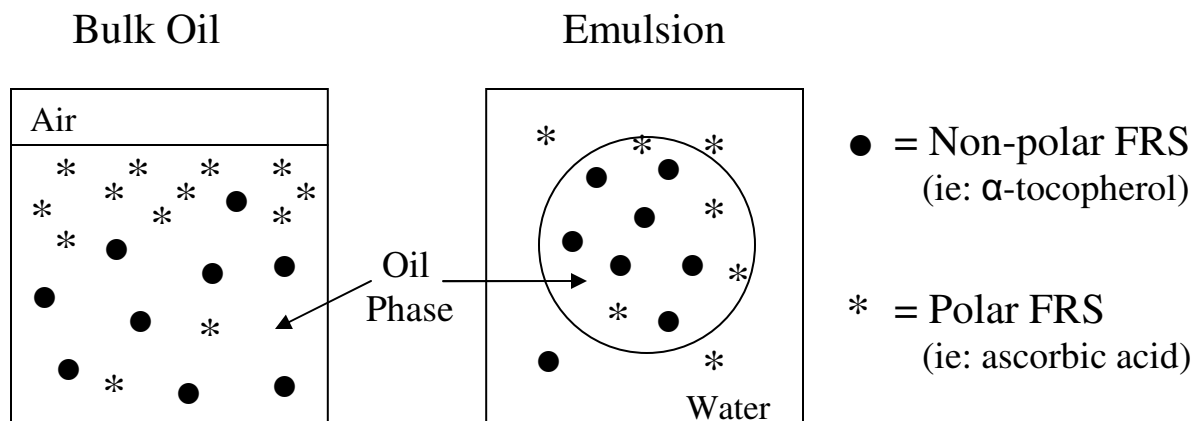
Free radical scavengers and chain breaking antioxidants have the ability to slow or inhibit oxidation by interfering with either chain initiation and/or propagation. The following reaction demonstrates the ability of FRS to interact with either peroxy ( $\text{LOO}\bullet$ ) or alkoxy ( $\text{LO}\bullet$ ) radicals (Decker, 2005; Frankel, 2005):



Peroxy radicals are found in the greatest concentration of all radicals in a system and have lower energy than other radicals therefore they preferentially react with the low energy hydrogens of the free radical scavenger rather than the unsaturated fatty acid resulting in the formation of a free radical scavenger radical ( $\text{FRS}\bullet$ ). The resulting low

energy FRS• will be less likely to catalyze the oxidation of unsaturated fatty acids. The inactivation of the FRS• occurs during a termination reaction with another FRS• or lipid radical (Decker, 2005; Buettner; 1993, Frankel; 2005).

Free radical scavengers can be physically classified into two groups: 1. hydrophilic (water loving/polar) and 2. lipophilic (oil loving/non-polar). The difference in the behavior of these two types of FRS in food systems is referred to as the *antioxidant polar paradox* (Figure 2.2). The premise of this theory is based on the observation that, in emulsified oils, non-polar FRS are more effective than polar FRS, while polar FRS are more effective than non-polar FRS in bulk oils (Frankel, 2005; Chaiyasit et al., 2005; Porter, 1993; Decker, 1998b). The key to this phenomenon is the ability of the FRS to concentrate where lipid oxidation is most prevalent. Polar FRS concentrate at oil-air or oil-water interfaces in bulk oils, where the majority of oxidation occurs due to high concentrations of oxygen and prooxidants. In emulsions, non-polar FRS accumulate in the lipid phase and at the oil-water interface where interactions between hydroperoxides at the droplet surface and prooxidants in the aqueous phase occur (Decker, 1998b; Chaiyasit et al., 2005; Decker, 2005).



**Figure 2.2.** Partitioning of polar and non-polar free radical scavengers (FRS) in bulk oil and oil-in-water emulsions as defined by the polar paradox. (Modified from Frankel, 2005 and Decker, 1998b).

To be used in food applications, synthetic FRS must be sufficiently active enough to be used at low concentrations (below 0.02%) and can not be toxic. They must also be stable to processing and cooking conditions. Compared to natural FRS, synthetic FRS are more effective, can be used at lower concentrations, are less expensive and can be prepared with consistent quality without an effect on flavor, color and aroma of the food product (Frankel, 2005; Pokorny, 2007). However, synthetic FRS are “label unfriendly” additives (Chaiyasit et al., 2007). Some of the most commonly used synthetic FRS in food systems are propyl gallate, butylated hydroxyl toluene (BHT), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) (Decker, 2005; Frankel, 2007).

In the past couple decades; use of natural FRS has increased due to worries about the possible hazardous effects of synthetic FRS and also current trends against the use of regulated/artificial food additives. The benefits of using natural FRS include

GRAS (generally recognized as safe) status, allowance to use higher concentrations and worldwide acceptance. The negative side of natural FRS includes wide variation in concentration of active components (due to source and extraction methods) and undesirable effects on flavor, color and aroma of foods (Frankel, 2005; Pokorny, 2007). Potentially active compounds from natural sources such as fruit, herbs, roots and leaves have been extensively studied since there is much interest on their FRS activity in relation to human health. Natural compounds that possess FRS activity are polyphenols, such as flavonoids, bioflavonoids, isoflavones, and tannins, as well as some vitamins including vitamin A, C and E. The role of these compounds is to interrupt the free radical chain reaction involved in oxidation. Polyphenols have strong FRS properties which can help protect cells against adverse effects of reactive oxygen species, free radicals and prooxidative metal ions (Dufresne and Farnworth, 2001; Aviram et al., 2002). The following are several examples of natural FRS. Aviram and others (2002) found that pomegranate juice contained a higher concentration of total polyphenols than red wine and other fruit juices and also exhibited a low IC<sub>50</sub> (the concentration needed to inhibit LDL oxidation by 50%). The polyphenolic constituents of teas can act as scavengers of reactive oxygen species and prevent damage to cellular macromolecules (Wei et al., 1996). Of the tea catechins, green tea extracts have been found to have higher phenol content and greater chain-breaking activity than black tea extracts (Manzocco et al., 1998). Carotenoids, found in fruits and vegetables, are another major group of natural compounds which have FRS properties. Lycopene has a high FRS potential due to its capacity to inactivate free radicals in lipid phases (Sies et al., 1992; Ribeiro et al., 2003) and by interfering with reactions of damaging oxidizing

agents and free radicals (Ribeiro et al., 2003; Henry et al., 1998). If a better understanding of the properties and numerous health benefits of natural free radical scavengers can be obtained it could lead to the development of food additives that may well both prevent lipid oxidation in foods and provide health benefits (Chaiyasit et al., 2007).

### **2.2.2 Metal inactivators and chelators**

Metal inactivators and chelators are compounds that can inhibit lipid oxidation by mechanisms that do not involve the deactivation of free radical chains. The most important of these are the metal inactivators, which decrease the ability of metal ions to promote initiation reactions and the decomposition of hydroperoxides into secondary aldehydes (Frankel, 2005, Pokorny, 2007). Chelators inhibit metal-catalyzed reactions by: prevention of metal redox cycling, formation of insoluble metal complexes, steric hindrance of metal-lipid interactions or oxidation intermediates (e.g. hydroperoxides) and/or occupation of all metal coordination sites (Decker, 1998a). Most chelators accumulate in the aqueous phase of foods, however, in order to inactivate lipid-soluble metals some chelators must also partition into the lipid phase (Decker, 2005).

Conversely, under certain conditions, some chelators can increase metal solubility or alter the redox potential of metals thus increasing oxidative reactions (Decker, 1998a). Ethylenediamine tetraacetic acid (EDTA), one of the most effective metal chelators, along with citric, tartaric and phosphoric acids are just a few examples of compounds which can deactivate metals by forming stable coordination complexes with prooxidant metals, thus effectively inhibiting both metal-catalyzed initiation and decomposition of

hydroperoxides (Frankel, 2005). However, the antioxidative and/or prooxidative properties of metal chelators are often concentration dependant. It has been found that, when present at an EDTA:iron ratio of  $> 1$ , EDTA will perform as a strong metal chelator, in contrast, at an EDTA:iron ratio of  $\leq 1$ , EDTA can behave as a prooxidant (Mahoney and Graf, 1986).

### **2.2.3 Singlet oxygen quenchers**

The use of quenching agents is an effective way to reduce singlet oxygen oxidation. Quenching agents may decrease singlet oxygen promoted oxidation by quenching the excited triplet sensitizer or single oxygen by chemical or physical means. Chemical quenching involves the reaction of singlet oxygen with the quenching agent to produce stable products. Physical quenching returns singlet oxygen to triplet oxygen without the consumption of oxygen and without the quenching agent undergoing any chemical changes (Min and Boff, 2002; Frankel, 2005). Natural food components such as carotenoids, tocopherols and ascorbic acid have been found to be effective quenching agents (Min and Boff, 2002). Carotenoids have the ability to quench singlet oxygen by both physical and chemical means, the most effective being physical quenching. Carotenoids can chemically quench singlet oxygen when the singlet oxygen attacks the double bonds of the carotenoid, resulting in carotenoid breakdown products such as aldehydes and ketones. Physical quenching does not lead to breakdown products. During physical quenching, there is a transfer of energy from the singlet oxygen to the carotenoid, producing an excited state carotenoid and ground state triplet oxygen. The energy from the excited carotenoid is dissipated by vibrational and rotational

interactions with the surrounding solvent to return it to the ground state (Decker, 2005). Lycopene has been found to be one of the most efficient singlet oxygen quenchers of the biological carotenoids (Di Mascio et al., 1989).

#### **2.2.4 Multiple antioxidant functions**

Antioxidant compounds may reinforce each other in multi-component systems by cooperative effects known as synergism. Synergists impart more protection against lipid oxidation than the sum of the activities of the compounds when used separately (Coleman and Williams, 2007). In addition, the use of synergistic antioxidant mixtures can allow for a reduction in the concentration of each antioxidant (Abdalla and Roozen, 1999). If both initiation and propagation are suppressed, successful synergistic inhibition can be achieved. A commonly used combination of synergistic compounds in foods is pairing metal inactivators with chain breaking antioxidants (Nawar, 1996; Frankel, 2005). An example of synergism between two compounds is the combined antioxidative effect of ascorbic acid and butylated hydroxy toluene (BHT). Ascorbic acid has the capability to chelate metals, therefore limiting their ability to initiate lipid oxidation. BHT, a phenol and a chain breaking antioxidant, has been shown to be much more effective at retarding lipid oxidation in the presence of ascorbic acid (Coleman and Williams, 2007).

#### **2.3 Measurement of antioxidant capacity**

In order to accurately evaluate the potential of antioxidants in food systems, the ideal antioxidant evaluation method should be conducted under the chemical, physical,



and environmental conditions expected in biological tissues. However, in food products, these conditions are not consistent; therefore, individual methods are needed (Decker et al., 2005). Many simplistic one-dimensional methods that use a broad range of conditions, oxidants and methods to measure end points of oxidation have been developed to measure the free radical scavenging or “antiradical” ability of antioxidants (Frankel, 2005; Brand-Williams et al., 1995).

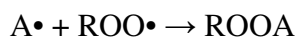
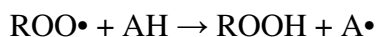
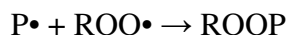
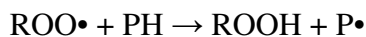
The composition of food is complex and separating each antioxidant compound and studying it individually would be inefficient and costly. Since antioxidant capacity methods are nonspecific and one-dimensional they can not be used to investigate the multiple protection mechanisms of natural antioxidants or synergistic effects between antioxidants. In addition, there is much confusion in understanding and interpreting the significance of the results of antioxidant capacity assays and possible biological implications (Huang et al., 2005; Frankel, 2007). Therefore, having a convenient method for the quick quantitation of antioxidant effectiveness is appealing to researchers and industry professionals (Huang et al., 2005).

Antioxidant capacity assays can be roughly classified into two types: hydrogen atom transfer (HAT) reactions or electron transfer (ET) assays. HAT assays, such as oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP), apply a competitive reaction scheme where antioxidant and substrate compete for thermally generated peroxy radicals through the decomposition of azo compounds. ET assays involve two components in the reaction mixture: antioxidants and the oxidant (which is the probe). The probe will abstract an electron from the antioxidant causing a color change of the probe. The color change is used to

monitor the reaction and works as an indicator of the reaction endpoint. Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP) and 2,2-diphenyl-picrylhydrazyl (DPPH) are examples of ET assays. HAT based methods are more relevant to the radical chain-breaking antioxidant capacity based on their quantification of hydrogen atom donating capacity and involvement of peroxy radicals versus ET based methods which measure an antioxidant's reducing capacity (Huang et al., 2005; Frankel, 2007; Sanchez-Moreno, 2002).

### 2.3.1 HAT methods

Hydrogen atom transfer (HAT) methods apply a thermal radical generator to give a steady flux of peroxy radicals in an air-saturated solution. When the antioxidant is added to the solution it competes with the probe (or substrate) for the radicals and inhibits probe oxidation. HAT assays have the following components: 1.) an azo radical initiator, typically 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH), 2.) a molecular probe for monitoring the reaction progress, 3.) an antioxidant and 4.) reaction kinetic parameters which are collected for antioxidant capacity quantification (Huang et al., 2005; Frankel, 2007). When a probe (PH) competes with an antioxidant (AH) for a constant flux of peroxy radicals (assuming one PH or AH scavenges two ROO• and the reaction is under steady state) the reaction is as follows (Huang et al., 2005):



HAT assays include the oxygen radical absorbance capacity (ORAC) assay and the total radical trapping antioxidant parameter (TRAP) assay. The major differences between these two assays are their quantitation approach and the type of probe utilized. ORAC uses the area under the kinetic curve (AUC) approach for quantification and applies fluorescein (FL) as the probe. TRAP quantifies with a lag time approach and uses R-phycoerythrin (R-PE) for a probe (Huang et al., 2005).

### **2.3.1.1 ORAC**

Oxygen radical absorbance capacity (ORAC) assay measures the ability of antioxidants to scavenge peroxy radicals (Kuti and Konuru, 2004). Cao et al. (1993) developed the method which measures antioxidant scavenging activity against peroxy radical production induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37°C (Ou et al., 2001). Various probes can be utilized as the fluorescent probe including phycoerythrin and fluorescein. The loss of fluorescence of the probe is an indication of the extent of damage from its reaction with the peroxy radical. The protective effect of an antioxidant is measured by calculating the area under the time recorded fluorescence decay curve (AUC) and the antioxidant capacity is expressed as  $\mu$ moles of Trolox equivalents (Ou et al., 2001; Frankel, 2007; Huang et al., 2005).

The advantage of ORAC is in its AUC approach. The AUC approach applies equally well for both antioxidants that exhibit distinct lag phases and those that have no lag phases. It unifies the lag time method and initial rate method and it can be applied to food samples. The ORAC assay has been widely used in academics and the food and

supplement industries as the method of choice for quantifying antioxidant capacity (Huang et al., 2005).

One drawback of the ORAC method is the assumption that the oxidative deterioration of the fluorescent substrate can simulate food substrates (Sanchez-Moreno, 2002; Frankel and Meyer, 2000). Measuring the effects of an antioxidant by the integrated areas under the decay curves, including total oxidation period, can be misleading because it does not distinguish between the initiation and propagation phases which are significant in relation to oxidative deterioration in foods. ORAC uses Trolox as a reference compound, which is not structurally related to any phenolic compounds found to be sources of antioxidants in foods (Frankel, 2007). In addition, by using an artificial water soluble azo compound as a radical generator, ORAC does not provide a useful estimate of the important protective activities of metal chelators and lipophilic antioxidants in food systems (Frankel, 2007; Sanchez-Moreno, 2002; Frankel and Meyer, 2000).

#### **2.3.1.2 TRAP**

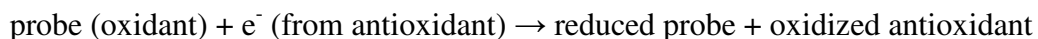
The total radical trapping antioxidant parameter (TRAP) assay was introduced by the Ingold group for the determination of the antioxidant status of human plasma. The thermal decomposition of a water soluble azo-initiator, such as 2,2'-azobis(2-amidinopropane) dihydrochloride (ABAP), generates peroxy radicals at a controlled rate (Wayner et al., 1985). When ABAP is introduced, the oxidation of plasma components is monitored by measuring the oxygen consumed during the reaction (Wayner et al., 1985). Delange and Glazer (1989) later modified this method

replacing the lipid substrate with R-phycoerythrin (R-PE), a phycobili-protein containing a red photoreceptor pigment, and correlated the oxidation kinetics of the fluorescent decay of R-PE with the activity of an antioxidant (excitation wavelength 495 nm and emission wavelength 575 nm). The induction period, or lag phase, is compared to Trolox and expressed as TRAP value ( $\mu\text{mol/L}$ ). This assay was developed to measure the total antioxidant capacity of plasma or serum (Wayner et al., 1985; Delange and Glazer, 1989; Sanchez-Moreno, 2002; Huang et al., 2005).

Like the ORAC assay, a drawback of the TRAP assay is also the assumption that the oxidative deterioration of the fluorescent substrate can replicate food substrates (Sanchez-Moreno, 2002; Frankel and Meyer, 2000). In addition, not all samples will yield a lag phase. Simulated effects of antioxidants on the lag phase of oxidation found that lag-time based measurements of antioxidant capacity overestimated the antioxidant capacity of weaker compounds (Huang et al., 2005). TRAP also uses Trolox as a reference compound, which is not structurally related to phenolic compounds found to be sources of antioxidants in foods (Frankel, 2007). By using an artificial water soluble azo compound as a radical generator, TRAP does not provide a useful estimate of the important protective activities of metal chelators and lipophilic antioxidants in food systems. In order to overcome another major problem of an unsteady electrode end point, several modifications have been made to the assay. The measurement of TRAP activity may be invalidated on the basis that free radical production would have to be sufficiently extensive enough to disturb the steady-state level of antioxidants (Sanchez-Moreno, 2002; Frankel and Meyer, 2000; Frankel, 2007).

### 2.3.2 ET methods

Electron-transfer (ET) based assays involve two components in the reaction mixture, the antioxidant and the oxidant (or probe). In itself, the probe is an oxidant that abstracts an electron from the antioxidant, causing color change of the probe. The degree of color change is proportional to the antioxidant concentration and the reaction end point is reached when the color change stops. The reaction is based on the following electron-transfer reaction (Huang et al., 2005):



Since there is no competitive reaction involved and there is no oxygen radical in the assays, it is unsure how the assay results would relate to the antioxidant capacity of a sample. Therefore, to make the correlation it is assumed that the antioxidant capacity is equal to the reducing capacity (Huang et al., 2005; Benzie and Strain, 1999). Grouped into the ET assay category are the Trolox equivalent antioxidant capacity (TEAC) assay, the ferric ion reducing antioxidant power (FRAP) assay and the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay (Huang et al., 2005; Frankel, 2007).

#### 2.3.2.1 TEAC

This method uses 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS), a metastable radical cation produced continuously by reacting the ferryl myoglobin radical generated from metmyoglobin, H<sub>2</sub>O<sub>2</sub> and peroxidase (Huang et al., 2005). The activation of metmyoglobin with hydrogen peroxide in the presence of ABTS (and the

presence or absence of antioxidants) to produce the radical cation has been criticized due to the fact that faster acting antioxidants might also contribute to the reduction of the ferryl myoglobin radical. The modified version, by Re and others, is a decolorization technique that involves the direct production of the blue/green ABTS chromophore through the reaction between ABTS and potassium persulfate (Re et al., 1999). The decay of the ABTS radical, due to the addition of antioxidants, is monitored by measuring its decrease in absorbance, with the main absorption maxima at 415 nm and secondary absorption maxima at 660, 734, and 820 nm (Re et al., 1999; Sanchez-Moreno, 2002). The amount of Trolox (mM) producing the same activity of 1 mM of the test compound is the Trolox equivalent antioxidant capacity (TEAC). The ABTS radical is formed during incubation with the test compound, therefore the activity measured is due not only to the prevention of ABTS radical formation but also to the scavenging of the radical (Frankel, 2007; Huang et al., 2005; Re et al., 1999).

The TEAC assay, due to its operational simplicity, has been used in many research laboratories for studying antioxidant capacity (Frankel, 2007). It is applicable to the study of both water-soluble and lipid-soluble antioxidants (Re et al., 1999). However, like other antiradical methods, the TEAC assay has limitations. This assay only measures reactivity towards artificial ABTS radicals and does not involve a substrate; hence, it does not test antioxidants for their ability to inhibit oxidation. For pure antioxidant compounds, there is no clear correlation between TEAC values and the number of electrons an antioxidant can give away. The ability of phenolic compounds to scavenge the artificial ABTS radicals can be measured; however it may not reflect their antioxidant activity by other mechanisms, such as metal chelation. The TEAC

assay is an end-point assay therefore reaction rate differences between antioxidant and oxidant are not reflected in TEAC values (Frankel and Meyer, 2000; Frankel, 2007; Huang et al., 2005). TEAC can also be limited by sample interference of pigmented compounds causing the activity of an antioxidant to be significantly underestimated (Frankel, 2007).

### **2.3.2.2 FRAP**

In the ferric reducing antioxidant power (FRAP) assay, direct measurement of the ability of antioxidants to reduce a ferric tripyridyltriazine complex to its ferrous complex at low pH is determined. Similar to the TEAC assay, except for the decreased pH, this method is based on the redox potential of a ferric complex and the resulting blue color is measured spectrophotometrically at 593 nm at 0.5 s and every 15 s for 4 minutes. The change in absorbance is linearly related to the total reducing power of the electron-donating antioxidants present in the system (Benzie and Strain, 1999; Frankel, 2007; Huang et al., 2005).

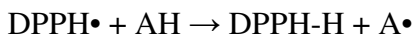
The FRAP assay is simple, speedy and the total antioxidant power can be directly measured (Benzie and Strain, 1999), however there are limitations to the assay. The major limitation to this assay is that the measured reducing capacity reflects the total antioxidant concentration, not necessarily specific antioxidant activity. In addition, there is no information provided regarding the protective properties of antioxidants since no oxidizable substrate is included in the assay (Frankel and Meyer, 2000; Frankel, 2007). It has been found that in some polyphenols, including caffeic acid, ferulic acid and ascorbic acid, absorption did not stop at 4 min.; instead it slowly



increased even after several hours. If the FRAP 4 min. reaction time is followed, the values of these compounds can not be accurately acquired. Chelators can bind Fe (III) and form complexes that are capable of reacting with antioxidants. This is a problem because the oxidant, ferric tripyridyltriazine, also contains other Fe (III) species that may be bound by chelators therefore the values obtained may not be precise (Huang et al., 2005).

### **2.3.2.3 DPPH•**

One of the earliest synthetic radicals to be used in a substrate-free assay in order to study the effects of structures on the activity of antioxidants is 2,2-diphenyl-1-picrylhydrazyl (DPPH•). DPPH• serves as both the oxidizing radical to be reduced by the antioxidant (AH) and the color indicator for the reaction (Frankel, 2007, Brand-Williams et al., 1995):



The effect of an antioxidant on decreasing the absorption of DPPH• in a methanol solution at 515-517 nm is measured spectrophotometrically until the absorbance reaches a steady state. The assay time could take anywhere from 5 min. to 8 hrs. (Wettasinghe and Shahidi, 2000; Lee et al., 2007; Frankel, 2007). Different types and concentrations of antioxidants can significantly vary the decay slope and the absorbance level reached by the remaining DPPH• radicals. The antioxidant concentration and the time necessary to reach the steady state to 50% of the initial DPPH• concentration are referred to as the antiradical efficiency (Frankel, 2007; Huang et al., 2005; Frankel and Meyer, 2000).

The DPPH• method is considered a valid and easy assay to evaluate the scavenging activity of antioxidants since the radical compound is stable and does not have to be generated, as is necessary in other radical scavenging assays (Sanchez-Moreno, 2002). However, there are limitations to this assay. First, it does not use a substrate, hence providing no information on the protective activity of antioxidants towards food systems. Secondly, DPPH• radicals are artificially generated therefore the assay is not relevant to real food lipid radicals (Frankel, 2007, Lee et al., 2007). Lastly, DPPH• radicals are long-lived nitrogen radicals that are relatively more stable than and bear no similarity to peroxy radicals which are involved in lipid oxidation (Huang et al., 2005; Frankel, 2007; Lee et al., 2007).

#### **2.4 Measurement of lipid oxidation**

Numerous tests exist to evaluate the oxidative stability of a sample. Oxidation measurements are typically carried out under standardized conditions to a suitable end point (Frankel, 2005). Primary lipid oxidation compounds are the first oxidation products produced by the initiation and propagation steps of lipid oxidation. They can appear early in the oxidative deterioration of lipids. In the later stages of oxidation the concentrations of primary compounds decrease because their formation rates become slower than their decomposition rates. Lipid hydroperoxide measurements are typically used to determine primary oxidation products. Secondary lipid oxidation products are compounds that are formed from the decomposition of fatty acid hydroperoxides by means of  $\beta$ -scission reactions. Since these reactions can generate hundreds of volatile and nonvolatile compounds which would be impossible to measure simultaneously,

methods such as GC detection are necessary and generally focus on analyzing a single compound or class of compounds. Since the formation of secondary products relies on the decomposition of lipid hydroperoxides, the presence of antioxidants can cause the concentrations of secondary products to be low while concentrations of primary products are high (Frankel, 2005; Decker, 2005; Chaiyasit et al., 2007; Lea, 1962; Vercellotti et al., 1992).

#### **2.4.1 Lipid hydroperoxides**

Peroxide value is one of the most commonly used methods for measuring the extent of oxidation in oils. It is expressed as millimole of hydroperoxide per kg of lipid (mmol/kg) (Nawar, 1996; Frankel, 2005). The ferric thiocyanate method is more sensitive than other peroxide methods and requires a smaller sample size. This method is based on the oxidation of ferrous to ferric ions, which are determined colorimetrically as ferric thiocyanate (Shantha and Decker, 1994). In bulk oils, the peroxide value can be analyzed directly. In food systems, such as emulsions and muscle tissues, the lipid must first be extracted by mixing with solvents (Frankel, 2005). The peroxide value is an empirical measure of oxidation which is useful for samples that are oxidized to relatively low levels under mild conditions so that the hydroperoxides are not appreciably decomposed. During oxidation, the peroxide value reaches a maximum peak and then begins to decrease at more advanced stages of oxidation (Nawar, 1996; Frankel, 2005). The maximum peroxide value can occur at earlier or later stages depending on the fatty acid composition of the oil and the conditions of oxidation. For more polyunsaturated oils, such as fish oils, the peroxide value maximum will occur at

an earlier stage because their hydroperoxides decompose more rapidly. Hydroperoxides will also rapidly decompose during oxidation conditions involving temperatures over 100°C, exposure to light and the presence of metals (Frankel, 2005).

#### **2.4.2 Thiobarbituric acid test**

The thiobarbituric acid reactive substances, or TBARS, method is used to measure the extent of secondary lipid oxidation products. The basis of this test is the absorbance of a pink color complex at 532-535 nm which is formed between thiobarbituric acid (TBA) and oxidation products of polyunsaturated lipids. This color complex is formed from the condensation of two moles of TBA with one mole of malonaldehyde under thermal acidic conditions (Nawar, 1996; Frankel, 2005). The determination of TBA value is expressed as the mg of malonaldehyde per kg of sample. Temperature, time of heating, pH, metal ions and antioxidants are all factors that can affect the production of the pink color complex. Variations to the TBA test are designed to increase the sensitivity, including heating in acids and adding ferric ions, or reducing production of decomposition materials during the assay by adding antioxidants or metal chelators (Frankel, 2005; McDonald and Hultin, 1987).

#### **2.4.3 Chromatographic Methods**

Various chromatographic techniques, including gas, liquid and thin-layer, have been used to determine oxidation in oil and lipid containing foods. These methods are based on the separation and quantification of specific fractions or individual components that are typically known to be produced during autoxidation (Nawar, 1996).

The dynamic headspace method, commonly known as purge-and-trap, includes the following steps: 1) adding a sample to a sealed tube or vessel, 2) trapping the vaporized volatiles into a short column without cooling, 3) desorbing the volatiles from the trap and transferring by back flushing with a carrier gas into the capillary inlet of the gas chromatograph (GC) and 4) separating the compounds by GC. In this method, the recovery of a suitable internal standard subjected to the same conditions as the sample is the basis for the quantification of the volatile compounds. Volatile profiles can be greatly affected by the sampling temperature. Lower temperatures can yield a smaller percentage of volatiles contributing to the total peak area, whereas, higher temperatures yield larger percentages of volatiles contributing to the total peak area (Frankel, 2005). Hexanal, an important volatile product; has been proven a useful analytical marker for the oxidative decomposition of n-6 PUFA's (Abdalla and Roozen, 1999; Frankel, 1982).

## **2.5 Omega-3 Fatty Acids**

There are three main types of fatty acids: 1. saturated (SFA), 2. monounsaturated (MUFA) and 3. polyunsaturated (PUFA). SFA and MUFA are synthesized endogenously in humans; however, PUFA needs to be supplied exogenously. PUFAs are considered the “good fats” of the fatty acids (Patil and Gislerod, 2006). The important omega-3 PUFAs are eicosapentaenic acid (EPA, 20:5) and docosahexaenoic acid (DHA 22:6) which are found from marine sources.

In the past, our ancestors are believed to have consumed equal amounts of omega-6 ( $\omega$ -6) and omega-3 ( $\omega$ -3) fatty acids. However, with advances in modern

agriculture and the increased availability of refined fats, the profile of dietary lipids in Western societies has changed dramatically, shifting our dietary intake ratio of  $\omega$ -6 and  $\omega$ -3 fatty acids to 7:1, respectively. This rapid change and lowered amounts of  $\omega$ -3 fatty acids in the diet is significant from a nutritional standpoint (Decker, 2005). Current nutritional studies focus on the numerous health benefits of maintaining sufficient levels of fatty acids in the diet, in particular the very long chain PUFA, especially  $\omega$ -3 fatty acids (Patil and Gislerod, 2006; Klurfeld, 2002). There are many studied and proven health benefits of dietary  $\omega$ -3 fatty acids, including protection against cardiovascular disease, cancer, hypertension, diabetes and rheumatoid arthritis, improved brain and retinal function in infants and reduced susceptibility to tumors (Tong et al., 2000; Simopoulos, 1991; Mori and Beilin, 2001; Patil and Gislerod, 2006).

Although  $\omega$ -3 fatty acids have many health benefits, they are extremely sensitive to lipid oxidation, which results in alteration of nutritional composition and quality of the lipid (Nuchi et al., 2001). These fatty acids are subject to rapid and/or extensive oxidation and other chemical changes by means of exposure to air, light, or heat during processing (Lytle et al., 1992). It is of great interest to food manufacturers to use  $\omega$ -3 fatty acids as functional ingredients to improve the nutritional profile of food products. Numerous food companies are using direct incorporation of  $\omega$ -3 fatty acids into foods and farmers are feeding  $\omega$ -3 fatty acids to livestock. These approaches are hindered by the oxidative deterioration of the  $\omega$ -3 fatty acids during processing and storage (Decker, 2005; Tong et al., 2000). If problems with the susceptibility of  $\omega$ -3 fatty acids to

oxidative rancidity can be overcome, they would be ideal functional food ingredients due to their many health and nutritional benefits (Alamed et al., 2006).

## **2.6 Lipid oxidation in food systems**

Food chemists have been studying the development of rancidity in foods for many decades. There are numerous reactions that can alter food safety and quality, such as heating, metal interactions and oxidation. In food systems these reactions can have a negative effect on texture, flavor, color, nutritive value and safety (Fennema and Tannenbaum, 1996). Progress has been made in controlling lipid oxidation by improving processing and packaging techniques and the use of antioxidants (Frankel, 2007). The activity of an antioxidant will vary between bulk oil and multiphase emulsion systems (Frankel, 2007) as well as in muscle foods. Therefore, one-dimensional tests that rank an antioxidant's capacity to scavenge free radicals in a simple system can not be directly related to the activity of the antioxidant in a complex food system.

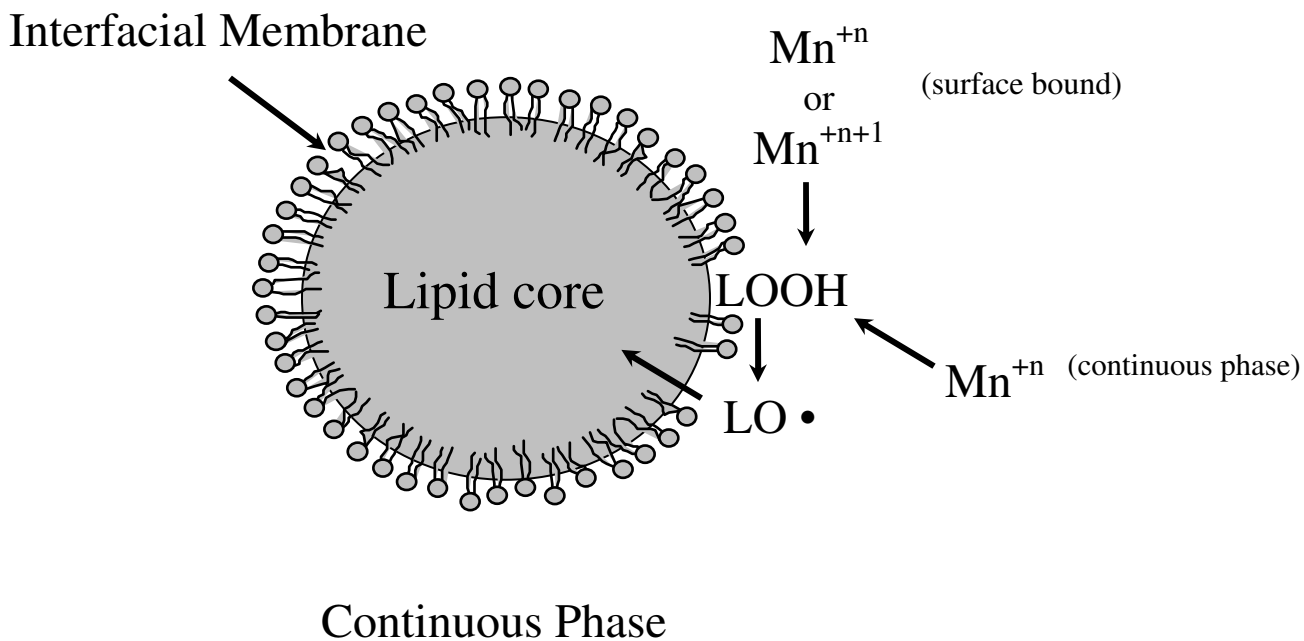
### **2.6.1 Emulsions**

An emulsion consists of two immiscible liquids, usually oil and water, where one liquid is dispersed as small spherical droplets in the other. Emulsions can be classified according to the distribution of the oil and aqueous phases. An oil-in-water (O/W) emulsion consists of oil droplets dispersed in an aqueous phase, examples of which include milk, dressings, mayonnaise and beverages. When water droplets are dispersed in an oil phase the system is called a water-in-oil (W/O) emulsion, such as

butter and margarine (McClements, 2005; McClements and Decker, 2000; Walstra, 1996). In emulsions, the dispersed phase is the substance that makes up the droplets and the continuous phase is the substance that makes up the surrounding liquid. Homogenization is necessary to convert the two separate immiscible liquids into an emulsion, however, a homogenized emulsion of pure oil and pure water would rapidly separate into two layers due to the fact that contact between oil and water molecules is thermodynamically unfavorable. A stabilizer, such as an emulsifier, is needed to improve the stability of an emulsion. Emulsifiers are surface active molecules that absorb to the surface of droplets creating a protective layer around the droplet preventing droplets from aggregating (McClements, 2005; Coupland and McClements, 1996). The membrane surrounding the droplet can consist of surface-active substances or proteins or both (Frankel, 2005) and the thickness and composition of the surface layer can impact the physical and chemical stability of the emulsion. In general, the most important variables determining emulsion properties are: 1) type of emulsion (O/W or W/O), 2) droplet size distribution, 3) volume fraction of dispersed phase and 4) composition and thickness of the surface layer surrounding the droplets (McClements, 2005; Walstra, 1996). There are various physical and chemical mechanisms that can lead to emulsion instability. Some of the most common physical mechanisms are creaming, sedimentation, gravitational separation, flocculation and phase inversion (McClements, 2005). Chemical processes that occur in food emulsions which have a negative effect on stability include enzyme hydrolysis and, more importantly, lipid oxidation (McClements, 2005).



Oil-in-water emulsions contain distinct environments, including the lipid core, droplet interfacial membrane and aqueous phase, in which molecules that participate in lipid oxidation can concentrate. Useful tools to deter lipid oxidation are the addition of antioxidants and/or metal chelators, engineering the droplet interface and adjustment of pH. Lipophilic antioxidants concentrate in the oil droplet, where lipid oxidation takes place, and are more effective at inhibiting oxidation than hydrophilic antioxidants which partition into the water phase (Decker, 1998b; Huang et al., 1997). Emulsifiers can create a protective barrier around lipid droplets deterring the penetration and diffusion of metals and radicals, which initiate lipid oxidation, into the lipid. The addition of metal chelators can decrease metal reactivity by binding metals and physically removing them from the lipid core and/or droplet interface, thus inhibiting the decomposition of lipid hydroperoxides (Figure 2.3), in turn retarding lipid oxidation (Decker et al., 2002). At increased pH lipid oxidation is generally slowest, whereas, the oxidation rate accelerates as the pH decreases. This could be due to the greater solubilization of metal catalysts at low pH. As pH decreases, metals that are located in the aqueous phase can become more hydrated and reactive with polar hydroperoxides and water-soluble radicals at the oil droplet surface (Frankel, 2005).



**Figure 2.3.** A schematic of the physical environments of an oil-in-water emulsion including the possible physical location of reactants in the transition metal-promoted decomposition of lipid hydroperoxide.  $Mn^{+n}$  and  $Mn^{+n+1}$  are transition metals in their reduced and oxidized states, respectively. LOOH is a lipid hydroperoxide and  $LO\bullet$  is an alkoxy radical. The components and phases are not drawn to scale (Modified from Decker et al., 2002).

Electrostatic charge of droplets and the pH have a major affect on the physical location and effectiveness of antioxidants and prooxidants in emulsions. Droplets stabilized with an anionic emulsifier are capable of accelerating lipid oxidation due to the electrostatic attraction that occurs between the negatively charged oil-water interface and the positively charged metal ions present (Frankel, 2005; Mei et al., 1999). The effectiveness of antioxidants can be influenced by the attractive/repulsive electrostatic

interactions between charged antioxidants, which will affect their location and activity within the system. Electrostatic forces can also have an effect on charged prooxidants, in turn, affecting the location and activity of transition metals in the system. In addition, pH can alter the location of ionic antioxidants by altering their charge and solubility and, in addition, can alter the ionic interactions between prooxidants and other components (Mei et al., 1999).

### **2.6.2 Edible (bulk) oils**

Lipids that are purchased as food components are generally stored in bulk (Porter, 1993). Edible oils consist primarily of a mixture of polyunsaturated fatty acids, which are greatly susceptible to lipid oxidation, and saturated and monounsaturated fatty acids, as either free fatty acids or as glycerol esters, which are fairly stable to lipid oxidation, plus assorted other minor components (Coupland and McClements, 1996), some of which are desirable and some of which are not. In order for manufacturers to meet consumer demand for light-colored, neutral flavored and both physically and oxidatively stable oils, numerous non-triacylglycerol compounds in the crude oil must be removed through a process called refining. There are two types of refining processes: chemical and physical. Chemical refining involves numerous steps which include degumming, neutralization, bleaching and deodorization. Physical refining entails the removal of free fatty acids and flavors through distillation, combining the neutralization and deodorization steps into one procedure (Johnson, 2002). The refining process is meant to remove undesirable constituents from the oil with the least possible amount of damage to the desirable constituents, for instance, natural antioxidants such

as tocopherols, carotenoids, and other phenolic compounds which can protect oil against autoxidation and provide vitamin activity. However, since the refining process is not 100% selective and efficient, not all desirable compounds are removed while some beneficial compounds are removed (Johnson, 2002).

The rate of lipid oxidation in bulk oil is dependent on a range of factors. In regards to association colloids, the rate and mechanism at which lipid oxidation occurs is dependent on a variety of factors including, total amount of substrate or reactant or antioxidant concentrated at the water-lipid interface, location and orientation of reactants, temperature, water content and mobility and concentration and reactivity of reactants or antioxidants in the inner core (Chaiyasit et al., 2007). As the degree of lipid unsaturation increases, the rate of lipid oxidation increases (McClements and Decker, 2000; Nawar, 1996). Unsaturated vegetable oils, unlike animal fats which are primarily saturated, are more reactive with other compounds, especially oxygen, and have higher susceptibility to lipid oxidation. Exposure to air, heat, light, trace metals and water enhances the chemical reactivity of bulk oils and leads to off-flavors, nutritional losses and other deteriorative changes (Naz et al., 2005).

Crude oils contain natural antioxidants making them more stable towards oxidation than refined oils where some of the natural antioxidants have been removed during the refining process (Johnson, 2002). The most preferable way to reduce lipid oxidation in refined bulk oil is the addition of antioxidants (Naz et al., 2005), which has been greatly studied. As discussed previously, the “*antioxidant polar paradox*” is based on the theory that in oils with a low surface-to-volume ratio (bulk vegetable oils) polar antioxidants are more effective than non-polar antioxidants because they have the ability

to concentrate at locations where oxidative reactions are the greatest (Chaiyasit et al., 2005). In contrast, in oils with high surface-to-volume ratios (emulsified oils), non-polar antioxidants are strongly favored (Frankel, 2005; Frankel et al., 1994; Porter, 1993; Huang et al., 1997).

### **2.6.3 Muscle Foods**

Throughout the animal kingdom muscle function is similar; however, there exist compositional differences in muscle and lipid between species and even differences within the same species. Disparities in muscle include muscle color, fiber length and concentration of lipids and myoglobin (Erickson, 2002; Foegeding et al., 1996). Lipids are the most variable component in meats and there are differences between species, among muscles of one species and amid animals of different ages. They can be categorized by their location in the muscle (Foegeding et al., 1996). In adipose tissue, stored triglycerides are arranged in large globules within the cell and they vary greatly in amount and composition within species and as a function of diet. In muscle tissue, muscle lipids are integral parts of various cellular structures, including the cell membrane, mitochondria and microsomes. These intracellular fats include most of the phospholipids of the tissue and have partial association with proteins (Erickson, 2002). In fatty fish muscle, fat occurs as extracellular droplets in the muscle tissue. In red muscle, distinct fat droplets exist within the cells, where as in white muscle; the fat is well dispersed outside the muscle cells (Foegeding et al., 1996).

One of the major causes of quality deterioration in muscle foods is lipid oxidation (Erickson, 2002). The level of lipid present does not determine the oxidative

susceptibility of the muscle; rather it is the type of lipid and the lipid's relative reactivity that comprise the major determinants for lipid oxidation in muscle foods (Frankel, 2005). The two classes of lipids, stored triglycerides and muscle lipids, are capable of oxidizing independently of each other. The ability to oxidize independently of each other may be due to differences in the distribution of lipid within the tissue (Watts, 1962). Lean beef muscle consists of about 2-4% triacylglycerols and 0.8-1% phospholipids containing 44% polyunsaturated fatty acids. These are the main lipids that are subject to oxidation (Frankel, 2005). In vitro studies of individual lipid classes have been invaluable at defining the contribution of each class to the oxidative stability of a food system. It's been found that free fatty acids oxidize faster than triacylglycerols while the reactivity of membrane lipids is greater than the reactivity of emulsified triacylglycerols (Erickson, 2002). Poultry muscle is more susceptible to lipid oxidation than beef because of their higher polyunsaturated phospholipid fraction (Frankel, 2005). Fish muscle is noticeably different than mammalian or avian muscles because they contain a larger percentage of unsaturated fatty acids making them the most unstable towards lipid oxidation (Erickson, 2002; Foegeding et al., 1996).

The presence of antioxidants and/or catalysts and food processing operations can alter the oxidative stability of muscle foods (Lee et al., 1997). Antioxidants are the most important defense mechanism for lipid oxidation in muscle foods. They inhibit oxidation by reducing the rate of oxidation or by reducing the maximal level of oxidation (Erickson, 2002). In muscle foods many oxidative reactions occur in the aqueous environment, hence, water-soluble antioxidants should be highly effective (Decker, 1998b). Free radical scavengers have shown to be effective inhibitors of meat

flavor deterioration (We and Brewer, 1994). Prooxidants in muscle foods include transition metal ions, singlet oxygen generation systems and enzymic initiation systems (Erickson, 2002). Biological oxidation is due almost exclusively to metal ion-promoted reactions (Kanner, 1992). All food products are subjected to numerous processing treatments prior to storage. During processing, an opportunity to alter patterns of oxidation arises. Mincing of muscle tissues disrupts cellular integrity and exposes more of the lipids to prooxidants. It will also dilute the antioxidant concentration, increase the exposure of tissue to oxygen, and increase surface area, accelerating degradation (Erickson, 2002). Heating modifies lipid oxidation by dislodging iron from heme compounds, disrupting cellular integrity, breaking down preexisting hydroperoxides and inactivating enzymes associated with lipid oxidation (Erickson, 2002; Watts, 1962).

Iron is essential for all living things because it's necessary for oxygen transport, respiration and the activity of many enzymes (Decker and Hultin, 1992). Muscle foods contain prominent amounts of iron, a known prooxidant. Lipid oxidation can be initiated by the presence of soluble chelates of low molecular weight iron which produce hydroxyl radicals from hydrogen peroxide (Decker and Hultin, 1992; Wu and Brewer, 1994). Aldehydes are the major contributors to off-flavors, off-odors and meat flavor deterioration (Wu and Brewer, 1994).

## CHAPTER 3

### INFLUENCE OF HEAT PROCESSING AND CALCIUM IONS ON THE ABILITY OF EDTA TO INHIBIT LIPID OXIDATION IN OIL-IN-WATER EMULSIONS CONTAINING OMEGA-3 FATTY ACIDS

#### 3.1 Abstract

The nutritional benefits of  $\omega$ -3 fatty acids make them excellent candidates as functional food ingredients if problems with oxidative rancidity can be overcome. Oil-in-water emulsions were prepared with 2% salmon oil, stabilized by 0.2% Brij 35 at pH 7. To determine the effects of heating (50-90°C), ethylenediaminetetraacetic acid (EDTA), and calcium on the oxidative and physical stability of salmon oil-in-water emulsions, particle size, thiobarbituric acid reactive substances (TBARS), and lipid hydroperoxides were measured. The heat-processed emulsions showed no significant difference in particle size, TBARS or hydroperoxides during storage compared to unheated emulsions. Above 2.5  $\mu$ M, EDTA dramatically decreased lipid oxidation in all samples. Addition of calcium to emulsions containing 7.5  $\mu$ M EDTA significantly increased both TBARS and hydroperoxide formation when calcium concentrations were 2-fold greater than EDTA concentrations. These results indicate that heat processed salmon oil-in-water emulsions with high physical and oxidative stability could be produced in the presence of EDTA.



### 3.2 Introduction

The omega-3 ( $\omega$ -3) fatty acids found in fish oil have been found to be clinically beneficial to health (Akoh & Min, 2002). The health benefits of dietary omega-3 fatty acids include reduced susceptibility to mental illness, protection against heart disease, and improved brain and eye function in infants (Simopoulous, 1991; Innis, 1991).

Although  $\omega$ -3 fatty acids have many health benefits, they are extremely sensitive to lipid oxidation, resulting in potential alteration in nutritional composition and food quality (Nuchi et al., 2001). These fatty acids are subject to rapid and/or extensive oxidation and other chemical changes by exposure to air, light, or heat during processing (Lytle et al., 1992). It is of great interest to food manufacturers to use  $\omega$ -3 fatty acids as physiologically functional ingredients to improve the nutritional profile of food products; however, lipid oxidation limits the utilization of these oils in processed foods (Frankel et al., 2002). The nutritional benefits of  $\omega$ -3 fatty acids make them excellent candidates as functional food ingredients if problems with oxidative rancidity can be overcome.

Successful incorporation of  $\omega$ -3 fatty acids into processed foods would most likely be in the form of lipid dispersions (Tong et al., 2000). Lipid dispersions which consist of oil dispersed in an aqueous phase in the form of small spherical droplets are referred to as oil-in-water emulsions (McClements and Decker, 2000). Oil-in-water emulsions consist of three distinct physical environments: the droplet's lipid core, the interfacial membrane, and an aqueous continuous phase. The differences in the physical environment of the lipids, and in the type and location of prooxidants and antioxidants,

mean that there can be large differences in the rate and extent of lipid oxidation between bulk and emulsified oils (McClements and Decker, 2000).

Lipid oxidation in oil-in-water emulsions has been extensively studied and it is believed that the interaction between lipid hydroperoxides located at the droplet surface and transition metals originating in the aqueous phase are the most common cause of oxidative instability (McClements and Decker, 2000). Incorporating antioxidants into foods is one of the most effective means of retarding lipid oxidation. In oil-in-water emulsions, the most successful type of antioxidant is one that chelates transition metal ions. A chelate is a complex that results from the combination of a metal ion and a multidentate ligand such that the ligand forms two or more bonds with the metal, resulting in a ring structure that includes the metal ion (Miller, 1996). Chelators that act as antioxidants can inhibit metal-catalyzed reactions by a variety of different mechanisms, including prevention of metal redox cycling, occupation of metal coordination sites, and steric hinderance of interactions between metals and lipid substrates (McClements and Decker, 2000). Ethylenediaminetetraacetic acid (EDTA), a transition metal chelator, has been shown to dramatically retard lipid oxidation in salmon oil-in-water emulsions by removing iron from the droplet surface (Mei et al., 1998). EDTA has been reported to be an inhibitor of lipid oxidation when the EDTA:iron ratio is greater than one. High concentrations of EDTA in relation to iron will inhibit lipid oxidation by surrounding the metal and preventing interaction with peroxides (Mahoney and Graf, 1986).

Many foods contain relatively high concentrations of multivalent ions in addition to iron (e.g. calcium). The effectiveness of EDTA as an antioxidant could be

diminished by the presence of calcium because it can compete with the iron for binding to EDTA. EDTA has a high calcium ion binding constant, although less than that for iron ions. Relatively high concentrations of calcium may bind up the EDTA in the system leaving iron the opportunity to associate with the emulsion droplets, resulting in oxidation of the lipid and instability of the emulsion.

The objective of this paper is to determine the effects of heat processing and calcium ions on the ability of EDTA to inhibit lipid oxidation in Brij 35 stabilized salmon oil-in-water emulsions at pH 7.

### **3.3 Materials and Methods**

#### **3.3.1 Materials**

Salmon fillets were purchased at a local grocer (Stop & Shop, Hadley, MA). Brij 35 was acquired from Aldrich Chemical Company, Inc. (Milwaukee, WI). Ethylenediaminetetraacetic acid disodium salt (EDTA), 2-thiobarbituric acid (TBA), ferrous sulfate, butylated hydroxytoluene, barium chloride, sodium acetate, and imidazole were obtained from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid was purchased from Acros Organics (Pittsburg, PA). All other chemicals and solvents were reagent or HPLC grade and were obtained from Fisher Scientific (Pittsburg, PA) or Sigma Chemical Company (St. Louis, MO).

### **3.3.2 Methods**

#### **3.3.2.1 Preparation of Salmon Oil**

To obtain fresh salmon oil, salmon fillets were skinned, hand chopped into small pieces and minced in a food processor. The mince was then centrifuged at 10,000 rpm for 20 min. at 5°C in a Sorval superspeed RC2-B automatic refrigerated centrifuge (Newtown, CT). The liquid lipid layer was decanted, placed into capped glass test tubes (16 x 125 mm; Fisherbrand) and stored at -80°C until use. The resulting salmon oil consisted of (99.5 ± 0.2%) triacylglycerol (Mei et al., 1998). The level of oxidation products initially in the oil was 0.32 mmol of lipid peroxide/kg of oil as determined by a modification of the method of Shantha and Decker (1994) and 0.04 mmol of TBARS as determined by the method of McDonald and Hultin (1987).

#### **3.3.2.2 Preparation of Emulsion**

A coarse emulsion consisting of 2 wt% salmon oil, 0.2 wt% Brij 35 (a non-ionic surfactant), and 10 mM sodium acetate/imidazole buffer (pH 7) was made by homogenizing the lipid and aqueous phases for 2 min. using a 2-speed hand held homogenizer (Biospec Products, Inc. Bartlesville, OK) at the highest speed setting. The coarse emulsion was passed three times through an APV-Gaulin model mini-lab 8.30H high-pressure valve homogenizer (APV Americas, Wilmington, MA) at 5000 psi. The final mean droplet diameter of the emulsion ( $d_{43}$ ) was 1.1 ± 0.1 μm, as determined by laser light scattering (LA-900, Horiba Instruments, Irvine, CA and LS-230, Coulter Corp., Miami, FL).

For heat processing studies a Brij 35 stabilized salmon oil-in-water emulsion was used in all experiments. The emulsion was separated into 30 mL allocations, which were heated in a water bath (NESlab GP-200, Fisher Scientific, Suwanee, GA) for a total of 10 min., and then immediately cooled in an ice bath for a total of 30 min.

### **3.3.2.3 Preparation of EDTA Containing Samples**

Ethylenediaminetetraacetic acid disodium salt (EDTA) solutions were made by dissolving EDTA in double distilled water obtained from a water purification system (Barnstead NANOpure infinity ultra pure, Dubuque, Iowa). EDTA solutions of varying concentrations were added to 30 mL of emulsion at a volume of 100  $\mu$ L and the emulsion was stirred for 1 minute. EDTA was added to non-heated samples and to heated samples either prior or post heat processing, respectively.

### **3.3.2.4 Preparation of Calcium Components**

Calcium chloride solutions were made by dissolving calcium chloride in double distilled water. Calcium solutions of varying concentrations were added to 30 mL of emulsion at a volume of 100  $\mu$ L and the emulsion was stirred for 1 min. Calcium was added to samples containing no EDTA and to samples containing 7.5  $\mu$ M EDTA, all of which were non-heated samples.

### **3.3.2.5 Lipid Oxidation Measurements**

Emulsions (10 mL) were placed in capped glass test tubes (16 x 125 mm; Fisherbrand) and incubated in the dark at 20°C for 8 days. Controls contained the salmon oil emulsion only and were not heat processed. Lipid hydroperoxides were

determined daily using a method adapted from Shantha and Decker (1994). Emulsion (0.3 mL) was added to a mixture of 1.5 mL of isooctane/2-propanol (3:1v/v), vortexed (10 s, 3 times), and the organic solvent phase was isolated by centrifugation at 1000 x g for 2 min. The organic solvent phase (200  $\mu$ L) was added to 2.8 mL of methanol/1-butanol (2:1v/v), followed by 15  $\mu$ L of 3.94 M ammonium thiocyanate and 15  $\mu$ L of ferrous iron solution (prepared by adding equal amounts of 0.132 M BaCl<sub>2</sub> and 0.144 M FeSO<sub>4</sub>). After 20 min., the absorbance was measured at 510 nm using a UV-vis scanning spectrophotometer (Shimadzu UV-2101PC UV-VIS, Kyoto, Japan). Hydroperoxide concentrations were determined using a standard curve made from cumene hydroperoxide.

Thiobarbituric acid reactive substances (TBARS) were determined daily using the method of McDonald and Hultin (1987). Emulsion (0.05 mL) was combined with 0.95 mL of water and 2.0 mL of TBA reagent (15% w/v trichloroacetic acid and 0.375% w/v thiobarbituric acid in 0.25 M HCl mixed with a 2% BHT in ethanol solution) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min. and then centrifuged (1000 x g) for 15 min. The absorbance was measured at 532 nm. Concentrations of TBARS were determined from a standard curve prepared using 1,1,3,3-tetraethoxypropane.

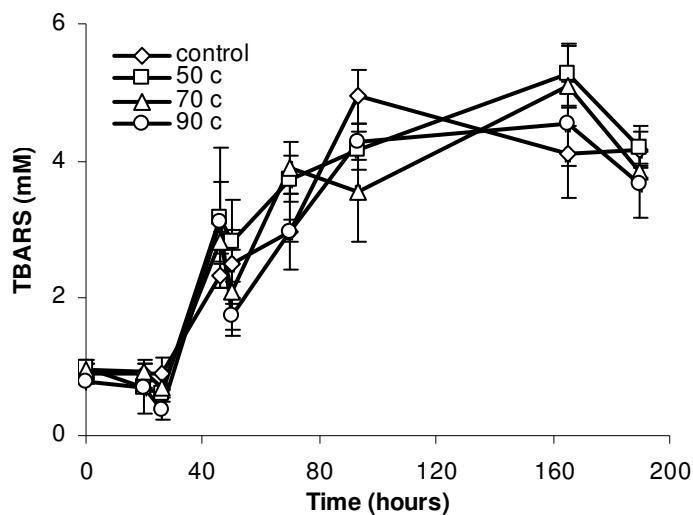
### **3.3.2.6 Statistics**

All experiments were conducted twice and measurements were performed on triplicate samples. Differences between means were determined with the least-squares means procedure at  $p < 0.05$  (Snedecor and Cochran, 1989).

## **3.4 Results and Discussion**

### **3.4.1 Heat Processing and Holding Time**

The effect of holding temperature on the physical and oxidative stability of the emulsion was tested by heating 30 ml of emulsion in a water bath heated to 50°C, 70°C, and 90°C, and holding for 10 min. A control was prepared which was not heated. The emulsions were then cooled to 20°C and stored for 8 days in capped glass test tubes (16 x 125 mm; Fisherbrand) in the dark. The droplet size distribution was monomodal and the mean droplet diameter remained stable ( $d_{43} = 1.1 \pm 0.1 \mu\text{m}$ ) as a function of storage time, indicating that heat processing had little effect on the physical stability of the emulsions. The effects of thermal processing on oxidative stability were initially screened using TBARS. Over 8 days, TBARS concentrations (Figure 3.1) of all samples increased at similar rates. These results indicate that emulsions that were heat processed at temperatures up to 90°C for 10 min. did not oxidize at faster rates during storage than the unheated control sample.



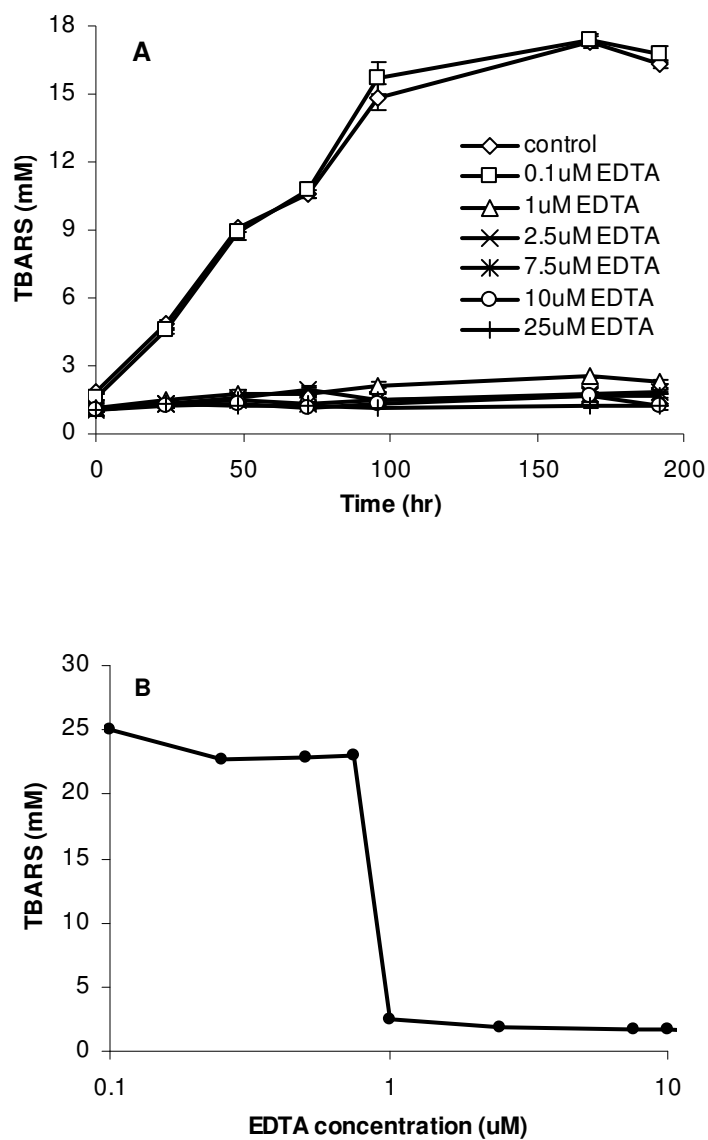
**Figure 3.1.** TBARS concentration of salmon oil-in-water emulsions exposed to heat treatments of 50°C, 70°C, 90°C and a control (not heated) stored at 20°C and measured over 8 days.

### 3.4.2 Influence of EDTA concentration on lipid oxidation

Various concentrations of EDTA (0-150  $\mu\text{M}$ ) were added to non-heated emulsions in order to determine the minimum amount of EDTA needed to retard lipid oxidation in the system. TBARS were used to initially screen the effects of EDTA concentration on oxidative stability. TBARS values showed the control sample containing no EDTA oxidized at the fastest rate over 8 days (Figure 3.2.a). EDTA at 0.1  $\mu\text{M}$  was not able to retard lipid oxidation and this emulsion oxidized at the same rate as the control. Samples containing 1-25  $\mu\text{M}$  EDTA successfully retarded lipid oxidation over 8 days, with  $\geq 2.5$   $\mu\text{M}$  EDTA being more effective at inhibiting oxidation than the sample containing 1.0  $\mu\text{M}$  EDTA after 5 days of storage. As little as 2.5  $\mu\text{M}$  EDTA was found to be sufficient in inhibiting oxidation after 7 days of storage (Figure 3.2.b). The



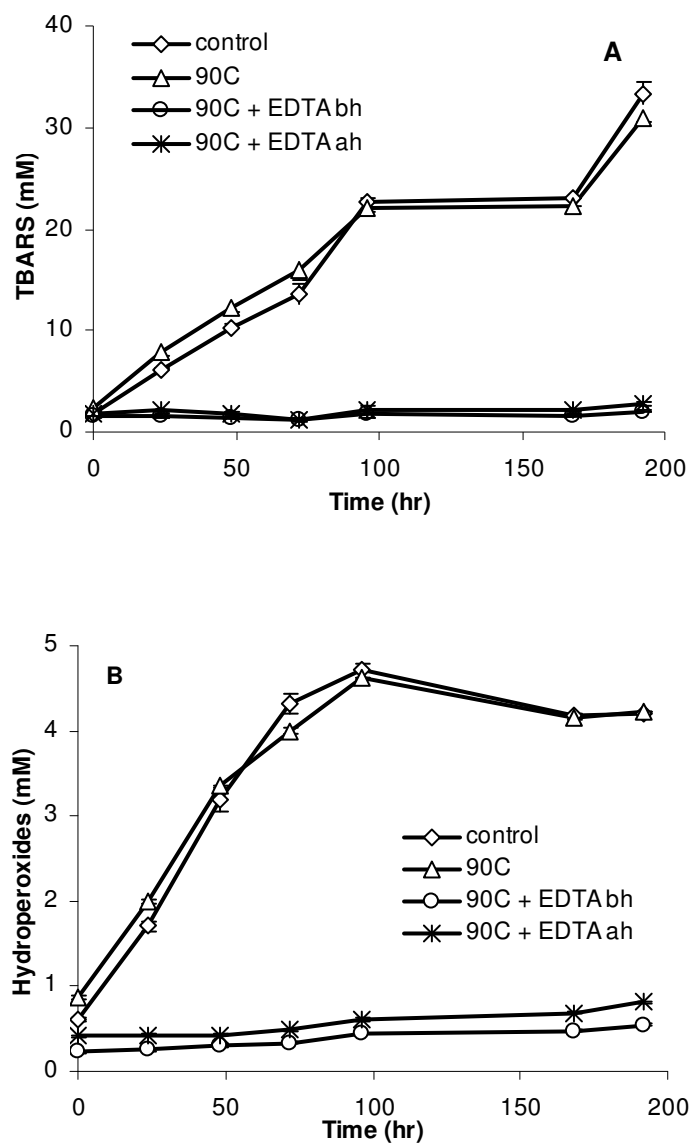
ability of EDTA to completely inhibit oxidation suggests that the transition metals naturally present in the oil and/or water were promoting lipid oxidation (Cuvelier et al., 2003). EDTA has been reported to promote oxidation by increasing both the solubility and the oxidation-reduction potential of iron when added at EDTA to iron ratio  $< 1$  (Mahoney and Graf, 1986). This prooxidant effect was not seen in the salmon oil-in-water emulsion used in this study. EDTA will act as an antioxidant at an EDTA to iron ratio of  $> 1$  (Mahoney and Graf, 1986). The results in figure 3.2.b showing inhibition of lipid hydroperoxides at EDTA concentrations  $\geq 2.5 \mu\text{M}$  suggests that the iron level in the emulsion was between 0.1 and 2.5  $\mu\text{M}$ .



**Figure 3.2.** TBARS concentration (a) of salmon oil-in-water emulsion stored at 20°C and measured over 8 days. Samples contain varying amounts of EDTA as well as one control with no EDTA added. TBAR values comparison of EDTA concentration and its effect on lipid oxidation at day 7 (b).

### **3.4.3 Influence of adding EDTA before or after heating**

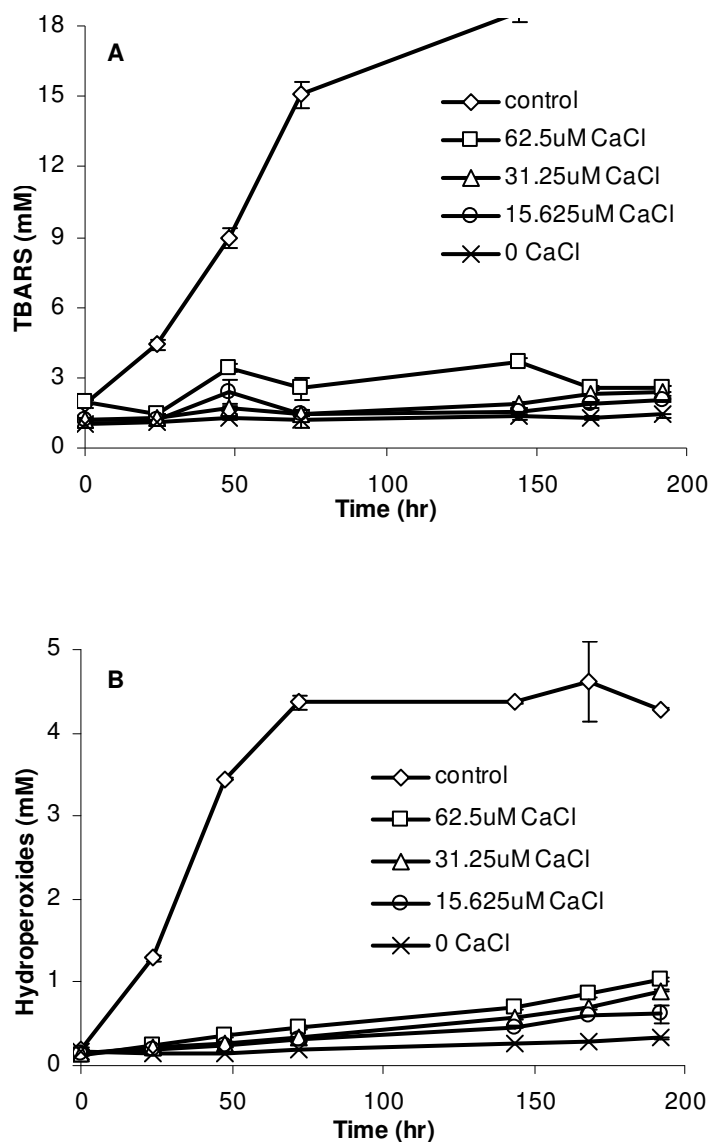
Since samples heated to 50, 70, and 90°C oxidized at similar rates, (see Figure 3.1), we chose to heat all samples at 90°C for 10 min. EDTA at a concentration of 10 µM was added to samples either before or after heat processing, in order to determine if time of addition had any influence on chelating activity. TBARS (Figure 3.3.a) and lipid hydroperoxide (Figure 3.3.b) values illustrate that the control and the sample heated to 90°C both oxidized, with the heated sample oxidizing at a similar rate to unheated emulsion as seen in Figure 3.1. EDTA (10 µM) was able to dramatically decrease lipid oxidation in the samples to which it was added. Samples where EDTA was added before heat treatment had slightly lower TBARS and hydroperoxide values than samples where EDTA was added after heating ( $p \leq 0.05$ ). This experiment showed that addition of EDTA either before or after heating did not have a major impact on oxidation, but the best protection is obtained by adding it before thermal processing.



**Figure 3.3.** TBARS concentration (a) and hydroperoxide concentration (b) of salmon oil-in-water emulsions heat processed at 90°C, stored at 20°C and measured over 8 days. EDTA (10 $\mu$ M) was added to samples before (bh) or after (ah) heat processing.

#### **3.4.4 Influence of calcium on antioxidant effects of EDTA**

It is possible that the effectiveness of EDTA as an antioxidant can be diminished in the presence of calcium. Calcium has a lower affinity to bind with EDTA than iron but it may compete with iron for EDTA if it is present at a sufficiently high concentration. Therefore, when calcium is added to an emulsion some unchelated, reactive iron could be left in the system, possibly overcoming the inhibitory effects of EDTA leading to lipid oxidation. In this set of experiments 7.5  $\mu\text{M}$  EDTA was added to all samples (except the control) and calcium was added at concentrations ranging from 1.6-62.5  $\mu\text{M}$ . Addition of calcium to emulsions was found to significantly ( $p < 0.05$ ) increase both TBARS (Figure 3.4.a) and lipid hydroperoxide (Figure 3.4.b) formation after 3 days of storage when calcium concentrations were 2-fold greater than EDTA concentrations. EDTA containing samples that contained less than 15.6  $\mu\text{M}$  added calcium showed the same low levels of lipid oxidation, as in the absence of calcium.



**Figure 3.4.** TBARS concentrations (a) and hydroperoxide concentration (b) of non-heated salmon oil-in-water emulsions stored at 20°C and measured over 8 days. Samples contain 7.5 μM EDTA and varying amounts of CaCl<sub>2</sub>, added after homogenization, as well as a control with no EDTA and no CaCl<sub>2</sub> added.

### 3.5 Conclusions

We have examined the effects of heat processing and calcium ions on the ability of EDTA to inhibit lipid oxidation in Brij 35 stabilized salmon oil-in-water emulsions at pH 7. Heat processing had no effect on the physical or oxidative stability of emulsions in the absence of EDTA. EDTA at a concentration of 2.5  $\mu\text{M}$  was able to almost inhibit oxidation completely. However, the addition of calcium at concentrations 2-fold higher than that of the concentration of EDTA resulted in higher oxidation values presumably due to its ability to compete with the chelating agent and release iron. The addition of EDTA to samples before heat processing had a greater effect on their overall ability to inhibit lipid oxidation than samples where EDTA was added after heat processing. These results indicate that heat processed salmon oil-in-water emulsions with high physical and oxidative stability could be produced in the presence of EDTA. These emulsions could be an excellent source of oxidatively stable  $\omega$ -3 fatty acids that could be used as a functional food ingredient.

## CHAPTER 4

### THE RELATIONSHIP BETWEEN A COMPOUNDS FREE RADICAL SCAVENGING ACTIVITY AND ITS ABILITY TO INHIBIT LIPID OXIDATION IN FOODS

#### 4.1 Abstract

The ability of compounds to inhibit lipid oxidation in foods has been postulated to be dependent on both its physical and chemical properties. Despite these hypotheses, there have been numerous attempts to relate the free radical scavenging capacity of a compound to its ability to act as an antioxidant in foods and other biological systems. The objective of this study was to compare how the free radical scavenging activity of a variety of compounds relates to their ability to inhibit lipid oxidation in cooked ground beef and oil-in-water emulsions. Free radical scavenging activity was measured with the oxygen radical absorbance capacity (ORAC) or the 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) assays for polar and non-polar compounds, respectively. The order of free radical scavenging activity of the polar compounds was: ferulic acid > coumaric acid > propyl gallate > gallic acid > ascorbic acid. The free radical scavenging activity of the non-polar compounds was rosmarinic acid > butylated hydroxytoluene (BHT)  $\geq$  tert-butylhydroquinone (TBHQ) >  $\alpha$ -tocopherol. Of these compounds only propyl gallate, TBHQ were found to inhibit the formation of thiobarbituric acid reactive substances (TBARS) in cooked ground beef while propyl gallate, TBHQ, gallic acid and rosmarinic acid were able to decrease lipid hydroperoxides and hexanal in the oil-in-water



emulsion. These data indicate that a compound's free radical scavenging activity did not directly correlate with their ability to inhibit lipid oxidation in cooked ground beef and emulsions suggesting that free radical scavenging assays have limited value in predicting the ability of a compound to act as an antioxidant in complex foods.

## **4.2 Introduction**

Lipid oxidation is a serious problem in the foods because it produces rancid odors and flavors, decreases shelf life, alters texture and color and decreases nutritional value. For example, lipid oxidation has been found to be one of the major causes of quality deterioration in processed muscle foods (Erickson, 2002). Processes such as grinding disrupt the cellular integrity of muscle tissues exposing lipids to oxidative catalysts and oxygen (Decker and Xu, 1998; Erickson, 2002). Thermal processing causes even more rapid acceleration of lipid oxidation of muscle foods by dislodging iron from heme proteins, disrupting cellular integrity, inactivating endogenous antioxidants and breaking down preexisting hydroperoxides (Decker and Xu, 1998; Erickson, 2002; Watts, 1962). Food emulsions are another example of a food that can rapidly degrade by lipid oxidation reactions. Lipid oxidation chemistry in oil-in-water emulsions is highly dependent on the interfacial membrane of the emulsion droplet since this is where prooxidants such as iron can interact with surface active lipids such as hydroperoxides (Decker et. al., 2002; McClements, 2005; McClements and Decker, 2000).

There have been numerous methods developed to control the rate and extent of lipid oxidation in foods, with one of the most effective being the addition of

antioxidants. In brief, an antioxidant is a synthetic or natural compound that has the ability to slow lipid oxidation. Most commercial food antioxidants work by scavenging free radicals or chelating metals (Decker and McClements, 2008). Free radical scavengers, such as tocopherols, butylated hydroxy toluene (BHT) and plant phenolics, inhibit lipid oxidation by reducing peroxy and alkoxy radicals into stable compounds. Through these pathways, free radical scavengers can inhibit chain propagation and fatty acid scission thus decreasing formation of the volatile fatty acid decomposition products (e.g. aldehydes and ketones) that cause rancidity (Frankel, 1996; Decker and McClements, 2008). In foods, the effectiveness of an antioxidant is dependent on both its chemical reactivity and physical properties which can determine the environment in which the antioxidant partitions (Schwartz et al., 1996; Frankel, 1996; Decker and McClements, 2008).

Many simplistic one-dimensional assays which use a wide range of conditions, oxidants and methods to measure end points of oxidation have been developed to investigate the free radical scavenging or “antiradical” ability of natural and synthetic compounds. Free radical scavenging capacity assays can generally be classified into two types: hydrogen atom transfer (HAT) reactions or electron transfer (ET) assays. HAT assays, such as oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP), utilize a competitive reaction scheme where a thermal radical generator is used to generate a steady production of peroxy radicals that in turn oxidize a probe which is used to monitor the peroxy radicals in the assay. When the test compound is added to these assays it competes with the probe for the peroxy radicals thus inhibiting probe oxidation thereby allowing determination of the free

radical scavenging activity of the test compound. In ET assays, including the Trolox equivalence antioxidant capacity (TEAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assays, the colorimetric probe is also a free radical. The test compound reduces the probe radicals causing a color change which is used to determine free radical scavenging activity (Huang et. al., 2005; Frankel, 2007; Sanchez-Moreno, 2002).

While there are many publications on the ability of HAT and ET assays to measure the free radical scavenging activity of natural and synthetic compounds, very little research has been conducted to determine if these assays can be used to predict the ability of a compound to inhibit lipid oxidation in a complex food system. Such comparisons are important because the ability of a compound to inhibit lipid oxidation in foods is thought to not only be related to its free radical scavenging activity but also its physical location (e.g. does the compound concentrate where oxidative reactions are most prevalent) and ability to participate in other oxidative pathways (e.g. metal inactivation and regeneration of endogenous food antioxidants). Therefore, the objective of this research was to utilize the oxygen radical absorbance capacity (ORAC) and the 2,2-diphenyl-1-picrylhydrazyl free-radical (DPPH•) assays to determine the free radical scavenging activity of polar and non-polar compounds, respectively. The ability of each compound to inhibit lipid oxidation in cooked ground beef and oil-in-water emulsions was also evaluated to determine if the free radical scavenging activity of the tested compounds could be used to predict their ability to inhibit lipid oxidation in complex food systems.

## **4.3 Materials and Methods**

### **4.3.1 Materials**

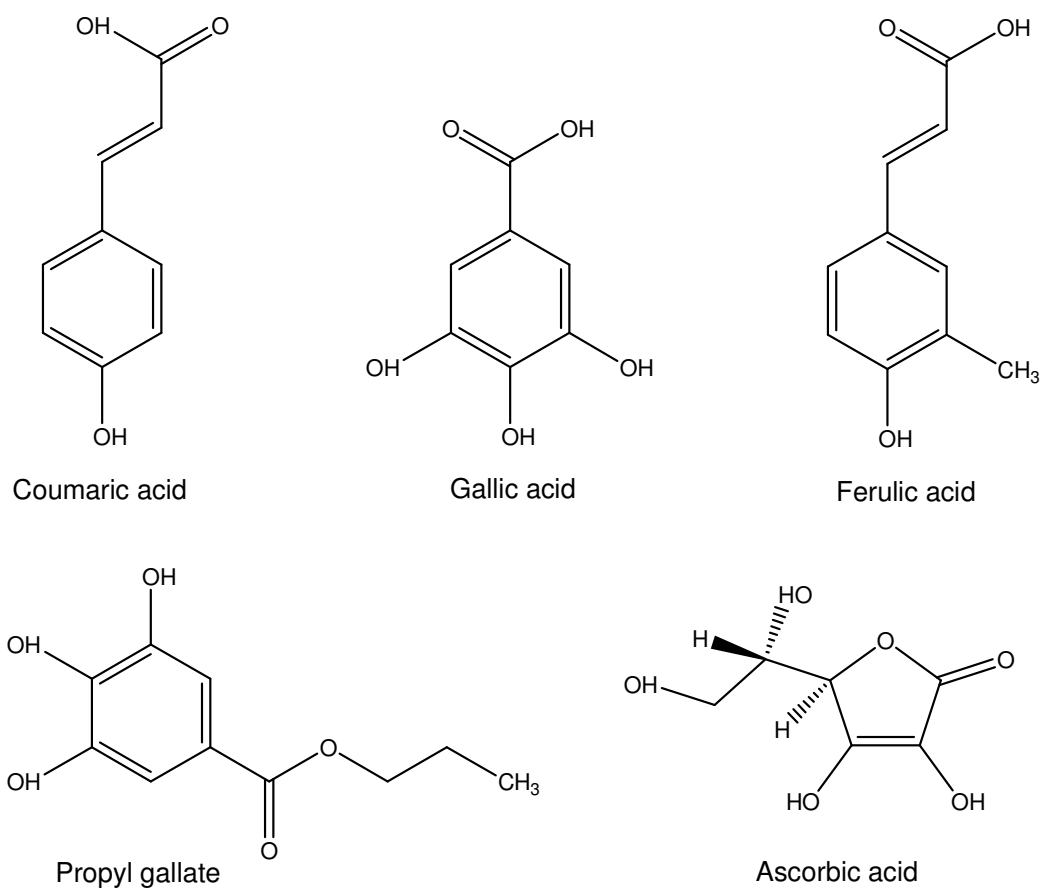
Fresh ground beef (15% fat) and corn oil were purchased from a local grocery store. Brij 35, ferulic acid and rosmarinic acid were attained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Coumaric acid, propyl gallate, gallic acid, ascorbic acid, 2-thiobarbituric acid (TBA), ferrous sulfate, barium chloride, imidazole, ammonium thiocyanate, hexanal, ethylenediammetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), fluorescein sodium salt, 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and  $\alpha$ -tocopherol were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO). Trichloroacetic acid (TCA) and tert-butylhydroquinone (TBHQ) were obtained from Acros Organics (Pittsburgh, PA). Sodium acetate, sodium phosphate dibasic and monobasic, hydrochloric acid, other reagent grade chemicals, test tubes, GC vials, seals and septa were obtained from Fisher Scientific (Pittsburgh, PA).

### **4.3.2 Methods**

#### **4.3.2.1 Free Radical Scavenging Assays**

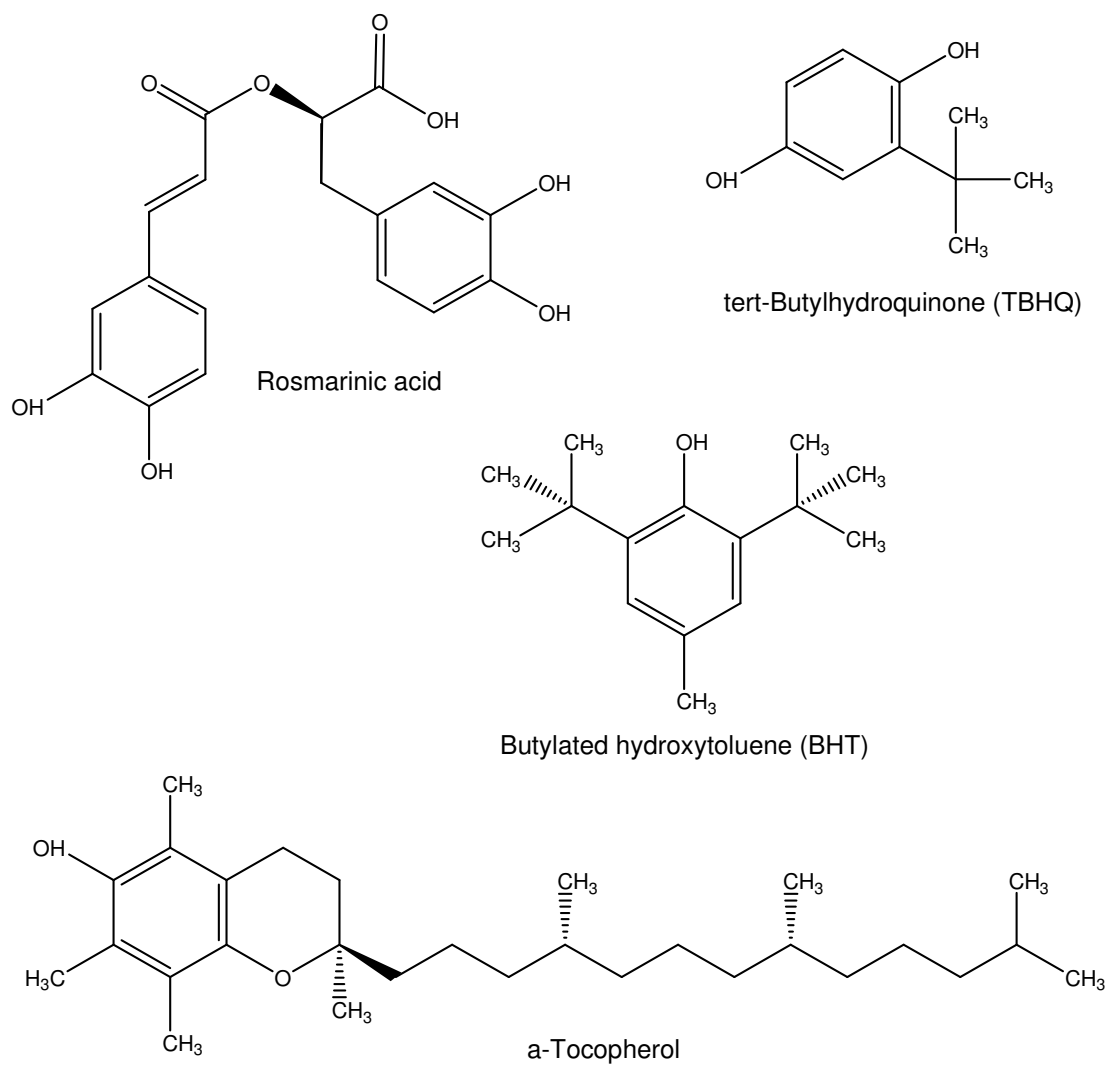
The free-radical scavenging activity of selected polar compounds (ascorbic acid, ferulic acid, gallic acid, propyl gallate and coumaric acid) (Figure 4.1) was determined using a modified oxygen radical absorbance capacity (ORAC) assay (Ou et al., 2001). First, a 75 mM phosphate buffer (pH 7.0) solution containing 100  $\mu$ M EDTA and 300

mM 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) was prepared and kept on ice. A separate fluorescein solution (50 nM) in 75 mM phosphate buffer (pH 7.0) was prepared immediately before each experiment. Stock solutions of the test compounds (500  $\mu$ M) were prepared in the 75 mM phosphate (pH 7.0) buffer. For each experiment, 2.7 mL of the fluorescein solution was added to a capped glass test tube (13 mm x 100 mm) and held at 37°C in a Forma Scientific 2095 water bath (Marietta, OH) for 15 min. Then 0.1 mL of the test compound solution (final concentration of 5  $\mu$ M) was added followed by 0.2 mL of the AAPH solution. Analyses were performed in a Hitachi F2000 fluorescence spectrophotometer (San Jose, CA) containing a heating/stirring unit, where the sample was kept at a controlled temperature of 37°C, mixed at 50% speed and kept in the dark. The excitation wavelength was 493 nm, and emission was 515 nm. Fluorescence was recorded every minute for 40 min, and the fluorescence relative to the initial time ( $F/F_0$ ) was calculated from the fluorescence decay curve. Trolox was used as a reference and data are expressed as micromoles of Trolox equivalents (TE) per gram (g) of sample ( $\mu$ mol of TE/g).



**Figure 4.1.** Structures of polar test compounds.

The free-radical scavenging activity of the selected non-polar compounds [butylated hydroxy toluene (BHT), tert-butylhydroquinone (TBHQ), rosmarinic acid and  $\alpha$ -tocopherol] (Figure 4.2) was determined using the 2,2-diphenyl-1-picrylhydrazyl free-radical (DPPH•) method. Stock solutions of the test compounds (3.84 mmol/L) were prepared in methanol and were added to a methanolic DPPH• solution to make the final DPPH• concentration 0.06 mmol/L. Loss of DPPH• was measured at 515 nm using an Ultrospec 3000 *pro* UV/visible spectrophotometer (Biochrom Ltd., Cambridge, England) every 15 min until the reaction reached completion (e.g., no more loss of DPPH•). The exact DPPH• concentration at the completion of the reaction was determined using a DPPH• standard curve. The median effective concentration of the test compound needed to decrease the DPPH• concentration by 50% was calculated and expressed as the EC<sub>50</sub> (Brand-Williams et al., 1995; Chaiyasit et al., 2005).



**Figure 4.2.** Structures of non-polar test compounds.



#### 4.3.2.2 Lipid Oxidation in Cooked Ground Beef

Raw ground beef was mixed for 2-3 min in a Hobart N-50 mixer (Troy, OH) to obtain a homogeneous sample and then 100 g of beef was weighed into 250 mL beakers. Test compounds (a 50 mM stock solution in methanol) were mixed into the ground beef by hand to achieve a final AO concentration of 0.5 mmol/kg muscle. The control sample contained only methanol. Next, 10 g of the raw ground beef samples were placed into test tubes (16 x 125 mm) and cooked in a water bath (NESlab GP-200; Thermo Fisher Scientific; Waltham, MA set at 90°C), until an internal temperature of 77°C was reached. The cooked beef was then immediately cooled in cold tap water and transferred to a refrigerator for 20 min. Cooled, cooked beef samples were removed from the test tubes, crumbled and mixed by hand to obtain a homogeneous consistency. The cooked beef was placed in plastic sample bags and stored in a refrigerator (4-8°C) in the dark for 96 hrs.

Thiobarbituric acid reactive substances (TBARS) were measured using a modified method of Srinivasan and Xiong (1996). A buffer solution containing 50 mM dibasic sodium phosphate, 0.1% (EDTA) and 0.1% propyl gallate was prepared and kept cold (4-8°C). A 30% trichloroacetic acid (TCA) solution and a 0.02 M thiobarbituric acid (TBA) solution were also prepared and kept cold (4-8°C). Cooked ground beef (2 g) was added to 16 x 100 mm glass test tubes and the weights recorded. Blanks contained 2 g of de-ionized water. The cold buffer solution (8 mL) was added to each cooked beef sample followed by homogenization for 20 sec. with a Tekmar Tissumizer (Cincinnati, OH). TCA solution (2 mL) was then added and the tubes were capped and centrifuged at 2000 x g for 5 min in a Fisher Scientific Centrifuge

225A (Waltham, MA). A 2 mL aliquot from the upper supernatant layer was added to a glass 16 x 125 mm screw cap test tube and mixed with 2 mL of TBA solution. The tubes were capped and vortexed for 5 sec. The samples were incubated in a boiling water bath (NESlab GP-200; Thermo Fisher Scientific; Waltham, MA) for 15 min. The samples were cooled in an ice water bath for 1 min. to stop the reaction and transferred to a refrigerator to cool for 30 min. Absorbance was measured at 533 nm using a Thermo Spectronic Genesys 20 Spectrophotometer (Waltham, MA) and TBARS were expressed as mg TBARS/kg muscle using the molar extinction coefficient of malondialdehyde ( $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ) and the weight of each sample.

#### **4.3.2.3 Lipid Oxidation in Oil-in-Water Emulsion**

An oil-in-water emulsion consisting of 5 wt% corn oil, 0.5 wt% Brij 35 (a non-ionic surfactant), and 5 mM sodium acetate/imidazole buffer (pH 7) was prepared by dissolving Brij 35 in the buffer and then combining the aqueous phase with the oil. A coarse emulsion was made by homogenizing the lipid and aqueous phases for 2 minutes using a 2-speed hand held homogenizer (Biospec Products, Inc.; Bartlesville, OK) at the highest speed setting. The coarse emulsion was then passed three times through an APV two stage high-pressure valve homogenizer (APV Americas; Wilmington, MA) at 3000 psi. The final mean droplet diameter of the emulsion ( $d_{43}$ ) was  $0.38 \pm 0.1 \mu\text{m}$ , as determined by laser light scattering (Mastersizer MSS; Malvern Instruments; Westborough, MA). The emulsion was separated into equal amounts and test compounds (50 mM stock solution in methanol) were added to achieve a final antioxidant concentration of 50  $\mu\text{M}$ . The control contained only methanol. One

milliliter of emulsion was pipetted into 10 mL headspace vials and sealed for hexanal and lipid hydroperoxide measurements. The vials were stored (37°C) in the dark for 24 days.

A method adapted from Nuchi et al. (2001) was utilized to determine lipid hydroperoxides in oil-in-water emulsion. Emulsion (0.3 mL) was added to a mixture of 1.5 mL of isooctane/2-propanol (3:1 v/v), vortexed (10 s, 3 times), and the organic solvent phase was isolated by centrifugation at 1000 x g for 2 min. The organic solvent phase (200 µL) was added to 2.8 mL of methanol/1-butanol (2:1), followed by 15 µL of 3.94 M ammonium thiocyanate and 15 µL of ferrous iron solution (prepared by adding equal amounts of 0.132 M BaCl<sub>2</sub> and 0.144 M FeSO<sub>4</sub>). After 20 minutes of incubation at room temperature, the absorbance was measured at 510 nm using an Ultrospec 3000 pro UV-vis spectrophotometer (Cambridge, England). Hydroperoxide concentrations were determined using a standard curve prepared from cumene hydroperoxide.

For headspace hexanal analysis, emulsion (1 mL) was placed into 10 mL headspace vials and sealed with poly (tetrafluoroethylene) butyl rubber septa. Headspace hexanal was determined using a Shimadzu 17A gas chromatograph equipped with a Hewlett-Packard 19395A headspace sampler (Chaiyasit et al., 2005). The headspace conditions were the following: incubation time, 15 min; sample temperature 55°C; sample loop and transfer line temperature, 110°C; pressurization, 10 s; venting, 10 s; injection, 1 min; and sample run time, 9 min. The volatile headspace components were separated isothermally at 65°C on a HP methyl silicone (DB-5) fused silica capillary column (50 m, 0.31 mm i.d., 1.03 µm film thickness). The splitless injector

temperature was 180°C and the flame ionization detector temperature was 250°C.

Concentrations were determined using a standard curve made from hexanal.

#### **4.3.2.4 Statistics**

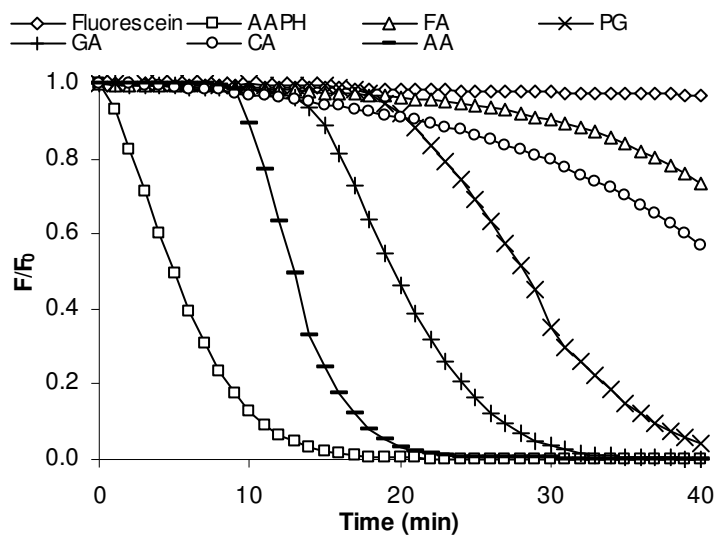
ORAC and DPPH• measurements were performed on duplicate samples.

TBARS, lipid hydroperoxides and headspace hexanal measurements were performed on triplicate samples. Differences between means were determined with the least-squares means procedure at  $p < 0.05$  (Snedecor and Cochran, 1989).

### **4.4 Results**

#### **4.4.1 Free Radical Scavenging Capacity Assays**

The free radical scavenging capacity of polar compounds as determined by the ORAC assay were ferulic acid > coumaric acid > propyl gallate > gallic acid > ascorbic acid (Figure 4.3, Table 4.1). The ORAC value of the compounds which is represented by area under the fluorescence decay curve (AUC) is expressed as  $\mu$ moles of Trolox equivalents. Other researchers have also studied the free radical scavenging activity of several of these compounds using the ORAC assay. In these studies, Nenadis and others (2007) found gallic acid to have a higher ORAC value than ascorbic acid. Gomez-Ruiz et al. (2007) found that ferulic acid was more active than coumaric acid while Davalos et al. (2004) found that ferulic and coumaric acids had similar radical scavenging activity.

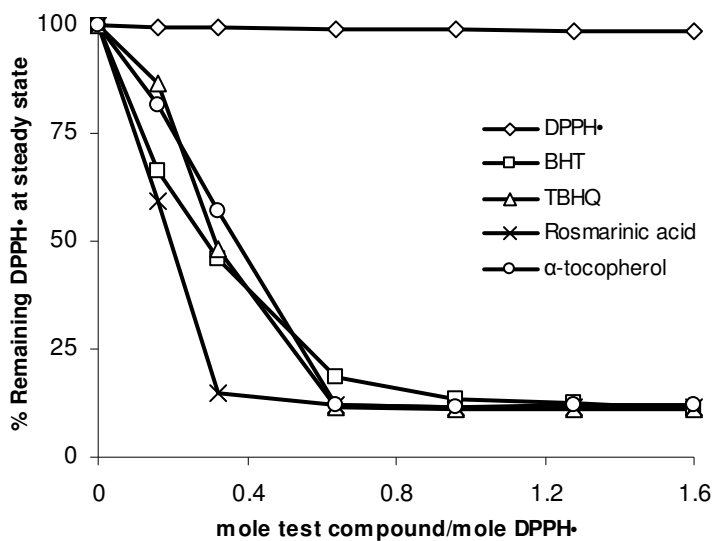


**Figure 4.3.** Changes in the relative fluorescence intensity of 45 nM fluorescein ( $\lambda_{EM}$  493 nm,  $\lambda_{EX}$  515 nm) in the presence of 20 mM 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) and 5  $\mu$ M ferulic acid (FA), propyl gallate (PG), gallic acid (GA), coumaric acid (CA) or ascorbic acid (AA) at 37°C.

**Table 4.1.** The oxygen radical absorbance capacity (ORAC) values of selected compounds expressed as  $\mu$ mol of trolox equivalents (TE)/mL. A higher ORAC value represents greater free radical scavenging capacity.

Test Compound	ORAC <sub>FL</sub>
Ferulic acid	13.75 $\pm$ 0.23
Coumaric acid	12.18 $\pm$ 0.15
Propyl gallate	10.75 $\pm$ 0.16
Gallic acid	8.22 $\pm$ 0.08
Ascorbic acid	5.15 $\pm$ 0.11

DPPH• produces a nonpolar free radical therefore it was used to evaluate the free radical scavenging activity of the nonpolar compounds in methanol. The DPPH• assay results indicate that the order of free radical scavenging activity of the nonpolar compounds was rosmarinic acid > BHT  $\geq$  TBHQ >  $\alpha$ -tocopherol (Figure 4.4, Table 4.2). Sun and Ho (2005) tested various compounds using the DPPH• assay and also found that BHT and TBHQ (0.1 - 1.0 mg/ml) had similar free radical scavenging activities. Conversely, Devi and Arumughan (2007) found TBHQ to have a higher free radical scavenging capacity than BHT. Chen and Ho (1997) looked at numerous compounds and determined that rosmarinic acid had a higher free radical scavenging capacity than  $\alpha$ -tocopherol and BHT however they found  $\alpha$ -tocopherol to be a more effective free radical scavenger than BHT.



**Figure 4.4.** The ability of butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), rosmarinic acid, or  $\alpha$ -tocopherol to inactivate the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•).

**Table 4.2.** Free radical scavenging activity of non-polar antioxidants as determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) assay.

Antioxidant	EC <sub>50</sub>
Rosmarinic acid	0.2 ± 0.02
BHT	0.29 ± 0.01
TBHQ	0.31 ± 0.03
α-Tocopherol	0.38 ± 0.03

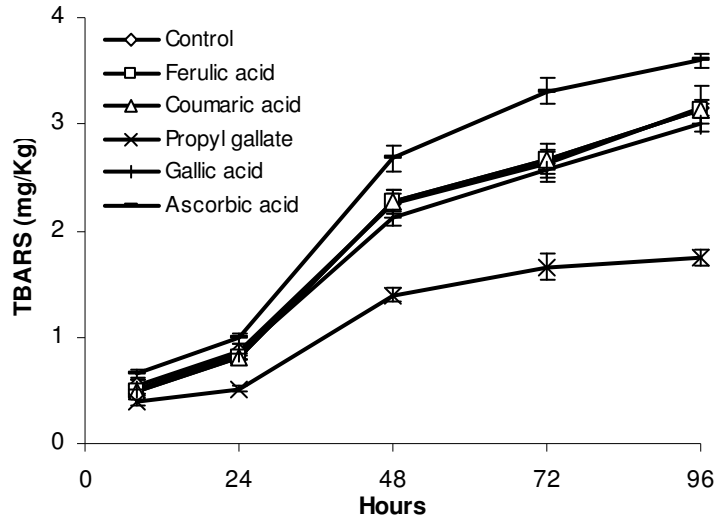
#### 4.4.2 Inhibition of Lipid Oxidation in Foods

The ability of the selected compounds to inhibit lipid oxidation in a complex food was tested in cooked ground beef and oil-in-water emulsions. Cooked ground beef was chosen because it is extremely susceptible to lipid oxidation and because it represents a heterogeneous food with different lipid phases (e.g. phospholipid membranes and neutral lipids). Propyl gallate (0.05 mmol/kg beef) was the only polar compound tested that could inhibit the formation of TBARS in the cooked beef. Propyl gallate (0.2 mmol/kg beef) has also been found to inhibit lipid oxidation in cooked restructured beef steaks (Stika et al., 2007) as well as cooked ground beef, lamb and pork at a concentration of 0.2 mmol/kg meat (Jayathilakan et al., 2007). Coumaric acid, gallic acid and ferulic acid had no effect on lipid oxidation compared to the control even though the ORAC assay indicated that they could scavenge free radicals. Ascorbic acid was prooxidative increasing TBARS formation compared to the control (Figure 4.5). Ramanathan and Das (1992) also found that ascorbic acid (0.17 and 1.14 mmol/kg meat) acts as a prooxidant in ground fish.

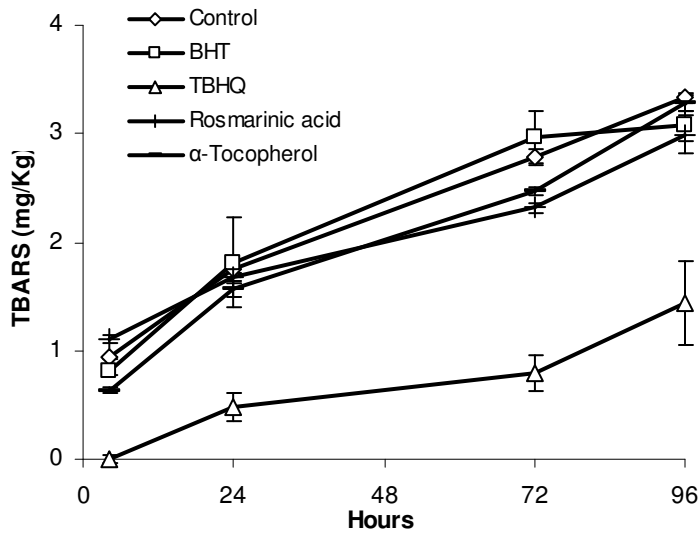
In cooked ground beef containing non-polar compounds, only TBHQ (0.05 mmol/kg beef) was able to inhibit TBARS formation. TBHQ has been found to inhibit lipid oxidation in cooked beef, lamb, pork (0.2 mmol/kg muscle Jayathilakan et al., 2007; 1.2 mmol/kg muscle Saleemi et al., 1993) and cooked herring (1.2 mmol/kg muscle Kamil et al., 2002). Rosmarinic acid,  $\alpha$ -tocopherol and BHT had no effect on lipid oxidation (Figure 4.6), although they were able to scavenge free radicals as determined by the DPPH• assay (Figure 4.4). Higher concentrations of BHT was reported to inhibit lipid oxidation in cooked ground pork (0.14 or 0.45 mmol/kg muscle,



Saleemi et al., 1993) and cooked ground beef (1.0 mmol/kg muscle Vasavada et al., 2006). While dietary  $\alpha$ -tocopherol is an effective antioxidant in beef (Faustman et al., 1989), it has been reported to be an ineffective antioxidant when added exogenously to cooked ground beef (Mitsumoto et al., 1993).

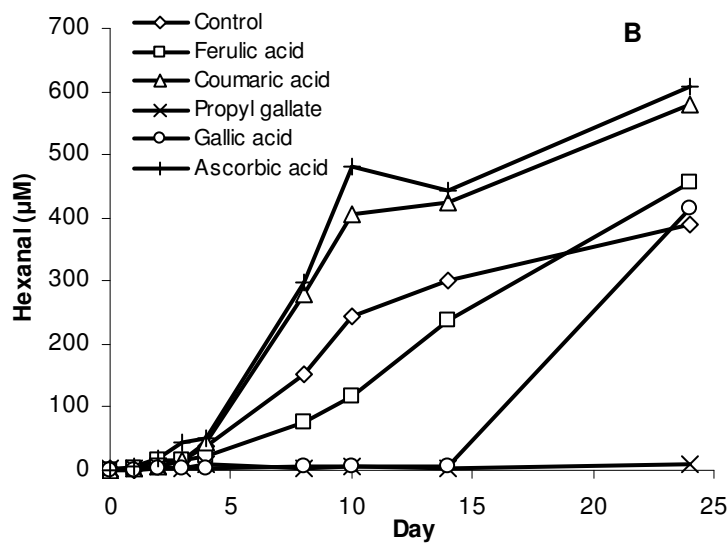
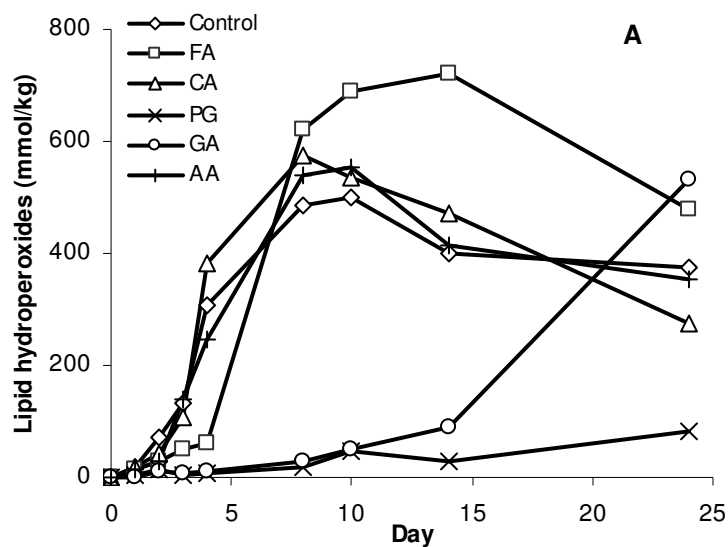


**Figure 4.5.** The formation of thiobarbituric acid reactive substance (TBARS) in cooked ground beef containing ferulic acid (FA), propyl gallate (PG), gallic acid (GA), coumaric acid (CA) or ascorbic acid (AA) (50  $\mu$ M) during storage at 4°C for 96 hrs.



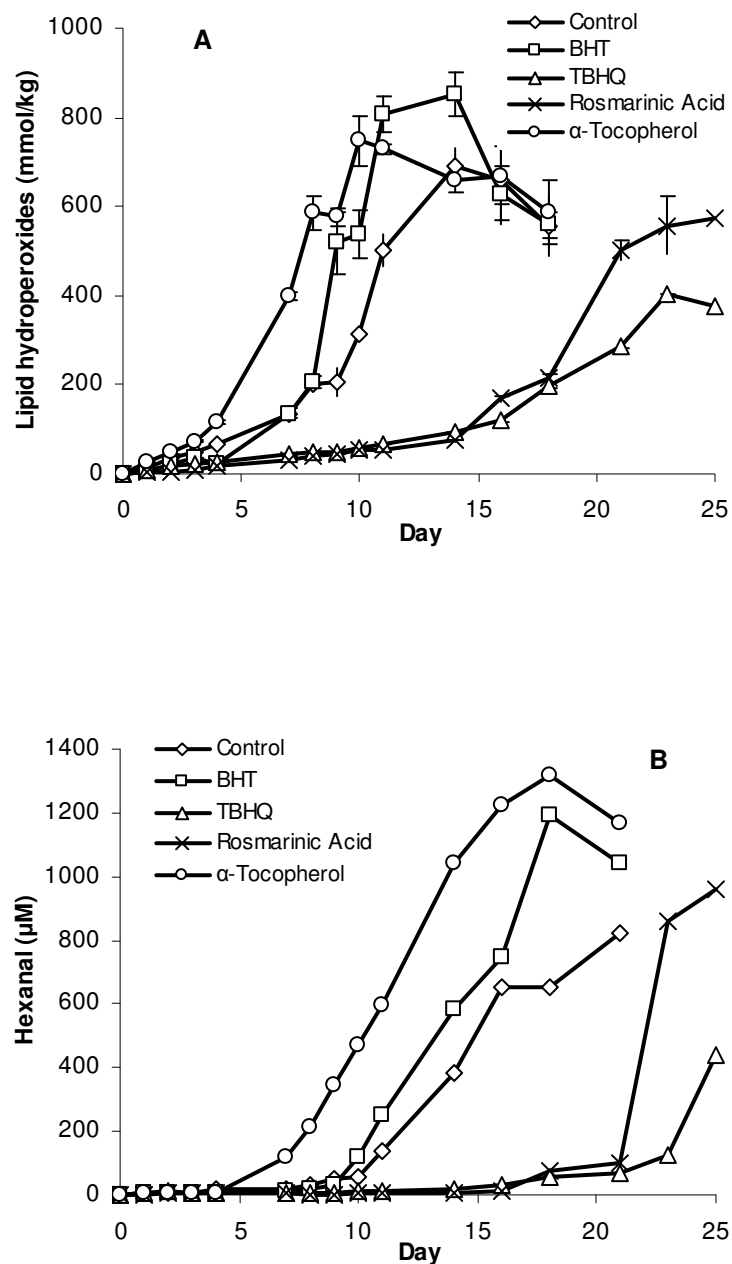
**Figure 4.6.** The formation of thiobarbituric acid reactive substance (TBARS) in cooked ground beef containing 50  $\mu$ M butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), rosmarinic acid, or  $\alpha$ -tocopherol during storage at 4°C for 96 hrs.

The ability of the selected compounds to inhibit lipid oxidation in foods was also tested in a corn oil-in-water emulsion. Emulsion was selected because it is a heterogeneous, multiphase food system where oxidation chemistry can occur at the oil-water interface. Propyl gallate, gallic acid and ferulic acid (50  $\mu\text{M}$ ) were the only polar compounds tested that could prolong the formation of lipid hydroperoxides and hexanal in the model emulsion (Figure 4.7). The order of effectiveness was propyl gallate > gallic acid > ferulic acid. Chang and coworkers (2003) found that propyl gallate and gallic acid (200  $\mu\text{M}$ ) could inhibit lipid oxidation in stripped corn oil-in-water emulsions. Stockmann and others (2000) found that propyl gallate but not gallic acid could inhibit lipid oxidation in a stripped corn oil-in-water emulsions oxidation at 1  $\mu\text{M}$ . On the contrary, Huang and Frankel (1997) reported that in stripped corn oil-in-water emulsions both gallic acid and propyl gallate (5 and 20  $\mu\text{M}$ ) accelerated the formation of lipid hydroperoxide and hexanal. Nenadis et al. (2003) reported that ferulic acid (150  $\mu\text{M}$ ) inhibited lipid hydroperoxide formation in a Tween 20 stabilized triolein oil-in-water emulsion. In our study, coumaric and ascorbic acids (50  $\mu\text{M}$ ) had no effect on lipid hydroperoxides but increased hexanal formation compared to the control. Sorensen et al. (2008) reported that coumaric acid (61  $\mu\text{M}$ ) was found to have no effect on hydroperoxide levels in fish oil-in-water emulsions. Mahoney and Graf (1986) and Porter (1993) reported that ascorbic acid (8  $\mu\text{M}$ ) was prooxidative in regards to the oxidation of 160  $\mu\text{M}$  arachidonic acid suspended in Tris buffer.



**Figure 4.7.** Formation of lipid hydroperoxide concentration (a) and hexanal (b) in corn oil-in-water emulsion containing 50  $\mu\text{M}$  ferulic acid (FA), propyl gallate (PG), gallic acid (GA), coumaric acid (CA) or ascorbic acid (AA) during storage at 55°C in the dark for 24 days.

In the corn oil-in-water emulsions containing non-polar compounds, TBHQ and rosmarinic acid (50  $\mu\text{M}$ ) were able to inhibit both lipid hydroperoxide and hexanal formation with TBHQ being more effective than rosmarinic acid. In oil-in-water emulsions containing stripped corn oil, 200  $\mu\text{M}$  TBHQ was effective at inhibiting lipid hydroperoxides (Chang et al., 2003). Li and others (2006) found that 20  $\mu\text{M}$  TBHQ inhibited lipid hydroperoxide formation in a stripped soybean oil-in-water emulsion. Rosmarinic acid (8  $\mu\text{M}$ ) was reported to exhibit slight antioxidant activity but was prooxidative at 14  $\mu\text{M}$  when the oxidation of stripped corn oil-in-water emulsions was monitored by lipid hydroperoxides and hexanal (Frankel et al., 1996). In this study, BHT and  $\alpha$ -tocopherol (50  $\mu\text{M}$ ) increased lipid hydroperoxides and hexanal formation compared to the control (Figure 4.8). Li and others (2006) found that BHT (200  $\mu\text{M}$ ) inhibited lipid hydroperoxide formation in a stripped soybean oil emulsion. Cillard and Cillard (1980) reported that in systems containing linoleic acid dispersed with Tween 20,  $\alpha$ -tocopherol was prooxidative at high levels (50 mM) while antioxidative at low amounts (25  $\mu\text{M}$ ) while Frankel and coworkers (1996) found  $\alpha$ -tocopherol (23  $\mu\text{M}$ ) to be effective at inhibiting hydroperoxide and hexanal formation in stripped corn oil-in-water emulsions.



**Figure 4.8.** Formation of lipid hydroperoxide concentration (a) and hexanal (b) in corn oil-in-water emulsion containing 50  $\mu\text{M}$  butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), rosmarinic acid, or  $\alpha$ -tocopherol during storage at 55°C in the dark for 24 days.

## 4.5 Discussion

Results from the free radical scavenging assays were not able to consistently predict which compounds were the most effective antioxidants in either cooked ground beef or corn oil-in-water emulsion (Figure 4.9). The most effective free radical scavengers were ferulic acid as determined by the ORAC (Figure 4.3, Table 4.1) and rosmarinic acid as determined by the DPPH• assay (Figure 4.4, Table 4.2). In the cooked ground beef, neither ferulic nor rosmarinic acids were able to inhibit lipid oxidation (Figure 4.5 and 4.6). Conversely, propyl gallate and TBHQ which were found to be intermediate free radical scavengers (Figure 4.3 and 4.4, Table 4.1 and 4.2) were effective at inhibiting lipid oxidation in the cooked ground beef (Figure 4.5 and 4.6). Ascorbic acid increased TBARS formation in cooked beef (Figure 4.5) even though the ORAC assay indicated that it could scavenge free radicals (Figure 4.3, Table 4.1).

In the oil-in-water emulsion, rosmarinic acid, TBHQ, gallic acid and propyl gallate were able to decrease lipid oxidation (Figure 4.7 and 4.8). Of these compounds, only rosmarinic acid was a strong free radical scavenger while the others had intermediate radical scavenging capacity. Ferulic acid (a strong free radical scavenger as determined by ORAC; Figure 4.3, Table 4.1), was less effective than gallic acid and propyl gallate while coumaric acid, ascorbic acid, BHT and  $\alpha$ -tocopherol were prooxidative as they increased hydroperoxide and hexanal formation in oil-in-water emulsion (Figure 4.7 and 4.8).

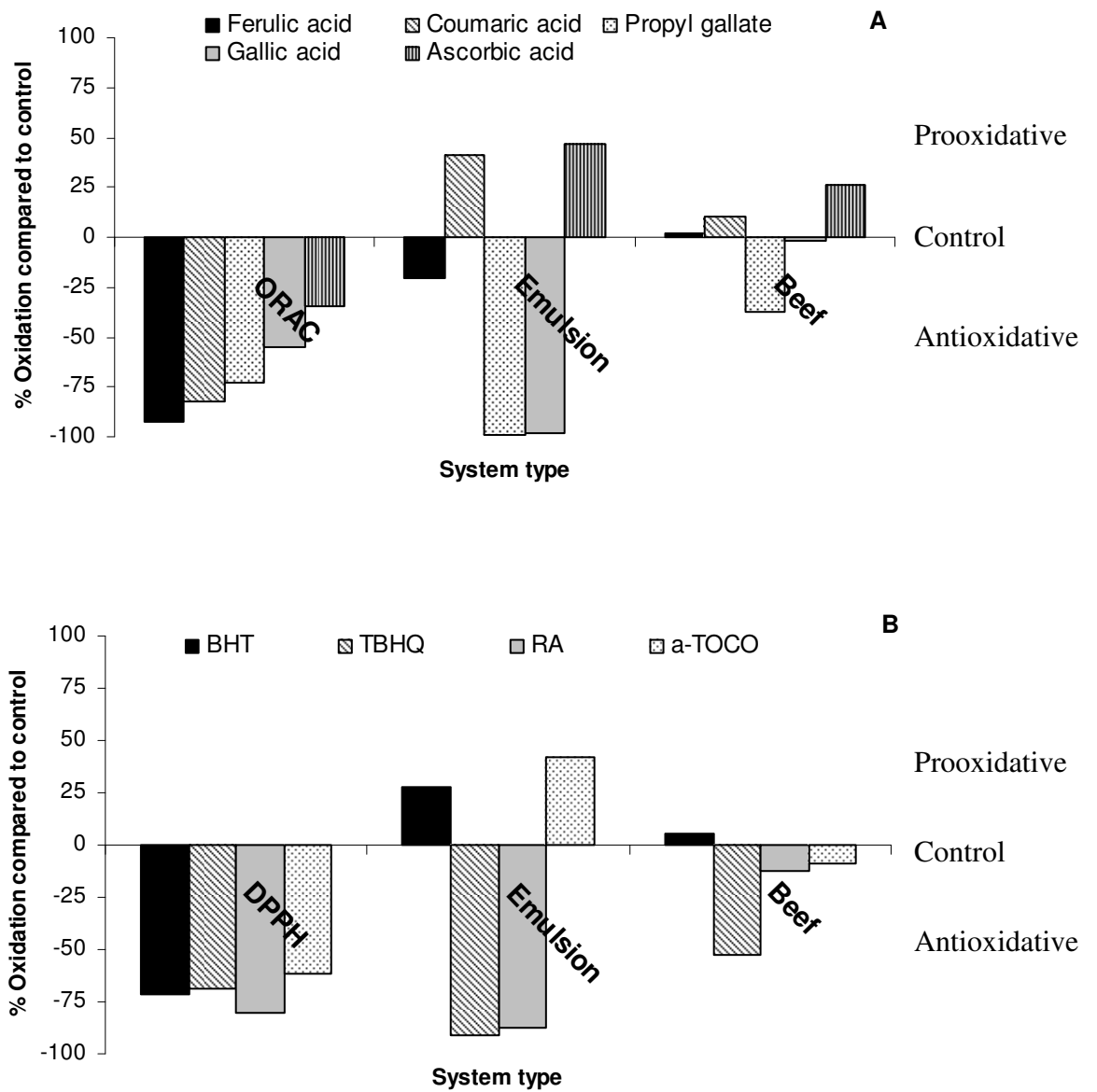


Figure 4.9. Comparison of free radical scavenging assay and lipid oxidation results of polar (a) and non-polar antioxidants (b) in oil-in-water emulsion (day 14) and cooked ground beef (day 3). Zero value was set to equal the no additive control. Negative values represent antioxidant activity while positive values represent prooxidant activity.



There are several possible explanations why the ORAC and DPPH• assays were inconsistent in predicting the ability of compounds to inhibit lipid oxidation in cooked beef and corn oil-in-water emulsions. Some compounds have the ability to inhibit lipid oxidation through mechanisms in addition to free radical scavenging. Iron is a major prooxidant in both cooked muscle foods and oil-in-water emulsions (Watts 1962; Decker and Xu, 1998; Erickson, 2002; Morrissey et al., 2003; Decker and McClements, 2001). Some phenolic compounds are able to chelate iron while others like ferulic acid which do not have a galloyl moiety do not bind iron (Andjelkovic et al., 2006). Lack of chelating activity could help explain why compounds like ferulic and coumaric acid which are good free radical scavengers did not inhibit lipid oxidation in cooked, ground beef and oil-in-water emulsions as effectively as compounds such as propyl gallate and TBHQ.

Another possible reason why free radical scavenging activity did not consistently relate to inhibition of lipid oxidation in cooked ground beef and oil-in-water emulsions could be due to the ability of some compounds to participate in redox reactions with iron resulting in the formation of ferrous ions which are stronger prooxidants than their oxidized counterpart, ferric ions (Decker and Hultin, 1992; Mei et al., 1999; Decker and McClements, 2001). Ascorbic acid is very effective at reducing ferric to ferrous ions (Decker and Hultin, 1992; Mahoney and Graf, 1986) which could help explain why it promoted lipid oxidation in cooked ground beef and oil-in-water emulsions (Figure 4.5 and 4.7) even though it is capable of scavenging free radicals (Figure 4.3). The ability of phenolics such as gallic acid to reduced iron (Mei et al., 1999) could also have decreased their antioxidant activity in foods.

The effectiveness of a compound at inactivating free radicals can also be dependent on its physical location in a food (e.g. water phase, lipid droplet, membrane phospholipids or adipose lipid). The site where a compound partitions can dictate whether it is present at the location where free radicals are promoting oxidation (Frankel, 2007). Lipid oxidation in muscle foods primarily occurs in cellular membranes (Decker and Hultin, 1992; Decker and Xu, 1998; Morrissey et al., 2003) and in oil-in-water emulsions it occurs in the lipid droplet or at the lipid-water interface (McClements and Decker, 2000). Therefore, if a compound were to preferentially partition at these locations they might inhibit lipid oxidation more effectively. Since assays such as ORAC and DPPH• are unable to determine how the physical location of a compound influences its antioxidant activity, this could explain why the free radical scavenging activity of a compound did not relate to its ability to inhibit lipid oxidation in foods.

The ability of a compound to inhibit lipid oxidation could also be influenced by its interactions with prooxidants or other antioxidants. One example of this type of relationship is the ability of ascorbic acid to regenerate oxidized  $\alpha$ -tocopherol to reactivate  $\alpha$ -tocopherol in biological membranes (Buettner, 1993; Porter, 1993). Another example of multiple compounds inhibiting lipid oxidation better than single compounds is when the compounds partition into different phases where they inhibit different oxidation pathways. For example a water-soluble compound could inactivate hydroxyl radicals generated from hydrogen peroxide while a cell membrane-soluble compound could inactivate peroxy radicals generated from phospholipid hydroperoxides. Finally, combinations of free radical scavengers and chelators could be more effective than individual compounds since a metal chelator could decrease metal-promoted free radical

generation thus decreasing the oxidation of free radical scavengers so they are effective for longer periods of time.

Finally, the ability of a compound to inhibit lipid oxidation can be concentration dependent. Concentration dependent reactivity can be due to the ability of compounds to participate in more than one reaction. For example, a compound that can reduce a metal to make it more prooxidative can often also donate an electron to inactivate a free radical. In situations such as this, a compound could act as a prooxidant at a low concentration where metal reduction is prevalent but be an antioxidant at high concentrations where there are sufficient electrons to inactivate numerous free radicals including those produced by the prooxidative metals. This is the case for compounds that are strong reducing agents such as ascorbic acid (Decker and Hultin, 1992). For weaker reducing agents, low concentrations may not cause significant metal reduction but can still result in free radical inactivation. However, if the concentration of the antioxidant increases, metal reduction could become significant thus diminishing the activity of the antioxidant. Similar scenarios could also be envisioned for compounds that can inactivate free radicals and chelate metals since chelation can often increase the water solubility of a metal making it more prooxidative. Finally, the effectiveness of some antioxidants can increase with increasing concentration. However, this only occurs up to a certain concentration where further addition of the antioxidant does not further decrease lipid oxidation. Therefore, if an antioxidant like tocopherol is added to an oil that has been stripped of its naturally occurring tocopherols, the tocopherol is found to be antioxidative while if the same amount of tocopherol is added to unstripped oil, the tocopherol is ineffective.

While the free radical scavenging activity did not consistently relate to antioxidant activity, it is interesting to note that the antioxidant polar paradox hypothesis (e.g. nonpolar antioxidants are most effective in oil-in-water emulsions) was also not able to consistently predict the ability of a compound to inhibit lipid oxidation in the oil-in-water emulsions. For example, compounds such as BHT and  $\alpha$ -tocopherol, that have essentially no water solubility (Huang et al., 1997), did not inhibit lipid oxidation in the corn oil-in-water emulsions while gallic acid, of which 70% partitions into the aqueous phase of a 10% corn oil-in-water emulsions (Huang et al., 1997; Stockmann et al., 2000) was an effective antioxidant. This suggests that while hypothesis such as the antioxidant polar paradox are helpful in understanding how antioxidants behave in model food systems, it may be very difficult to develop a system that allows for the accurate prediction of a compound's antioxidant effectiveness in all foods.

#### **4.6 Conclusions**

Free radical scavenging assays such as ORAC and DPPH• were not able to accurately predict the ability of compounds to inhibit lipid oxidation in cooked ground beef. The lack of correlation between free radical scavenging and antioxidant activity in a complex food is likely due to the multitude of factors that can impact the ability of a compound to inhibit lipid oxidation. The major drawback of the free radical scavenging assays is that they do not measure the ability of a compound to chelate metals, partition into lipids where oxidation is prevalent or interact with other antioxidants and prooxidants (e.g. metals) in a food product. Therefore, while simple one-dimensional

free radical scavenging assays can be helpful in evaluating the antioxidant mechanisms of a compound, the data from these assays should not be used to imply that compounds with high free radical scavenging capacities are good antioxidants in food systems in general.

## CHAPTER 5

### OVERALL CONCLUSIONS

The composition of foods is complex and contains a variety of fatty acids that differ in chemical and physical properties and susceptibility to oxidation. The greater the number of double bonds a fatty acid contains the more rapidly it will oxidize. Unfortunately, nutritionally desirable unsaturated fatty acids often contain multiple double bonds and thus are prone to oxidation. Numerous factors common to foods such as heating, metal interactions, light and exposure to oxygen will accelerate the decomposition of unsaturated fatty acids resulting in development of rancidity. In order to improve the nutritional quality of foods by incorporation of unsaturated fatty acids, technologies must be developed to inhibit rancidity development.

Metal is an important prooxidant in foods so one possible way to control lipid oxidation is through the addition of metal chelators. Ethylenediaminetetraacetic acid (EDTA) is a common metal chelator used in foods. The ability of EDTA is dependent on metal concentrations since EDTA only inhibits lipid oxidation when it is present at concentration greater than prooxidative metals. In a Brij 35 stabilized salmon oil-in-water emulsions at pH 7, an EDTA concentration of 2.5  $\mu\text{M}$  was able to inhibit the majority of oxidation suggesting that prooxidant metal concentrations were less than 2.5  $\mu\text{M}$ . The activity of EDTA can be diminished by the presence of other cations that could compete with prooxidant metals for binding with EDTA. In the Brij 35 stabilized salmon oil-in-water emulsions, calcium at concentrations 2 fold greater than EDTA decreased the ability of EDTA to inhibit lipid oxidation suggesting that calcium could displace prooxidant metals from EDTA allowing them to promote oxidation. Increasing

temperatures are well known to accelerate metal-promoted lipid oxidation. Therefore, it was not surprising to find that EDTA was more effective at inhibiting lipid oxidation when added to the emulsions prior to heat processing. These results indicate that heat processed salmon oil-in-water emulsions with high physical and oxidative stability could be produced in the presence of EDTA. When using EDTA to control oxidation, it should be added to the emulsion prior to heating at concentrations greater than levels of prooxidant metals found in the food. In addition, if a food contains high levels of cations, higher levels of EDTA may be necessary to control oxidation.

Lipid oxidation in foods can also be controlled by antioxidants that scavenge free radicals. Unfortunately, it is often difficult to predict which antioxidants will be effective in different foods. In an attempt to characterize the ability of compounds to act as antioxidants, simplistic one-dimensional assays such as ORAC and DPPH• have been developed to investigate the free radical scavenging or “antiradical” ability of selected compounds. To determine if these antiradical assays can predict the ability of compounds to inhibit lipid oxidation in foods, the free radical scavenging activity of series of polar and non-polar compounds were determined by the ORAC and DPPH• assays, respectively. The ability of the same compounds to inhibit lipid oxidation was then tested in cooked ground beef and corn oil-in-water emulsions. Overall, the ability of a compound to scavenge free radical did not consistently predict whether that compound effectively inhibited lipid oxidation in cooked ground beef and the corn oil-in-water emulsion. The inability of free radical scavenging assays to predict the ability of a compound to inhibit lipid oxidation in foods is likely due to a multitude of factors in addition to free radical scavenging that can impact the ability of a compound to

inhibit lipid oxidation. These factors include the ability of a compound to chelate metals, partition into lipids where oxidation is prevalent, reduce metal and/or interact with other antioxidants found naturally in foods. Therefore, while simple one-dimensional free radical scavenging assays can be helpful in evaluating the antioxidant mechanisms of a compound, the data from these assays should not be used to imply that compounds with high free radical scavenging capacities are good antioxidants in food systems.

Addition of nutritionally beneficial unsaturated fatty acids to foods will continue to be a challenge since these lipids can cause quality deterioration due to oxidative rancidity. The oxidative degradation of nutritionally beneficial fatty acids can be controlled by compounds that scavenge free radicals and/or chelate prooxidant metals. However, there are numerous chemical and physical factors that influence the ability of a compound to be an effective food antioxidant. A better understanding of how these factors impact the ability of compounds to inhibit lipid oxidation reactions could lead to novel antioxidant technologies for foods.



## CHAPTER 6

### FUTURE WORK

Many researchers have created model systems and one-dimensional free radical scavenging capacity assays in order to predict how a compound will behave in food. However, from this research we have established that it is still not possible to accurately predict how a compound will work in complex food systems (e.g. oil-in-water emulsion and cooked ground beef). Therefore, there exists a need for a more comprehensive understanding of the complexity of food matrixes, the chemistry of antioxidant compounds and novel antioxidant capacity methodology permitting for more accurate predictions of a compounds ability to function as an antioxidant in complex foods.

There are multiple factors that influence lipid oxidation between various types of foods (e.g. bulk oil, emulsion, and muscle foods). One factor is the heterogeneous environment of foods. Oil-in-water emulsions and cooked ground beef contain various phases including the aqueous and lipid phases and interfacial regions and membranes. The “polar paradox” theory states that in an oil-in-water emulsion non-polar compounds will partition into the lipid phase and are more apt to protect against lipid oxidation whereas in bulk oil polar antioxidants will partition at the oil-air or oil-water interface and are more apt to protect against lipid oxidation. However, from this research we have determined that not all compounds comply with the polar paradox theory. Our research indicated that gallic acid, a more polar compound, worked well at decreasing lipid oxidation in oil-in-water emulsions whereas BHT, a non-polar compound, had a prooxidative effect on lipid oxidation in emulsions. These findings differ from the polar paradox theory hypothesis.

In addition, the partitioning of compounds between the multiple environments present in foods has a significant effect on its chemical reactivity and can dictate how lipids interact with prooxidants (e.g. transition metals). Our research has shown that EDTA, a strong metal chelator, works well to protect against iron catalyzed oxidation of bioactive unsaturated fatty acids (e.g. omega-3 fatty acids) in a model system. However, when added to an emulsion in the presence of salt at concentrations higher than those of EDTA, our research indicated that the chelation effects of EDTA were diminished by the competitive binding of salts rather than iron, allowing for iron catalyzed oxidation to take place. Further understanding of antioxidant/prooxidant interactions and partitioning and the development of novel methods to protect against the oxidative destruction of lipids in food is needed. Such knowledge will aid in the development of foods with longer shelf lives and improve the stability of foods containing bioactive lipids.

Many simplistic one-dimensional methods that use a broad range of conditions, oxidants and methods to measure end points of oxidation have been developed to measure the free radical scavenging or “antiradical” ability of antioxidants (Frankel 2005, Brand-Williams et al., 1995). However, the major drawback of the free radical scavenging assays is that they do not measure the ability of a compound to chelate metals, partition into lipids where oxidation is prevalent or interact with other antioxidants and prooxidants (e.g. metals) in a food product. For foods the ideal antioxidant evaluation method should be conducted under the chemical, physical, and environmental conditions expected in food systems in order to accurately evaluate antioxidant potential. In order to create methods to evaluate the potential of compounds

to work as food antioxidants some general considerations must be observed for all food products including the avoidance of high temperatures during storage, non-oxidized beginning lipid, measurement of primary and secondary oxidation products, use of crude extracts or pure test compounds, lipid source with natural endogenous antioxidants and prooxidants, pH close to the food product and standardized times and conditions. In food products, these conditions vary so widely that individual evaluation methods are needed for foods (Decker et al., 2005).

In order to measure a compound's antioxidant effectiveness in oil-in-water emulsions or muscle products methodology utilizing systems similar to those found in food is needed and oxidation testing conditions need to be standardized. For example, in oil-in-water emulsions, standardized methodology for the creation of emulsions needs to be determined. Factors such as homogenization, surfactant type and concentration, particle size, and order of addition of test compounds must be established. Oxidation measurements for primary (e.g. lipid hydroperoxides or conjugated dienes) and secondary oxidation products (e.g. volatile compounds or TBARS) should also be determined. In muscle foods, antioxidant activity can be affected by prooxidants in the different stages of preparation including raw muscle foods, washed muscle foods and cooked muscle foods therefore standardized preparation methods need to be created. Oxidation measurements of primary (e.g. lipid hydroperoxides) and secondary (e.g. TBARS) oxidation products need to be established and solvent types need to be standardized. Establishing and standardizing assays that more accurately predict a compound's effectiveness at inhibiting oxidation in foods will be a useful tool in the food industry.

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