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thesis entitled

Antioxidant and antimicrobial activities of 1,2,3,4-tetrahydroxybenzene and 2,3,4,5-tetrahydroxybenzoic acid

presented by

Andrea Sue Molengraft

has been accepted towards fulfillment
of the requirements for the

Master of Science	degree in	Food Science and Human Nutrition

Gale M Strasburg
Major Professor's Signature

6/30/2003
Date

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ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF 1,2,3,4-TETRAHYDROXYBENZENE AND 2,3,4,5-TETRAHYDROXYBENZOIC ACID

By

Andrea Sue Molengraft

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

2003

ABSTRACT

ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF 1,2,3,4-TETRAHYDROXYBENZENE AND 2,3,4,5-TETRAHYDROXYBENZOIC ACID

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The objectives of this study were to compare the antioxidant activities of 1,2,3,4,-tetrahydroxybenzene (THB) and 2,3,4,5,-tetrahydroxybenzoic acid (THBA) to commercial antioxidants currently used in the food industry and to determine whether these compounds display antimicrobial activity. The compounds were tested for antioxidant activity in bulk oils, liposomes and ground beef using peroxide value, fluorescence and the thiobarbituric acid test, respectively. THBA displayed high antioxidant activity in each system, comparable or superior to all of the commercial antioxidants tested. THB was superior to the antioxidants tested in bulk oil and in cooked ground beef, but showed little activity in the liposome assay. A disk diffusion screening assay using *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp, showed that only THB possessed antimicrobial activity. Minimum inhibitory concentrations (MIC) of THB were determined against three bacteria in typticase soy broth (0.6% yeast extract) media for 24 and 48 h at 35°C. MICs for THB ranged from 2.8-8.4 mM at 24 h and 5.6-11.2 mM at 48 h. Given both antioxidant and antimicrobial activity, THB may serve as a multifunctional food ingredient, whereas THBA may serve as an alternative antioxidant.

This thesis is dedicated to my family for all their love and support

ACKNOWLEDGMENTS

I would like to express my gratitude to my major professor, Dr. Gale Strasburg, for his knowledge, guidance, and support through my studies at Michigan State University.

I am also grateful to my committee members for their prolonged support. I would like to acknowledge Dr. John Frost for all his support and enthusiasm for this project, and especially for providing the antioxidant samples used in this research. I would also like to thank Dr. Elliot Ryser for his guidance with the project, for use of the equipment in his laboratory, and help with data analyses and interpretation. I also want to express my thanks to Dr. Arzu Cargi for her guidance in the laboratory as well.

I am also very grateful to Dr. Enayat Gomaa for her assistance with training and TBARS analysis. I would also like to thank Dr. Alden Booren for his suggestions with the project and for providing me with more than enough lard than one could hope for! I would also like to thank past and present colleagues in my laboratory, especially John Rodgers for his insight and friendship. A special thanks to my friends Vareemon Tuntivanich, Hyo-Jung Yoon, Jin-Shan Shie and Dr. Wen Chiang for all their support and long days in the lab.

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INTRODUCTION

Naturally occurring antioxidants, including rosemary and sage extracts, polyphenolic catechins of green tea, flavonoids and other phenolic compounds are often superior to synthetic antioxidants in terms of activity, safety, and consumer acceptance (Mukhopadhyay 2000). In addition, significant human health benefits such as anti-cancer, anti-mutagenic and anti-inflammatory activities have been attributed to consumption of plant-derived compounds (Duthie 1991). The prospect of using naturally derived compounds to improve human health while stabilizing lipid-containing foods, offers opportunities to enhance both the diets of consumers and the quality of food products.

Even though natural antioxidants are widely accepted by consumers, their adoption by the food industry has been limited. Fluctuations in both supply and cost of the antioxidant product are major concerns for food processors. Problems during harvest, such as low yield or contamination of crops, may also result in a substandard product. Plant material extracts rich in antioxidants are frequently used rather than more costly purified antioxidants. However, these extracts contain variable concentrations of antioxidants, undesirable colors, odors and flavors. In addition, natural antioxidants are subjected to less scrutiny and scientific evaluation than synthetic antioxidants. These natural antioxidants must be used cautiously due to the potential presence of mutagens, carcinogens and/or other pathogens (Pratt 1996). Therefore, synthetic antioxidants are usually preferred due to their lower cost, higher effectiveness and consistent availability.

An alternative approach to use of natural-product extracts could involve the use of abundant, low-value, by-products of food processing as raw material for the synthesis of novel antioxidant compounds. Disposal of steeping liquor from cereal grain processing is problematic due to the presence of phytic acid, an anti-nutritive oligophosphate compound with strong metal-chelating activity (Wood 2000). While steeping liquor can be concentrated and sold for animal feed, environmental concerns raised by high levels of phosphorus in animal excrement limit the value of steeping liquor. Removal of phytic acid from the steeping liquor for use in synthesis of novel antioxidants could enhance its value as well as that of the steeping liquor, which could be used as phytate-free animal feed.

Phytic acid can be isolated from the steeping liquor of grain processing and dephosphorylated by phytase to produce *myo*-insitol. In a simple two- or three-step conversion, *myo*-inositol is converted to 1,2,3,4-tetrahydroxybenzene (THB) (Hanson and others 1999) and 2,3,4,5-tetrahydroxybenzoic acid (THBA) (Stueben 2002), respectively. The polyhydroxylated aromatic structures of these compounds suggest that they may function as highly effective, free-radical-scavenging antioxidants.

A phenolic compound may function as an antioxidant by donating a hydrogen atom to a free radical; the resultant phenolic radical remains relatively stable due to delocalization of the unpaired electron. Previous studies suggest that compounds with multiple hydroxyl groups attached to the aromatic nucleus will be potent antioxidants (Cuvelier and others 2000). We hypothesized that the polyphenolic compounds, THB and THBA, will show equivalent or greater antioxidant activity compared to commercial antioxidants used currently in the food industry. As with all antioxidants, activity will

vary depending on the nature of the lipid substrate and type of food system to which they are added. Thus, the first objective of this study was to evaluate the antioxidant activities of THB and THBA in three different experimental systems; bulk oils, liposomes and ground beef. Antioxidant activities of these compounds were compared with activities of structurally related compounds.

The phytic acid derivatives also share structural similarities with the antimicrobial compounds fumigatin and aurantiogliocladin. We hypothesized that like the former compounds, THB and THBA would possess antimicrobial activity. To test this hypothesis, the second objective of this study was to evaluate the antimicrobial activity of THB and THBA using a disk diffusion assay as a screening tool. If a compound shows activity in this assay, it is analyzed further in broth culture to determine its minimum inhibitory concentration against common foodborne pathogens.

Compounds displaying both strong antioxidant and strong antimicrobial activity with negligible human toxicity have an enormous potential for use as multifunctional food ingredients. They could be used in multistage or combination spray washing of animal carcasses or fruits and vegetables, thereby enhancing the safety and quality of the final product. Utilization of these compounds by the food industry may also reduce water and energy costs, minimize color degradation, and maintain structural integrity of the food. Moreover, these compounds may be safer alternatives to more toxic compounds such as hydrogen peroxide and chlorine.

This thesis is organized into five chapters. The following chapter provides an overview of the literature for this study. The proceeding chapters each cover a specific objective and were prepared as manuscripts for publication. Chapter 2 is devoted to the

first objective, i.e., evaluation of antioxidant activity and Chapter 3 describes the antimicrobial activity of THB and THBA. Overall conclusions and suggestions for future research are also provided in Chapters 4 and 5.

CHAPTER ONE

LITERATURE REVIEW

1.1 Lipid Autoxidation

Lipid autoxidation is a term that describes a series of free-radical mediated reactions, which include the direct reaction of atmospheric oxygen with lipids. Autoxidation products are responsible for off-flavors and odors in lipid-containing foods as well as a reduction of the nutritional value of the lipid, loss of fat-soluble vitamins, carotenoids or proteins, or bleaching of food color (Jacobson 1999; Mukhopadhyay 2000). These deteriorative reactions decrease shelf-life and consumer acceptance of food products. In addition, there may be consequences of autoxidation which are less readily recognized. Products of lipid autoxidation, including cholesterol oxides and 4-hydroxy-2-nonenal (Nawar 1996), may lead to pathological processes including premature aging, tumorgenesis and atherosclerosis (Ladikos and Lougovois 1990; Ramarathnam and others 1995). Free-radical mediated human diseases may be prevented or delayed by stabilizing food products with antioxidants as well as by consumption of diets rich in natural antioxidants. Thus, through the judicious use of antioxidants, human health may be enhanced in conjunction with the production of a more stable, desirable and wholesome, healthy product.

Among the various classes of lipid molecules, the two types most commonly associated with autoxidation are triglycerides and phospholipids. Triglycerides are neutral lipids typically found in adipose tissue of animals or as storage oils in plant seeds. They consist of a glycerol backbone to which fatty acids are esterified. Fatty acids are

hydrocarbon chains with a terminal carboxylic acid moiety. Saturated fatty acids are fully reduced or saturated with hydrogen, whereas unsaturated fatty acids found in foods typically contain one to six double bonds between the carbon atoms.

Autoxidation of fatty acids generally occurs alpha to the double bonds, and the degree of unsaturation affects the kinetics of autoxidation. Fatty acids with higher numbers of double bonds on the fatty acyl chain are more susceptible to lipid autoxidation. Oxidation rates for oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) relative to stearic acid (18:0), which contain no double bonds, are 100, 1,200 and 2,500, respectively (Belitz and Grosch 1987). Arachidonic acid (20:4) was reported to oxidize 2.9 times faster than linoleic acid (Porter and others 1981). Based on these data, fish oils, soy oil, and other lipids enriched in polyunsaturated fatty acids will oxidize faster than less unsaturated lipids, such as beef fat or tropical oils.

Heating of oils, during frying of foods, accelerates autoxidation of lipids, promoting the dominant major reactions, which include both polymerization and fragmentation of oxidized lipids. These reactions are responsible for physical changes of the lipid including darker color and increases in average molecular weight, viscosity, foam and refractive index. Concomitantly, there are decreases in polyunsaturated fatty acid content and smoke point (Bennion and Park 1968; Nawar 1996).

Phospholipids are lipid molecules that consist of glycerol, fatty acids, phosphate and a polyhydroxyl compound or organic base. The parent compound of the phospholipid is phosphatidic acid, which consists of fatty acids esterfied to the *sn*-1 and *sn*-2 hydroxyl groups of the glycerol backbone, and a phosphate at the *sn*-3 position. In more complex phospholipid systems, the phosphate group is usually linked through a

diester bond to the basic or polyhydroxy moiety. Examples of common phospholipids include phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol. The amphiphilic nature of these compounds enables them to form the bilayer structure of cellular membranes. The high surface-to-volume ratio of membrane structures makes this lipid class highly susceptible to oxidation. Thus, phospholipids are the dominant lipid substrate for autoxidation in products having intact membranes, such as muscle foods and whole plant tissue.

Products of autoxidation in lipid-containing foods are responsible for the rancid off-flavors, odors and undesirable color changes in foods that have undergone lipid autoxidation. Thus, it is important to understand the nature of the chemical reaction involved in autoxidation of these foods and the mechanisms of preventative measures that may be taken to ensure a more desirable product.

1.1.2 Mechanism of Lipid Autoxidation

Autoxidation is an irreversible, free-radical-mediated series of reactions (Figure 1.1) which occurs in three stages: initiation, propagation, and termination. During the initiation step, a hydrogen atom (H·) is abstracted from an unsaturated fatty acyl group (RH) by an initiator to form a fatty acyl radical (R·) (Equation 1):

$$RH + \text{initiator} \quad R\cdot + H\cdot \qquad \text{(Eq. 1)}$$

Initiators include hydroxyl free radicals (·OH), superoxide ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), ionizing radiation, singlet oxygen ($^1O_2$), transition metal ions (M^{n+}) or other reactive species. Transition metals react with H_2O_2, forming

Initiation

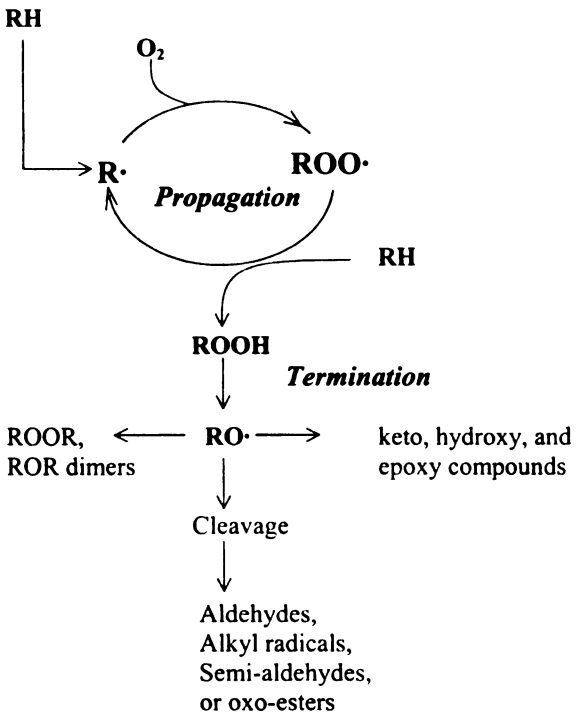


Figure 1.1 Scheme of Lipid Autoxidation (adapted from Schmidt 2000; Hsieh and Kinsella 1989)

strong oxidizing agents, which are believed to be ·OH, $MO^{(n+1)+}$ or $MOH^{(n+1)+}$ (Equations 2 and 3):

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + \cdot OH + OH^- \quad \text{(Eq. 2)}$$

$$MOH^{(n+1)+} \leftrightarrow MO^{n+} + H^+ \quad \text{(Eq. 3)}$$

The reaction of H_2O_2 with transition metals is referred to as Fenton's Reaction (Equations 4 and 5) (Aust and others 1985; Hsieh and Kinsella 1989), in which a hydroxyl radical is produced.

$$O_2^{\cdot-} \cdot + M^n \rightarrow O_2 + M^{n-1} \quad \text{(Eq. 4)}$$

$$M^{n-1} + H_2O_2 \rightarrow M^n + \cdot OH + OH^- \quad \text{(Eq. 5)}$$

Trace amounts of metals are often found in edible oils, originating from the soil in which the plant was grown, leaching or flaking of metal from processing equipment, or as natural components of biological tissue. The oxidation state of the metal is important in determining the rate of induced autoxidation. Reduced iron (Fe^{2+}), for example, is a much more effective pro-oxidant than the oxidized form (Fe^{3+}) (Graf and Empson 1987; Arora and Strasburg 1997). Oxidized metal ions become effective pro-oxidants in the presence of a reducing agent (e.g. ascorbic acid), which regenerates the reduced form of the metal.

Initiation of lipid oxidation requires an active form of oxygen. The direct reaction of oxygen with unsaturated fatty acids is thermodynamically unfavored due to differences in spin state of oxygen and the fatty acid. The ground state of unsaturated fatty acids corresponds to the singlet state, whereas the ground state of oxygen is the triplet state. Therefore, the reaction of unsaturated fatty acids with triplet oxygen ($^3O_2$) is forbidden (Harel and Kanner 1985; Hsieh and Kinsella 1989). Spin restriction of molecular oxygen is overcome by sufficient activation of energy to form $^1O_2$. This can be achieved by the reaction of $O_2^{-}\cdot$ with H_2O_2 or the reaction of $^3O_2$ with $\cdot OH$ or sensitizers, such as plant pigments, to form $^1O_2$.

In propagation, the fatty acyl radical reacts with diatomic oxygen (O_2) to form a peroxy radical (ROO·) (Equation 6) which can then initiate autoxidation of another fatty acid by abstracting an allylic hydrogen atom (rate-limiting step) to form a hydroperoxide (ROOH) (Equation 7):

$$R\cdot + O_2 \quad ROO\cdot \quad \text{(Eq.6)}$$

$$ROO\cdot + R\ H \quad ROOH + R\ \cdot \qquad \text{(Eq. 7)}$$

The kinetics of hydroperoxide formation is proportional to the number of double bonds present in the fatty acid. Polyunsaturated fatty acids are more sensitive to oxidative damage than monounsaturated fatty acids due to lowered bond dissociation energy of the allylic hydrogen (Aust and others 1985). The peroxy radical can abstract a hydrogen more readily from a 1,4-pentadiene system than from a single allyl group due to resonance stablization of the 1,4-diene radical generated (Belitz and Grosch 1987).

Hydroperoxides are common intermediates of autoxidation that are unstable and spontaneously decompose, especially in the presence of metal ions, into acids, aldehydes, alcohols, carbonyls and ketones. These low-molecular-weight, volatile compounds, commonly referred to as secondary oxidation products, are responsible for the rancid off-flavors, odors and subsequent undesirable color changes in food products that have undergone lipid autoxidation.

Hydroperoxides break down through a series of decomposition steps (Nawar 1996). The hydroperoxide first undergoes scission at the oxygen-oxygen bond, resulting in an alkoxy radical and a hydroxyl radical (Equation 8):

$$ROOH \quad RO\cdot + \cdot OH \qquad \text{(Eq. 8)}$$

In the second step the carbon-carbon bond is cleaved on either side of the alkoxyl group. Cleavage on the carboxyl side results in the formation of an aldehyde and an acid, whereas scission on the methyl side produces a hydrocarbon and an oxoacid.

Termination of autoxidation, shown in equations 9, 10, and 11, is defined as the combination of radicals to form non-radical, stable compounds:

$$ROO\cdot + R\cdot \quad ROOR \qquad \text{(Eq. 9)}$$

$$RO\cdot + R\cdot \quad ROR \qquad \text{(Eq. 10)}$$

$$ROO\cdot + ROO\cdot \quad ROOR + O_2 \qquad \text{(Eq. 11)}$$

1.2. Role of Antioxidants

An antioxidant is defined as 'any substance that when present in low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits autoxidation of that substrate' (Gutteridge 1994). Antioxidants operate by different mechanisms depending on their structure, and are classified as free radical terminators, oxygen scavengers (or reducing agents), or chelating agents (Giese 1996).

1.2.1. Free Radical Terminators

Free radical terminators interfere with the free-radical chain of oxidative reactions by donating a hydrogen atom from phenolic hydroxyl groups. Effective free radical terminators (AH) compete with the unsaturated lipid substrate (Hsieh and Kinsella 1989) by inhibiting free radical formation in the initiation step or by interrupting propagation (Equations 12 and 13):

$$R\cdot + AH \quad RH + A\cdot \qquad \text{(Eq. 12)}$$

$$ROO\cdot + AH \quad ROOH + A\cdot \qquad \text{(Eq. 13)}$$

The most effective free radical terminators are phenolic compounds. When a phenolic compound donates a hydrogen atom to a lipid free radical formed during autoxidation, a phenolic radical is formed, which is stabilized by electron delocalization over the aromatic nucleus (Figure 1.2). The resulting antioxidant radical (A·) is less reactive than the fatty acid radical due to this stabilization.

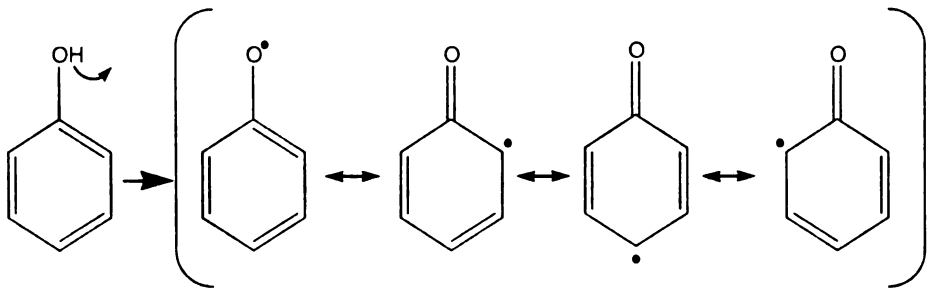


Figure 1.2 Resonance Stabilization of a Phenolic Radical

The reactivity of an antioxidant compound and the stability of the phenolic radical are influenced by the presence or absence of substituents on the phenolic nucleus. Depending on their nature, number, and location, these substituents may either enhance or suppress antioxidant activity. A second hydroxyl group in the *ortho* or *para* position of a phenolic compound increases antioxidant activity by further electron delocalization of the aromatic ring (Cuvelier and others 1992). Compounds with *ortho* or *para* substitution are stabilized because the initial semiquinone radical produced by reaction with a lipid radical can further oxidize to a quinone by reaction with another lipid radical (Figure 1.3 (a)) or by reacting with another semiquinone radical, thereby regenerating the antioxidant (Figure 1.3 (b)). The presence of a hydroxyl group at the *ortho* position, such as 1,2-dihydroxybenzene derivative, is believed to also stabilize the radical through intramolecular hydrogen bonding (Figure 1.4) (Baum and Perun 1962).

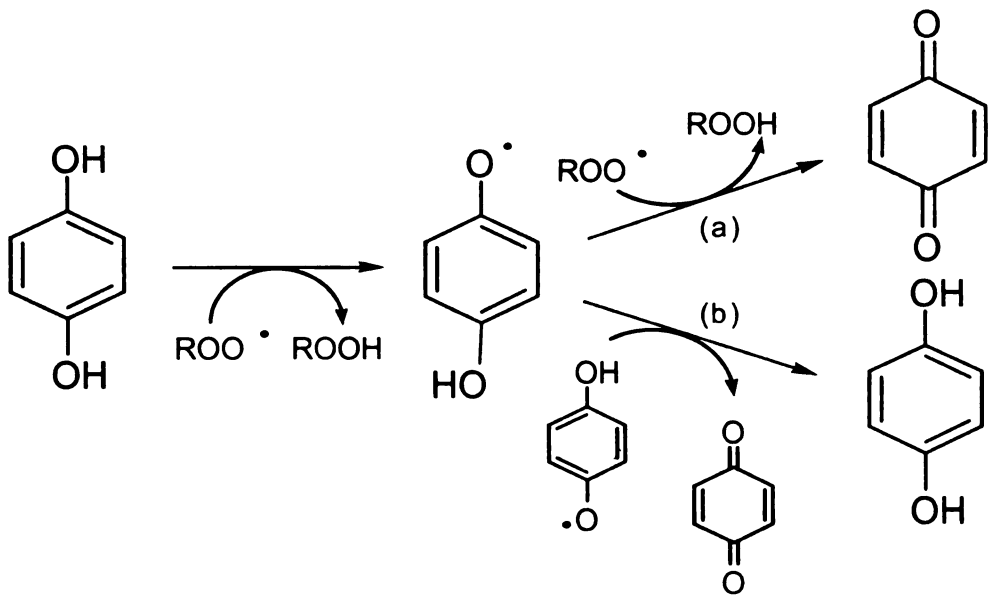


Figure 1.3 Quinone formation by a *para*-substituted phenolic compound upon reaction with free radicals

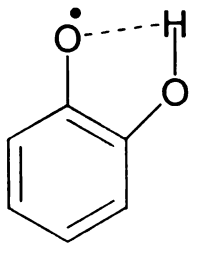
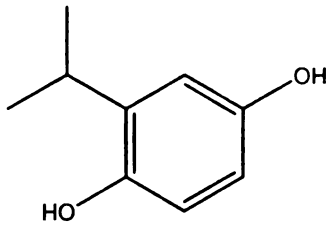


Figure 1.4 Stabilization of a phenolic free radical by hydrogen bonding of an *ortho* hydroxyl substituent

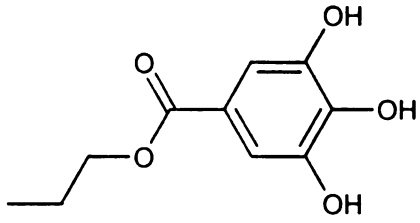
Substitution of phenolic compounds with three hydroxyl groups generally increases antioxidant activity compared to compounds with only two hydroxyl groups. For example, gallic acid, with three hydroxyl groups, is more effective than protocatechuic acid with only two hydroxyl groups (Pokorny 1987; Cuvelier and others 1992). Protocatechuic acid and caffeic acid (*o*-diphenols) have better antioxidant activity than their respective monophenols, *p*-hydroxybenzoic acid and *p*-coumaric acid (Cuvelier and others 1992).

Alkyl substitution also contributes to increased antioxidant activity. Phenolic compounds with methyl groups attached to the benzene ring have been shown to increase antioxidant activity by electron donation to the ring. Thus, the phenolic hydrogen atom is more labile for donation to a lipid free radical. Steric effects of alkyl substituents also act to stabilize the antioxidant radical (Baum and Perun 1962). Substitution of a *tert*-butyl group *ortho* to the hydoxyl group makes butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) effective antioxidants. Steric hindrance of the bulky *tert*-butyl group decreases reactivity of the radical. The most effective free radical terminators are typically aromatic compounds including BHT, BHA, 2-*tert*-butylhydroquinone (TBHQ), propyl gallate, Coenzyme Q_{10}, and the tocopherols (Figure 1.5).

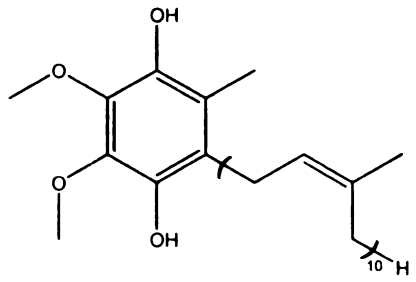
Hydroxycinnamic acid compounds, which are widely distributed in nature, show similar structure-activity relationships to the phenolic compounds discussed above (Figure 1.6). Methoxy substitution *ortho* to a hydroxy group has shown to decrease antioxidant effectiveness for most acid phenols. For example, caffeic acid, with *ortho* substitution of a hydroxy group, possesses greater stability of the phenoxy radical and



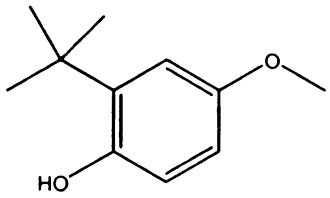
2-tert-butylhydroquinone (TBHQ)



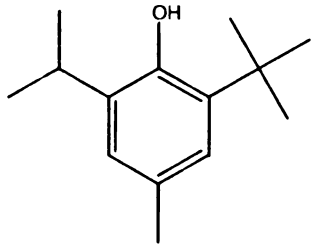
Propyl Gallate



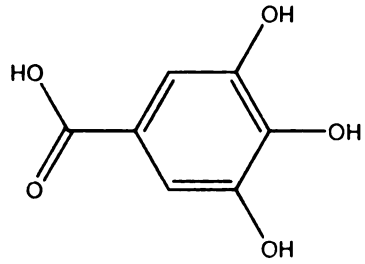
CoEnzyme Q_{10}



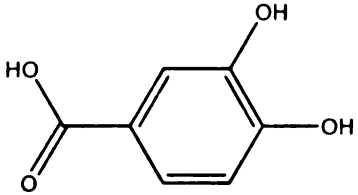
Butylated Hydroxyanisole (BHA)



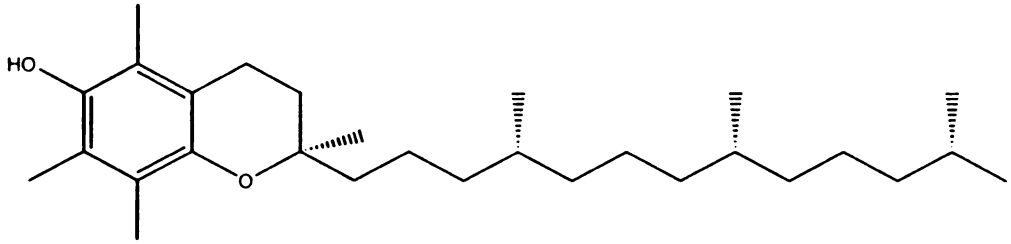
Butylated Hydroxytoluene (BHT)



Gallic Acid

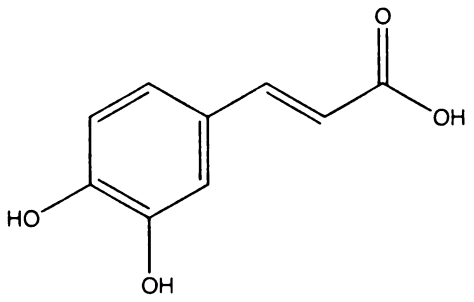


Protocatechuic Acid

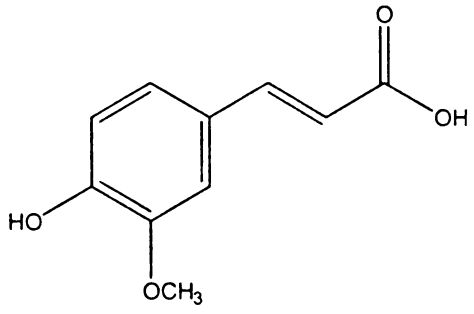


alpha–tocopherol

Figure 1.5 Structures of common antioxidants: Free radical terminators



Caffeic Acid



Ferulic Acid

Figure 1.6 Structures of some hydroxycinnamic acid compounds

increased antioxidant activity compared to ferulic acid with methoxy *ortho* substitution (Cuvelier and others 1992). Rosmarinic acid, with four hydroxyl groups, shows greater antioxidant activity than compounds with only one or two hydroxyl groups, such as ferulic acid and caffeic acid (Chen and Ho 1997). This is believed to be due to the increased resonance stabilization and/or *o*-quinone or *p*-quinone formation (Chen and Ho 1997; Frankel 1998).

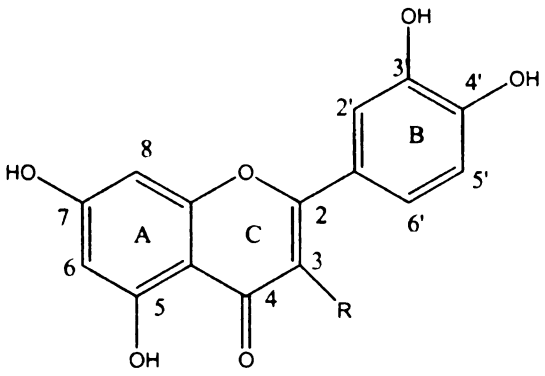
Flavonoids are another class of widely distributed plant compounds, some of which possess potent antioxidant activity (Figure 1.7). The basic structure of a flavonoid molecule consists of two aromatic rings, identified as rings A and B, linked by a three-carbon aliphatic chain which condenses to form a pyran ring, C. Substitution with hydroxyl groups, especially on the B-ring, increases antioxidant activity, whereas substitution with methoxy groups decreases antioxidant activity (Arora and others 1998). For example, quercetin, rutin and luteolin, each having 3 ,4 -dihydroxy phenyl substitution, shows stronger antioxidant activity than naringenin and hesperetin. This is likely due to increased electron delocalization by the presence of the *o*-dihydroxy substitution on the B-ring.

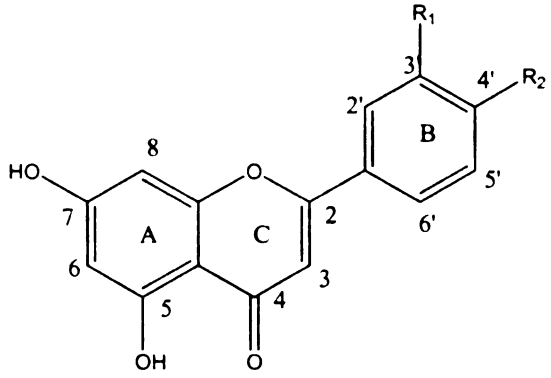
The antioxidant radical may further react with another peroxyl radical, convert back to AH by reacting with another molecule, or dimerize to A_2 (Gutteridge and Halliwell 1990). Equations 14, 15, and 16 show the termination steps with the addition of antioxidants.

$$ROO\cdot + A\cdot \quad ROOA \qquad \text{(Eq. 14)}$$

$$A\cdot + RH \quad AH + R\cdot \qquad \text{(Eq. 15)}$$

$$A\cdot + A\cdot \quad A_2 \qquad \text{(Eq. 16)}$$





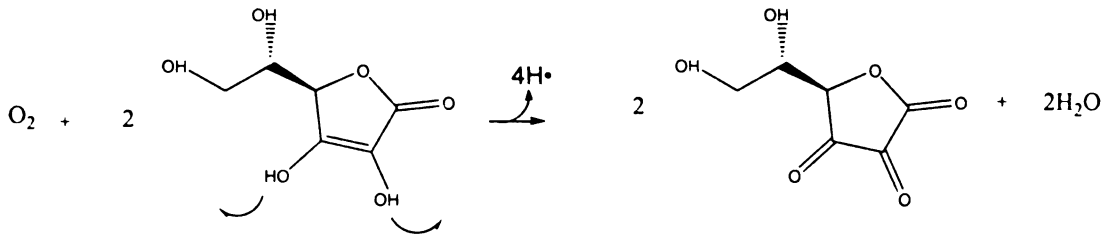
R= OH	Quercetin
R=O-rutinose	Rutin
R=H	Luteolin

R_1=H, R_2=OH	Naringenin
R_1=OH, R_2=OCH3	Hesperetin

Figure 1.7 Structures of commonly occurring flavonoids

1.2.2. Oxygen Scavengers

Oxygen scavengers reduce the partial pressure of oxygen, an essential component of lipid autoxidation, by transferring hydrogen atoms. Examples of oxygen scavengers include ascorbic acid and erythorbic acid, which reduce molecular oxygen producing two molecules of water (Figure 1.8) (Giese 1996).



Ascorbic Acid Dehydroascorbic acid.

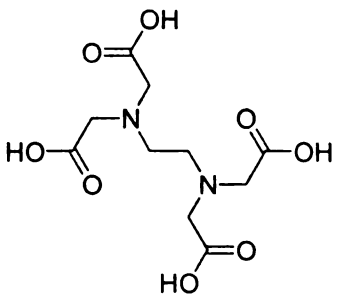
Figure 1.8 Oxygen scavenging activity of ascorbic acid

When used in combination with other antioxidants, they function as synergists by promoting their antioxidative effects. For example, ascorbic acid and its sodium salt, sodium ascorbate, regenerate phenolic antioxidants by donating hydrogen atoms to phenoxyl radicals produced by lipid oxidation (Frankel 1998).

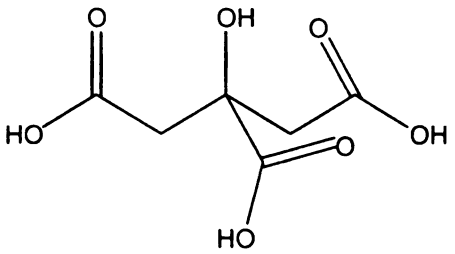
1.2.3. Chelating Agents

Chelating agents [e.g. citric acid or ethylenediaminetetraacetic acid (EDTA)] are often added to a food product to form complexes with endogenous pro-oxidant metal ions, such as iron or copper, to delay autoxidation (Figure 1.9). A chelate is a complex formed by combining a metal ion with a multidentate ligand.

A monodentate ligand, forms only one bond with the metal while a polydentate ligand forms multiple bonds, which increases the stability of the complex. For example, EDTA has six donor atoms, in which it encloses a metal ion, making the metal less available for autoxidation reactions (Gordon 1990). In addition, chelating agents lower the M^{2+}/M^{3+} redox potential, thereby limiting the ability of the metal to act as an oxidizing agent. In contrast, large bulky ligands tend to be less stable exposing the metal to solution giving rise to prooxidant effects (Miller 1996)



Ethylenediaminetetraacetic acid (EDTA)



Citric Acid

Figure 1.9 Chelating agents

1.2.4. Antioxidants in Food Systems

In addition to recognizing the structure-activity relationship of antioxidants, it is important to understand that an antioxidant's efficacy varies depending on the type of food system to which it is added. According to Porter and others (1989), lipophilic antioxidants such as BHT and BHA are more effective in emulsion and liposome systems than in bulk, dry oils when compared to more hydrophilic antioxidants, such as THBQ. These findings led to the formation of the "polar paradox" principle, which states that non-polar antioxidants function best in polar lipid emulsions and membranes, whereas polar antioxidants are relatively more effective in non-polar lipids such as bulk oils.

The mechanism underlying this paradox is based on differences in distribution or partitioning of antioxidants with respect to the aqueous and lipid phases (Frankel 1998). For an oil/water emulsion, hydrophilic antioxidants dissolve in the water phase and become diluted, while the lipophilic antioxidants partition into the oil-water interface. In a bulk oil system, hydrophilic antioxidants are oriented at the air-oil interface, where oxidation of lipids is most likely to occur, while lipophilic antioxidants are dispersed throughout the oil phase. Antioxidants that are polar, hydrophilic or amphiphilic with high hydrophile-lipophile balance (HLB) are more effective in low surface-to-volume ratio (LSV) products, such as bulk oils and fats (either vegetable or animal), or synthetic esters of fatty acids (Porter and others 1989). In contrast, antioxidants that are non-polar, lipophilic or amphiphilic with low HLB are more effective in high surface-to-volume ratio (HSV) lipid structures. HSV matrices include emulsions, micelles and membranes of whole tissue foods.

1.2.5. Regulation of Antioxidant Use

Food preservation strategies were developed long before the science of antioxidants was understood. Many natural spices and herbs are classified as *GRAS (Generally Recognized as Safe)* substances because of their historical use in food preservation. Examples of *GRAS* substances possessing antioxidant activity include rosemary and sage extracts (21 CFR 182.10). Newly discovered antioxidants and extracts from plant sources without a long history of consumption as foods must be approved by either the Food and Drug Administration (FDA) or the United States Department of Agriculture (USDA), depending on the food to which it is added. The FDA oversees all domestic and imported foods sold in interstate commerce, including shell eggs, bottled water, and wine beverages with less than 7 percent alcohol. In contrast, domestic and imported meat, and poultry and related products, such as meat- or poultry- containing stews, pizzas and frozen foods, and processed egg products (generally liquid, frozen and dried pasteurized egg products) are under jurisdication of the USDA. (U. S. Food and Drug Administration 1998).

Based on weight of the fat or oil content of a food, the FDA allows the addition of antioxidants (BHT, BHA, for example), either alone or in combination at concentrations up to 0.02% w/w (200 ppm) when used in emulsion stabilizers for shortenings (21 CFR 170) and the USDA allows the addition of BHA, BHT, propyl gallate and TBHQ, for example, up to 0.01% w/w (100 ppm) or 0.02% in combination, with no individual antioxidant exceeding 0.01% (9 CFR 424.21). Tocopherols can be added up to 0.03%, when added to rendered animal fat or vegetable oils (9 CFR 424.21).

1.3. Methodologies for Evaluation of Lipid Autoxidation

Antioxidant efficacy of new compounds is determined by comparing their effect on the rate of lipid oxidation to those of antioxidants used presently in a food or model system. Lipid autoxidation and antioxidant efficacy are typically determined by using an acceleration method such as heat, light or addition of metal catalysts, followed by measurement protocol.

1.3.1. Acceleration Methods

1.3.1.1. Heat

Heating is the most common and effective means of accelerating autoxidation in order to determine an antioxidant's efficacy in a relatively short time. The mechanism of autoxidation changes with temperature as described by the Arrhenius equation (Equation 17):

$$k = k_A \exp\left(\frac{-E_A}{RT}\right) \qquad \text{(Eq. 17)}$$

where k = rate constant, k_A = Arrhenius equation constant, E_A = activation energy (J), T = absolute temperature (K), and R = 8.3144 (J/mol·K).

When comparing the rate constants of autoxidation with and without the addition of an antioxidant, an Arrhenius plot of log induction period versus temperature shows that with the addition of an antioxidant, the log induction period decreases with an increase in temperature (Taoukis and Labuza 1996). The induction period at ambient storage temperatures conditions can then be predicted by extrapolating the results from elevated temperatures to lower temperatures. However, in more complex food systems, the rate of autoxidation is a composite of multiple dominant reactions, making high

temperature testing less reliable. Different changes in rate constants for one series of reactions in a food may result in their dominance above a certain temperature, while other reactions may predominate at lower temperatures (Taoukis and Labuza 1996).

At ambient temperature the rate of autoxidation is independent of oxygen partial pressure. As the temperature increases the rate of autoxidation becomes dependent on oxygen partial pressure due to a decrease in oxygen solubility (Frankel 1998). In addition, peroxides begin to decompose at higher temperatures with the scission products distilling off (Frankel 1993). For example, at 100°C primary hydroperoxides rapidly decompose, making high temperature tests less reliable (Frankel 1993).

1.3.1.2. Light

Lipid autoxidation also can be accelerated by the exposure to light. Initiation by photooxidation results from the presence of sensitizers, classified as Type I and Type II (Equations 18 and 19, respectively) (Frankel 1998).

$$^3\text{Sens} + \text{RH} \xrightarrow{h\nu} [\text{Intermediate}] + O_2 \rightarrow \text{ROOH} + \text{Sens} \qquad \text{(Eq. 18)}$$

$$^3\text{Sens} + O_2 \xrightarrow{h\nu} [\text{Intermediate}] + {}^1\text{Sens} \rightarrow {}^1O_2 + \text{RH} \rightarrow \text{ROOH} \qquad \text{(Eq. 19)}$$

Type I sensitizers (e.g. riboflavin) are in the triplet state (^{3}Sens) and react with the lipid substrate by abstracting hydrogen atoms to form decomposition products of autoxidation (e.g. hydroperoxides). Type II sensitizers (e.g. chlorophyll) are also in the triplet state, but react with oxygen to produce $^1O_2$, which in turn reacts directly with the lipid substrate.

Photooxidation of lipid-containing food products is usually not a concern since the reaction only occurs when the lipid is exposed to direct sunlight or fluoresent light (Frankel 1998). Therefore, photooxidation is often not used as an acceleration method for lipid oxidation studies.

1.3.1.3. Transition metals

Transition metals increase the rate of autoxidation by activating hydroperoxide decomposition, reacting with an unoxidized substrate or by activating molecular oxygen to form $^1O_2$, $O_2^{-}\cdot$, or RO· (Equations 20, 21 and 22):

$$M^{n+} + ROOH \rightarrow M^{(n+1)+} + OH- + RO\cdot \quad \text{(Eq. 20)}$$

$$M^{n+} + RH \rightarrow M^{(n-1)+} + H^{+} + R\cdot \quad \text{(Eq. 21)}$$

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{-} \rightarrow {}^1O_2 + HO_2\cdot \quad \text{(Eq. 22)}$$

Acceleration methods yield valuable information on shelf-life and antioxidant activity in a much shorter time frame than experiments performed under ambient conditions. Careful consideration of the acceleration method and the type of evaluation for antioxidant activity must be done in order to have valid results.

1.3.2. Methods for Measurement of Lipid Autoxidation

1.3.2.1. AOM and Rancimat

In the active oxygen method purified air is bubbled through an oil or fat sample at 110°C-130°C, and with acidic volatile products are passed into a deionized water trap (Pike 1998). The induction period is determined by periodic measurements of either peroxide value or sensory evaluation of the rancid odor. The Rancimat method is based

on increased electrical conductivity of small molecules formed as a consequence of lipid autoxidation (Pike 1998). These tests suffer from the disadvantage that at elevated temperatures, the reaction rate of autoxidation changes (e.g. decrease in oxygen solubility).

1.3.2.2. Schaal oven test

The Schaal oven test is an example of a low temperature test in which results correlate most closely with ambient temperature storage (Pike 1998). The milder temperatures used in this test do not substantially change the mechanism of autoxidation relative to that of room temperature storage. In this test a known amount of oil or fat is heated at 60-70°C with autoxidation determined by quantifying peroxides present in the sample. The peroxide value, commonly used to measure primary autoxidation products in bulk lipid substrates (Frankel 1993), serves to predict the formation of secondary products that are responsible for undesirable off-flavors, odors and undesirable color changes in food products that undergo lipid autoxidation.

Lipid peroxides are measured by reaction with potassium iodide (KI) in acid solution; iodine is generated in direct proportion to the amount of peroxides in the sample and is quantified by reaction with sodium thiosulfate. Starch is used as the indicator for the presence of iodine (Equations 23 and 24) (Pike 1998).

$$ROOH + K^+I^- \xrightarrow{H^+} ROH + K^+OH^- + I_2 \qquad \text{(Eq. 23)}$$

$$I_2 + \text{starch} + 2\ Na_2S_2O_3 \quad \underset{\text{(colorless)}}{2NaI + \text{starch} + Na_2S_4O_6} \qquad \text{(Eq.24)}$$
(blue)

Peroxide value is usually reported as milliequivalents (mEq) of lipid peroxide per kg lipid.

Lipid autoxidation can be evaluated by determining the induction period (I.P.), which is the time required to produce a detectable level of rancidity or for onset of a sudden increase in the rate of autoxidation (Frankel 1993). Figure 1.10 shows peroxide formation and decomposition as a function of time. The I.P. of an antioxidant-treated substrate is compared to that of a control (no antioxidant added), with longer induction periods indicating better antioxidant activity. The protection factor is defined as the ratio of the I.P. of the antioxidant treated sample compared to the control.

The Schaal oven test is highly empirical and subject to some laboratory-to-laboratory variation. Determination of the endpoint of titration is subjective and thus, may lead to variations in data. Iodine also can react with carbon-carbon double bonds as well as with peroxides, which may result in an apparent decrease in peroxide value. Nevertheless, peroxide value is widely used because it is applicable to most fats and oils and easy to perform.

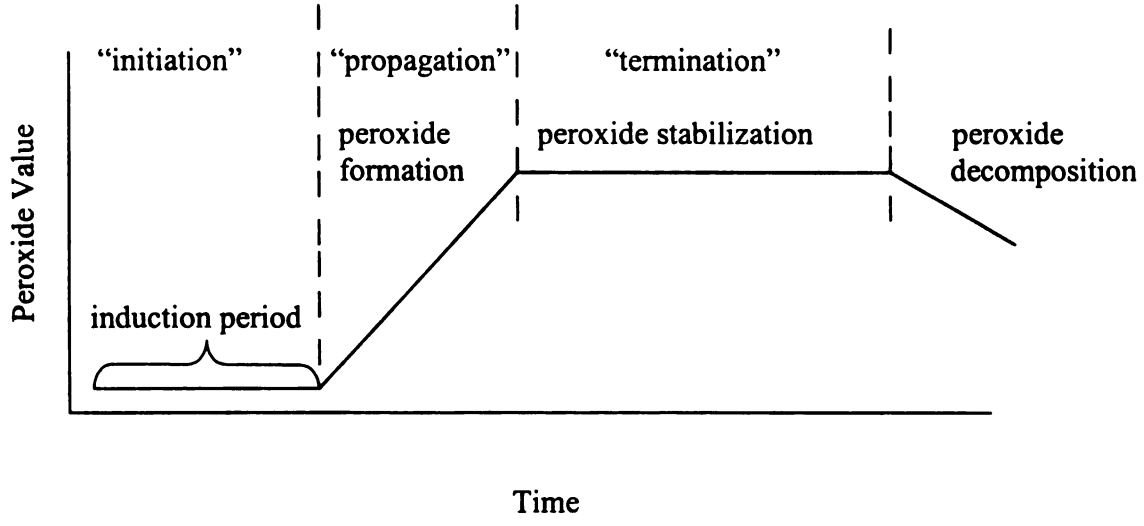


Figure 1.10 Peroxide formation and decomposition as a function of time (adapted from DeMan 1999)

1.3.2.3. Analysis of Carbonyl Compounds by Gas Chromatography

Gas chromatography (GC) has been used to quantify volatile, secondary oxidation products of autoxidation. Carbonyl products correspond to the decomposition of hydroperoxides in which acids, aldehydes, ketones or other carbonyl products are formed. Carbonyl content is often used to evaluate autoxidation because these compounds contribute to flavor deterioration of a lipid-containing food, thus correlating to sensory evaluation.

Headspace analysis is commonly used to monitor lipid autoxidation by the chromatographic analysis of the sample held in a closed container (Frankel 1998). After the sample is heated and vaporized into the gas phase, an aliquot of the gas phase headspace is injected directly into a GC column. The concentration of volatile compounds in the headspace is proportional to the concentration of volatiles in the sample. Identity of compounds is tentatively based on the co-elution with standards.

The benefits of GC are generally enhanced when coupled with mass spectrometry (GC-MS). In GC-MS, the identity of compounds within peaks is confirmed on the basis of mass. Mass spectral analysis of peaks also provides supporting evidence for purity of the material within a peak. Gas chromatography can be used to assess a wide variety of food samples without altering the product by extraction. One disadvantage of GC, however, is that volatiles may be lost during heating. Additionally, GC analysis of carbonyls requires costly equipment which must be maintained by a trained operator.

1.3.2.4. Anisidine value

Anisidine value is a spectrophotometric technique that determines the amount of aldehydes and related carbonyl compounds formed during autoxidation of fats and oils (Pike 1998). The aldehydes react with *p*-anisidine to form a chromagen, the concentration of which is determined by absorbance at 350 nm. Anisidine values are advantageous because non-volatile carbonyl compounds can be detected. Anisidine value and peroxide value are often used together to give a totox value (anisidine value + (2 x peroxide value)) as an indicator of total autoxidation of a sample by evaluating both the primary and secondary oxidation products. Anisidine and totox values are widely used throughout Europe, whereas peroxide value is used most commonly in the U.S. (Stauffer 1996).

1.3.2.5. Thiobarbituric acid

The 2-thiobarbituric acid (TBA) test is one of the oldest and most commonly used tests for measuring lipid autoxidation, especially in determinating *warmed-over-flavor* of cooked meats (Melton 1983; Crackel and others 1988; Hoyland and Taylor 1991). Cooked meats oxidize at an accelerated rate compared to raw products, and primary autoxidation products (lipid hydroperoxides) rapidly decompose into secondary oxidation products (e.g. aldehydes) associated with rancidity.

The formation of malondialdehyde (MDA) in cooked meat products is often used as in indicator of lipid autoxidation. The hydrogen β to the peroxide group of unsaturated peroxides is believed to undergo cyclization to a five-membered ring, which then decomposes to form MDA (Figure 1.11) (Dahle and others 1962). In later studies, it was

reported that other primary and secondary lipid oxidation products, such as five-membered hydroperoxy epidioxides and 1,3-dihydroperoxides, are precursors of MDA (Frankel and Neff 1983).

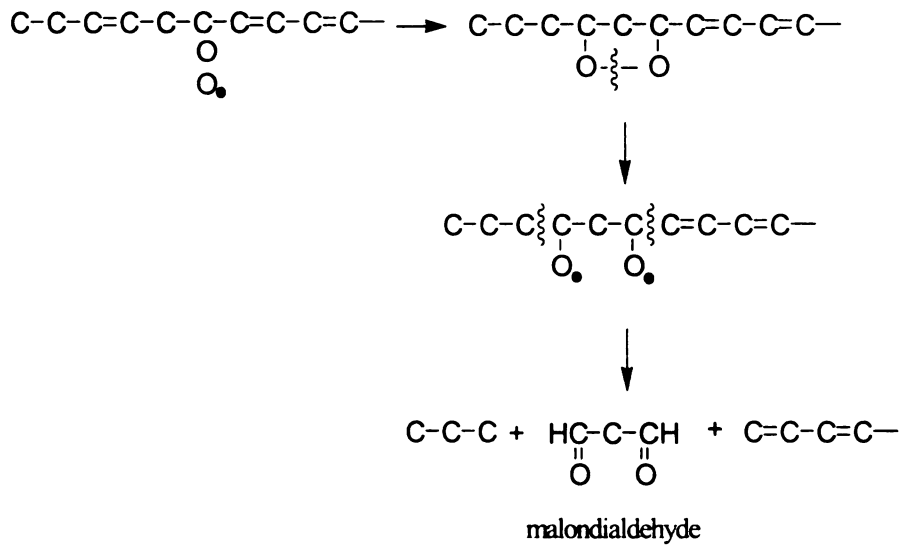


Figure 1.11 Malondialdehyde formation

One molecule of MDA reacts with two molecules of TBA with the elimination of two molecules of water (Figure 1.12) to yield a pink compound, which is quantified spectrophotometrically at 532-535 nm (Antolovich 2001) and used as an indicator of autoxidation.

In addition to MDA, there are many other secondary autoxidation products that can react with TBA, including ketones and other carbonyls, which are responsible for rancid off-flavors and odors (Tarladgis and others 1960; Crackel and others 1988; Hoyland and Taylor 1991). Therefore, the results of this reaction are often reported as

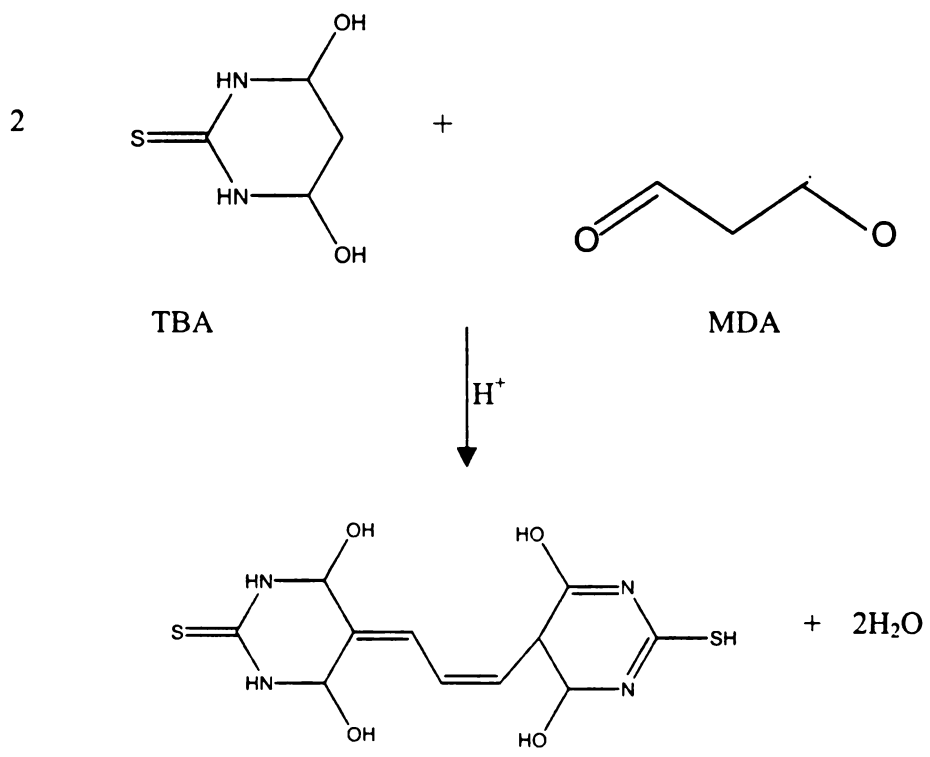


Figure 1.12 Chromophore formed by the condensation of MDA with TBA

TBA reactive substances (TBARS) (mg MDA/kg sample). TBARS are calculated as shown in Equation 25:

$$\text{TBARS} = A_{532nm} \times K \qquad \text{(Eq. 25)}$$

$$\text{where, } K = \frac{\text{conc. in moles/5mL of distillate}}{\text{Absorbance}} \times \text{M.W. MDA} \times \frac{10^7}{\text{wt. of sample}} \times \frac{100}{\%\text{ recovery}}$$

The distillation constant, K, is calculated from a standard calibration curve using tetramethoxypropane (TEP), a MDA precursor.

The whole sample, or less frequently, a lipid extract is examined using the TBA test. By using the whole sample, oxidation products of protein-bound lipids and phospholipids can be measured. While the whole sample method is the most quantitative, it requires numerous solvent extractions which can be very time consuming (Sinnhuber and Yu 1958). In the past, volatiles from the oxidized sample were collected by passing steam through the slurry. Since then, the distillation technique has been simplified by directly heating the samples in Kjeldahl distillation flasks with simultaneous collection of the volatile compounds, including MDA. A clear, aqueous distillate of the sample is collected, which is reacted with TBA and the product is measured spectrophotometrically. With this procedure, interference mechanisms/products are minimized and the accuracy of the test is improved.

Advantages of the TBA test include sensitivity, accuracy and reproducibility. However, the TBA test has limited specificity (e.g. MDA may react with other oxidation products), requires a large sample size and can be very labor-intensive. Numerous reports have correlated TBA numbers with sensory evaluation, many of which contradict each other (Hoyland and Taylor 1991). This may be due to different training practices of

the panel. Additionally, variation in TBARS value may also be due to different sources of meat, storage conditions, processing and packaging. Therefore, the TBA test should only be used in relative terms when comparing antioxidant activity between laboratories (Tarladgis and others 1960; Gray and Monahan 1992). The TBA distillation procedure is still the most commonly used method for evaluation of lipid autoxidation in ground beef and has been chosen for this study.

1.3.2.6. Liposome Screening Assay for Lipid Oxidation

Evaluating large numbers of antioxidants in food products can be quite time consuming. By using a screening method coupled with an appropriate model system, analysis time can be reduced. Numerous model systems have been used including linoleic acid and methyl linoleate micelles and liposmes. The liposome screening assay used in this study is a rapid and sensitive method to evaluate antioxidant compounds in a model system which is representative of biological membranes (Arora and others 1997).

Other model systems, such as linoleic acid and methyl linoleate micelles, are not favored as food models because they are not structurally representative of membrane phospholipids or triglycerides (Frankel 1993). Liposomes, however, mimic membrane lipid structures dispersed in foodstuffs. Liposomes are a type of emulsion system in which "phospholipids, when dispersed in water, form multi-layers consisting of bilayers between the water phase and the innermost layer next to the oil phase" (Frankel 1998) (Figure 1.13). They mimic biological membranes without the presence of pro-oxidant enzymes or endogenous antioxidants that could confound interpretation of results (Arora and others 1997). In addition, their composition and structure can be carefully controlled. Liposomes, therefore, are often used as models when determining antioxidant efficiency.

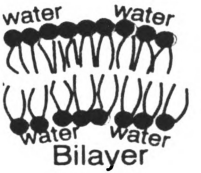
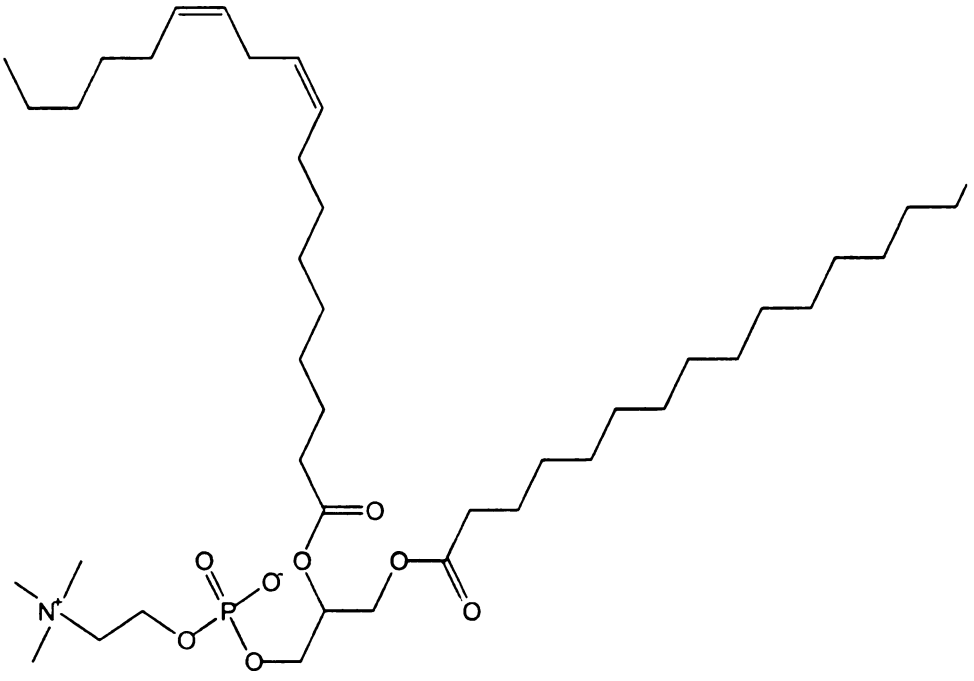
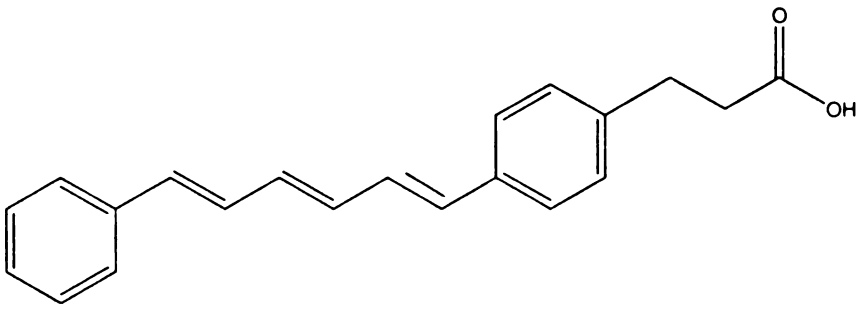


Figure 1.13 Structure of liposome bilayer

Liposomes are classified by size and number of bilayers: small unilamellar vesicles (SUVs) (diameter from 20-50 nm) and large unilamellar vesicles (LUVs) (diameter 60 nm) (Chapman 1984). In addition, multilamellar vesicles (MLVs) form spontaneously when dry phospholipid films swell in water or buffer resulting in the formation of both SUVs and LUVs. Due to the heterogeneity in size and the small proportion of lipid exposed to the aqueous medium of the internal bilayers, the MLV system is a poor model for lipid autoxidation in membranes. In contrast, approximately 70% of the total lipid is located in the outer leaflet of SUVs (Chapman 1984). Due to less ordered packing of the SUV, the structure is constrained between the phospholipid head groups, thus, allowing penetration of pro-oxidants. Therefore, LUVs consisting of phospholipids found in biological membranes are the preferred membrane model for lipid autoxidation studies. The lipid substrate used in this model system is 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC), with the saturated fatty acid at the *sn*-1 position, an unsaturated fatty acid at the *sn*-2 position and a phosphate containing polar group at the *sn*-3 position (Figure 1.14).



1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine



3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA)

Figure 1.14 Structures of molecules used in liposomal assay : 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine and 3-(p-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA)

Oxidation in the liposomal suspension is determined by fluorescence spectroscopy which offers great advantages as an analytical tool when combined with a defined lipid substrate. The analysis can be run using <1 μmol of pure lipid, and a small concentration of fluorescent probe to lipid (1:350), allowing for high sensitivity and minimal membrane perturbation. In addition, fluorescence spectroscopy is 100-1000 times more sensitive than other spectrophotometric techniques (Arora and others 1997; Strasburg and Ludescher 1995). The speed of the assay is also beneficial in that iron or other free-radical generators can be used to induce lipid autoxidation, thus avoiding high-temperature conditions.

In an aqueous phase, lipophilic and amphilic membrane probes are generally non-fluorescent, but when embedded in the hydrophobic environment of a lipid bilayer, they are highly fluorescent. Arora and Strasburg (1997) incorporated the probe, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA), (Figure 1.14), into liposomes to serve as a reporter of lipid autoxidation. The conjugated double bonds of this probe are highly susceptible to react with free radical intermediates of lipid autoxidation. The polar substituent of the probe is localized at the lipid-water interface of the membrane and the hydrophobic tail portion lies parallel to the acyl chains of the membrane. In addition, DPH-PA is relatively photostable and exhibits strong fluorescence in lipid environments. Reaction of the probe with free radicals produced by membrane autoxidation results in a loss of conjugation and consequencly, a decrease in fluorescence. Presence of an antioxidant decreases the rate of fluorescence decay with the rate of fluorescence decay reflecting the rate of autoxidation as well as antioxidant efficacy.

1.4. Natural vs. Synthetic Antioxidants

Demand for extended-shelf-life foods in which antioxidants are the basis for protecting flavor and color in lipid containing foods has grown (Langen 2002). Natural antioxidants such as rosemary and sage extracts, polyphenolic catechins of green tea, tocopherols and flavonoids are more accepted by consumers in that perception of the risk of potential toxicity issues is minimized. (Mukhopadhyay 2000). Even though natural antioxidants are more widely accepted than synthetic antioxidants by consumers and the food industry, their adoption has been limited. Inconsistent availability of the plant source and cost of the crop are major concerns. Problems during harvest, such as contamination or taints in the finished product may also result in a substandard product. Extracts of plant materials rich in antioxidants are frequently used rather than more costly purified antioxidants. However, extracts may have variable concentrations of antioxidants, and also suffer from off-colors or odors. Caution must be taken when using extracts for antioxidants in that potential mutagens, carcinogens and/or pathogens may be present (Pratt 1996). Therefore, synthetic antioxidants are used more commonly throughout the food industry because they're relatively inexpensive, highly effective, readily available and have a well defined toxicity profile.

Despite the advantages of synthetic antioxidants, heightened concerns have been raised regarding the environmental consequences of using petroleum-based products as starting materials for chemical synthesis. Use of petroleum-based products in organic synthesis produces volatile organic compounds, which can contribute to ground level smog and depletion of the ozone layer. An environmentally friendly alternative to these

petroleum-based products involves the use of plant-derived ingredients for the synthesis of novel antioxidants.

Phytic acid (inositol hexaphosphate) (Figure 1.15) is a naturally occurring component of plant fiber with antioxidant properties. It chelates iron and/or other pro-oxidant metals, thus retarding hydroxyl radical formation via the Fenton reaction in lipid-containing foods (Lee and Hendricks 1982). It is found in highest concentration in corn (6%), followed by sesame (5%), wheat (3%) and rice (2%) (Quillin and Quillin 2001).

Phytic acid is also found in high abundance in the steeping liquor from grain processing, which presents a disposal problem for the industry (Hoseney 1986; Wood 2000). Steeping liquor may be concentrated and sold for animal feed, but environmental concerns about high levels of phosphorus derived from phytic acid in animal excrement limit the value of this product. High levels of phosphorus in water run-off from fields enhances biological oxygen demand in lakes, rivers and streams leading to a decline in water activity.

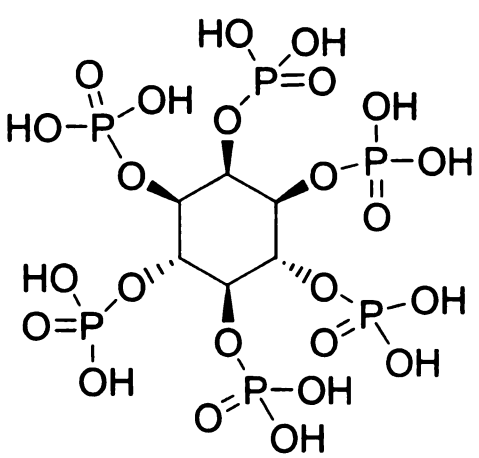


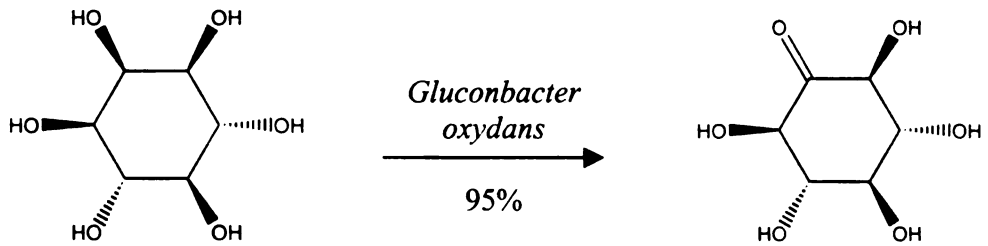
Figure 1.15 Phytic Acid

One option for limiting the level of phytic acid in the environment is to extract it from the steeping liquor and convert it to a compound or compounds with increased value. Phytic acid can be isolated from the grain, dephosphorylated, and hydrolyzed to *myo*-inositol from which 1,2,3,4-tetrahydroxybenzene (THB) (Hanson and others 1999) and 2,3,4,5-tetrahydroxybenzoic acid (THBA) (Stueben 2002) are formed. Figures 1.16 (a) and (b) show the reaction scheme from which THB and THBA are synthesized. This synthesis can be done relatively quickly, giving consistent results at low cost.

The characteristics of an effective antioxidant are found in the compounds derived from phytic acid. Ideally, effective antioxidants are considered to have good solubility in the fat or water phase, good dispersion throughout the food, no harmful physiological effects, and no objectionable flavors, colors or odors. In addition, they should be effective in low concentrations, readily available, stable throughout processing and economical (Dugan 1976; Giese 1996).

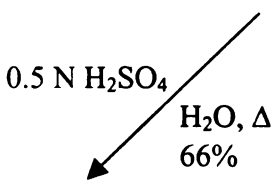
Phenolic compounds donate a hydrogen atom to free radicals and remain relatively stable due to delocalization of the unpaired electron. Previous studies suggest that compounds with multiple hydroxyl groups attached to the aromatic nucleus will be potent antioxidants (Cuvelier and others 2000). We hypothesized that the polyphenolic compounds, THB and THBA, will show equivalent or greater antioxidant activity compared to commercial antioxidants used currently in the food industry. In this study, THB and THBA are compared to alpha-tocopherol, gallic acid, protocatechuic acid, propyl gallate and TBHQ for antioxidant activity in bulk lipids, ground beef and liposome systems.

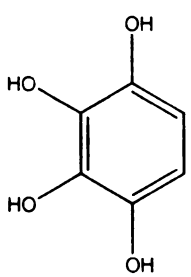
(a)



myo-inositol

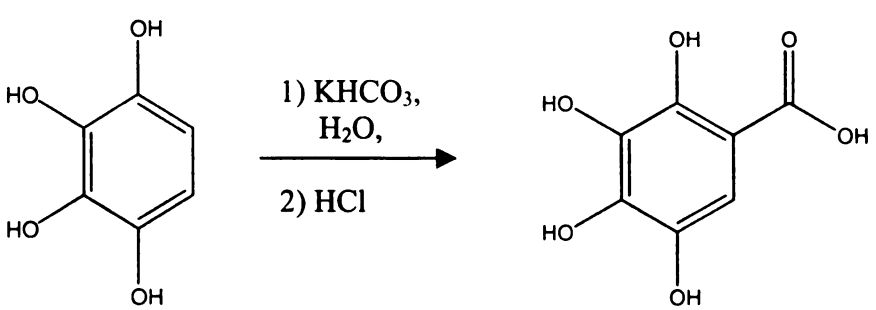
myo-2-inosose





1,2,3,4-tetrahydroxybenzene (THB)

(b)



1,2,3,4-tetrahydroxybenzene (THB) 2,3,4,5-tetrahydroxybenzoic acid (THBA)

Figure 1.16 (a) Synthesis of THB and THBA from phytic acid (Hanson and others 1999) (b) Synthesis of THBA from THB (Stueben 2002)

1.5. Antioxidants as Multi-functional Food Preservatives.

An estimated 76 million cases foodborne disease results in illnesses, including 325,000 hospitalizations and 5,200 deaths occurring in the United States each year (Mead and others 1999). According to the Economic Research Service, the estimated cost (medical costs, productivity losses from missed work, and losses from premature deaths) of foodborne diseases caused by *Campylobacter*, *Salmonella* (nontyphoidal serotypes only), *Escherichia coli* O157:H7, *E. coli* non-O157:H7 STEC, and *Listeria monocytogenes,* is $6.9 billion annually (U.S. Department of Agriculture 2002). Based on heightened interest from consumers and the food industry, we have chosen to test the antimicrobial activity of THB and THBA against *E. coli* O157:H7, *Salmonella* and *L. monocytogenes*.

Food preservatives include food additives that are antimicrobial agents, anti-browning agents and antioxidants (Davidson 2001). Antimicrobial agents are chemical compounds, either added to or already in food, that inhibit microbial growth or inactivate microorganisms, thus preserving safety and quality of the product.

Most antimicrobial agents are bacteriostatic or fungistatic rather than bactericidal or fungicidal. Many microbial factors, including inherent resistance, growth rate, antagonism and cellular composition must be considered when using an antimicrobial agent. In addition, many intrinsic and extrinsic factors associated with the food are responsible for an antimicrobial agent's effectiveness. There are many interacting reactions which take place simultaneously, making it difficult to pinpoint the exact target of the antimicrobial agent in a food system (Davidson 2001). It is thought, however, that the main targets of antimicrobial agents are the cell wall, cell membrane, metabolic

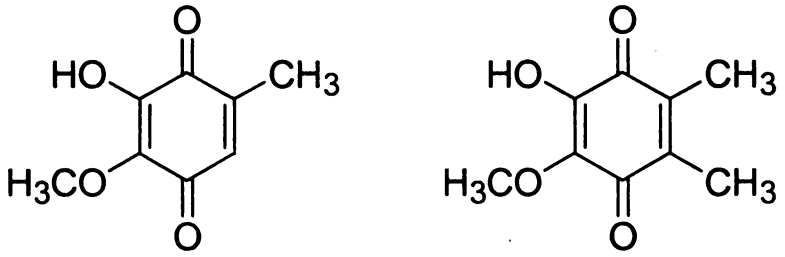
enzymes, protein synthesis, and genetic systems. For example, organic acids have been shown to be most effective in their undissociated form, in which they penetrate the lipid bilayer of the cell membrane to disrupt cell integrity (Davidson 2001).

1.5.1. Antimicrobial Evaluation of Phenolic Compounds

Phenolic compounds were first recognized for their antimicrobial activity in 1867, when Joseph Lister used phenol for sanitizing surgical equipment (Davidson 1993). Phenol is currently used as a disinfectant and antiseptic and is also a component of mouthwash and throat lozenges. However, skin exposure to high levels of phenol can lead to liver damage, diarrhea, dark urine and hemolytic anemia (Agency for Toxic Substances and Disease Registry 1999). More recently, phenolic compounds that are commonly used as antioxidants have been assessed for possible antimicrobial activity. Parabens including *p*-hydroxybenzoic acid are the most common examples of antimicrobial phenolic compounds. They are believed to increase membrane permeability (Davidson 2001). Phenolic antioxidant compounds including BHT, BHA, propyl gallate and TBHQ also show antimicrobial activity (Davidson 1993). For example, Tuncel and Nergiz (1993) found that BHA at a concentration of 400 μg/mL inhibited *Staphylococcus aureus, Bacillus cereus, E. coli, and S. typhimurium* when grown in trypticase soy broth at 37°C for 18 h. BHA also showed antifungal activity at 200 ppm against *Aspergillus*, *Penicillium*, and *Geotrichum* in glucose salt broth when incubated at 32°C, for 85 h (Ahmad and Branen 1981).

We hypothesized that THB and THBA would have antimicrobial activity, based on structural similarity to the other phenolics and to the antimicrobial compounds

fumigatin and aurantiogliocladin (Figure 1.17). The latter compounds consist of quinone structures similar to those that could be formed by phenolic compounds THB and THBA.



Fumigatin Aurantiogliocladin

Figure 1.17 Structures of common antimicrobial agents

1.5.2. Bacteria Cell Wall Composition and Susceptibility to Antimicrobial Agents

Antimicrobial activity is partly related to polarity. An antioxidant compound must be sufficiently lipophilic to pass through the cell membrane and once inside, it must also be sufficiently water-soluble to interact with cell components. Parabens have been shown to effectively disrupt cell integrity. The mechanism by which phenolic antioxidant compounds inhibit microbial growth is likely similar to that of parabens (Davidson 2001).

Gram positive bacteria are generally more sensitive to antioxidants than Gram negative bacteria (Tuncel and Nergiz 1993). Davidson (1993) reported that hydrophobic compounds are more effective antimicrobial agents against Gram positive bacteria whereas Gram negative bacteria are more susceptible to hydrophilic compounds. These differences between Gram positive and Gram negative bacteria result from differences in cell wall composition.

The cell wall in Gram positive bacteria contains a thick layer of peptidoglycan which is tightly bound to teichoic acid and lipoteichoic acid. The cell wall is pressed tightly against the cell membrane with very little space between, making such organisms easy to kill. In contrast, Gram negative bacteria have a layer of lipid polysaccharide as part of their cell wall. The outer membrane is a thin shell of peptidoglycan, lipoproteins and liposaccharides.

Unlike Gram positive bacteria, Gram negative organisms have extensive space between the peptidoglycan layer and the cell membrane. The outer membrane serves as a partial chemical sieve by allowing only relatively small molecules to penetrate through the porins. As a result, Gram negative bacteria are more resistant to antibiotics which can't penetrate the lipid polysaccharide layer as readily.

1.5.3. Bacterial Pathogens

1.5.3.1. Listeria monocytogenes

L. monocytogenes is a Gram positive, psychrotrophic, facultative anaerobe that is motile by means of flagella at ambient temperatures. It is of special concern to the food industry because of its ability to grow at refrigeration temperatures (2-4°C), although optimum growth occurs between 30-37°C (Seeliger and Jones 1984; Swaminathan 2001). As a result, ready-to-eat foods such as soft cheeses, frankfurters, and delicatessen meats are among the most common foods implicated in outbreaks of listerious. *L. monocytogenes* can also multiply and survive in or on foods for extended periods of time under adverse conditions including low pH and high salt concentrations. *L. monocytogenes* is unique in that it is more acid-tolerant than many other foodborne

pathogens, with growth occurring at pH values as low as pH 4.3 (Lou and Yousef 1999), although most strains grow best at neutral to slightly alkaline pH (Seeliger and Jones 1984). It is also salt-tolerant, capable of growing at salt concentrations up to 10% and surviving in 23% (a_w 0.83) brine solutions for extended periods (Shahamat and others 1980). Finally, *L. monocytogenes* is unique in that it resists the effects of freezing, drying and heating without forming spores (Center for Food Safety and Applied Nutrition 2003).

Manifestations of listeriosis, the foodborne disease caused by ingestion of *L. monocytogenes*, are serious and include septicemia, meningitis and spontaneous abortion. Complications include encephalitis, endocarditis, abscesses and local purulent lesions (Seeliger and Jones 1984). The onset time for systemic listeriosis varies, from as few days to 70 days. Less commonly, this organism causes gastroenteritis with an onset time of 20-27 hours (Swaminathan 2001). The infectious dose of *L. monocytogenes* varies with bacterial strain and with susceptibility of the individual. It is believed, however, that as few as 100 CFU/g may cause disease in susceptible persons such as pregnant women, the elderly and immunocompromised adults (Swaminathan 2001).

Cases of listeriosis are usually sporadic with at least 2,518 illnesses, 2,322 hospitalizations, and 504 deaths occurring each year in the U.S. (Swaminathan 2001).

1.5.3.2. Escherichia coli O157:H7

Escherichia coli is a Gram negative, motile, oxidase-negative, non-spore forming lactose fermenter. There are five types of *E.coli* isolates which can cause disease. They are differentiated based on three major surface antigens, which permit serotyping: the O (somatic), H (flagella), and K (capsule) antigens. Among these isolates,

enterohemorrhagic *E. coli* (EHEC) is the most significant group based on severity of disease, and will be the focus of this discussion.

E. coli O157:H7 predominates in the United States and many other countries (Meng and others 2001). It has been reported that *E. coli* O157:H7 produces large quantities of one or more related potent toxins: the verotoxin and the shiga-like toxin (Meng and others 2001), which may cause a severe and sometimes fatal condition known as hemolytic uremic syndrome (HUS) (kidney failure). *E. coli* O157:H7 is unique compared to other stains of *E.coli* in that is unable to grow at temperatures 44.5°C in *E. coli* broth, unable to ferment sorbitol within 24 hrs, and is unable to produce β-glucuronidase (Meng and others 2001). Unlike many *E.coli* strains, *E.coli* O157:H7 is acid tolerant (growth at pH minimum 4.0-4.5), and thus is able to grow in fermented and other low acid foods.

Vehicles most commonly associated with *E.coli* O157:H7 infections are undercooked ground beef and less frequently, fresh produce, unpasteurized milk and juice (e.g. apple cider). The organism is commonly found in the intestinal tract of cattle; thus, slaughter practices must be carefully monitored so that the meat does not become contaminated. Raw milk may become contaminated if fecal matter containing *E.coli* is present on the udders at the time of milking (Center for Food Safety and Applied Nutrition 2003). Produce may become contaminated by exposure to manure used as fertilizer, and juice prepared from fruit which has fallen to the ground may likewise become contaminated by fecal matter. The infectious dose of *E.coli* O157:H7 is unknown, but it is believed that as few as 10 cells (dose similar to *Shigella*) (Center for Food Safety and Applied Nutrition 2003) may cause disease, with an onset time of 1-2

days. Symptoms include severe cramping and bloody diarrhea lasting approximately eight days. All individuals are at risk, but children and the elderly are most susceptible. Up to 15% of infected children develop HUS whereas the elderly usually develop thrombotic thrombocytopenic purpura (TTP) (abnormal decrease in platelets), fever and neurologic symptoms. Hemolytic uremic syndrome is the primary cause of kidney failure in children in the United States with most cases caused by *E.coli* O157:H7 (Center for Food Safety and Applied Nutrition 2003).

The CDC has reported that approximately 73,000 cases of infection and 61 deaths occur each year in the US (Center for Food Safety and Applied Nutrition 2003). Prevention of *E.coli* O157:H7 infection is based on its sensitivity to heat. Cooking meat products to an internal temperature of at least 74°C (165°F) for several seconds will inactivate the pathogen (Meng and others 2001). Pasteurization of milk and juices is sufficient to eliminate *E.coli* from these products. However, some food products such as lettuce or alfalfa spouts, will lose their acceptability upon excessive heating. Therefore, it is important to understand the growth and development of the organism and to find ways to reduce contamination.

1.5.3.3. Salmonella spp.

Salmonella spp. are Gram-negative, motile, non-sporeforming, facultative anaerobic, catalase-positive, oxidase-negative rods belonging to the family *Enterobacteriaceae.* The ability of *Salmonella* to metabolize nutrients by both respiratory and fermentative pathways makes this pathogen a concern in modified-atmosphere and vacuum-packaged foods, and fermented raw milk products (high acid

tolerance). *Salmonella* spp. are widespread in animals, especially in poultry and swine. Food sources include eggs, raw meats, and poultry. Infection occurs primarily through the fecal/oral route (Center for Food Safety and Applied Nutrition 2003). *S.* Enteritidis is unique in that the organism is present inside of the egg (in the yolk) suggesting vertical transmission from chicken to egg (Center for Food Safety and Applied Nutrition 2003). The CDC estimates 75% of *S.* Enteritidis outbreaks are associated with consumption of raw or inadequately cooked Grade A whole shell eggs (Center for Food Safety and Applied Nutrition 2003).

Though the organism grows optimally at 37°C, some strains grow at elevated temperatures (54°C). The pH range for growth of *Salmonella* extends from 4.5-9.5, with an optimum pH for growth of 6.5-7.5 (D'Aoust 1989). Although *Salmonella* growth is inhibited in 3-4% brine solutions, some salt tolerance has been observed at increasing temperatures (D'Aoust 1989). *Salmonella* also survive for prolonged periods of time in foods stored at freezing and ambient temperatures (D'Aoust 1989). Thus, the organism can adapt to and survive under many conditions.

Typhoid and enteric fevers as well as gastroenteritis are common diseases caused by *Salmonella*. The infectious dose of *Salmonella* is as few as 15-20 cells with usual onset time of 48 hours. Acute symptoms include nausea, vomiting, abdominal cramps, bloody diarrhea, fever and headache. More chronic consequences include reactive arthritis 3-4 weeks after onset of acute symptoms. Acute symptoms may last 1-2 days or longer, depending on dose and the individual. Disease is caused by the penetration and passage of *Salmonella* from the gut lumen into the epithelium of the small intestine where inflammation occurs. All age groups are susceptible, but symptoms are most severe in

children and the elderly. Among the elderly in hospitals/nursing homes, *Salmonella* exhibits a mortality rate of approximately 3.6% (Center for Food Safety and Applied Nutrition 2003).

1.6. Methodology for Evaluating Antimicrobial Activity

The rise in foodborne illnesses is due partly to changes in food preparation (e.g. microwave ovens), increases in meals prepared outside the home (e.g. fast food), more susceptible populations, and increased antibiotic resistance. These changes have led to increased consumer concerns for the safety of food; in response, microbial analysis of food samples is studied more readily. Various measures have been implemented to reduce risk of contracting foodborne illness. For example, the development of Hazard Analysis and Critical Control Point (HACCP) programs and guidelines for safe handling and food preparation minimize potential contamination.

Conventional methods for evaluating microbial growth include isolating and enumerating cultures on selective media. More advanced methods include antibody-based and nucleic acid-based detection assays, many of which are now semi-automated for more rapid detection.

Methods for testing antimicrobial resistance include agar screening, disk diffusion, broth dilution and the E test® (Horseheath, Cambridge CB1 6RG, United Kingdom). Agar screening assesses the growth of bacteria when the antimicrobial agent is added to the agar. In the disk diffusion method, a paper disk containing a known amount of the antimicrobial agent is added to a seeded agar plate. After incubation, the zone of bacterial inhibition around the disc is measured. The broth dilution test is

commonly used in laboratories to determine the minimum inhibitory concentration (MIC) (the lowest concentration that prevents bacterial growth at 35°C for 24 and 48 hours). Some factors which influence diffusion testing include inoculum density, agar composition and depth, time and temperature of incubation, and concentration of the antimicrobial agent.

The E test® is a newer method that combines dilution and diffusion. A plastic strip with various dilutions of the antimicrobial agent is placed on a seeded agar plate. After incubation, the antimicrobial agent from the strip that inhibits bacterial growth will form an elliptical zone of inhibition. The E test® generates an accurate and reproducible MIC. Testing parameters, such as different bacterial strains, diluents, incubation conditions and media formulations all account for lab to lab variability. Therefore, results from different labs need to be carefully interpreted.

In this portion of our study, our objective was to determine if THB and THBA possess antimicrobial activity. They share structural similarities with the antimicrobial compounds fumigatin and aurantiogliocladin; therefore, we hypothesize they will also have antimicrobial properties. The diffusion disk assay was used initially to screen for activity, followed by MIC determination.

In this study we illustrate how phytic acid, a by-product of grain processing, could be used to synthesize active antioxidant and antimicrobial compounds. These compounds can stabilize the shelf life of lipid-containing foods, and give rise to a value-added product. By expressing both antimicrobial activity and antioxidant activity, THB and THBA offer several advantages to the food industry. These antioxidants derived from

by-products of grain processing provide an inexpensive and efficient tool for food preservation.

CHAPTER TWO

ANTIOXIDANT ACTIVITY OF 1,2,3,4-TETRAHYDROXYBENENE AND 2,3,4,5-TETRAHYDROXYBENZOIC ACID

2.1 INTRODUCTION

Lipid autoxidation is the primary mechanism responsible for deterioration of low moisture foods and is a major contributor to deterioration of other food products including pre-cooked convenience foods. Free radicals generated by lipid autoxidation give rise to off-flavors and odors in lipid-containing foods as well as a reduction of the nutritional value of the lipid, and loss of fat-soluble vitamins, carotenoids, or proteins (Jacobson 1999; Mukhopadhyay 2000). In some cases, free radical-mediated lipid oxidation may yield toxic autoxidation products (Nawar 1996). The onset of perceptible rancidity resulting from autoxidation may be delayed by judicious use of antioxidants.

Demand for extended-shelf-life foods in which antioxidants are the basis for protecting flavor and color in lipid containing foods has grown (Langen 2002). Natural antioxidants are preferred by the food industry over synthetic antioxidants in terms of safety and consumer acceptance (Mukhopadhyay 2000). Even though natural antioxidants are more widely accepted than synthetic antioxidants by consumers, their adoption by the food industry has been limited. Volatility in supply of the plant source and cost of the crop are major concerns. Problems during harvest, such as contamination or taints in the finished product may also be responsible for a substandard product. Extracts of plant materials rich in antioxidants are frequently used rather than more costly purified antioxidants. However, extracts may have variable concentrations of

antioxidants, or they may suffer from off-colors or odors. Caution must be taken when using extracts for antioxidants in that potential mutagens, carcinogens and/or pathogens may be present (Pratt 1996). Therefore, synthetic antioxidants are used more commonly throughout the food industry because they're relatively inexpensive, highly effective, consistently available and have a well defined safety/toxicity profile.

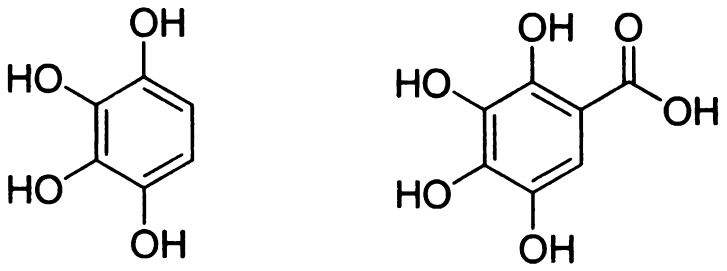
Despite the advantages of synthetic antioxidants, there has been rising concern with respect to environmental consequences of using petroleum-based products as starting material for chemical synthesis. Use of petroleum-based products in organic synthesis results in production of volatile organic compounds, which have been found to contribute to ground level smog as well as to the depletion of the ozone layer. In addition, chronic exposure of workers in the chemical industry to these compounds places their health in jeopardy. An environmentally friendly alternative to this use of petroleum-based products involves the use of plant-derived ingredients for the synthesis of novel antioxidants.

Phytic acid is a naturally occurring component of plant fiber with antioxidant properties. It chelates iron and or other pro-oxidant metals, thus retarding hydroxyl radical formation via the Fenton reaction in lipid-containing foods (Lee and Hendricks 1995). It is found in highest concentration in corn (6%), followed by sesame (5%), wheat (3%) and rice (2%) (Quillin and Quillin 2001).

Phytic acid is also found in high abundance in the steeping liquor from grain processing, which presents a disposal problem for the industry (Wood 2000). Steeping liquor may be concentrated and sold for animal feed, but environmental concerns about high levels of phosphorus derived from phytic acid in animal excrement limit the value of

this product. High levels of phosphorus in water run-off from fields enhances biological oxygen demand of lakes, rivers and streams and contributes to deterioration of water quality.

In this study, we show the potential for use of phytic acid as starting material for the synthesis of the novel compounds 1,2,3,4-tetrahydroxybenzene (THB) and 2,3,4,5-tetrahydroxybenzoic acid (THBA) (Figure 2.1). Previous studies suggest that increasing the number of hydroxyl groups attached to the aromatic nucleus increases antioxidant activity (Cuvelier and others 2000). Substituting phenolic compounds with three hydroxyl groups generally increases antioxidant activity compared to those having only two hydroxyl groups. Gallic acid with three hydroxyl groups is more effective than protocatechuic acid (PCA) with only two hydroxyl groups (Pokorny 1987; Cuvelier and others 1991). The polyhydroxylated aromatic structures of THB and THBA suggest that they may function as highly effective free-radical scavenging antioxidants.



THB THBA

Figure 2.1 1,2,3,4-tetrahydroxybenzene (THB) and 2,3,4,5-tetrahydroxybenzoic acid (THBA)

The objectives of this study were to compare antioxidant efficacy of THB and THBA with several commonly used commercial antioxidants, and to evaluate activity with structurally related compounds. Antioxidant activity was evaluated in three lipid systems: bulk oil, ground beef, and liposomes. The results suggest that both compounds possess high potential for use as antioxidants in food products, provided they have a favorable safety profile.

2.2 EXPERIMENTAL PROCEDURES

2.2.1. Materials

1,2,3,4-tetrahydoxybenzene (THB) and 2,3,4,5-tetrahydroxybenzoic acid (THBA) were obtained from the laboratory of Dr. John Frost, Department of Chemistry, Michigan State University (East Lansing, MI). Fresh ground beef was purchased from the Michigan State University Meat Lab (East Lansing, MI) immediately after grinding. The cattle were harvested 2 days prior to boning. Stripped corn oil was purchased from Acros Organic (Pittsburgh, PA). Lard was purchased from Hasselbach Meats Inc. (Fremont, OH). Synthetic 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine (SLPC) and polycarbonate membranes (diameter 0.75", pore diameter 100 nm) were obtained from Avanti Polar Lipids (Alabaster, AL). The fluorescent probe, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA) was obtained from Molecular Probes (Eugene, OR).

Propyl gallate, protocatechuic acid, gallic acid, alpha-tocopherol, ethylenediaminetetraacetic acid (EDTA), 2-thiobarbituric acid (TBA), celite (acid-washed), calcium phosphate, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), (3-[N-morpholine]propanesulfonic acid) (MOPS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), iminodiacetic acid chelating resin and 1% starch indicator solution were purchased from Sigma Chemical Company (St. Louis, MO). Tert-butylhydroquinone (TBHQ) was purchased from Eastman Chemical Company (Rochester, NY). Dimethylsulfoxide, (DMSO), sodium sulfate, sodium chloride, potassium iodide (KI) (A.C.S. grade) and methanol were purchased from J.T. Baker Co. (Phillipsburg, NJ)). Glacial acetic acid (reagent grade), dichloromethane, hydrochloric

acid, and chloroform (U.S.P. grade) were purchased from EM Science, (Gibbstown, NJ). A saturated solution of potassium iodide was prepared daily using freshly boiled, distilled water, and was kept out of direct light. A 0.1 N standardized sodium thiosulfate solution was purchased from Aldrich Chemical Co. (Milwaukee, WI). A 0.01N sodium thiosulfate solution was prepared by making a ten-fold dilution of the 0.1N standard solution using distilled water. Tris(hydroxymethyl)aminomethane (Tris) base was purchased from GibcoBRL Life Technologies (Grand Island, NY).

Antifoam and glass wool were purchased from Thomas Scientific (Swedesboro, NJ). Ethyl alcohol (200 proof) was purchased from Pharmco Products (Brookfield, CT). Boileezers (non-volatile granules) were purchased from Fisher Scientific (Pittsburgh, PA).

2.2.2. Methods

Fat Determination of Ground Beef: Fat content of the meat was determined using the method of Marmer and Maxwell (1981). Briefly, a dry column was prepared by placing a glass wool plug at the bottom of a glass column (diameter 1.25"), followed by the addition of 10 g celite/calcium phosphate (9:1), which was packed firmly into the column. The sample (5 g) was ground with 20 g sodium sulfate, followed by 15 g celite, using a mortar and pestle. This mixture was then added to the column slowly, packing firmly. The mortar and pestle were rinsed with dichloromethane/methanol (9:1). Approximately 50-60 mL of dichloromethane/methanol (9:1) was added to the column. After the first drop was eluted, an additional 150 mL dichloromethane/methanol (9:1) was added to the column. The filtrate, containing total lipids, was filtered into a 500-mL round bottom flask of predetermined weight and was evaporated using a Büchi

Rotoavapor-R (Flawil, Switzerland) until completely dried before weighing. Percent lipid was determined by the following equation:

% Lipid = dry lipid weight/sample weight x 100 (Eq. 1)

Schaal Oven Test

Peroxide value: Peroxide value measurements were calculated using A.O.C.S. Official method Cd 8-53 with the modification that the starch indicator (1.0 mL) was added prior to titration. All samples were tested in triplicate. Peroxide value (milliequivalents/kg lipid) was calculated using the following equation:

$$PV = \frac{(S-B)(N)(1000)}{\text{weight of sample}} \quad \text{(Eq. 2)}$$

where: B = titration of blank (mL), S = titration of sample (mL) and
N = normality sodium thiosulfate (equivalents/L).

Each antioxidant (TBHQ, propyl gallate, alpha-tocopherol, protocatechuic acid, gallic acid, THB, or THBA) was dissolved in 500 μL DMSO and then mixed into either stripped corn oil or lard to yield final concentrations of 0.01%, 0.005% or 0.001% (wt/wt). The control treatment consisted of 500 μL DMSO without antioxidant. All treatments and controls were incubated in 500-mL beakers at 60^0 +/- 3°C in the dark using a Blue M Electric Company Oven (Blue Island, Illinois). Each data point is reported as the mean, ± 1 SD, of triplicate measurements.

Antioxidant Evaluation: The induction period (I.P.) corresponds to either the time required to reach a level of detectable rancidity or a sudden increase in the rate of autoxidation (Frankel 1993) and indicates antioxidant efficacy. Antioxidant treated samples were compared to the control, in which nothing was added. The protection

factor, defined as the ratio of the I.P. of the antioxidant-treated sample compared to the control) was also determined and used for evaluation.

Thiobarbituric Acid (TBA) Test

Sample preparation: The ground beef was thawed at 4°C for 24 h at which time the sample were prepared. Antioxidant stock treatments (1%) were prepared by dissolving compounds in 2.1 mL ethyl alcohol. Solutions were then mixed into the meat to yield a final antioxidant concentration of 0.01% based on the fat content of the meat (17.5%). The antioxidant stock solutions were first mixed with approximately 5-10 grams of ground beef, which was, in turn, thoroughly mixed with the rest of the sample (total 210 g) by hand. The meat was divided into patties (105 g) that were stored 1-2 hours at 4°C in Petri dishes (9 cm diameter x 1.5 cm thickness) until frying. The patties were cooked on a West Bend electric grill (West Bend, WI) at the highest heat setting to an internal temperature of 72°C ± 2°C (frying time 3 minutes/side). The cooked patties were stored at 4°C covered with plastic wrap and aluminum foil.

TBARS Analysis: Lipid oxidation was determined using the TBA test as described by Tarladgis (1960) and modified by Crackel and others (1988). A 10-g portion of each cooked patty was analyzed daily for TBARS over 5 days, beginning with the zero-time value taken immediately after frying. The sample was homogenized in 10 mL H_2O and 10 mL 1% antioxidant solution (0.5% propyl gallate plus 0.5% EDTA) using a Polytron PCU-2-110 (Brinkman Instruments, Westbury, NY) at speed 5 for 10 seconds. The resulting homogenate was transferred quantitatively to a 250-mL Kjeldahl distillation flask containing 67.5 mL H_2O and 2.5 mL 4 N hydrochloric acid. Boiling beads and

antifoam spray (5 pumps) were added to the distillation flask, and the samples were distilled using a Labconco distillation apparatus (Kansas City, MO) adjusted to a heat setting that kept all flasks at a constant boiling level. A 50-mL aliquot of distillate was collected.

Five mL of the distillate and 5 mL of freshly prepared TBA solution (0.02 M) were mixed in a screw capped test tube. The blank was prepared using 5 mL H_2O + 5 mL TBA (0.02 M). Samples were capped, boiled for 30 minutes, and cooled to room temperature for 10 minutes. Absorbance was then read at 532 nm using a Cary | 3E | UV-Visible spectrophotometer (Varian Analytical Instruments, Walnut Creek, CA). TBARS were calculated using the following equation:

$$TBARS = A_{532nm} * K \text{ (mg MDA/kg sample)} \qquad \text{(Eq. 3)}$$

where, $K = \frac{\text{conc. in moles/5mL of distillate}}{\text{Absorbance}} \times \text{M.W. MDA} \times \frac{10^7}{\text{wt. of sample}} \times \frac{100}{\%\text{ recovery}}$

The distillation constant used for this experiment was determined by previous experiments performed in our lab: K=6.8.

Statistical Analysis: Statistical analysis was performed using the pairwise multiple comparision procedure, Dunnett's method (SAS® Version 7, Cary, NC). Treatment samples were analyzed for significant differences ($p < 0.05$) from the control, in which no antioxidant was added, and to each other. Results are expressed from triplicate experiments.

Liposomal Assay

Large unilamellar vesicles (LUVs) containing the probe DPH-PA were prepared each day, using the extrusion procedure of MacDonald and others (1991) as modified by Arora and others (1997).

Preparation of LUVs: The liposomes [780 μL of 5mg/mL SLPC chloroform stock + 4.5 μL probe of 1 mg/mL stock] were prepared by drying under vacuum using a Büchi Rotoavapor-R (Flawil, Switzerland). The dried lipid was then resuspended in 500 μL MOPS buffer (0.15 M sodium chloride, 0.1 mM EDTA, and 0.01 M MOPS; pH 6.8). After 10 freeze-thaw cycles using a dry ice/ethanol bath, the suspension was passed 29 times through a polycarbonate filter (100 nm pore size) using a Liposofast extruder apparatus (Avestin, Ottawa, Canada). The suspension was transferred to a microcentrifuge tube and kept on ice, out of direct light during analysis.

Sample preparation: A control sample was prepared by mixing 100 μL HEPES-Tris (50 mM, pH 7), 200 μL sodium chloride (1M), and 1.68 mL nitrogen-sparged water, to which a 20-μL aliquot of the LUV suspension was added. The suspension was mixed continuously using a magnetic stir bar for 5 minutes at room temperature, out of direct light, then transferred to a thermostated cuvette holder (24°C) in the spectrofluometer and incubated for 5 minutes. The suspension was then mixed by inversion and fluorescence was read (0 time). The fluorescence intensity was read at 1 minute and thereafter at 3 minute intervals up to 21 minutes.

A Fe (II)-containing pro-oxidant sample, in which no antioxidant was added, was prepared by mixing 100 μL HEPES-Tris (50 mM, pH 7), 200 μL sodium chloride (1 M), and 1.66 mL nitrogen-sparged water, to which a 20-μL aliquot of the LUV suspension

was added. The suspension was mixed continuously using a magnetic stir bar for 5 minutes at room temperature out of light, then transferred to a thermostated cuvette holder (24°C) in the spectrofluometer and incubated for 5 minutes. Oxidation was initiated by the addition of 20 μL of 0.5 mM $FeCl_2$ to yield a final concentration of 5 μM. The suspension was immediately mixed by inversion and fluorescence was read (0 time). The fluorescence intensity was read 1 minute after addition of $FeCl_2$ and every 3 minutes thereafter up to 21 minutes.

Antioxidant treatment samples were prepared by mixing 100 μL HEPES-Tris (50 mM, pH 7), 200 μL sodium chloride (1 M), and 1.64 mL nitrogen-sparged water, to which a 20-μL aliquot of the LUV suspension was added. After 5 minutes of continuous stirring of the sample at room temperature, out of light, an aliquot of antioxidant stock solution (1 mM) was added to yield the desired final antioxidant concentration. The suspension was then transferred to a thermostated cuvette holder (24 °C) in the spectrofluometer and incubated for 5 minutes. Oxidation was initiated by the addition of 20 μL of 0.5 mM $FeCl_2$ to yield a final concentration of 5 μM. The suspension was immediately mixed by inversion and fluorescence was read (0 time). Fluorescence intensity was read 1 minute after addition of $FeCl_2$ and every 3 minutes thereafter up to 21 minutes.

The final concentrations of components in the cuvette were 2.5 mM HEPES-Tris (pH 7), 100 mM sodium chloride, 2.5 μM lipid and 7.4 nM probe. The fluorescence signal was followed with an SLM instruments (Model 4800) spectrofluorometer (Urbana, IL) equipped with data acquisition hardware and software from On-Line Instrument Systems (Bogart, GA). The excitation wavelength used was 384 nm with a slit width of 2

nm. The emitted light was passed through optical filters (KV 418, Schott, Duryea, PA), prior to detection.

Antioxidant Evaluation: The rate of fluorescence decay is an indication of rate of autoxidation. Therefore, the rate in which fluorescence decay is inhibited by the addition of a particular compound is an indicator of its antioxidant efficacy. Relative fluorescence (F_{rel}) was calculated by the following equation:

$$(F_{rel}) = \frac{F}{F_o} = \frac{\text{fluorescence intensity at a given time}}{\text{fluorescence intensity at 0 min}} \qquad \text{(Eq. 4)}$$

2.3 RESULTS

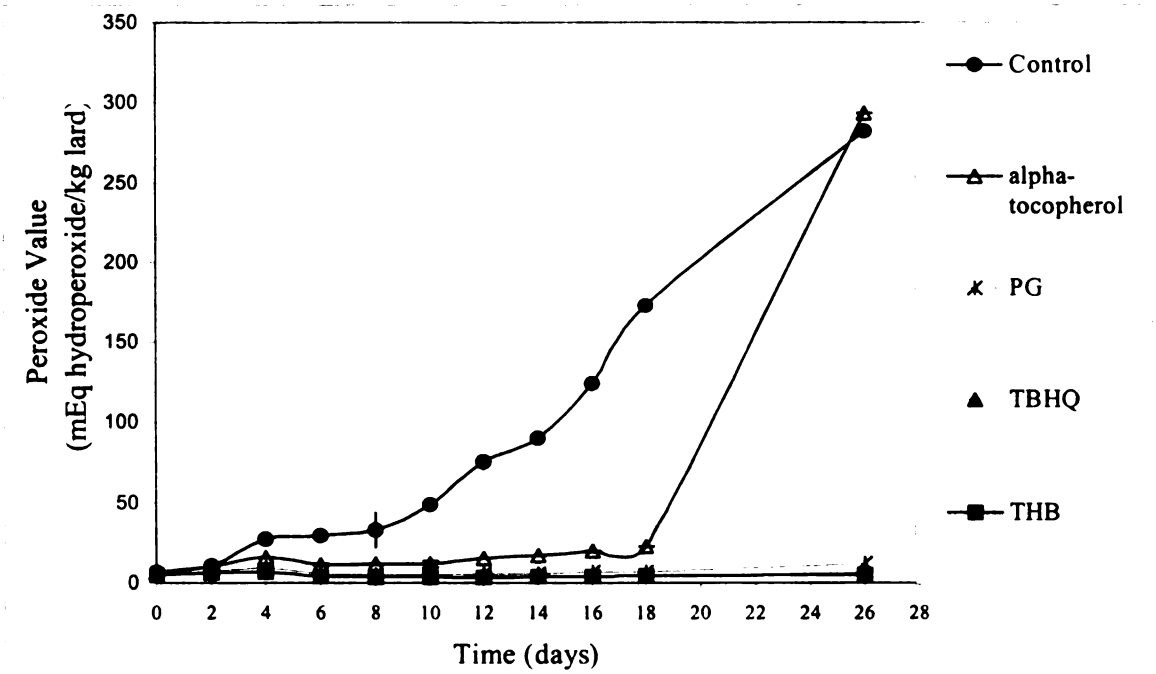
Preliminary experiments were conducted to determine antioxidant activity of THB in bulk oil using the Schaal oven test. The antioxidant activity of THB was compared to alpha-tocopherol, TBHQ and propyl gallate (0.01% w/w) using lard and stripped corn oil as the lipid substrates (Figure 2.2). Antioxidant activity was evaluated by determining peroxide value of the lard or oil as a function of time.

In lard (Figure 2.2 A), the control had an induction period of approximately 10 days, followed by alpha-tocopherol at 18 days, thus giving a protection factor of 1.8 for alpha-tocopherol. TBHQ, propyl gallate and THB did not reach the end of the induction period within 26 days, at which time the substrate was totally consumed. Therefore, we concluded that the antioxidant activity from greatest to weakest in lard is TBHQ THB propyl gallate > alpha-tocopherol > control.

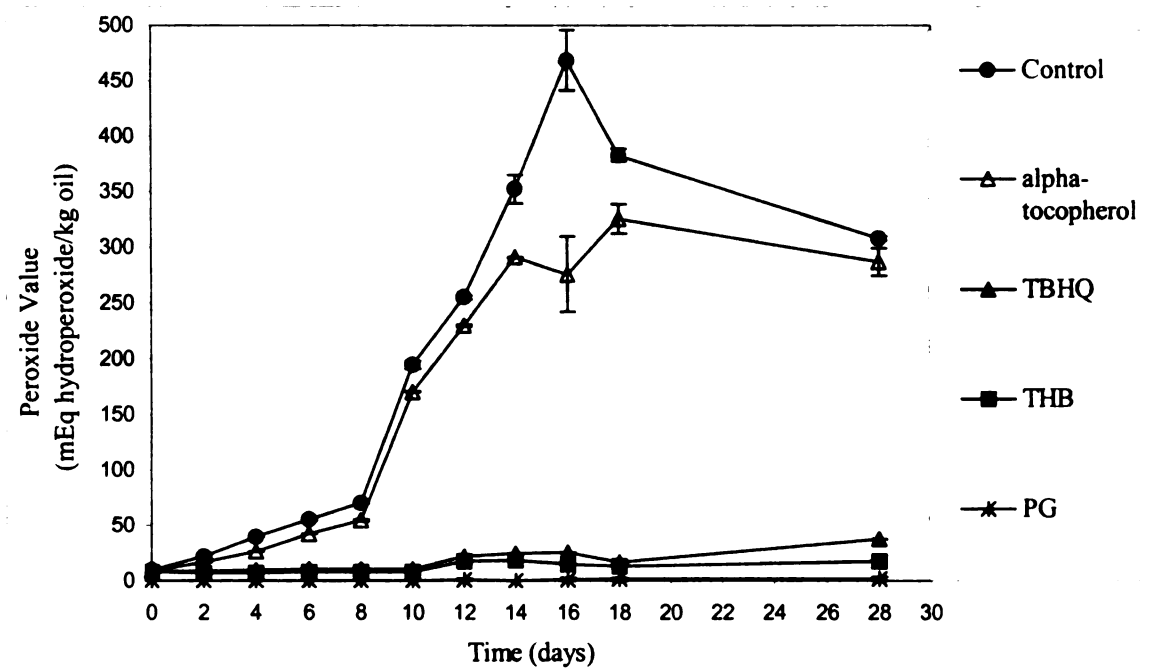
These compounds were also evaluated for antioxidant activity in corn oil stripped of endogenous antioxidants (Figure 2.2 B). In this lipid substrate, the control and alpha-tocopherol samples both had induction periods of approximately 8 days compared to the experiments using lard. As with the lard experiments, TBHQ, propyl gallate and THB did not reach the end of the induction period within 28 days. Therefore, we concluded that the antioxidant activity in stripped corn oil from greatest to weakest is TBHQ THB propyl gallate > alpha-tocopherol control at 28 days.

Figure 2.2 Antioxidant activities of alpha-tocopherol, propyl gallate (PG), 2-*tert*-butylhydroquinone (TBHQ) and 1,2,3,4-tetrahydroxybenzene (THB) (0.01% wt/wt) in (A) lard and (B) stripped corn oil.

Each data point is the mean of triplicate measurements. Error bars correspond to ± 1 SD. Error bars not shown are smaller than the symbols.


Peroxide Value
(mEq hydroperoxide/kg lard)
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (days)
Control
alpha-tocopherol
PG
TBHQ
THB

(A)


Peroxide Value
(mEq hydroperoxide/kg oil)
500
450
400
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time (days)
Control
alpha-tocopherol
TBHQ
THB
PG

(B)

Figure 2.2

The purpose of these experiments was to do a preliminary screening of THB antioxidant activity in comparison to antioxidants commonly added to foodstuffs. The results indicated that THB possesses antioxidant activity in bulk lipid that is comparable, as far as could be measured within the limits of these experiments, to that of TBHQ and propyl gallate. In order to distinguish the antioxidant properties of THB and THBA from the other commonly used antioxidants, a series of experiments was designed with the aim of determining structure activity relationships within a group of phenolic compounds comprising two, three, or four hydroxyl groups, and with or without a carboxyl group. Protocatechuic acid and gallic acid were chosen for these studies based on their structures, each comprising a carboxylic acid group and two or three hydroxyl groups, respectively, attached to a benzene ring. THBA also comprises a carboxylic acid group and four hydroxyl groups attached to a benzene ring.

Three experimental systems were chosen for subsequent detailed analysis of antioxidant activity of these compounds: bulk oil (lard), ground beef, and liposomes. Selection of the ground beef and lard systems was based on the observation of Porter and others (1989) that antioxidants display different activities depending on the food matrix. Polar antioxidant compounds are generally more effective in bulk oils than non-polar compounds, whereas non-polar compounds are more effective than polar compounds in emulsions and membrane-containing food products such as muscle foods. The compounds were also tested in the liposomal screening assay developed previously in our laboratory (Arora and Strasburg 1997) to further evaluate the assay's efficacy as a rapid screening tool for antioxidant activity of novel compounds.

2.3.1. Evaluation of Structurally Related Antioxidants in a Bulk Lipid System

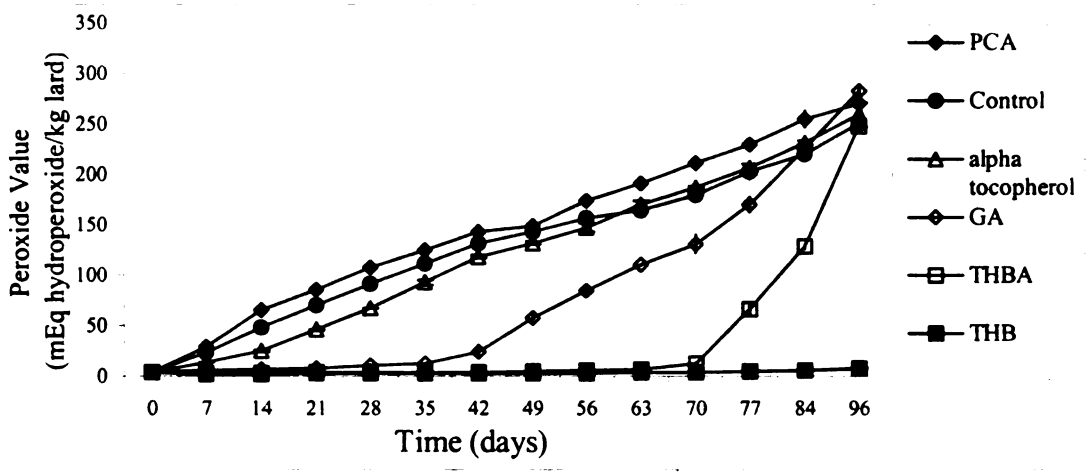
Our preliminary studies suggested that within the limited time frame of the experiments, THB possessed antioxidant activity similar to that of TBHQ and propyl gallate. In order to distinguish the antioxidant properties of the phenolic compounds, we conducted subsequent experiments at 0.01%, 0.005% and 0.001% (w/w) using the Schaal oven test (Figure 2.3). Antioxidant efficacies of the compounds were determined by comparison of protection factors (Table 2.1).

At the 0.01% level, THB showed the highest antioxidant activity followed by THBA, gallic acid, alpha-tocopherol, and protocatechuic acid. After 96 days, the end of the induction period for the THB sample still was not reached. THBA and gallic acid had induction periods of 70 and 42 days, respectively, yielding protection factors of approximately 10 and 7, respectively. Alpha-tocopherol showed similar activity to the control, whereas protocatechuic acid showed slight pro-oxidant activity.

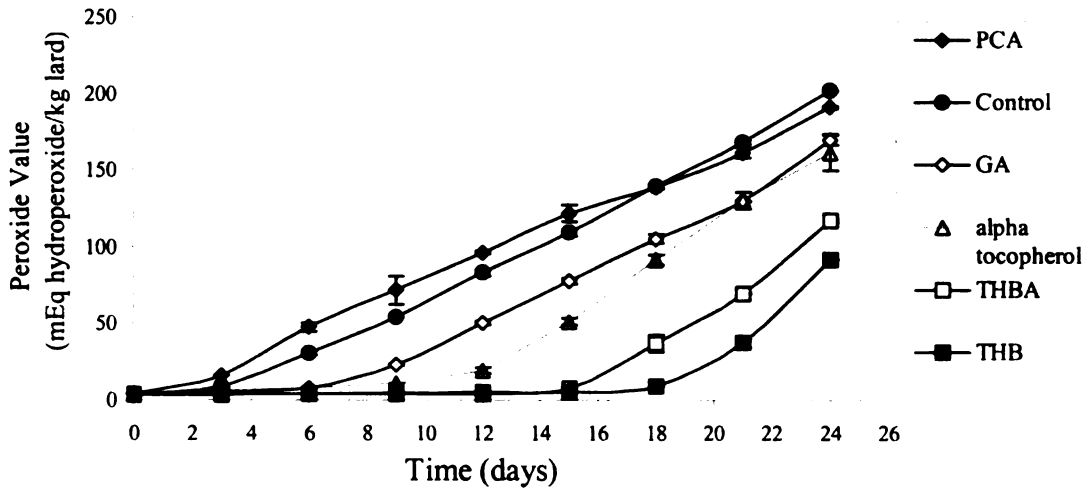
At the 0.005% level, all samples oxidized more readily. THB had an induction period of 18 days, followed by THBA with 15 days, alpha-tocopherol with 12 days, and gallic acid with 6 days. Protocatechuic acid was indistinguishable from the control at approximately 3 days. Based on the protection factors of the antioxidants at 0.005%, the antioxidant efficacy beginning with the highest activity was TBA followed by, in decreasing order, THBA, alpha-tocopherol, gallic acid and protocatechuic acid, respectively.

Figure 2.3 Antioxidant activities of gallic acid (GA), protocatechuic acid (PCA), alpha-tocopherol, 1,2,3,4-tetrahydroxybenzene (THB), and 2,3,4,5-tetrahydroxybenzoic acid (THBA) in lard. Concentrations of antioxidants were: (A) 0.01%, (B) 0.005% and (C) 0.001% (wt/wt).

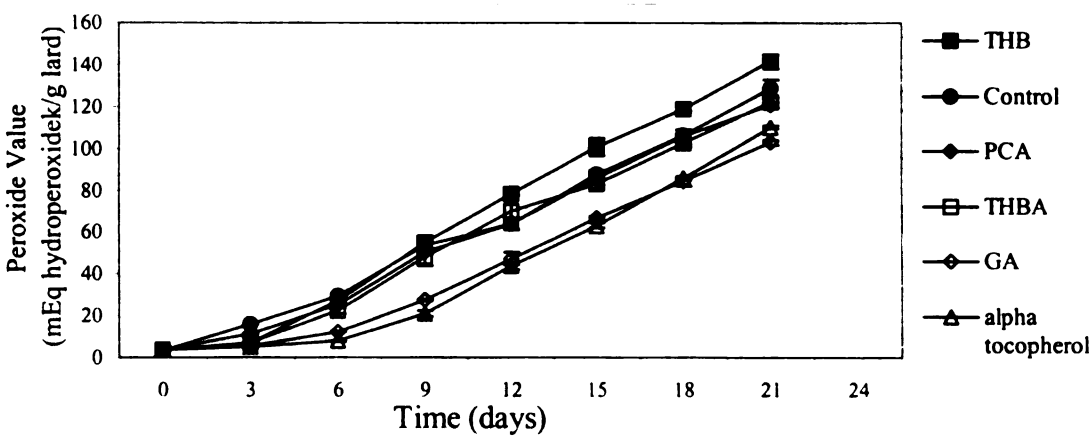
Each data point is the mean of triplicate measurements. Error bars correspond to ± 1 SD. Error bars not observed are smaller than the symbols.



(A)



(B)



(C)

Figure 2.3

Table 2.1 Protection Factors of Antioxidant Compounds in Bulk Oil

	Protection Factor[a]		
Compound	0.01%(wt/wt)	0.005%(wt/wt)	0.001%(wt/wt)
Gallic Acid	7	2	~2
Protocatechuic Acid	<1	1	~1
Alpha-tocopherol	1	4	~2
THB	>14	6	1
THBA	10	5	~1

[a] ratio of the induction period of the antioxidant-treated sample compared to the control

At the 0.001% level, THB showed slight pro-oxidant effects, whereas THBA and protocatechuic acid showed no activity, and gallic acid and alpha-tocopherol showed the highest antioxidant activity. Based on these data, we conclude that antioxidant activity of THB and THBA is concentration-dependent in a bulk lipid substrate, and are superior to the other compounds tested over the range of 0.005% to 0.01%.

2.3.2. Evaluation of Antioxidants in a Meat System

Antioxidant activities of THB and THBA were compared with the other phenolic compounds in a cooked ground beef system using the 2-thiobarbituric acid (TBA) test. Antioxidant activity was evaluated by calculating TBARS at 0, 24, 48, 72, and 96 hours. As shown in Figure 2.4, antioxidant activities of THB and THBA were both significantly greater ($p<0.05$) than the control at each time point, except 0 time, when only THB had statistically significant activity ($p<0.05$). The antioxidant activity of THB was significantly greater than alpha-tocopherol, protocatechuic acid and gallic acid at each time point, and was significantly greater than THBA at 48, 72 and 96 hours. Based on the overall TBARS values, the antioxidant activity of the compounds tested were: THB > THBA > gallic acid > alpha-tocopherol > protocatechuic acid.

2.3.3.Evaluation of Antioxidants in a Liposome System

Antioxidant activity of the phenolic compounds in a suspension of liposomes was evaluated using fluorescence spectrophotometry. Lipid autoxidation was initiated by adding Fe (II) with the resulting free radicals reacting with the fluorescent probe, which is localized within the lipid bilayer. The rate of decay of

Figure 2.4 Antioxidant activities of gallic acid (GA), protocatechuic acid (PCA), alpha-tocopherol (aT), 1,2,3,4-tetrahydroxybenzene (THB), and 2,3,4,5-tetrahydroxybenzoic acid (THBA) in cooked ground beef. Concentration of antioxidants were 0.01% based on fat content.

Treatments with different superscripts within a specific time point are significantly different ($p < 0.05$). Results are expressed as averages of triplicate measurements. Error bars correspond to ± 1 SD.

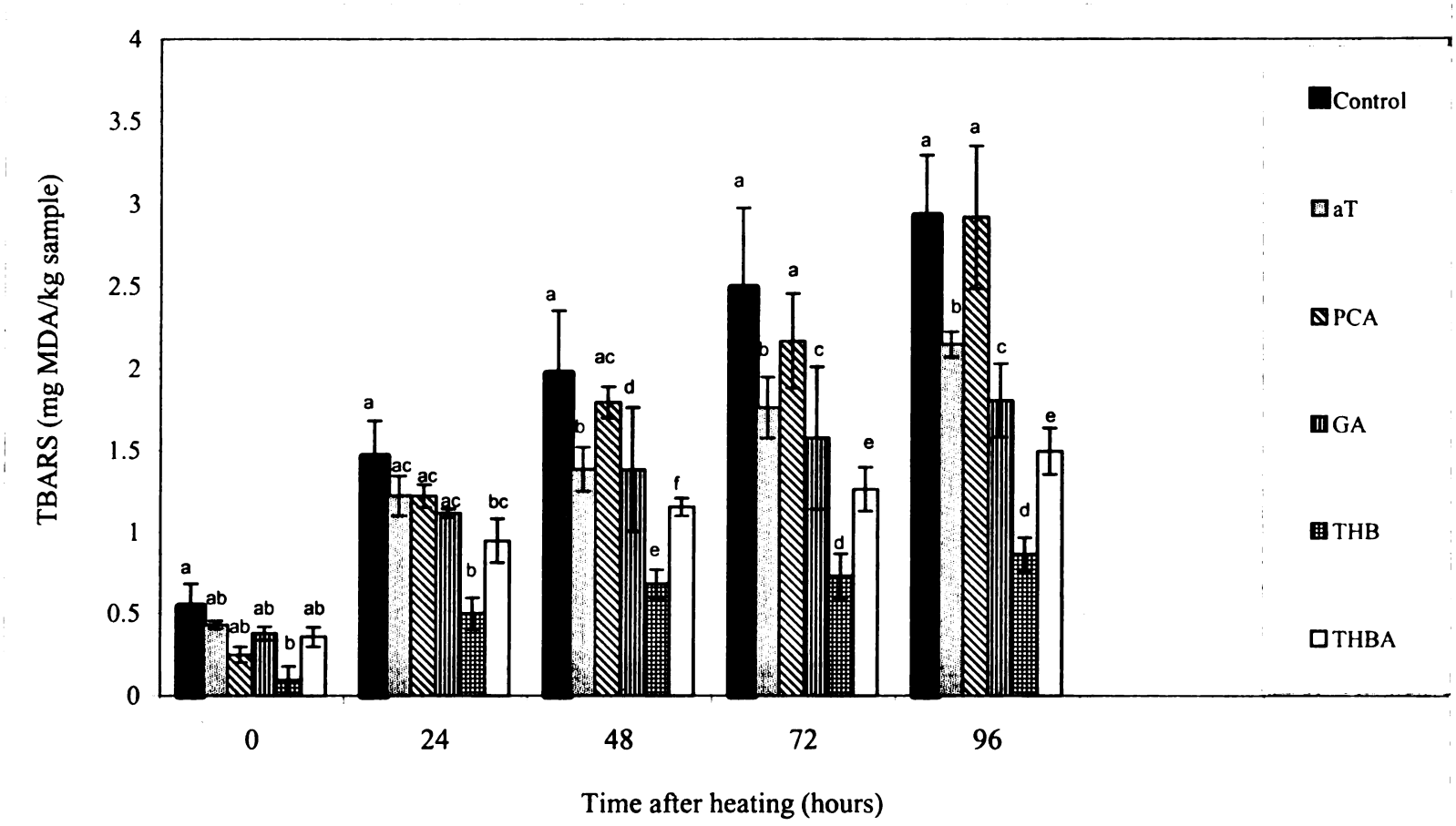

TBARS (mg MDA/kg sample)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
24
48
72
96
Time after heating (hours)
Control
aT
PCA
GA
THB
THBA

Figure 2.4

fluorescence intensity is an indication of the rate of autoxidation. Therefore, the rate in which fluorescence decay is inhibited by the addition of a particular compound is an indicator of its antioxidant efficacy.

When comparing relative antioxidant effectiveness, gallic acid > propyl gallate protocatechuic acid > THBA > THB > alpha-tocopherol (Figure 2.5). Fluorescence in the more lipophilic compounds remained just below that of the control indicating high antioxidant activity. THB, a polar compound and alpha-tocopherol showed minimal activity. THBA shows biphasic decay in fluorescence with the signal first dropping slowly and more rapidly at approximately 9 minutes. From this observation, we decided to vary the concentration of THBA and re-evaluate its antioxidant activity in the liposome system. Variability of these results is uncertain, but it is speculated THBA is not as stable at lower concentrations. Further studies will need to be carried out in order to verify this.

Figure 2.6 shows the concentration dependence of THBA in this system. At the highest level tested, 50 μM, THBA showed a steady rate of fluorescence equivalent to that of gallic acid (10 μM). At 20 μM THBA, fluorescence did not decrease until approximately 9 minutes. The lower concentrations tested, 0.1 and 1 μM, showed an immediate decay of fluorescence similar to that of THB (10 μM) and the Fe (II) prooxidant.

Figure 2.5 Antioxidant activity of gallic acid (GA), propyl gallate (PG), protocatechuic acid (PCA), 2,3,4,5-tetrahydroxybenzoic acid (THBA), alpha-tocopherol (aT), and 1,2,3,4-tetrahydroxybenzene (THB), 10μM using Fe (II)-induced peroxidation of LUVs.

Relative fluorescence represents the fluorescence intensity at a given time point divided by the fluorescence intensity at time = 0 min. Results are expressed as averages of triplicate measurements, except aT, n=1. Error bars correspond to ± 1 SD.

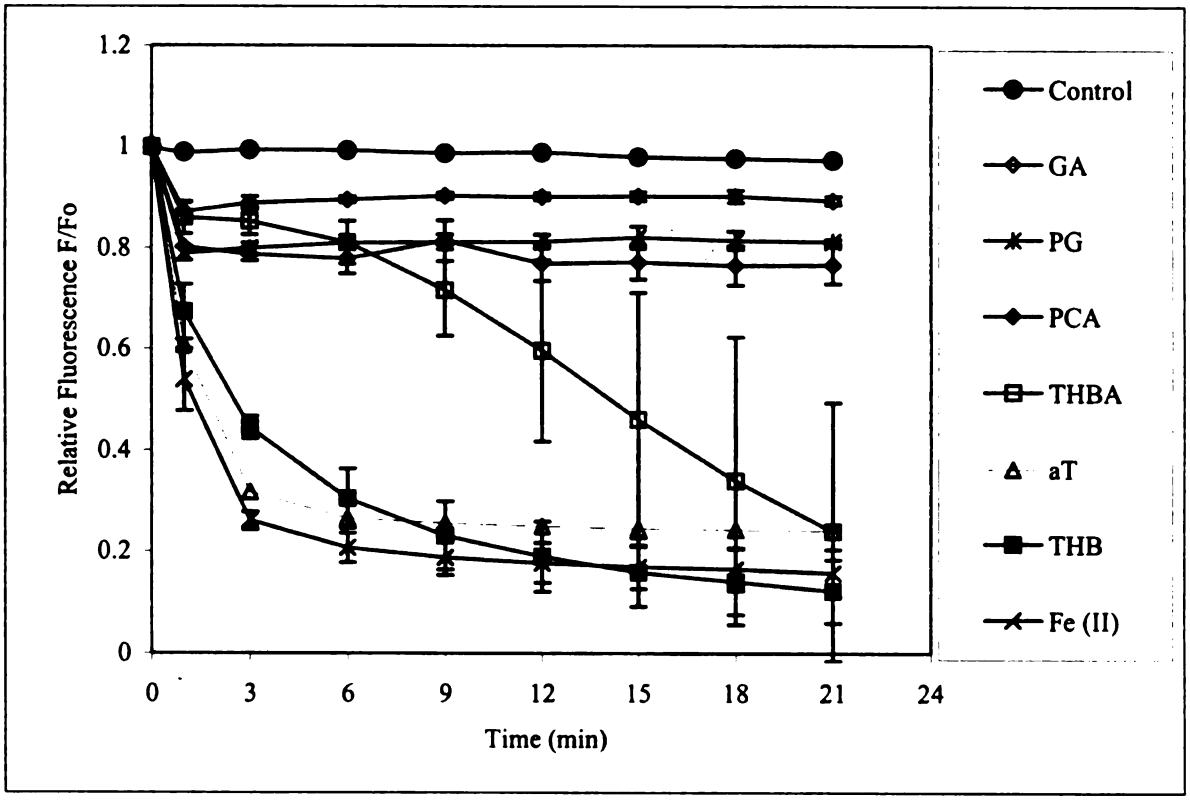

1.2
1
0.8
0.6
0.4
0.2
0
Relative Fluorescence F/Fo
0
3
6
9
12
15
18
21
24
Time (min)
Control
GA
PG
PCA
THBA
aT
THB
Fe (II)

Figure 2.5

Figure 2.6 Concentration dependence of antioxidant activity of 2,3,4,5-tetrahydroxybenzoic acid (THBA) using Fe (II)-induced peroxidation of LUVs. THBA was tested at various concentrations as indicated in the figure legend.

Relative fluorescence represents the fluorescence intensity at a given time point divided by the fluorescence intensity at time = 0 min. n=1.

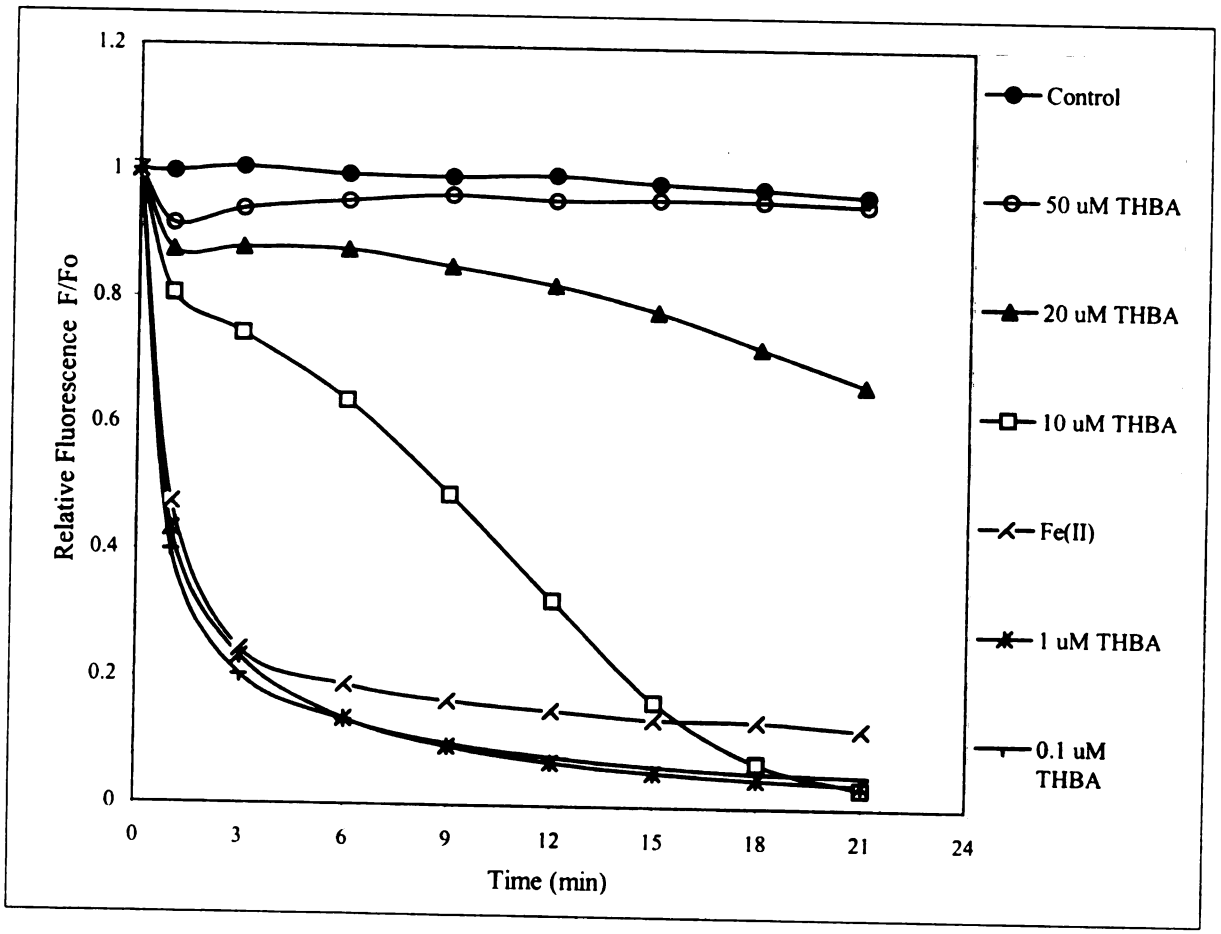

1.2
1
0.8
0.6
0.4
0.2
0
Relative Fluorescence F/Fo
0
3
6
9
12
15
18
21
24
Time (min)
Control
50 uM THBA
20 uM THBA
10 uM THBA
Fe(II)
1 uM THBA
0.1 uM THBA

Figure 2.6

2.4 DISCUSSION

Previous studies suggest that compounds with multiple hydroxyl groups attached to the aromatic nucleus will be potent antioxidants. We hypothesized that the polyphenolic compounds, THB and THBA, would show equivalent if not greater antioxidant activity compared to commercial antioxidants currently used in the food industry.

Our results support the structure-activity relationships reported by other investigators. Cuvelier and others (1992) and Chen and Ho (1997) showed that polyphenols are more active antioxidants than monophenols in a bulk lipid system. Gallic acid, having three hydroxyl groups, showed greater antioxidant activity than protocatechuic acid, having only two hydroxyl groups, at each of the concentrations used in this study. Baum and Perun (1962) showed that 1,2,3,-trihydroxybenzene was a stronger antioxidant than 1,2-dihydroxybenzene, which in turn, was more effective than phenol.

Similar trends in antioxidant activity were found in each substrate (corn oil versus lard) when comparing induction periods. However, the induction period for alpha-tocopherol was longer in lard than in stripped corn oil. This is similar to what Chen and Ho (1997) found in their studies, but the reason for this is not clear. For all antioxidants tested, the induction period was shorter in corn oil than lard. This was expected because there are more unsaturated fatty acids in corn oil than in lard, making corn oil more susceptible to oxidation (Love 1995; Orthoefer 1995).

In ground beef, our results showed that THB and THBA were the most effective antioxidants of those tested. Overall antioxidant activity increased as the number of

hydroxyl groups increased. Alpha-tocopherol was relatively ineffective in this system as reported in other studies. Benedict and others (1975) also found alpha-tocopherol to be an ineffective antioxidant when added at 0.005% (wt/wt) to ground beef.

The liposome system was designed as a screening assay to rapidly evaluate extracts and pure compounds before testing in food products, which is much more time- and labor-intensive. While tested and validated for several types of structurally related compounds, this assay may still require further modifications for screening different types of compounds. THB and THBA were compared in the liposomal assay with other well-characterized antioxidant compounds. Results obtained for propyl gallate, alpha-tocopherol, and protocatechuic acid compare favorably with those of Richman and others (1996) and Chang and others (2003).

The THBA results from the liposomal assay also compare favorably with those obtained in ground beef. However, based on the results from the liposomal assay, THB is predicted to be a poor antioxidant in ground beef, whereas the results in cooked ground beef show it is highly effective. One possible explanation for the low apparent antioxidant activity of THB in the liposome system is that THB could be regenerating Fe (II) from Fe (III) in a manner similar to that of ascorbic acid, and thus accelerating oxidation.

Diphenols have greatest antioxidant activity when hydroxyl groups are attached at the *ortho* or *para* positions. The second hydroxyl group in the *ortho* or *para* position of phenolic compounds increases antioxidant activity by further electron delocalization of the aromatic ring (Cuvelier and others 1992). Adding a hydroxyl group at the *ortho*

position, such as a 1,2-dihydroxybenzene derivative, is believed to stabilize the radical through intramolecular hydrogen bonding (Figure 1.4) (Baum and Perun 1962).

Compounds with *para* substitution, such as 1,4-dihydroxybenzene derivatives, are also believed to have increased antioxidant activities. This is due to production of a semiquinone radical that can be further oxidized to form a quinone by reaction with a lipid radical (Figure 1.3 (a)). Alternatively, the semiquinone radical may react with another semiquinone radical to regenerate a molecule of antioxidant, and form a quinone (Figure 1.3 (b)).

THB and THBA share structural similarity with other phenolic antioxidants such as alpha-tocopherol, BHT, BHA, THBQ, propyl gallate, gallic acid, and protocatechuic acid, and thus share a similar mechanism of action. THB and THBA each have four hydroxyl groups, which suggest they may function as effective free radical scavenging antioxidants because of their ability to stabilize the antioxidant radical by electron delocalization. Figures 2.9 and 2.10 show the possible resonance structures of THB upon reaction with a lipid free radical. The order in which the hydrogen atoms are donated is unknown at this point. Baum and Perun (1962) suggest that a hydroxyl group *ortho* to a phenolic radical may stabilize the radical through hydrogen bonding. We hypothesize that the hydrogen atoms in the 2 or 3 position are more labile because of dual stabilization of the resultant radical through hydrogen bonding by the two *ortho* hydroxyl groups. Thus, the increased antioxidant activity of THB may result from dual stabilization of the free radicals by intramoleculer hydrogen bonding and increased electron delocalization via the ring (Figure 2.9).

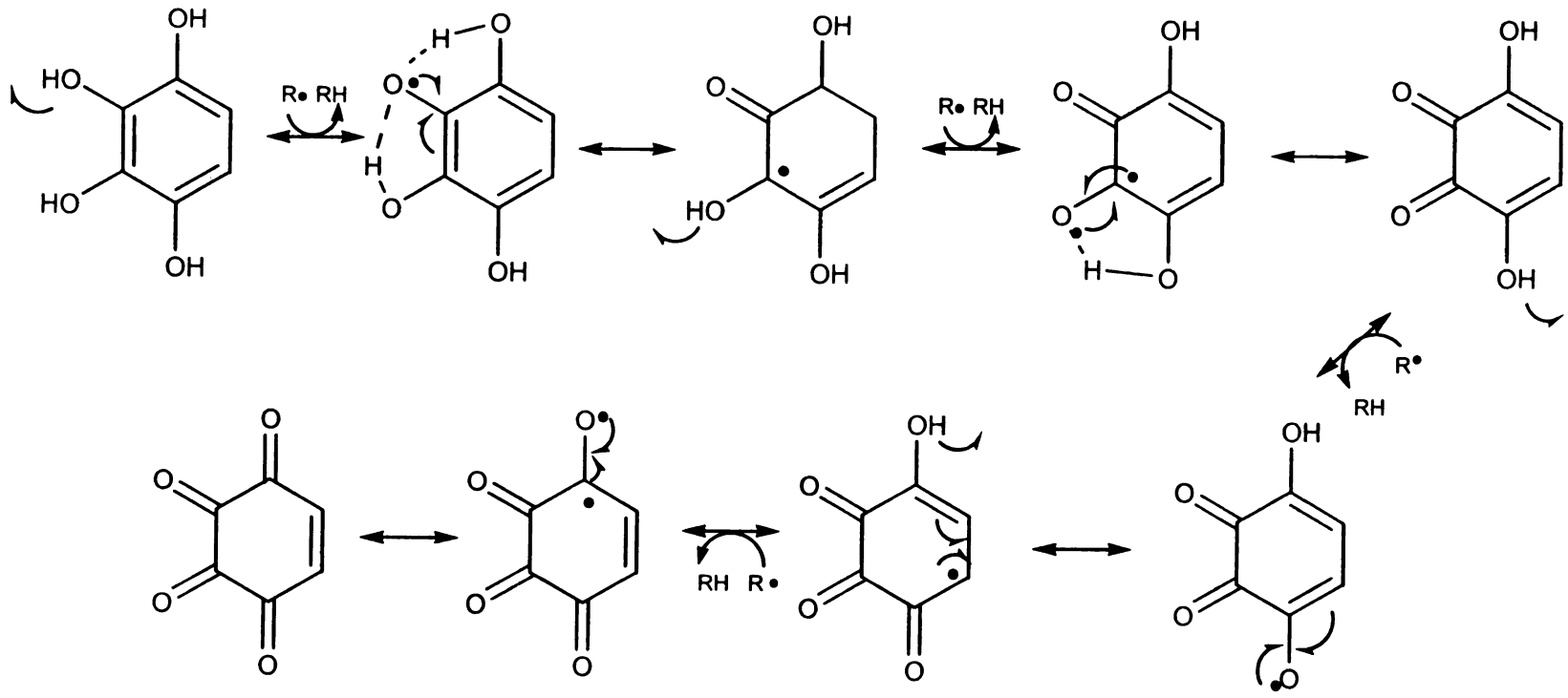


Figure 2.9 Predicted reaction scheme of THB with free radicals

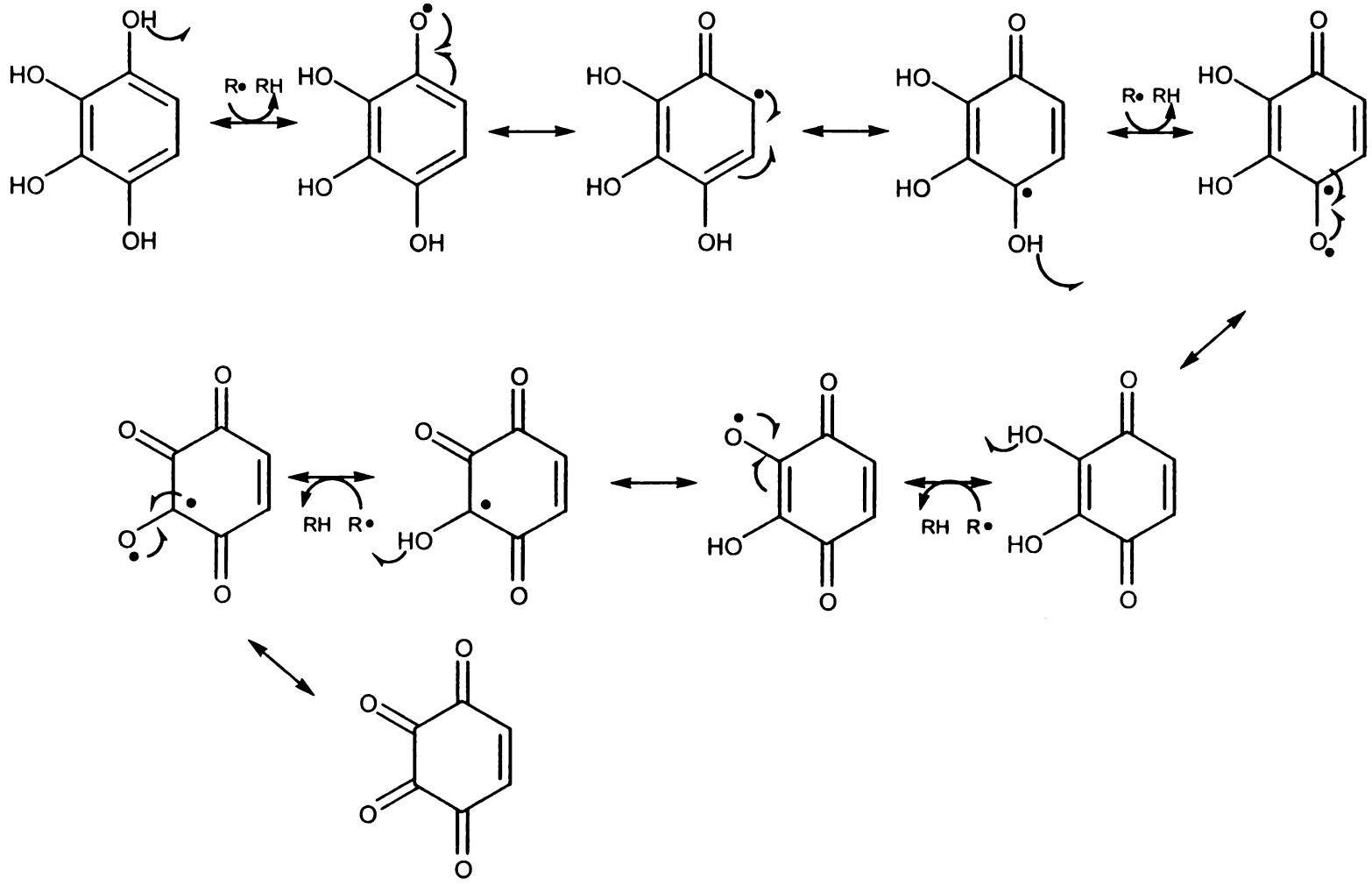


Figure 2.10 Alternative predicted reaction scheme of THB with free radicals

An alternate mechanism is suggested by studies on the fate of propyl gallate (Dziedzic and others 1986). Ellagic acid is formed from two molecules of propyl gallate, suggesting that the hydrogen atom at the *para*-hydroxyl position is not lost, and therefore may by less labile than the hydrogen atoms on the *meta*-hydroxyl groups. Figure 2.10 shows an alternative reaction pathway of THB with lipid free radicals with resonance stabilization in which the hydrogen atoms are donated initially at the *para* positions.

Our findings indicate that THBA and THB are the most effective of the compounds tested contradicting Pokorny (1987) who states that, pyrogallol is the most active trihydroxybenzene, and that the presence of more than three hydroxyl groups does not improve activity. No supporting data was provided in his paper.

In this study we have demonstrated the potential for use of low-value, by-products of food processing as a source of potent antioxidant compounds, while enhancing the value of the steeping liquor, which can be used as phytate-free animal feed. These antioxidants could provide an inexpensive and efficient tool for food preservation provided they have a favorable safety/toxicity profile.

CHAPTER THREE

ANTIMICROBIAL ACTIVITY OF 1,2,3,4-TETRAHYDROXYBENZENE AND 2,3,4,5-TETRAHYDROXYBENZOIC ACID

3.1 INTRODUCTION

Food safety concerns in the United States have risen over the past ten years, in response to several recently emerged pathogens that can cause serious illnesses and death (National Center for Infectious Diseases). In the United States, it is estimated that 76 million cases of foodborne illnesses, including 325,000 hospitalizations and 5,200 deaths occur each year (Mead and others 1999). According to the Economic Research Service, the estimated annual costs of foodborne disease caused by *Salmonella*, *Listeria monocytogenes,* and *Escherichia coli* O157:H7 are $2.4 billion, $2.3 billion and $659.1 million, respectively (U.S. Department of Agriculture 2002).

Phenolic antioxidants, such as BHA, BHT and TBHQ, have been studied as antimicrobial agents to inactivate, kill, or inhibit the growth of both pathogenic and spoilage organisms in food products (Branen 1993). Use of approved antioxidants that also express antimicrobial activity would reduce the amount of food additives introduced into a food system and would eliminate the need for further toxicological studies. Antioxidants with antimicrobial activity may also be used as rinses or sprays to lower contamination on food surfaces.

In our previous work, THB and THBA, derived from phytic acid, possessed high antioxidant activity, and thus showed significant promise as food additives. Since these compounds are structurally related to the antibiotics aurantiogliocladin and fumigatin, we

hypothesized that THB or THBA would also have antimicrobial activity. The objective of this study was to screen these compounds for inhibitory activity against *E. coli* O157:H7, *L. monocytogenes,* and *Salmonella* spp.

3.2 EXPERIMENTAL PROCEDURES

3.2.1. Materials

Listeria monocytogenes (CWD 95, CWD 246, CWD 201 and CWD 1503), *Escherichia coli* O157:H7 (AR, AD 305, and AD 317) *Salmonella* Typhimurium DT 104 (H3380 and 25-2D), *S.* Enteritidis (clinical isolate H3527, phage type 13A) and *S.* Heidelberg (clinical isolate F5038BG1) were obtained from Dr. Elliot Ryser (Michigan State University, East Lansing, MI). All strains were stored at -70°C in trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 10% (v/v) glycerol. Each strain was subcultured three times in TSB containing 0.6% (w/v) yeast extract (TSB-YE) (Difco) at 35°C, 18 to 24 h before use. The two phenolic antioxidant compounds, 1,2,3,4-tetrahydroxybenzene (THB) and 2,3,4,5-tetrahydroxybenzoic acid (THBA) obtained from Dr. John Frost (Michigan State University, East Lansing, MI), were synthesized as described in Figure 1.16.

3.2.2. Methods

Disc Diffusion Assay:

Treatment preparation: Saturated solutions of THB and THBA were prepared at room temperature in deionized water; the approximate concentrations of the saturated

solutions were 1.4 M. Dilutions of 1/10, 1/100, 1/1000, and 1/10000 were prepared from the saturated solutions.

Assay: Twenty mL of trypticase soy agar containing 0.6% yeast extract (TSA-YE) (Difco) was seeded with 200 µL of an 18-24 h-old culture of *S.* Typhimurium DT 104 (25-2D) (10^8 cfu/mL), *L. monocytogenes* 1503 (10^9 cfu/mL) or *E. coli* O157:H7 (AD 305) (10^9 cfu/mL) and transferred to a Petri dish (100 mm diameter x 15 mm thickness). After solidification, a 16-mm diameter Whatman #4 filter disk (Whatman Co., Maidstone, England) containing 50 µL of THB or THBA of the desired concentration was placed aseptically in the center of each agar plate. Disks were prepared by soaking them in 50 µL of treatment. After 24 h of incubation at 35°C, the inhibition zone diameters were measured perpendicularly to the nearest mm using a metric ruler. All tests were run in duplicate with duplicate measurements averaged. Filter disks containing only 50 µL of sterile distilled water served as negative controls.

Minimum Inhibitory Concentration (MIC)

All strains were evaluated for MIC. In addition, the following three-strain cocktails were prepared for MIC determination:

1. *L. monocytogenes* (CWD 95, CWD 246, and CWD 201)
2. *E. coli* O157:H7 (AR, AD 305, and AD 317)
3. *Salmonella* Typhimurium DT 104 (H3380), *S.* Enteritidis and *S.* Heidelberg

Culture preparation: All strains were cultured as described above.

Cocktails were prepared by combining 3-mL aliquots of each strain within each species. After centrifugation at 9740 x g for 15 minutes at 4°C (Sorvall Super T21, Newtown, CT), the pellet was resuspended in 9 mL peptone broth (0.1%).

Tubes of TSB-YE containing 0.0014, 0.0028, 0.0056, 0.0070, 0.0084, 0.0098, 0.0112, 0.0126, 0.0140 M THB were inoculated with 10 μL of each test organism (10^9 cfu/mL) or cocktail (10^{10} cfu/mL) to give a final count of 10^6 cfu/mL and 10^7 cfu/mL, respectively, in each tube. All tubes were incubated at 35°C and observed for growth after 24 and 48 h. All tests were run in triplicate with results reported as the minimum inhibitory concentration (MIC) at which no growth was visibly present. Tubes without THB served as negative controls.

Statistical Analysis: Statistical analysis was performed using a complete block design, where block = time (which has two levels 24 hours and 48 hours) and treatment is the main effect. The GLM generalized linear model (SAS® Version 7, Cary, NC) was used to perform the statistical analysis. Also LSMEANS was used in the GLM procedure to obtain pairwise comparison of the treatments.

3.3 RESULTS

3.3.1. Disk Diffusion Assay

THB and THBA were initially screened for antimicrobial activity using a disk diffusion assay. The 100% saturated solution of THB yielded inhibition zones of 28, 25, and 33 mm for *E. coli* O157:H7 (AD 305), *S.* Typhimurium DT 104 (25-2D) and *L. monocytogenes* (1503), respectively, and 19 mm for each of these strains when using a 10% saturated solution of THB (Figure 3.1). Concentrations less than 10% saturated solution of THB did not show antimicrobial activity. THBA was noninhibitory at all concentrations tested, even at 100% saturation.

Based on these results, the MIC of THB was evaluated for *L. monocytogenes* (CWD 95, CWD 246, and CWD 201), *E. coli* O157:H7 (AR, AD 305, and AD 317) and *Salmonella* Typhimurium DT 104 (H3380), *S.* Enteritidis and *S.* Heidelberg using a broth assay.

3.3.2. Minimum Inhibitory Concentration

In TSB-YE, *E. coli* O157:H7 (AR) and *L. monocytogenes* (CWD 95) were inhibited at 0.0042 M THB and 0.0056 M THB at 24 and 48 h, respectively (Table 3.1). *S.* Enteritidis and *S.* Heidelberg were both inhibited at 0.0056 M THB after 24 and 48 hours. The *Salmonella* cocktail was inhibited by 0.0028 M THB at 24 h, whereas the *E. coli* O157:H7 (AD 305, AD 317 and AR) and *L. monocytogenes* (CWD 201, CWD 246 and CWD 95) cocktails were inhibited by 0.0056 M THB at 24 h and 0.0084 and 0.0056 M at 48 h, respectively. The concentration and time effect of the L. monocytogenes (CWD 201, CWD 246 and CWD 95) cocktail is significantly different ($p<0.05$) from the

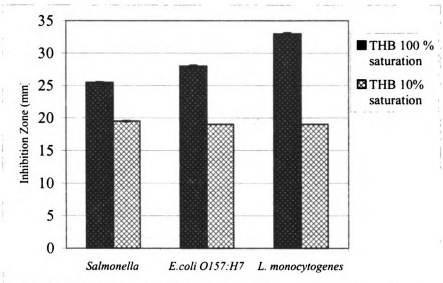


Figure 3.1 Inhibition of *S.* Typhimurium DT 104 (25-2D), *E. coli* O157:H7 (AD 305) and *L. monocytogenes* 1503 by THB in a disk diffusion assay.

Error bars correspond to ± 1 SD. Results are expressed as the average of duplicate experiments.

Table 3.1

Organism	MIC[1] (M x 10^{-3})	
	24 h	48 h
E.coli O157:H7		
AD 305[a]	7.0	11.2
AD 317[b]	8.4	9.8
AR[c]	4.2	5.6
Cocktail[b]	5.6	8.4
Salmonella		
Typhimurium[a]	5.6	7.0
Enteritidis[c]	5.6	5.6
Heidelberg[c]	5.6	5.6
Cocktail[b]	2.8	4.2
L. monocytogenes		
CWD 201[c]	5.6	5.6
CWD 246[c]	5.6	5.6
[2]CWD 95[b]	4.2	-
Cocktail[c]	5.6	5.6

[1] Minimum inhibitory concentration- the lowest concentration at which complete inhibition of growth was observed.
[2] No inhibition at 48 h

Table 3.1 Minimum inhibitory concentrations of THB for *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp.
Different letters show significant difference ($p < 0.05$) of a complete block design, where block = time (which has two level 24 hours and 48 hours) and treatment is the main effect.

cocktails for *Salmonella* and *E. coli*.

3.4 DISCUSSION

The aim of this study was to determine if the antioxidant compounds THB and THBA possess antimicrobial activity. Using the disk diffusion assay as a screening method, THB showed significant inhibitory activity against all three pathogens, whereas THBA showed no activity. In subsequent experiments the minimum inhibitory concentration (MIC), defined as the lowest concentration of which complete inhibition is observed, was determined for THB. The results indicate that THB is inhibitory for growth of the pathogens tested over a range of 2.8-8.4 mM at 24 h and 5.6-11.2 mM at 48 h.

While direct comparison of MIC for THB with other compounds is difficult, some generalizations can be made. The MIC found in this study using THB against the *Salmonella* cocktail was considerably lower to BHA, which is most recognized for its antioxidant activity, but is also used for its antimicrobial properties. Tuncel and Nergiz (1993) found that BHA at a concentration of 400 μg/mL inhibited *Staphylococcus aureus, Bacillus cereus, E. coli, and S.* Typhimurium when grown in trypticase soy broth at 37°C for 18 h. The *Salmonella* cocktail (10^7 cfu/mL) in our study was inhibited by THB with MICs of 2.8 and 4.2 mM THB after 24 and 48 h, respectively. The *L. monocytogenes* cocktail was inhibited by 5.6 mM THB at both 24 and 48 h. While the MICs in each of these studies are quite similar, our incubation times were longer. Other phenolic antioxidants with well documented antimicrobial activity include BHT and TBHQ (Raccach 1984; Davidson 2001).

The mechanism by which antioxidants inhibit microbial growth is not well defined (Davidson 2001). However, an antioxidant must be sufficiently lipophilic to pass through the cell membrane and once inside, it must also be sufficiently water-soluble to interact with cell components. Hydrophobic compounds are more effective antimicrobial agents against Gram positive bacteria, whereas Gram negative bacteria are more susceptible to hydrophilic compounds (Davidson and Branen 1980; Raccah 1984; Davidson 1993). THB was generally more effective against *L. monocytogenes* and *Salmonella* than *E. coli* O157:H7.

Steric effects also influence the antimicrobial activity of phenolic antioxidants. When several *n*-alkyl BHT deriviatives were studied by Kabara (1980) substitution of a bulky *tert*-butyl group *meta* to the phenolic group instead of *ortho*, as in BHT, lowered the MIC from 250 μg/mL to 25 μg/mL against *Streptococcus mutans* in trypticase soy broth. They concluded that BHT, with the hindered phenolic group, was far less effective than BHA, in which the hydoxyl group is much less hindered.

Even though the exact mode of action for antimicrobial agents is poorly understood, numerous studies suggest the antimicrobial mechanism of phenolic compounds is attributed to their action on the cellular membrane, as well as altered synthesis of nucleic acids, proteins and lipids (Eklund 1980; Raccach 1984; Davidson 2001). Parabens effectively disrupt cell membrane integrity leading to increased permeability and leakage of cell contents that can lead to death of the organism (Davidson 2001). The antimicrobial mechanism of THB may be similar to that of the parabens.

THB is a potent antioxidant with good antimicrobial activity, thus making the compound an attractive multifunctional food ingredient. Possible THB applications in the food industry include multistage or combination spray washing of meats, fruits and vegetables. Currently used sanitizers include organic acids, hydrogen peroxide, and hypochlorite compounds but their use is quite limited. Hydrogen peroxide is an effective antimicrobial agent, but a decrease in organoleptic quality of some produce is a consequence (Sapers and Simmons 1998). Chlorine is also often used, but dissipates quickly in water with high levels of organic residue in addition to creating high levels of hazardous fumes (Anon 1999).

Results from these studies suggest that THB has significant promise for use as a food ingredient. Further studies are warranted to determine more precisely its antimicrobial activity in food processing applications.

CHAPTER FOUR

SUMMARY AND CONCLUSIONS

In this study we hypothesized that the polyphenolic compounds, THB and THBA, would show equivalent or greater antioxidant activity compared to other commercial antioxidants used currently in the food industry. THB and THBA share structural similarities with the antimicrobial compounds fumigatin and aurantiogliocladin as well. Thus, our second hypothesis was that THB and THBA would also possess antimicrobial activity.

The first objective of this study was to evaluate the antioxidant activities of THB and THBA in three different experimental systems; bulk oils, ground beef and liposomes. THB and THBA displayed high antioxidant activity in the bulk oil, comparable or superior to all of the commercial antioxidants tested. THB and THBA also showed significant antioxidant activity in ground beef. THB did not show activity in the liposome system at the concentration tested. THBA showed moderate activity at the same concentration of the other antioxidants tested and its activity increased at higher concentrations. The antimicrobial activity of THB was comparable to other phenolic antioxidants used for antimicrobial application.

In this study we have shown an alternative use for natural-product extracts. Abundant, low-value by-products of food processing could be used as raw materials for the synthesis of novel antioxidant compounds. The antioxidant activity of THB and THBA offers an advantage to the food processing industry and these compounds are potential value-added products for cereal manufacture. Removal of phytic acid from the

steeping liquor for use in synthesis of novel antioxidants enhances its value as well as that of the steeping liquor, which can be used as phytate-free animal feed. By expressing both antioxidant and antimicrobial activity, THB may serve as a multifunctional food ingredient. Reduction in the amount and types of food additives introduced into a food system and the elimination of toxicological studies is reduced by using a compound which expresses both antioxidant and antimicrobial properties.

CHAPTER FIVE

FUTURE RESEARCH

Some suggestions for future research are listed below:

1. THB and THBA showed potent antioxidant activity in both bulk oil and meat systems. To demonstrate their potential as active food ingredients, their activities should be tested in other food products, including processed meats, emulsions and cereals.

2. 1,2-dihydroxyquinone, which is formed from THB upon reaction with a lipid free radical should be evaluated for potential antioxidant activity to help in clarification of the mechanism of THB activity.

3. In the liposome system, iron-induced oxidation was used to evaluate antioxidant effectiveness of THB and THBA. The results indicated that THB would not be an effective antioxidant in contrast to our findings in the oil and meat systems. One explanation for this is that upon oxidation of iron, THB might be regenerating Fe (II), thus accelerating oxidation. The effect of THB with iron may be better understood, if other prooxidant agents, such as a hydrophilic free radical initiator, e.g. 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), are used to initiate autoxidation in this same system. In this study, liposomes were formed using an electrically neutral lipid substrate. A negatively charged

substrate, (e.g. phosphatidyl serine) when used in combination with SLPC, would be more representative of a biological membrane and may help to improve validity of this assay.

4. We have shown that using fluorescence spectroscopy is a fast and easy way to evaluate lipid oxidation in liposomes. Development of a screening assay using a bulk lipid coupled with fluorescence would be very beneficial for evaluating antioxidant compounds.

5. THB shows promising antimicrobial activity. A more in depth study will help to fully understand its mechanism of action. By comparing THB to other phenolic antioxidants, a better comparison as its relative effectiveness would be useful. BHA has antifungal activity at 200 ppm against *Aspergillus*, *Penicillium*, and *Geotrichum* in glucose salt broth when incubated at 32°C, for 85 h (Ahmad and Branen 1981). The structural similarity of THB and BHA suggests that compounds, should be tested against other organisms such as spore & non-spore forming, spoilage bacteria, pathogenic bacteria and molds & yeasts. Evaluation at different temperatures may also help in understanding the mechanism in which THB expresses antimicrobial activity.

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