INVESTIGATING THE EFFICACY OF POMEGRANATE EXTRACT AS AN ANTIOXIDANT IN MULTIPLE HIGH-LIPID MODEL SYSTEMS

BY

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THESIS

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Abstract

Antioxidants are of paramount importance to the food industry. They confer benefits in the form of economic savings as well as drastic quality improvements to high-lipid products. Previous research has evaluated synthetic and natural compounds in a plethora of matrices to compare antioxidant efficacies. Because of the intricate nature of antioxidants, their solubility and differences in application, expansive research must be done to qualify antioxidants of similar chemical characteristics.

Research was conducted in Urbana, IL to examine the efficacy of an industrially-derived, natural antioxidant (pomegranate extract) using four direct analysis technqiues and three high-lipid model systems in contrast to well-known and highly utilized antioxidants. Direct analysis techniques evaluated several antioxidants on two highly informative parameters of antioxidant activity: reducing capacity and hydrogen atom donating capacity. The high lipid models evaluated antioxidant activity in visceral systems by looking at each compounds ability to prolong the induction period of oxidation from different prooxidants (heat-, hemoglobin-, and irradiation-induced oxidation). These results improve the understanding of antioxidant capacity in applicable systems, as well as suggest potential mechanisms by which pomegranate extract might work.

My thesis is dedicated to my friends and family who have assisted me both physically and mentally.

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Chapter 1: Introduction

Antioxidants provide invaluable benefits to society. Their uses span from the rubber industry into nutritional significance and extension of the shelf life of many high-lipid foods. Because of their diversity, confusion has developed in qualifying the efficacy of antioxidants in relation to each other. Often, industries and the media have conflated antioxidant use in food systems with antioxidant use in living, biological systems. In 2010, POM Wonderful, LLC received complaint charges by the Federal Trade Commission for deceptively advertising their products (extracts, juice and POMx pills) without proper scientific validation. This confusion is common between consumers, the media, health care professionals, academic agencies and industry. A primary reason for the confusion originates from the unclear definition and purpose of an antioxidant. This is further illustrated by removal of the Oxygen Radical Absorbance Capacity (ORAC) Database for Selected Foods by the U.S Department of Agriculture's Nutrient Data Laboratory. ORAC values are measures of antioxidant potency that are relevant to food matrices but, as the USDA's Nutrient Data Lab contests, "[these values] have no relevance to the effects of specific bioactive compounds, including polyphenols, on human health." (USDA, 2012). This was further expounded in a June 2011 issue of Nutritional Outlook in an article entitled, 'The Polyphenol Paradox'. While the structure of polyphenolic compounds act exceptionally well as antioxidants in food systems, their molecular size inhibits absorption and the interaction in the stomach and lumen destroys their ring structure. This causes their accepted mechanism of antioxidant activity, seen clearly in foods, to be abolished. In food systems, antioxidants act as chemical constituents that don't require metabolic transformation to interact with and ultimately delay the oxidative damage that radical species create in an environment.

It is necessary to define antioxidants by their function. Metabolically-activated antioxidants that reduce oxidative stress on living tissue must be distinguished from antioxidants that reduce oxidative stress on non-living systems (food, rubber, plastics, etc.). Hence, the evaluation of antioxidants is still of prime importance to many industries. To date, there is no single method for determining the efficacy of an antioxidant. Methods of direct analysis have been criticized at large (Frankel, 1993; Prior *et al.*, 2005; Apak *et al.*, 2007). Furthermore, consumer interest in 'clean labeling' has led to preference for naturally occurring antioxidants to replace those with more chemical-sounding names. Combining direct analysis techniques (which can offer clues to the underlying mechanism of antioxidation) with model systems allows for a more complete profile of a compounds antioxidant activity to be portrayed.

Meat model systems, such as ground beef, are often chosen because they have the highest amount of fat on a weight-by-weight basis (27.4%, Rhee, Seideman & Cross, 1986) and require antioxidants to maintain freshness. Lard and oils are also commonly mixed with antioxidants (such as BHT/BHA) to maintain freshness. Both the tallow from beef and the lard from pork contain similar unsaturated fatty acid profiles (Bitman, 1976). Of note, pork contains over twice (8 g compared to 3 g) as much linoleic acid as beef (a precursor to off-flavor and odor in oxidation), but beef contains linolenic acid (which highly prone to oxidation) while pork contain none. Where beef and pork contain 53 and 58% unsaturated fatty acids, respectively, canola oil contains 91% unsaturated fatty acids (USDA, 2012). Of the unsaturated and easily degraded fatty acids in canola oil, 22% is comprised of linoleic acid while 11% comprised of linolenic acid. Because the results of one model-system are rarely translatable among different matrices, some authors have found it useful to test the same antioxidants among multiple matrices.

Chapter 2: Literature Review

2.1 Lipids

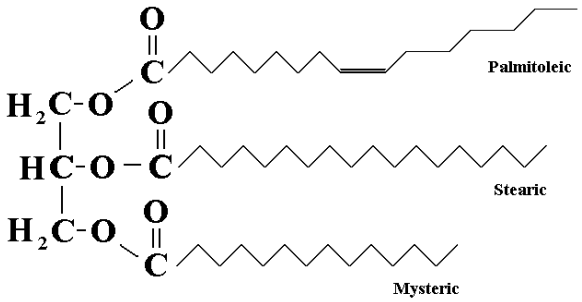
Of the macronutrients found in nature, lipids are important for a multitude of reasons. In terms of dietary considerations, lipids provide approximately 9 kilocalories on a per gram basis. Metabolically, lipids are necessary components for all living cells; they provide a structural basis (i.e. phospholipid bilayer) for cellular membranes, act as carriers for fat soluble vitamins (A, D, E and K), provide essential fatty acids to the diet (linoleic and linolenic acid), and on a larger macro provide insulation and protection to the human body. Beyond the metabolic attributes of lipids, they offer palatability to food, confer feelings of satiety, and offer a chemical oasis for hydrophobic compounds in a predominantly hydrophilic environment. Because of the diametric opposition of lipids to hydrophilic components of food products, they contain several advantages in terms of interactions with: hydrophobic side-chains of proteins (isoleucine, valine, alanine, and leucine), fat soluble antioxidants (vitamins A and E), formation of lipid-derived flavor compounds (trans,cis-2,6-nonadienal from linolenic acid in cucumbers (Tressl *et al*., 1981), fresh fish aroma (Josephson, Lindsay & Stuiber, 1983), etc.), and stability of select flavor compounds and flavor release (limonene, etc.) (Roberts *et al*., 2003).

Edible lipids are classically distinguished by their physical structure at room temperature. Oils are characteristically liquid at room temperature (20-25 ^{o}C) and are generally derived from plants (although fish lipids are also primarily liquid). Fats are lipids derived primarily from animal products that maintain a solid or semisolid structure at room temperature. The physical structure is determined predominantly by the degree of saturation and chain length each lipid contains; those with more unsaturated double bonds tend to be liquid, while those with high degrees of saturation remain solid at higher temperatures. Degree of saturation also determines

other functional aspects of the lipids, such as melting point, dispersal in a solvent and predominance to oxidize. Structural diversity occurs among lipids and lipid derived compounds beyond just saturation. The scientific community has found it useful to organize lipids into groups such as simple lipids, compound lipids and lipid derived structures (sterols, essential oils, etc.).

Simple lipids, such as waxes and acylglycerols, are esters of fatty acids alcohols.

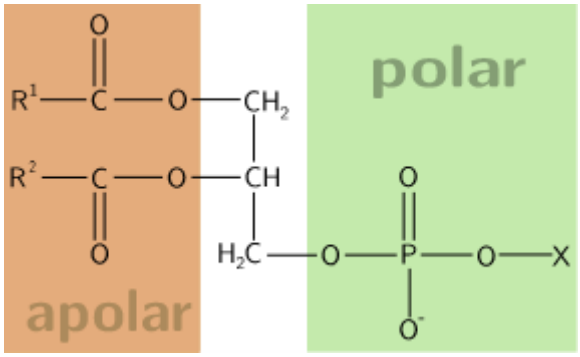
Figure 2.1: A Mixed Triacylglycerol.



Their characteristic hydrophobic nature and long chain length are easily seen in fatty acids (Figure 2.1). Specifically, acylglycerols make up 99% of lipids in animals and plants. Fatty acids can contain one, two or three acylglycerols and are described as aliphatic monocarboxylic acids. Hydrolysis, by enzyme, heat or acid, can liberate fatty acids from the glycerol backbone. While saturated fatty acids have no double bonds, unsaturated fatty acids may have one (monounsaturated fatty acids, MUFAs) or more (polyunsaturated fatty acids, PUFAs). In PUFAs, the double bonds are usually unconjugated and, more often than not, methylene interrupted. Further characterization of lipids indicates geometric isomerization; that is, these bonds can occur with the carbons *cis*- (on the same side) or *trans*- (across from). There is a natural predilection for the *cis*- configuration, while *trans*-fatty acids are generated industrially through hydrogenation.

Complex lipids have the same requisites as simple lipids, but differ by the addition of another functional group. Glycerophospholipids (GLP) and sphingolipids (SL) provide excellent examples of complex lipids. GLPs are a group of complex lipids that contain an *ortho*-acyl, -alkyl, or -alkenyl group attached to the glycerol backbone. Many GLPs (phosphatidlycholine, phosphatidylinositol) exist in the biological membranes of cells and are found in many foods.

Figure 2.2: General Chemical Structure of Glycerophospholipids



Source: http://en.wikipedia.org/wiki/Image: Phospholipid.svg

SLs differ from GLPs in that their core exists as a long-chain amino alcohol and their absence of a glycerol backbone.

2.2 Lipid Oxidation

Oxidation is a degradation process involving lipids and a reactive species, usually oxygen (ROS) or nitrogen (RNS). It is common in many industries, such as the rubber industry, metabolic systems, and the deterioration of food products. Economically, it is relevant to the food industry because of the development of off odors, off flavors, off colors, and structural degradation via acid formation, nutrient degradation, and mutagenic/carcinogenic/teratogenic by-products. Conversely, controlled lipid oxidation can be desirable in products like aged cheeses and fried foods.

2.2.1 Types of Oxidation in Lipids

When discussing lipid oxidation, it is important to distinguish which mechanism is at work. Autoxidation is the primary pathway of importance in the food industry, and will be discussed momentarily. Photooxidation is similar to autoxidation but differs by a number of features: (1) the reactive, singlet oxygen [$^1O_2$] is produced from a 'sensitizer' and triple oxygen [$^3O_2$]; (2) the reaction cascade is not a radical chain process but an "ene" reaction (Bradley & Min, 1992); (3) there is no induction period; (4) exclusion of light, O_2 or inhibition of $^1O_2$ by a quencher are the only known methods of inhibition, conventional antioxidants used in autoxidation show now effect; (5) it specifically attacks olefinic carbon atoms [MUFAs] and can be seen during the *cis*- to *trans*- isomerization of the double bond; (6) it happens more quickly than autoxidation [which can be measured by number of double bonds instead of number of doubly activated allylic groups]; (7) the end-products are similar in nature, but different in structure to those produced by autoxidation (Gunstone, 1999).

Enzymatic oxidation is common in many food products. Often, enzymatic oxidation is due to a lipoxygenase system. The system bears a similarity to autoxidation when considering the formation and degradation of 9- and 13-hydroperoxides, but differs due to the mechanistic production of these compounds by lipoxygenases and subsequent cleavage by bond-specific lyases (hydroperoxide lyase)(Galliard *et al*., 1976; Cai, 1997). These mechanistic differences bear great importance because they also render the conventional antioxidants used in autoxidation useless.

Autoxidation, as previously mentioned, is a radical chain process that has been studied extensively. Radicals are highly reactive, odd electron species. They are often hard to measure and quantify, especially in a dynamic environment. Classically, the process of oxidation is split

into three phases: initiation, propagation and termination. The induction period of autoxidation can be understood in two parts: the creation of a reactive species and abstraction of an α-methylenic hydrogen atom (initiation). While the creation of a reactive species is not fully understood, there exist some hypotheses: (1) radical hydroperoxides are produced by metal-catalyzed decomposition; (2) photooxidation [as described above] may produce radical hydroperoxides; (3) heat may initiate the propagation of a radical species.

2.2.2 Initiation

To understand initiation, it is important to understand and employ molecular orbital (MO) theory. During bonding, MO theory dictates that single bonds contain a sigma-bond, while higher order bonds contain one sigma- and a number of pi- or delta-bonds associated with the total number of bonds between two atoms. The strength of a sigma-bond is explained by the mixing of *s*- and *p*-orbitals, while the pi-bond is characteristically weaker due to a more limited overlap that the *p*-orbitals are allowed. However, the existence of both σ- and π-bonds in a compound is stronger than either two alone. In terms of PUFAs, such as linoleic acid, the two sigma- and pi-bonds interact so as to draw electron density away from adjacent carbon atoms, making it easier to abstract a hydrogen atom from one of the carbon-hydrogen bonds next to the double bond (which are electron-deficient). Abstraction of a hydrogen atom becomes easier when considering the pentadiene configuration of the methylene interrupted carbon in linoleic acid (due to the electron-rich double bonds on either side of that specific carbon). This interaction (including the unknown sensitizer or catalyst) can be described as:

(1) $$RH + Initiator \rightarrow R^{\bullet} + H^{\bullet}$$

The hydroxyl ($OH^{\bullet}$) or hydroperoxyl ($HOO^{\bullet}$) radical delivered by the sensitizer is considered to be the most important initiator of unsaturated lipid autoxidation (Aikens & Dix,

1991; Choe & Min, 2006; Gebickie & Bielski, 1981). The mechanism involving hydroxyl radicals and unsaturated fatty acids involve a hydrogen atom abstraction to complete the hydrolysis reaction as follows

$$(2) \qquad RH + OH^{\bullet} \rightarrow R^{\bullet} + H_2O$$

Alternatively, the hydroperoxy radical can also scavenge a hydrogen atom to produce an alkyl radical ($R^{\bullet}$), but requires that double allylic hydrogen atoms be present for the reaction to proceed (Bielski *et al*., 1983).

The substrates that have been observed to cause these highly reactive compounds have primarily been enzymatically- or heme-formed superoxide radical anions ($^{\bullet}O_2^-$) (Kanner *et al.*, 1987; Aikens & Dix, 1991, Winterbourn, 1990), photo-sensitized triplet oxygen ($^3O_2$) (Haseloff, Ebert & Roeder, 1989), hydrogen peroxide (H_2O_2) via fenton reactions (Kanner *et al*., 1987; Watanabe *et al*., 2002) or metal-catalyzed Haber-Weiss reactions (Kellog & Fridovich, 1975; MacManus-Spencer & McNeill, 2005) and water (H_2O) via ionization or excitation (Choe & Min, 2006, Schronerova *et al*., 2007).

In the autoxidation of meat, there are several proposed initiators. Harel and Kanner (1985) have proposed that species are activated by the interaction of hydrogen peroxide with metmyo- / methemoglobin. Alternatively, it has been suggested that microsomal oxidase systems that include Fe^{2+} and Fe^{3+} could initiate the process of lipid oxidation (Rhee, Dutson & Smith, 1984). Overall, whether authors agree that heme-proteins initiate lipid oxidation in meat, there is consensus that they continue the propagation process of meat (Ladikos, & Lougovois, 1989).

2.2.3 Propagation

Propagation is the mechanism by which oxidation both continues and exponentially increases the rate by which reactive products are produced. Because of both effects, this is the

stage by which the most damage to highly unsaturated lipid-based products can occur; control of this step can extend the induction phase previously described and greatly aid in the shelf-life and quality of many oxidation-prone food products.

The alkyl radical previously formed under the mechanism of initiation is highly reactive and can bond easily with triplet oxygen, forming a peroxy radical ($ROO^{\bullet}$) as detailed below:

(3) $$R^{\bullet} + {}^{3}O_2 \rightarrow ROO^{\bullet}$$

The formation of a hydroperoxy radical is highly unstable, making it only slightly less of an oxidizing agent than its precursor. In order to stabilize, the hydroperoxy radical abstracts a hydrogen atom from another unsaturated lipid to give a hydroperoxide and an additional alkyl radical (which can undergo the above mentioned reactions) (Bolland & Gee, 1946; Farmer *et al*., 1942; Frankel *et al*., 1961).

(4) $$ROO^{\bullet} + R'H \rightarrow ROOH + {}^{\bullet}R'$$

At this point, multiple outcomes can occur: (5) The new alkyl radical can abstract hydrogen atoms; (6) while stable at room temperature, hydroperoxides easily degrade under heat, ultraviolet radiation, or in the presence of a metal catalyst. These conditions can cause branching of the hydroperoxide (ROOH) back into a hydroperoxy radical ($ROO^{\bullet}$) and hydrogen ion (H^{+}), or (7) branching can occur under the same circumstances between the oxygen couplet to form a peroxy radical ($OH^{\bullet}$) and an alkoxy radical ($RO^{\bullet}$). All of which can abstract hydrogen atoms from nearby unsaturated fatty acids.

(5) $$R'^{\bullet} + R''H \rightarrow R'H + {}^{\bullet}R''$$

(6) $$ROOH \xrightarrow{\Delta,\upsilon} ROO^{\bullet} + H^{\bullet}$$

$$ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + Fe^{2+} + H^{+}$$

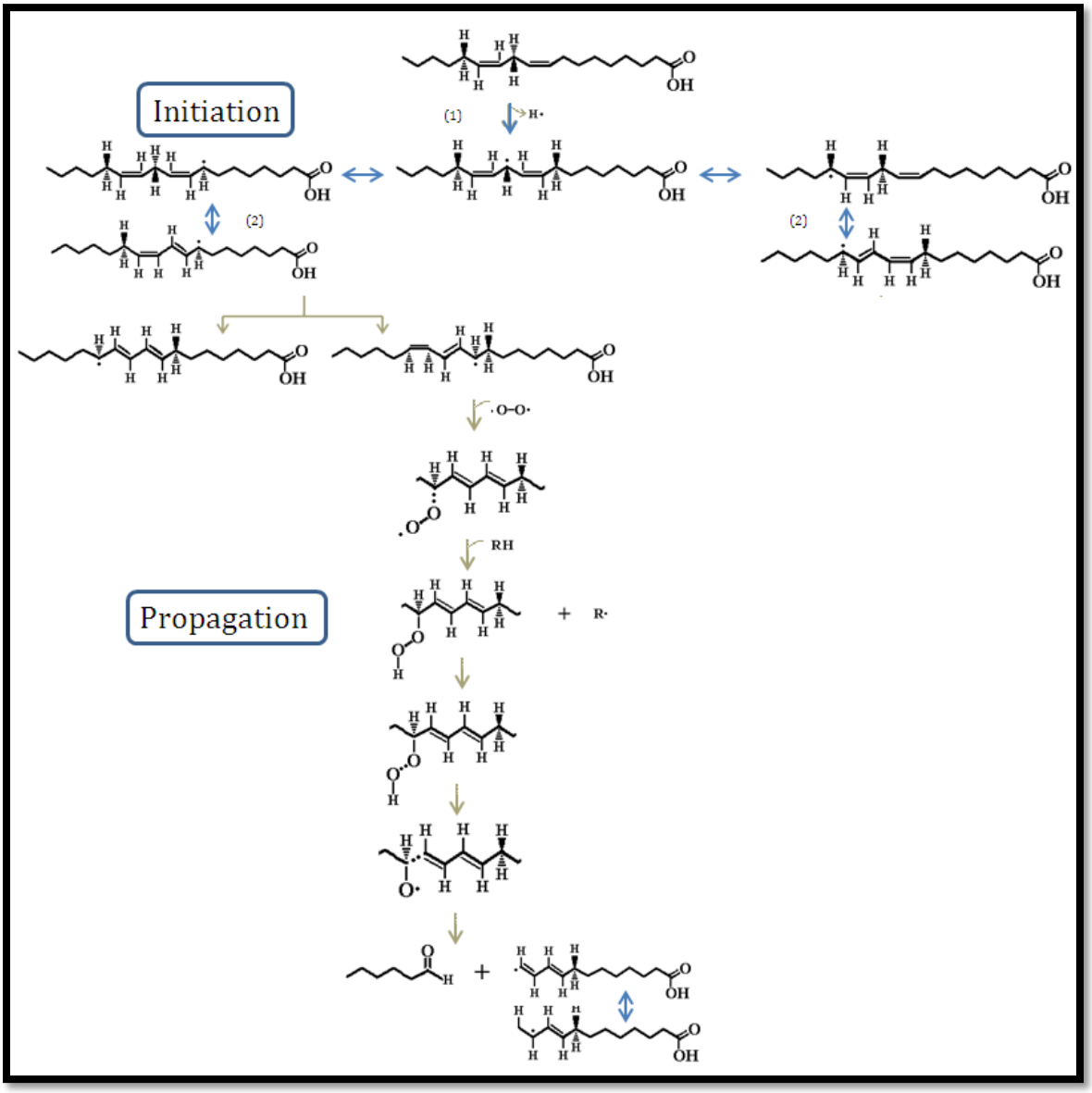
(7) $$ROOH \xrightarrow{\Delta,\upsilon} RO^{\bullet} + HO^{\bullet}$$

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + Fe^{3+} + OH^{-}$$

The greatest issue in understanding the mechanics of oxidation results from the complexity of the substrates (i.e. whole food systems). To more easily define, control and understand the mechanisms by which propagation occurs, simpler systems have been modeled, such as methylenic esters from fatty acids. Many authors (Frankel, 1980; Frankel, 1982; Frankel, 1991, Hseigh *et al.*, 1989) have detected the formation of hydroperoxides from methyl oleate, linoleate, linolenate and arachidonate and polyunsaturated fatty acids through qualitative and quantitative methods. The figure below shoes a representation of the steps in the oxidation of linoleic acid.

The 1,4-pentadiene structure makes the ester of linoleic acid roughly 10 times faster at oxidizing than the methylenic ester of oleic acid (Labuza, 1971). Additionally, the methylene group at position 11 (where the double bonds are often described as methylene-interrupted) is twice as active in terms of hydrogen atom abstraction due to the adjacent double bonds. This abstraction allows for carbon-9 and carbon-13 to form a mixture of 9- and 13-hydroperoxides. The geometric isomerization from a *cis,trans*-hydroperoxide to a *trans,trans*-hydroperoxide has been demonstrated through HPLC and ^{13}C-NMR (Labuza, 1971).

Figure 2.3 Oxidation of Linoleic Acid



Additional propagation can occur when alkoxy radicals can react with (8) unsaturated fatty acids to form stable alcohols, or they can (9) undergo fragmentation to form unsaturated aldehydes (RCHO).

(8) $RO^{\bullet} + RH \rightarrow ROH + R^{\bullet}$

(9) $RO^{\bullet} \rightarrow RCHO + R^{\bullet}$

Because of the formation of not just one, but in most cases two radical species, the propagation of autoxidation becomes a cascade that threatens the quality of high-lipid matrices. The primary byproducts of lipid oxidation are hydroperoxides. While these substrates are tasteless and odorless, their instability begets secondary byproducts (e.g. aldehydes, ketones, alcohols, acids, hydrocarbons) have a great impact on flavor. Aldehydes are often very easy to detect by humans, some at concentrations as low as 1 ppm. Ultimately, the degradation of hydroperoxides, unsaturated aldehydes and ketones, and volatile secondary bi-products of hydroperoxide degradation can interact with proteins, amino acids, and amines, altering sensory characteristics, environmental pH, and the nutritional status of the foods from which they are derived (Halliwell *et al*., 1990; von Sonntag *et al*., 1990).

2.2.4 Termination

The mechanism of termination includes a condensation reaction involving peroxy, alkoxy, or alkyl radicals. For reactive radical species: (10) the combination of two peroxy radicals at room temperature lead to the production of diatomic oxygen and a peroxy-linked dimer (ROOR). Under low oxygen pressures and high temperatures, (11) ether-containing dimers (ROR) can be produced from alkoxy radicals and (12) carbon-carbon linked dimers (RR) can be generated from alkyl radicals. Lastly, (13) alkyl radicals can combine with peroxy radicals to generate peroxy-linked dimers (ROOR) (Hseih *et al*., 1989, Frankel, 1980; Frankel, 1982).

(10) $ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$

(11) $RO^{\bullet} + R^{\bullet} \rightarrow ROR$

(12) $R^{\bullet} + R^{\bullet} \rightarrow RR$

(13) $ROO^{\bullet} + R^{\bullet} \rightarrow ROOR$

In conclusion, the oxidation of lipids is a complex process that undergoes several complex mechanisms. The environmental conditions and initial substrates often generate several pathways that lead to a diverse assortment of primary and secondary end products. These end products can lead to off-odors, off-flavors, nutrient degradation, environmental changes, and possibly even toxins.

2.3 Measurement of Lipid Oxidation

Because oxidative decomposition is of economic and nutritional importance to industries whose products have high lipid concentrations, multiple tests of lipid oxidation have been employed to detect the degree to which a sample has oxidized. The most intuitive and useful test of lipid oxidation is sensory analysis. The nature of the food industry is consumer-based. That is to say, if consumers detect an inferior product, consequences can be observed in the form of economic loss, loss of product credibility, and loss of reputable stature. Alternatively, chemical methodologies have been long employed to detect oxidation in food products. To date, there is no methodology or instrument that can detect all possible initial, primary (hydroperoxides), secondary and tertiary species of the oxidation process. Even with current methodologies and instruments, there is no perfect way to measure such species in terms of all lipids, all lipid-containing foods, nor under all processing conditions. At best, each method or instrument can measure a few changes under specific conditions for a specified period of time. In terms of oxidation of high-lipid foods, primary and secondary end products are of the highest value to the consumer and industry.

2.3.1 Sensory Evaluation

The consumer holds the power of discrimination between an acceptable product and an unacceptable product. This axiom combines with the reality that the human senses are more

sensitive than any machine to explain why sensory evaluation is the ultimate method when assessing the quality of a food product (Meilgaard *et al*., 1990). Humans can combine the structural, compositional and internal physical forces of a food matrix in a near-instant manner. At the same time, the human experience can combine physiological, psychological and cognitive data about that matrix; this latter facet accounts for the higher-quality of results from the sensory analysis in comparison to a machine. In fact, many attempts to use a machine to emulate a single human sense can, at best, only show correlation (Cook *et al.*, 2005; Kappes, Schmidt, & Lee, 2007) and, at worst, fail (Kappes, Schmidt, & Lee, 2006); Lee *et al*, 2005). Conversely, sensory evaluation has its disadvantages; physiological factors (e.g. adaptation error), psychological factors (e.g. expectation error), poor physical conditions (e.g. perfume, "supertaster" status), and poor environmental conditions can affect human responses in addition to it being costly (Meilgaard *et al*. 1990). For these factors, instrumental analysis and chemical assays have proven superior. Many authors have found reconciliation by combining both chemical assays (TBARS, POV, etc.) with instrumental analysis (gas chromatography, solid phase microextraction) and sensory analysis.

2.3.2 Chemical Methodologies

2.3.2.1 TBARS

Thiobarbituric reactive substances (TBARS) is generally considered the 'gold standard' in the measurement of lipid oxidation, with specific appreciation held by those using a muscle-food matrix (Gray & Monahan, 1992). The overall methodology involves the spectrophotometric measurement of a pink chromophore giving an absorption maximum between 530 and 533 nm (Miller, 1998). The chromophore is formed by the reaction of 2-thiobarbituric acid (TBA) with a secondary product of lipid oxidation, generally aldehydes and ketones. Often, 1,1,3,3-

tetraethoxypropane (TEP) is used to create a standard curve against which a TBARS-value can be calculated. This polymethinic pigment complexes with TBA and reacts to liberate malondialdehyde (MDA) in a hydrolysis reaction [Figure 2.2].

Figure 2.4: Reaction between TBA and MDA to form TBA pigment (Fernández, et al. 1996)

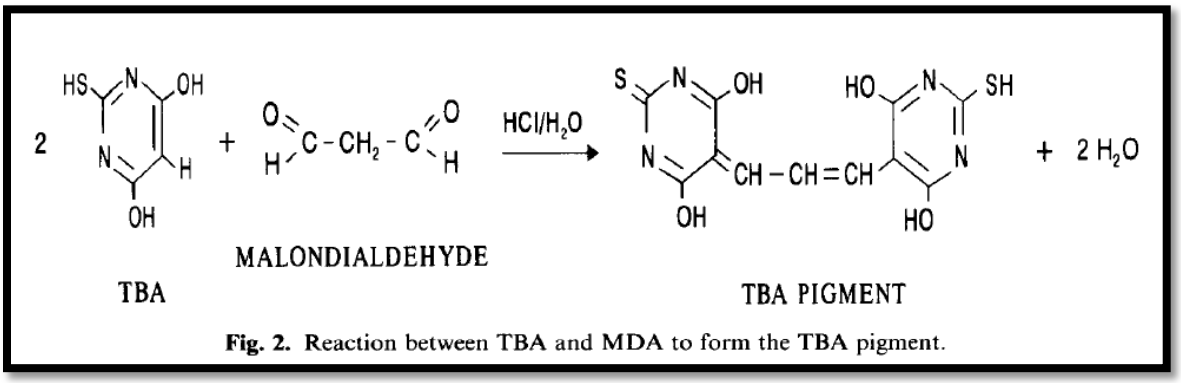


Fig. 2. Reaction between TBA and MDA to form the TBA pigment.

The extent of oxidation is frequently given in milligrams of MDA equivalents per kilogram of sample. Interfering agents have been cited as acids, esters, sugars, amino acids, oxidized proteins, pyrimidines and pyridines (Guillén-Sans & Guzmán-Chozás, 1998). The lack of chemical specificity of the assay has garnered the all-inclusive term "reactive substances" in the assays' name.

While the assay is used almost ubiquitously in the literature, it contains multiple limitations that need to be addressed. First, the assay is extremely sensitive to operator-use. Secondly, there may be discrepancy in the methodology of the experiment. The method described by Tarladgis, Watts and Younathan (1960) uses steam distillate, while methods described by Miller (1998) using acidic extracts are faster and easier. The advantage of an acidic extract has been recommended under conditions where the sample size is large (Pikul *et al*., 1989). Also, the acidic extraction is done under cold conditions, which inhibits thermally-generated products from distillation methodology. Third, while the test is capricious in detecting lipid oxidation products, it can falsely recognize substrates such as nitrites (e.g. cured meats)

(Shahidi, 1991). Lastly, the short chain carbon products that are produced (such as MDA) are not stable and degrade to organic alcohols and acids (Fernandez *et al*., 1997).

Taking these cautions into consideration, it is known that iron catalyzes hydroperoxides production in meat to MDA at physiological pH and temperature (Janero, Burghardt, 1989). Most importantly, sensory analysis has shown that off-flavors in meat are strongly associated with the production of TBARS, pentanal, and hexenal (Stetzer *et al.*, 2008; Poste *et al*., 1986; St. Angelo *et al*., 1987). In fact, panelists can detect oxidation off-flavors when the TBA values are as low as 0.5 to 2.0 μg / g sample (Gray, Gomaa & Buckley, 1996).

2.3.2.2 Carbonyl Value

As an alternative method to measure secondary oxidation products is the determination of carbonyls (Henick, Benca, & Mitchell, Jr., 1954). As mentioned previously, carbonyl compounds are responsible for most deleterious flavors; specifically, volatile carbonyl compounds participate in off-flavor production. As such, methods to determine these compounds, quantify them, and correlate them with sensory attributes have been developed.

Most methods to quantify volatile carbonyl compounds involve vacuum or steam distillation or extraction via hexane. The particularly well-known method by Henick, Benca & Mitchell (1954) measures the formation of 2,4-dinitrophenyl hydrozones in the presence of an acid catalyst (generally trichloroacetic acid) from carbonyl compounds at 340 nm under alkaline conditions. Disadvantages to this method involve the degradation of hydroperoxides due to the conditions of the test.

2.3.2.3 p-Anisidine value

The anisidine value is a determination of secondary oxidation products, specifically 2-alkenyl concentration (White, 1995). As a spectrophotometric assay, the value is defined at 100

× Absorbance at 350 nm of 1 gram of fat per 100 mL of p-anisidine:acetic acid solvent (AOCS, 1998). The p-anisidine value has often been correlated to the headspace of volatile analysis , polymer content and sensory evaluation (Tompkins & Perkins, 1999), as well as FTIR spectroscopic predictions in thermally stressed fats and oils (Dubois *et al.*, 1996). While it hasn't been considered particularly valuable for determining off-flavors in products stored at ambient or refrigerated temperatures (Holm & Ekbom-Olsson, 1972), it tends to be a rapid, widely reproducible assay in determining secondary oxidation products of thermally-stressed matrices.

2.3.2.4 Peroxide-Oxygen Value

As primary oxidation products, hydroperoxides are formed in low levels. As such, measurement of these products is directed towards uncooked products stored at low temperatures (Coxon, 1987). Herein lies multiple disadvantage in the measurement of primary oxidation products: (1) their predilection to degrade into volatile, secondary oxidation products; (2) after maximum levels are achieved, they decay as a function of temperature, environment, etc.; (3) any test measuring peroxides will only give values for that specific point in time, making comparison among samples difficult; (4) the peroxide value and negative sensory attributes have been inconsistent tend to be strictly matrix- and processing-dependent (Kanner, *et al.*, 1992; Fennema, 2008).

Derived from the AOAC method (1998), the peroxide value can be reported in milliequivalents of iodine per kilogram of fat. For muscle tissue, hydrophobic solvents are utilized to extract the lipid portion of the matrix. Caution must be taken when choosing solvents (which must be free of reducing/interfering agents) and when evaporating the solvent from the lipid. The most common POV method used for meat was developed by Folch *et al*. (1957); however, other methods include iodometric titration and other colorimetric changes (e.g. the

ferric thiocyanate method). When using this method, units can be given in milliequivalents of peroxide per kilogram fat.

In essence, POV can be used for determining early stages of oxidation, but fails as a method of analysis for prolonged storage (or to the point of consumer dissatisfaction). Regardless of the method, researchers must always be cognizant that values given are empirical, and caution should be taken when making conclusions on the monitoring of oxidation.

2.3.2.5 Conjugated Dienes

Geometric isomerization is one of the first noticeable changes that occur as lipids oxidize. Diene conjugation occurs primarily as a method of stabilization after hydrogen atom abstraction destabilizes the lipid, and appears again during hydroperoxide formation to again offer stabilization. This isomerization can be determined quantitatively via absorption at 232-234 nm (Dobagarnes & Velasco, 2002). When using a complex system (such as meat), lipid needs to be extracted using a bi-phasic solution. Hexane:Isopropanol in a 3:1 ratio is the most common solution employed (Juntachote *et al*., 2006) because of its ability to remain monophasic until the addition of a hydrophilic constituent causes separation.

There are several advantages to the conjugated diene methodology. In addition to being faster and simpler than assays such as POV, the conjugated diene assay does not require the use of chemical reaction and can be performed on smaller samples. Conversely, it does suffer from the same environmental conditions as Peroxide Oxygen Value. Also, the use of nature of these species stability causes a plateau during measurement (when the concentration of breakdown equals the concentration of newly formed conjugated dienes).

2.3.3 Instrumental Analysis

The use of a gas chromatograph (GC) and tools such as gas chromatography/ mass spectrometry (GS/MS) in characterizing and quantifying volatile compounds has been widely appreciated. These instrumental methodologies are highly sensitive, reliable and well suited for many organic compounds (James, 1995). Methods using these machines and techniques include solid phase microextraction (SPME)/ static headspace, dynamic headspace, solvent assisted flavor evaporation, in addition to direct gas chromatography. In static headspace analysis, gas above the sample (which has come to equilibrium) is transferred directly in a GC column which then separates compounds and quantifies them based on polarity and size. This is advantageous because it does not destroy the sample, is convenient and rapid as a method for analyzing volatile compounds. The disadvantage of this method is the requirement of elevated temperatures at which the sample must be held.

2.4 Antioxidants: Control of Lipid Oxidation

A common method to inhibit lipid oxidation is to employ the use of antioxidants. No antioxidant is capable of stopping / reversing the process of oxidation, but they can minimize the cascade of reactions. Also, antioxidants do not function to control hydrolytic rancidity (the cause of which is enzymatic). Compounding the issue, the variability of fats and fat composition in foods translates to multiple problems associated with antioxidants and measurements of their efficacy. To start, each system of lipid (plant-, monogastric-, or polygastric-based) contains different levels of oils and fats with different levels of endogenous antioxidants. Because of this intrinsic difference, each system must be evaluated specifically. Also, caution should be waved against comparing results from one matrix to a separate matrix. During experimentation, temperature variations and assays used can alter results. Depending on the nature of the species

being measured (geometric isomerization, primary, secondary or tertiary oxidation products), temperature can alter the mechanism of oxidation, volatility of antioxidant or breakdown of hydroperoxides (Gunstone, 1999). Mixtures of antioxidants have synergistic effects. Perhaps most importantly is the influence of solubility and distribution of the antioxidant in a matrix. From these factors, it becomes evident the complexity of the issue of qualifying and quantifying antioxidants. Before quantifying the efficacy of an antioxidant in a specific matrix, it is helpful to separate antioxidants based on their mechanisms of action. While many authors qualify antioxidants differently, the scientific community at large has agreed on 6 mechanistic actions (commonly divided into 3 categories, denoted below by the superscripts †,‡, and §) (Ruberto *et al.*, 2001; Athukorala *et al*., 2006):

a. Scavenging species that initiate lipid oxidation†

b. Chain breaking to prevent hydrogen atom abstraction†

c. Quenching singlet O_2 to prevent peroxide formation‡

d. Peroxide removal‡

e. Chelating free metal ions‡

f. Reduction of localized O_2 concentration§

2.4.1 Type I † (Chain Breaking Antioxidants)

The first, and most prevalent, group of antioxidants is the chain-breaking antioxidants. Mechanistically, this category promotes the termination process. Structurally, compounds with high unsaturation (usually conjugated) or many phenolic components fall into this category. These compounds act sacrificially, and only as long as the stability of their structures allow. These free radical acceptors are known to react with hydroperoxy radicals (ROO▪) and not alkyl

radicals (R▪), indicating a competition between the inhibition reaction (4') and the chain propagation reaction (4) (Bolland & Ten Have, 1947):

(4') $ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$

The characterization of these "primary antioxidants" is governed by their activation energy (E_A) , rate constants, red-ox potential, solubility, and length of activation. Because antioxidants of this category can donate a hydrogen atom and retain a stable structure through resonance, they prevent the cascade of oxidation from continuing for a modest amount of time. This is functionally seen by an increase in the induction phase of oxidation.

Type I antioxidants are prevalent in foods. Recently, Rojas & Brewer (2008) demonstrated that consumers have trended towards "natural" and "clean labeled" products, opting to trade synthetic antioxidants such as BHA/BHT and TBHQ with those from natural sources. Many plant sources contain a variety of natural phenolic antioxidants, and an enormous amount of research has been published on antioxidant and antimicrobial activities of their extracts. In addition to scavenging free-radicals, antioxidant components from plant sources can chelate metals (Type II) and absorb light in the ultraviolent region (a high-energy source of free radicals).

2.4.2 Type II ‡ (Free Radical Prevention)

The mechanisms that chelate metals or destroy hydroperoxides fall into the category of Type II antioxidants. Largely, metal chelators such as ethylenediamine-tetraacetic acid (EDTA) and ascorbic acid (vitamin C) are used in these functions. Their use relates to the matrix in which they are applied. In high water foods, they can act effectively in binding metals; in proteinaceous foods with highly bound metals, they tend to be less effective (Labuza *et al.*, 1991). Additional

environmental concerns are involved with thermally processed foods, presence of enzymes, and composition of the matrix.

Hydroperoxide destroyers also fall into the category of type II antioxidants. Tocopherol (vitamin E) and Trolox™ (a carboxylic acid analog of vitamin E) work to reduce hydrogen donation (Tappel, 1972). Meat-related foods also contain endogenous hydroperoxide and hydrogen peroxide quenchers, such as glutathione peroxidase and catalase (Niki, 1987). These enzymes are effective in reducing primary oxidation products without generating free radicals. Finally, it is of paramount importance to acknowledge that synergy between type I and type II antioxidants occurs.

2.4.3 Type III[§] (Environmental Control)

Environmental control is a mechanism of controlling oxidation that relates more to physical parameters than chemical modification of a matrix. Because the initiators of oxidation are varied, type I and II antioxidants are limited by their specificity. However, altering the environment of the matrix so that it has less unbound water, lower temperatures, exclusion of light or reduced access to oxygen has also been shown to prolong the induction period of lipid oxidation.

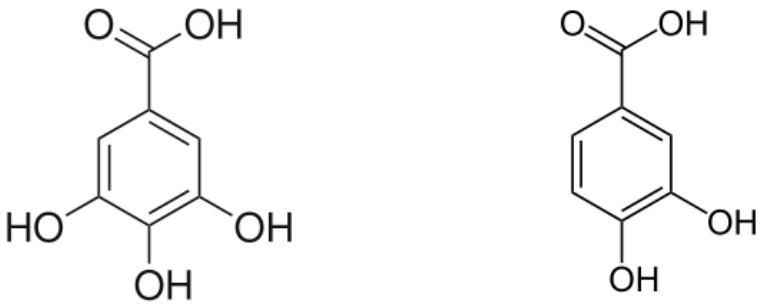
2.4.4 Natural Antioxidants

With the change in consumer perception of natural versus synthetic food additives (Rojas *et al.,* 2008), the food industry has had to make a drastic alteration in their methods of antioxidant activity. In the search for natural antioxidants, many botanical and algal agents have been researched. These biological substrates contain compounds such as sulfated polysaccharides, phenolics, tepenoids, lactones, sterols and fatty acids. (McDermid & Stuercke, 2003; Duan et al., 2006; Qi et al., 2006). From a chemical standpoint, the major plant phenolic

compounds can be broken into aromatic indoles (e.g. betalain, found in beets), reducing lactones (e.g. vitamin C), terpenes (e.g. lycopene, found in tomatoes), tocopherols (e.g. vitamin E) and polyphenolic compounds.

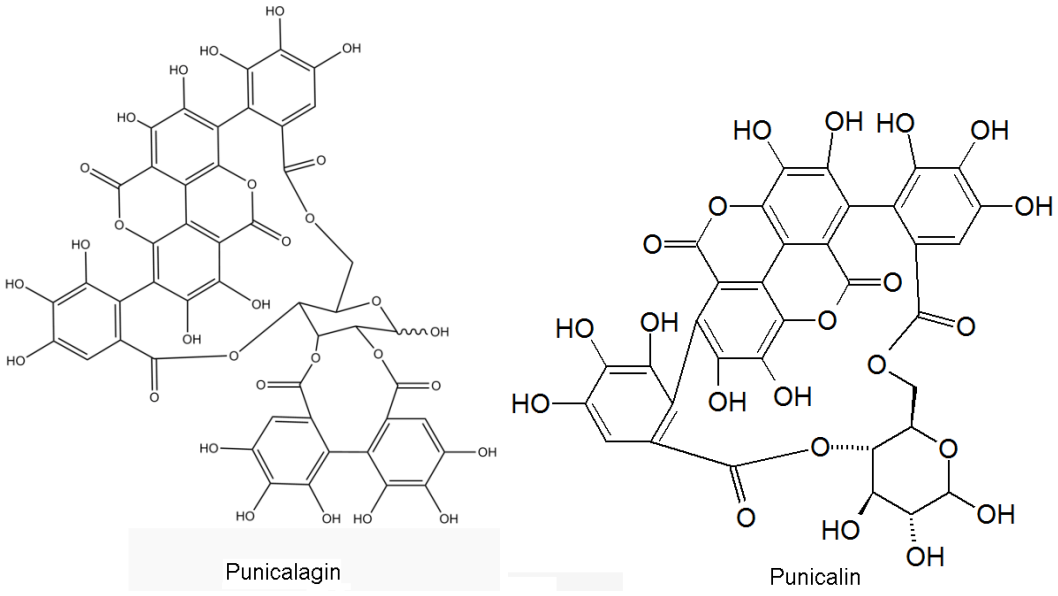
Phenolic compounds are of great interest. Of the phenolic compounds, four groups can be further subdivided: phenolic acids (gallic acid, rosmarinic acid,), phenolic diterpenes (e.g. carnosic acid), flavonoids (chatechin, epicatechin) and volatile oils (eugenol, methnol). These categories also dictate mechanistic antioxidative pathways. Where phenolic acids may trap free radical species, flavonoids can inhibit oxidation by chelating metals or scavenging free radicals (Geldof & Engeseth , 2002).Their efficacy in a system is dependent on their chemical characteristics (number of double bonds, number and placement of hydroxyl groups, etc.) as well as their physical location within a food (propinquity to emulsion interfaces, etc.) (Lupea *et al.*, 2008; Wanatabe *et al.*, 2010). Brown and Kelly (2007) proved that the location of hydroxyl substitutions on a compound affects the efficacy in phenolic compounds, while Gheldof and Engeseth (2002) verified this fact in flavonoids . Specifically ortho-dihydroxy substitutions were found to be more effective than trihydroxy-substituted compounds, potentially due to steric hindrance of the more-substituted phenolic compound (Figure 2.5).

Figure 2.5: Gallic acid (left) showing a higher degree of substitution than protocatechuic acid (right).



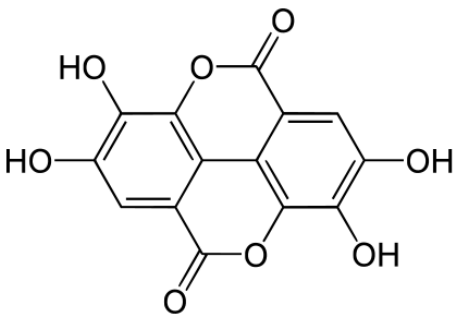
Of specific interest to this study is the amount of natural antioxidant compounds found in POMx (the source of pomegranate extract in this study). Rasheed *et al.* (2009) and Sartippour *et al.* (2008) both reported the contents of POMx to contain 86.0% ellagitannins (according to manufacturer's data) with 19% ellagitannins as punicalagins and punicalins (Figure 2.6), 4% free ellagic acid, and 77% oligomers composed of 2–10 repeating units of gallic acid, ellagic acid, and glucose in different combinations.

Figure 2.6: Punicalagin (left) and punicalin (right) constitute antioxidant components of POMx



Ellagitannins are polyphenolic derivitives of ellagic acid, formed through oxidative reactions. The galloyl groups found in ellagitanins are formed through depside bonds.

Figure 2.7: Ellagic Acid



2.5 Measurement of Antioxidant Potential

Due to the variety of mechanisms that can induce oxidation (photooxidation, enzymatic oxidation, autoxidation, etc.) and the complexity of matrices (food, cellular, etc.), comparison of antioxidants remains a challenge in both the fields of nutrition and food science. The use of model studies remains beneficial to both fields. However model systems are costly, labor intensive and rarely retain applicability across matrices. While there has yet to be a method of direct analysis that perfectly characterizes an antioxidant or can apply across all antioxidants, there are multiple assays in the literature that have attempted to measure total antioxidant capacity *in vitro*. While the ones discussed hereafter are not exhaustive, they are both common and display the advantages and disadvantages among published methods.

Briefly, the scientific community has developed experimental methods to measure antioxidation capability based upon mechanistic features that relate to the mechanism of oxidation. For the duration of this overview, only food matrices will be taken into account, so as to limit compounding issues of metabolic interconversion. Frankel and Meyer (2000), in their review on antioxidants, encouraged a multi-dimensional analysis when evaluating antioxidants. Furthermore, their article outlined basic considerations including the use of biologically relevant substrates and molarly equivalent antioxidants, using various oxidation conditions, measuring both primary and secondary oxidation products, and quantifying results based on induction period, percent inhibition, IC_{50} (antioxidant concentration to achieve 50% inhibition of oxidation) or rates of hydroperoxide formation/decomposition. From these tenets, a multitude of antioxidant assays have been developed that can roughly be broken into two categories: single-electron transfer (SET) assays and hydrogen-atom transfer (HAT) assays. Huang *et al.* (2005) defined 8 criteria that any approach to antioxidant measurement should possess:

a. Measures chemistry actually occurring in potential application
b. Utilizes a biologically relevant radical source
c. Simplicity of the assay,
d. Clarity of the end-point and mechanism
e. Readily available instrumentation
f. Good intra- and inter-assay reproducibility
g. Adaptability to simultaneously assay lipo- and hydrophillic antioxidants
h. High throughput for routine quality control analysis

2.5.1 Single-Electron Transfer Assays

The popularity and ubiquity of SET assays originates from their reproducibility, low cost, and drastically less labor-intensive procedures. Mechanistically, assays of this type are fundamentally based on reduction of one compound (usually a metal-alloyed species). A simple representation of the mechanism was described by Huang, Ou, and Prior (2005) as such:

Probe (oxidant) + e^- (from antioxidant) → reduced probe + oxidized antioxidant

(4') $\mathrm{ROO^{\bullet} + AH/ArOH \rightarrow ROO^{-} + AH^{\bullet+}/ArOH^{\bullet+}}$

(5') $\mathrm{AH^{\bullet+}/ArOH^{\bullet+} + H_2O \rightleftarrows A^{\bullet}/ArO^{\bullet} + H_3O^{+}}$

(6') $\mathrm{A^{\bullet}/ArO^{\bullet} + H_3O^{+} \rightleftarrows ROOH + H_2O}$

(Apak 2007)

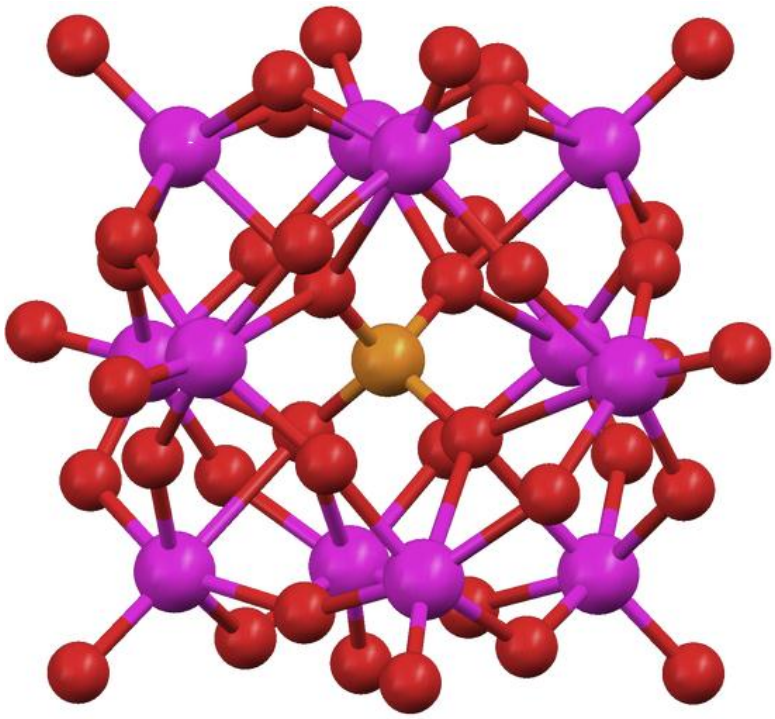
Often, SET assays employ the use of spectrophotometric color changes or classical redox titrations as a quantification of reducing ability of the antioxidant in question. Reducing ability

has been largely linked to antioxidant activity (Nawar, 1996). From this, it would fit that "electron based"-transfer assays would give comparable (but not identical) results. The reason for this is due to the diverse reaction conditions: red-ox potentials, pH, and kinetics of specific assays

2.5.1.1 Reduction by Folin-Ciocalteu Reagent

Proposed in 1912 and developed in 1927 (Ciocalteu, 1927) to measure phenolic content in proteins, the Folic-Ciocalteu Reagent (FCR) has been used often to correlate the phenolic profile of compounds with their antioxidant activity (on the basis of reducing capacity). Since then, the International Organization for Standardization (2005) has set forth a methodology for determining total polyphenols that many authors have adapted for alternate matrices. A major use of this assay is the oxidation of mono- and vicinal diphenols, species not always reactive in other SET assays. The assay uses an oxidant with an uncharacteristically high standard redox potential (0.7 V) and appears to be a powerful, nonspecific oxidant. Singletary *et al.* (1999) have indicated the assay is convenient, simple, has a large body of comparable data, and uses only common equipment (Singleton *et al.*, 1999). A critical look at the procedure and environmental conditions was published in 1999 (Singleton *et al.*). The currently accepted mechanism is the reduction of molybdenum in a sequence of one- and two-electron reactions on the heteropolyphospho-tungstates/molybdates (possibly, the Keggin anion (Shown in Figure 2.8) $[PMoW_{11}O_{40}]^{4-}$ or Dawson anion $[3H_2O\cdot P_2O_5\cdot 13WO_3\cdot 5MoO_3\cdot 10H_2O]$) (Huang, Boxin & Prior, 2005; Apak *et al.*,2007). They exist as hydrated octahedral complexes of the metal oxides caged around the central phosphate. The structure of the this compound shows a phosphorous situated at the center of a polyoxometallate cage.

Figure 2.8: Potential Keggin structure of the active agent in Folin-Ciocalteu reagent; color code: Molybdenum and Tungsten, pink; Oxygen, red; Phosphorous, orange. (Housecroft & Sharpe, 2005)

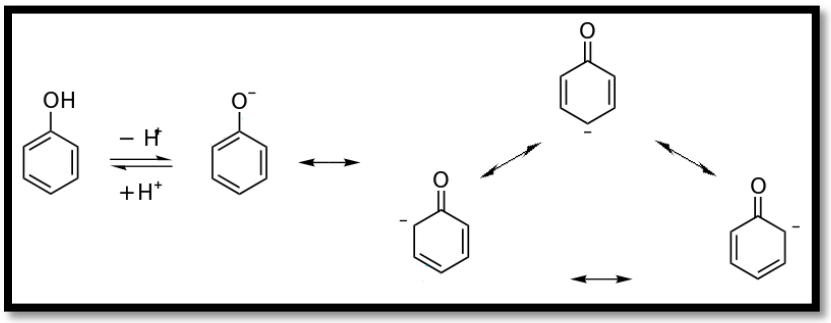


Since no alteration in the cage is observed by the addition of electrons, in lieu of the chemistry involving molybdenum and tungsten being nearly identical, the reaction is assumed to be described:

$$M^{6+} + e^{-} \rightarrow M^{5+}$$

The alkaline conditions under which the procedure is run can be concerning. Even in the methodology, the alkaline conditions destroy excess FC reagent. Without the presence of a reducing species and sufficient time to reduce FCR, the solution turns colorless. However, any phenolic species will dissociate into a phenolate anion, capable of reducing the FCR and creating a blue colored species whose absorbance can be determined between 730 and 760 nm.

Figure 2.9: Dissociation of phenol to phenolate resonance structure.

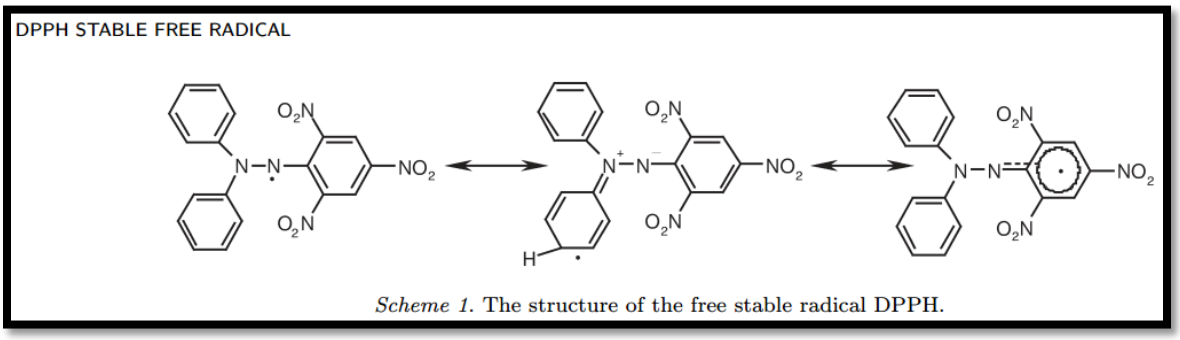


Depending on the nature of the antioxidant extract, considerations need to be taken into account, such as the presence of reducing sugars, phenolic amino acids, copper (I) complexes, vitamin C, etc. Additionally, the nature of the phenolate ion makes the pH important. While a basic environment is unusual for a food matrix, the phenolate ion requires a pH near it's pK to rapidly uptake oxygen to completion (Figure 2.9). Regardless, much data has been generated and as long as samples of similar

2.5.1.2 Free Radical Scavenging by DPPH

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical synthesized in 1922 (Goldschmidt & Renn, 1922) and used for the measurement of antioxidant activity (Blois, 1958). The stability is determined by the delocalized electron and nature of the molecule to resist dimerizing (as most radicals might) (figure 2.10). The overall reaction is a decolorized reduction of the dark violet solution (which gives a characteristic absorption band at 517 nm). It is advantageous for use over a wide pH range, while being mindful that its solubility is better in alcoholic environments and it is highly sensitive to atmospheric oxygen and light.

Figure 2.10: Resonance structure of DPPH (Ionita, 2005).



While scavenging DPPH was originally presumed to be an HAT-based assay, Ionita (2005) indicated that DPPH did not scavenge oxygen active species, and Foti *et al.*(2004) determined that it occurs as a *fast* electron transfer assay. Authors have positively correlated the scavenging of DPPH with antioxidant structure (namely increased hydroxyl groups and higher activity) (Sroka & Cisowski, 2003). With the ubiquity of the assay, multiple authors have attempted to set method parameters to follow (Molyneux, 2004; Sharma & Bhat, 2009). Abuse by many publications has come in the form of solvent chosen and concentration of DPPH. The extraction of an antioxidant using buffered methanol is preferred for non-polar/less polar and polar solvents (over water and acetone) and a DPPH concentration of 25-70 μM gives the most accurate results.

Absorbance results have been given in numerous ways, due to the ubiquity of the assay:

$$\%\text{DPPH}_{\text{rem}} = \frac{DPPH_{Remaining}}{DPPH_{T=0}} \times 100$$

$$\text{Antiradical Efficiency} = \frac{1}{EC_{50}} \times T_{EC_{50}}$$

Or in terms of a standard curve, often Trolox™, gallic acid, or ascorbic acid (Molyneux, 2004; Szabo, Idiţoiu, Chambre & Lupea, 2006).

Certain disadvantages are inherent due to the nature of the radical. Structurally, it is *stable*, which bears little resemblance to the transient and highly reactive peroxyl radicals it is meant to imitate (Huang, Ou & Prior, 2005). Like other reductive species, antioxidants that react with peroxyl radicals (rutin) react slowly with DPPH. Furthermore, the stoichiometry of the antioxidant must be known, as ascorbic acid reacts much faster than sulfur-containing molecules (Molyneux, 2004). It is also unreactive to monohydric phenols, simple sugars, purines and pyrimidines, while it has a tendency to precipitate proteins. Because its kinetics are not linearly correlated to DPPH concentration, results may be skewed heavily depending on the time of measurement.

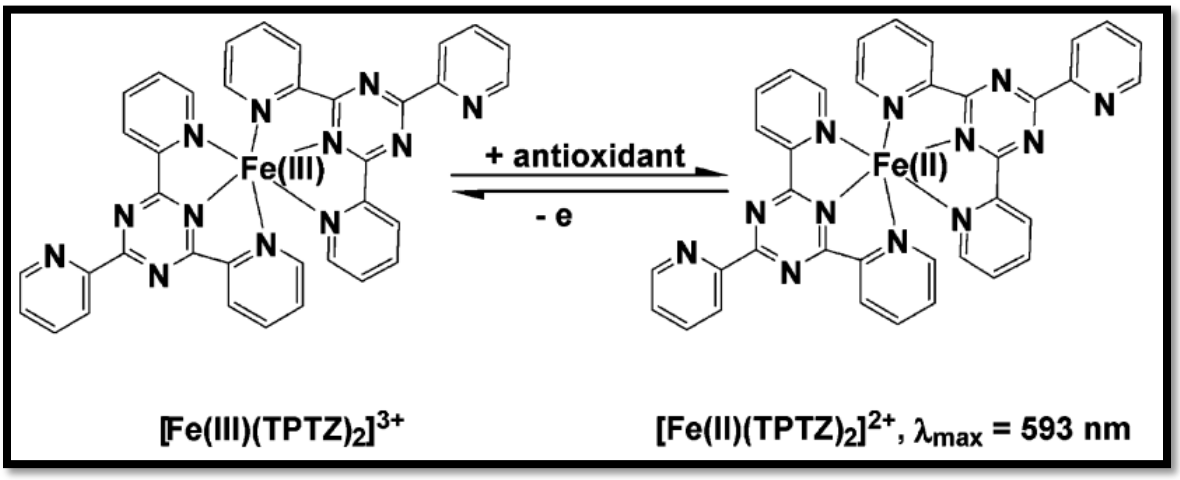
2.5.1.3 Colorimetric Determination by Iron-salts

Iron and iron complexes are advantageous in terms of direct analysis of antioxidants because they are biologically relevant. In meat, heme-iron is a common reducing agent that assists in the process of oxidation. Many metabolic reactions involve the reduction ferric compounds (Fe^{3+}) to ferrous (Fe^{2+}) ones, such as ferric tripydriyltriazine [$Fe(TPTZ)_2$, Figure 2.11] used in the popular Ferric Reducing Antioxidant Power (FRAP) assay.

$$Fe(TPTZ)_2^{3+} + ArOH \rightleftarrows Fe(TPTZ)_2^{2+} + ArO\cdot + H^+$$

(Berker *et al*., 2007)

Figure 2.11: Mechanism of FRAP Assay (Huang *et al.*, 2005)



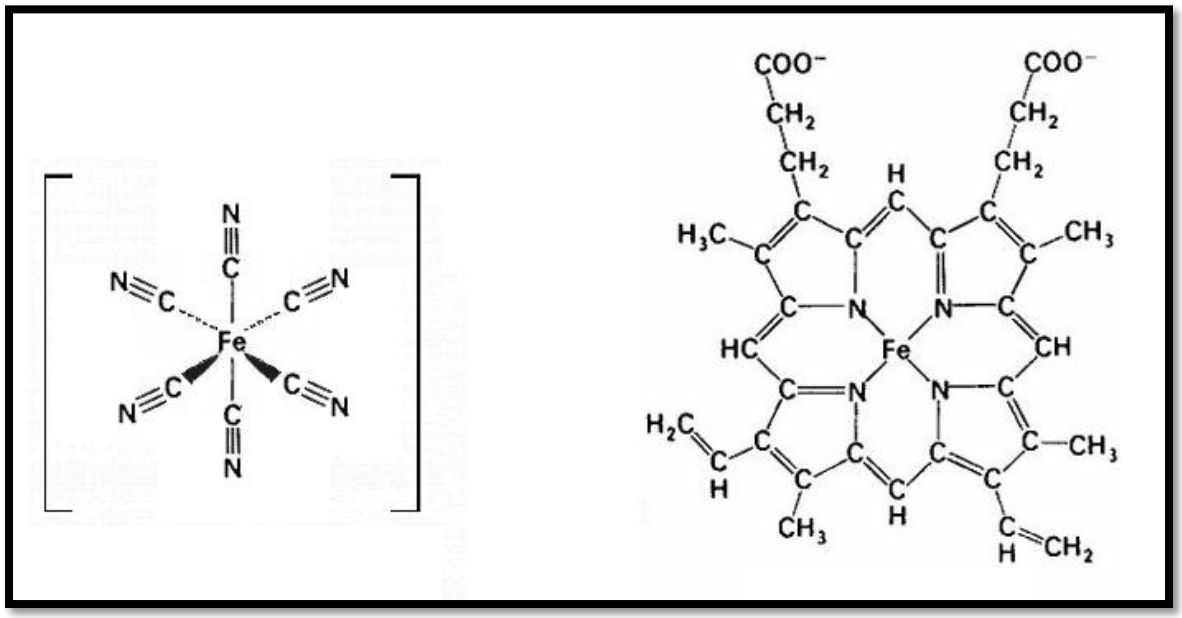
In the literature, there are two primary assays that utilize iron chemistry: FRAP and reducing power as determined by ferricyanide. Both are advantageous because they are simple colorization reaction that are speedy, inexpensive and robust (Benzie & Strain, 1999). Both use antioxidants as reducing agents and are measured by their colorimetric change at a given wavelength. In FRAP, the red-ox reaction between ferric 2,4,6,-tripyridyl-s-triazine (Fe^{3+}-TPTZ) to its divalent form liberates an intense blue color monitored at 593 nm. The reducing power in FRAP is determined as a μM value:

$$\text{FRAP } (\mu M)\text{value} = \frac{0\ to\ 4\ \min \Delta_{593\ nm} test\ sample}{0\ to\ 4\ \min \Delta_{593\ nm} standard} \times [FRAP]_{std}$$

The non-specificity of the assay is useful to compare multiple antioxidants, but can be problematic regarding antioxidants of different stoichiometric reactivities (e.g. vitamin C compared to bilirubin). Also, Pulido *et al.* (2000) demonstrated with carotenoids, and Cao & Prior (1998) with thiol-containing antioxidants, that certain structures may not show reactivity depending on the media used (i.e. albumin). Also, the production of hydroxyl radicals can be formed when ferrous iron (Fe^{2+}) reacts with H_2O_2 during red-ox cycling, due to the unbound nature of the iron in this assay (Ou *et al.*, 2001; Benzie & Strain, 1999).

While mechanistically similar, reducing power via ferricyanide relates even more closely in terms of biological relevance [Figure 2.12].

Figure 2.12: The ferricyanide octahedral coordination complex (left) bears structural similarity to Fe-protoporphyrin IX (right) found in hemoglobin.



Also of note, where the FRAP assay is held under acidic conditions, reduction by ferricyanide utilizes a buffer that carries out the reaction under neutral conditions. This is due to the hydrolysis of iron near neutral pH conditions; Ferricyanide, however, is stable to this hydrolysis due to the saturation of six monodentate ligands which simultaneously contribute to its biologically relevant conditions.

The chemistry associated with this assay results from the Fe^{3+} cation (a high-spin complex, where all 4 of the 5 *d*-orbitals are filled with lone electrons) being reduced to Fe^{2+} (where 3 of the 5 orbitals are doubly occupied). In the ferri-/ferrocyanide complexes, those of Fe^{2+} in $[Fe(CN)_6]^{4-}$ have shorter bond lengths and different stretching frequencies indicating stronger pi-bonding in the lower oxidation state (Housecroft & Sharpe, 2005). The transfer of

electrons from the 2^+ to the 3^+ state gives a characteristic "Prussian blue" color that is measured at 700 nm.

Additionally, the saturation of the complex reduces the likelihood of redox cycling of iron (since the reduced iron is always bound to a stable complex). While unlikely, redox cycling should be addressed, because of the addition of ferric chloride.

$$Fe^{3+} + antioxidant_{reduced} \rightleftarrows Fe^{2+} + antioxidant_{oxidized}$$

$$Fe^{2+} + Fe(CN)_6^{3-} \rightleftarrows Fe[Fe(CN)_6]^-$$

Or

$$Fe(CN)_6^{3-} + antioxidant_{reduced} \rightleftarrows Fe(CN)_6^{4-} + antioxidant_{oxidized}$$

$$Fe(CN)_6^{4-} + Fe^{3+} \rightleftarrows Fe[Fe(CN)_6]^-$$

These reactions give a standard redox potential of 0.77V. Berker *et al.* (2007) indicated that the reduction potential can be made greater by using (for antioxidants that may require it) *o*- or *batho*-phenanthroline.

Regardless of the method, certain drawbacks are common to iron-based assays: not all antioxidants can be assessed; bathochromic (specifically, solvatochromic) shifts are possible; interference of chelating agents can create synthetically-lowered values.

2.5.1.4 Cupric Ion Reducing Antioxidant Capacity

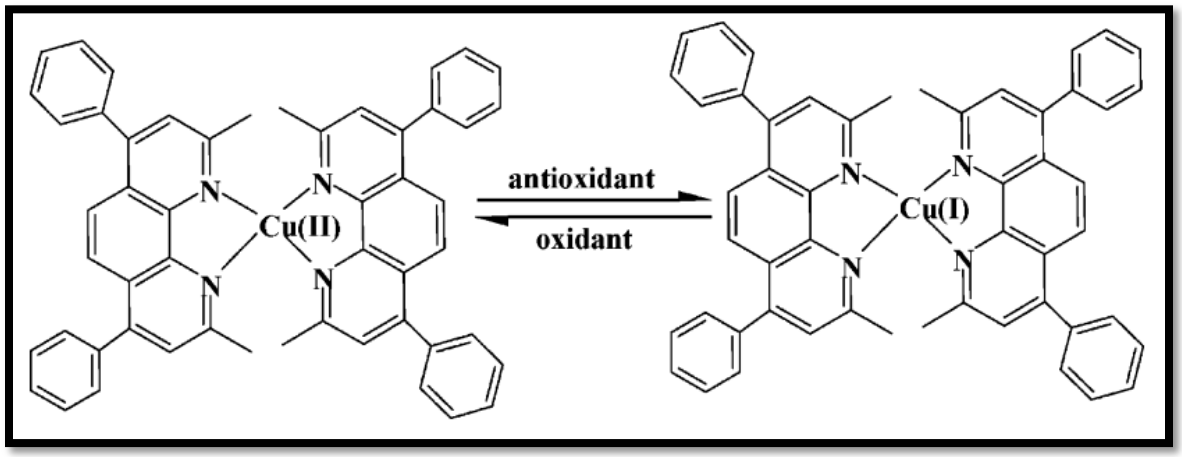
Developed to assay thiols in aqueous solution or β-carotene in dichloromethane and conquer certain other drawbacks to other reduction assays (FRAP, Folin, etc.), CUPRAC was designed as a low-cost and simple assay to measure dietary antioxidants. The chromogenic species of activity is $Copper^{2+}$ neocuproine (shown below) which bears great resemblance to the conjugated prophyrin ring in hemoglobin [Figure 2.13 similar to ferricyanide, mentioned above]. Because the reactive agent contains a macrocyclic ligand (with a redox potential of 0.6 V),

coordination to the copper center in unlikely; only electron transfer should occur. Similar to the ferricyanide method, this allows for the assay to be carried out at physiological condition, applicability to both hydrophilic and –phobic compounds, completion of redox reaction for most common flavonoids, selective oxidation of antioxidants without affecting sugar and citric acid (chelating agent), and the ability to characterize thiol-containing antioxidants (Apak *et al*., 2007).

$$n\ Cu(Nc)_2^{2+}\ Ar(OH)_n \rightarrow n\ Cu(Nc)_2^{+} + Ar(=O)_n + n\ H^{+}$$

where Cu(NC) represents cupric neocuproine and Ar(OH) represents a general aromatic antioxidant.

Figure 2.13: CUPRAC Mechanism (Apak *et al.*, 2007)



The CUPRAC assay is done under physiological conditions, and completed within 30 minutes. Using an ammonium acetate buffered solution controls the liberated hydrogen ions. It is often helpful to know the structure of the antioxidant tests, as flavonoids require acid hydrolysis to transform them into their aglycon form for complete antioxidant activity to be measured.

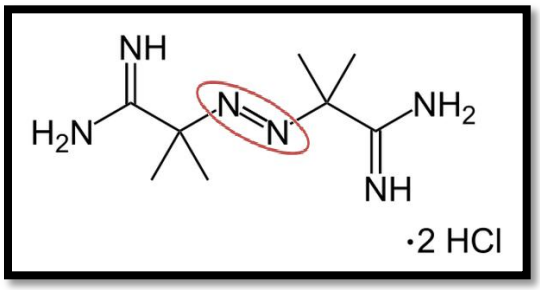
The advantage of using Copper over iron-based antioxidant assays stems from the mildly basic oxide that is formed in the cupric state. Because $Copper^{2+}$ is more readily reduced, it involves faster kinetics (in comparison with the ferric state of iron). Apak *et al.* (2007) determined a high correlation with results between CUPRAC, ABTS / TEAC, reduction by FCR,

FRAP, reduction by ferricyanide and other antioxidant assays. Potentially the most important aspect of the CUPRAC assay is its attempt to solve the 'antioxidant polarity paradox'[1]. By being able to assess antioxidants of either –philicity, CUPRAC offers a unique view towards qualifying antioxidant activity.

2.5.2 Hydrogen Atom Transfer Assays

In contrast to SET-based assays, most HAT-based assays are competitive reactions focused on the kinetic competition between the antioxidant and the substrate for peroxyl radicals. More simply, they qualify antioxidant activity by a species ability to quench free radicals via

Figure 2.14: Chemical Structure of AAPH



hydrogen atom donation. These radicals are generated through the decomposition of azo-compounds, such as 2,2'-azobis[2-amidinopropane] dihydrochloride (Figure 2.14). Assays of this mechanism work very similarly, such as the Oxygen Radical Absorbance Capacity (ORAC) assay, Total Radical Antioxidant Parameter (TRAP) assay and Crocin bleaching assay. As such, they also all suffer from a relevance-perspective, as they apply a radical reaction without a proper chain-propagation step (Prior *et al*., 2005). Similar to SET assays, the basic mechanism is defined as:

$$(4') \qquad ROO^{\bullet} + AH/ArOH \rightarrow ROOH + AH^{\bullet+}/ArOH^{\bullet+}$$

[1] Antioxidant Polarity Paradox: hydrophilic antioxidants are often less effective than lipophillic antioxidants in oil-in-water emulsions; lipophillic are less effective than hydrophilic in bulk oil.

2.5.2.1 ORAC

The hydrogen-atom transfer that occurs during the Oxygen-Radical Absorbance Capacity (ORAC) assay was first proposed by Glazer (1988, 1990). Since then, it has been modified and is currently a gold standard for identifying antioxidant activity in medical, nutritional, and industrial facilities. The most recent method proposed by Davalos *et al.* (2004) proceeds by the following theoretical schematic: a free radical generator (commonly AAPH) reacts with an indicator (fluorescein) to reduce its color over time. The addition of an antioxidant acts anti-catalytically and inhibits the effects of the generator. The more hydrogen atoms the antioxidant can donate, the longer the indicator remains active. Qualitative comparisons can be made by measuring the area under the curve (AUC) of any given antioxidant. The AUC is an attempt to measure the reaction rate and efficiency through the combination of inhibition percent and inhibition time as a single value (Davalos, Gomez-Cordoves, Bartolome, 2004). The advantage of *in vivo* antioxidant evaluation of antioxidants reflects the nature of oxidation and the nature of antioxidants (i.e. compounds capable of extending the induction period of oxidation). Similarly, the use of spectrofluorimetry is advantageous to spectrophotometry in that the former only 'excites' specific compounds, leaving other interfering agents at a ground-level 'excitation'. Often, authors find similarity between results from ORAC and results from other antioxidant assays: ABTS (Simonetti, Pietta & Testolin, 1997; Frankel *et al.*, 1995), DPPH (De Beer *et al*., 2003; Larrauri *et al*., 1999), and lipid oxidation (Kondo, Ohnishi & Kawaguchi, 1999).

Part in parcel with the advantages ORAC has (in terms of ambiphilicity) is the fact that fluorescein (the indicator and species on which the reaction is measured) is relatively polar, while the overall solvent is not. Furthermore, the environment reacts with an artificial radical initiator, while food systems have oxidation initiated by light, metals, heat and enzymes.

2.6 References

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Chapter 3. Evaluation of Antioxidant Capacity of Commercially Available and Natural Antioxidants

3.1 Abstract

The antioxidant activity of several natural less-common antioxidants extracts (pomegranate and grape seed) were studied in contrast to common natural antioxidants (d,l-α-tocopherol, ascorbic acid, and gallic acid) and butylated hydroxyanisole (BHA), a synthetic antioxidant. The antioxidants were dissolved in a methanolic solvent (0.01 g / 100 mL) for single electron transfer assays and acetone:water:acetic acid (0.5:70:29.5) for the hydrogen atom transfer assay. Antioxidants were evaluated via the reducing capacity as determined by the Folin-Ciocalteu reagent and ferricyanide method, free radical scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH), and oxygen radical absorbance capacity (ORAC). For each antioxidant, there was a high correlation between reducing ability and phenolic content. Reductive capacity for pomegranate and grape seed extract showed comparable antioxidant activity to two natural antioxidants (gallic acid and ascorbic acid) and always showed statistically significantly higher antioxidant activity than the synthetic antioxidant (BHA). Hydrogen atom donating ability of the synthetic antioxidant was significantly higher than all natural antioxidants. d,l-α-Tocopherol showed extremely low reductive activity while dietary selenium (produced by yeast metabolism) showed no antioxidant activity as measured by any of the assays.

Keywords: antioxidant activity; pomegranate extract; grape seed extract

3.2 Introduction

Lipid oxidation continues to be a problem for the food industry. Since 1994, multiple sources have indicated consumer rejection of synthetic, chemical-sounding food additives (Hillman, 2010) in favor of those that are familiar and “natural” (Jopin, 2006) in favor of those

that are familiar and "natural". While many synthetic antioxidants (BHT, BHA, TBHQ, EDTA, etc.) have been engineered to maximize their efficacy at lower doses, plants are abundant with endogenous antioxidants, such as polyphenols and vitamin E (Lee, Koo & Min, 2003). Considering consumer trends, it becomes increasingly important to find sources of concentrated antioxidants that function at the same capacity of their synthetic counterparts.

Pomegranates (*Punica granatum*) are a concentrated source of natural polyphenolic compounds, which can be further concentrated through solid-phase extraction. Rasheed *et al.* (2009) and Sartippour *et al.* (2008) both reported the contents of pomegranate extract (POMx) to have high antioxidant potency and contain 86% ellagitannins (according to manufacturer's data - Paramount Farms, CA, USA) with 19% ellagitannins as punicalagins and punicalins, 4% free ellagic acid, and 77% oligomers composed of 2–10 repeating units of gallic acid, ellagic acid, and glucose in different combinations. Ellagitannins are polyphenolic derivatives of ellagic acid, formed through oxidative reactions with galloyl groups formed through dipeptide bonds. Rasheed *et al.*(2009) noted that pomegranate extract suppressed inflammatory response in human cells, while Shukala *et al.*(2008) found the same extract worked to reduce inflammation in patients with rheumatoid arthritis. Both authors suggested reduction in inflammation was associated with suppression of reactive oxygen species. To date, there have been no studies on the use of pomegranate extract to delay the onset of oxidation in a high-lipid food matrix.

Phenolic compounds in grapes (*Vitis vinifera*) and their seeds have been shown to promote antioxidant activity when incorporated to reduce peroxidation *in vitro* (Jayaprakasha, Singh & Sakariah, 2001). The high amount of proanthocyanadins are continually credited for the antioxidant activity of grape seeds. Rojas & Brewer (2007, 2008) indicated that industrially-concentrated grape seed extract was highly effective in inhibiting oxidation in both cooked,

refrigerated and frozen, vacuum-packaged beef and pork. Furthermore, hydroperoxide and propanol formation was inhibited in an emulsion system due to grape seed extract, as shown by Hu & Skibsted (2002). In both plant extracts, the highly ringed structures that form polymeric compounds are composed of gallic or ellagic acid; both are highly effective reductants that are commonly employed in chemical assays utilized to show antioxidant activity. These constituents, which can make up anthocyanin fractions as well, have been found to be responsible for the antioxidant capacity (Rivero-Parez *et al.*, 2008). Additionally, these ringed-compounds are mechanistically correlated with electron transfer processes.

In both plant extracts, the highly ringed structures that form polymeric compounds are composed of gallic or ellagic acid. Both are highly effective reductants that are commonly employed in chemical assays utilized to show antioxidant activity. These constituents, which can make up anthocyanin fractions as well, are responsible for the antioxidant capacity (Rivero-Parez *et al.*, 2008). Additionally, these ringed-compounds are mechanistically correlated with stabilized radical species (e.g. phenolate anions) during electron transfer processes.

Selenium has been routinely proven to show antioxidant activity *in vivo*, through metabolic biotransformation. Its status as an essential trace mineral in the human diet has been well established (Holben & Smith, 1999). Mechanistically, it is well understood that selenomethionine (a metabolic configuration of selenium) can be incorporated with glutathione peroxidase to reduce hydrogen and organic peroxides during phase I metabolism. Because the efficacy of antioxidants as nutritional supplements does not have to be evaluated by the Food and Drug Administration, wholesale corporations often market and sell compounds, like selenium, without educating consumers about which metabolic forms are active and promote antioxidant activity. Many factors can destroy antioxidant activity, such as processing parameters, storage

conditions, and metabolically-inactive byproducts. This misnomer in education makes it imperative to compare the presumed antioxidant activity in certain "natural" antioxidants, such as dietary selenium, grape seed and pomegranate extract, with conventional and well-accepted antioxidants like gallic acid, l-ascorbic acid (vitamin c), and d,l-α-tocopherol (vitamin E).

Multiple authors (Frankel &Meyer, 2000; Huang, *et al.*, 2005; Apak *et al.*, 2007) have discussed the inability of a single methodology to evaluate antioxidant activity. While model systems appear to be the best method for testing antioxidant activity *in vivo*, they remain expensive, time-consuming, and untranslatable to other matrices. In an attempt to correct this problem, multiple approaches towards a direct analysis have been suggested. These approaches are commonly separated by their mechanisms into single-electron transfer (SET) and hydrogen-atom transfer (HAT) assays. SET-based assays measure a proposed antioxidants ability to reduce a chromogenic substrate (a literal definition of an antioxidant); HAT-based assays measure the capability of an antioxidant to donate a hydrogen atom and quench a free radical species (competition kinetics representative of the mechanism in lipid oxidation).

Multiple studies have taken an integrated SET/HAT assay or a multi-SET based approach to evaluate the antioxidant potential of extracted algae and plant material (Thaipong, *et al.*, 2006; Yildrim, A. *et al.*, 2001; Shon, M.-Y. *et al.*, 2003; Anesini, C. *et al*. 2008; Huo, L. *et al.*, 2011). In each of these studies, the authors compared some or all of the antioxidant results from reduction by FCR, reduction by ferricyanide, radical scavenging ability of DPPH, or oxygen-radical absorbance capacity (ORAC) to denote antioxidant capacity. While the first three assays are considered SET assays based on deprotenation and ionization potential, their experimental conditions and substrates provide benefits useful in comparing antioxidant reductive capacity. Conversely, ORAC measures the relative bond-dissociation energy and hydrogen donating

ability of a species, another important parameter of antioxidants. The Folin-Ciocalteu reagent is a non-specific oxidizer originally intended for polyphenol characterization that uses an unknown poly-molybdotungstate species to generate a chromogenic end-product characterized by its dark blue hue commonly read spectrophotometrically at 760 nm. This method, along with ORAC, has been standardized. Three methods have been standardized at the *First International Congress on Antioxidant Methods* in June 2004 (Orlando, FL) and are considered a method of standardization for antioxidant quantification: ORAC, Folin-Ciocalteu and ABTS/TEAC (Apak, 2007). The large amount of data generated from these assays make them ideal candidates in a multi-dimensional approach for comparing antioxidant potential.

It is important to note the deficiencies of these assays, to utilize alternative assays to partially resolve these issues. The Folin-Ciocalteu reagent is a non-specific oxidizer originally intended for polyphenol characterization that uses an unknown poly-molybdotungstate species to generate a chromogenic end-product characterized by its dark blue hue. The large amount of data generated from this assay makes it an ideal candidate in a multi-dimensional approach to comparing antioxidant potential. Additionally, the standardized procedure is simple, reproducible and minimizes matrix interference by being read at 760 nm. However, the assay is intrinsically laden with problems: its non-specificity measures non-antioxidant species, it doesn't evaluate thiol-related antioxidants in addition to inhibiting the measurement of certain compounds (such as flavonoids-glycosylates which require acid hydrolysis to measure full activity), and the alkaline pH required to generate the colored species enhances the reducing capacity of phenolic compounds. Measurement by ferricyanide reduction is comparable to reduction by the FC reagent as both SET assays have similar standard redox potentials (0.7 V and 0.77 V, respectively). In addition, the ferricyanide reduction can offset the problems associated with

alkalinity. Because the ferric ion is complexed with six monodentate ligands, the reduction of the assay can work at physiological conditions without concern of iron hydrolysis. Furthermore, the biologically-relevant environment and structural similarity the ferricyanide substrate shares structural with the heme-B structure in hemoglobin (which contrasts the molybdotungstate-complex used in FCR) promote this assay as tantamount to FCR reduction. .

In contrast to these metal-catalyzed, colorization reactions, free radical scavenging by DPPH uses an organic, stable free radical in a decolorization assay. Where the prior two assays utilize aqueous enviornments (selecting for hydrophilic antioxidants), DPPH is soluble in organic solvents, allowing for expanded measurement of hydrophobic antioxidants. In addition to its simplicity, Thaipong *et al*. (2006) found the assay to give more reproducible results than other assays (such as FCR). Floegel *et al.* (2011) found that DPPH had a high correlation ($\rho = 0.949$) with other antioxidant assays (ABTS) in regards to fruit and beverage matrices, while Huang (2005) found that reduction via ferricyanide and DPPH shared similar results in wild mushroom extracts. The DPPH assay has been used extensively in conjunction with ORAC (mentioned earlier as a standard method of antioxidant quantification) and shown comparative results in tea infusions (Roy, M.K. *et al.*, 2010) and honeys (Gheldof & Engeseth, 2002). The purpose of this study is to compare and validate the antioxidant potency of three "natural" consumer antioxidants (pomegranate and grape seed extracts and dietary selenium) in a multi-dimensional direct analysis approach against accepted natural antioxidants (gallic acid and vitamins C and E) and a synthetic antioxidant (BHA).

3.3 Materials and Methods

Reduction of Folin-Ciocalteu reagent, reduction by ferricyanide and free radical scavenging by 2,2-diphenyl-1-picrylhydrazyl assays were designed as 7 (antioxidants:

pomegranate extract, grape seed extract, dietary selenium, butylated hydroxyanisole, l-ascorbic acid, d,l-α-tocopherol, and gallic acid) one way analysis of variance with three replications. Standard curves were run in duplicate and controls (containing distilled water) were used as blanks. Oxygen Radical Absorbance Capacity assay was treated as a randomized, complete block design with seven antioxidant treatments and eight replications. Standard curves were run in quadruplicate and controls (containing acetone : water : acetic acid) were used as blanks.

3.3.1 Chemicals

Reagent-grade 2,2'-azobis(2-amidino-propane)dihydrochloride [AAPH] was purchased from Wako Chemical (Richmond, VA). Folin-Ciocalteu's phenol reagent, anhydrous sodium carbonate (purity 99%), 2,2-diphenyl-1-picryl hydrazyl [DPPH] (purity 90%,), methanol (HPLC grade), and trichloroacetic acid [TCA] were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). All other reagents (sodium phosphate, mono- and di-basic; Trolox™; fluorescein; ferric chloride; potassium ferricyanide) were purchased from Thermo-Fisher Scientific, Inc. (Fair Lawn, NJ, USA).

3.3.2 Antioxidants

Pomegranate extract and grape seed extract were donated by their respective manufacturers. Pomegranate extract (POMx, *Punica granatum* L., Wonderful variety; Paramount Farms, CA, USA) contained 86.0% ellagitannins (according to manufacturer's data). Preparation was a two-step process: first, fruit residue was extracted after pressing for juice; this was followed by a solid-phase extraction of the residue to produce a powder with a high concentration of polyphenols. This powdered extract was reported by the manufacturer to contain on average 86.0% ellagitannins, 2.5% ash, 3.2% sugars, 1.9% organic acids as citric acid equivalents, 0.8% nitrogen, and 1.2% moisture. The approximate percent distribution of

pomegranate polyphenols in POMx is as follows: 19% ellagitannins as punicalagins and punicalins, 4% free ellagic acid, and 77% oligomers composed of 2–10 repeating units of gallic acid, ellagic acid, and glucose in different combinations. Grape seed extract (Gravinol Super™, Kikkoman, Tokyo, Japan) contained 98% total flavanols (89% proanthocyanidins) based on manufacturer's data; Dietary selenium (Nature's Bounty™, Bohemia, NY, USA) containing 200 μg of selenium (selenomethionine) was derived from yeast origin. All further chemicals were reagent-grade: d,l-α-Tocopherol (purity 89%, Alpha Aeser, Ward Hill, MA, USA;), ascorbic acid (purity 99.2%, Fisher); gallic acid (99%, sigma); butylated hydroxyanisole (sigma); Standard solutions were made by dissolving all antioxidants in respective solvents (methanol or 70:29.5:0.5 acetone:water:acetic acid) at a concentration of 0.010 g Antioxidant per 100 mL solvent.

3.3.3 Reducing Power as Determined by Folin-Ciocalteu Reagent

The reducing capacity was determined by the antioxidant reduction of Folin-Ciocalteu (FC) reagent. For the given antioxidants, this was determined spectrophotometrically (Lambda 950 UV/Vis/NIR spectrophotometer, Perkin Elmer, Inc.), using gallic acid as a standard, according to the method described by the International Organization for Standardization (ISO) 14502-1. Each antioxidant extract was run in triplicate at 3 concentrations: 6.25, 10 and 20 mg antioxidant per liter methanol. Briefly, 0.4 mL of the diluted sample extract was transferred in triplicate to separate tubes containing 1.6 mL of dH_2O. To the samples, a 1:10 dilution of FC reagent in water was added. Then, 8.0 mL of sodium carbonate solution (20% w/v) was added. The tubes were placed in a room temperature (25^oC) incubator (Isotemp oven 516G; Fisher Scientific, Pittsburgh, PA, U.S.A.) for 110 minutes before the absorbance at 760 nm was measured against distilled water. The reducing capacity was expressed as gallic acid equivalents

(GAE) in mg per L solution. The concentration of polyphenols in the samples was derived from a standard curve (run in duplicate) of gallic acid ranging from 0 – 100 mg per L solvent (Pearson's correlation coefficient: $r^2 = 0.9996$).

$$A_{760} = 0.0091\ C + 0.0904$$

where A is the absorbance and C is the gallic acid equivalents

3.3.4 Ferricyanide Reducing Antioxidant Capacity

The antioxidant activity as correlated to the species reducing capacity of ferricyanide was performed by spectrophotometry, according to the method described by Berker *et al*. (2007). Each antioxidant was run in triplicate at 3 diluted concentrations: 2.75, 11 and 22 mg antioxidant per liter solution. Briefly, to 1 mL of methanolic extract was added 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ solution (1%); the mixture was vortexed for 5 sec and incubated at 50^{o}C in an incubation chamber (Isotemp oven 516G; Fisher Scientific, Pittsburgh, PA, U.S.A.) for 20 min. The incubated mixture was allowed to cool at 22^{o}C for 20 min, after which 2.5 mL of TCA (10%) was added. The solution was vortexed again for 5 sec and placed in a centrifuge for 10 min at 3000 rpm. An aliquot of 2.5 mL of the mixture was removed into a separate 15 mL screw-cap test tube and 2.5 mL dH_2O was added. Following this addition, 0.5 mL $FeCl_3 \cdot 6H_2O$ solution (0.1%) was added to make a final volume of 5.5 mL. After a 2 min reaction time, the resulting solution was read at 700 nm (A_{700}) against a reagent blank. Results were expressed by adapting an equation provided by Benzie & Strain (1999), using the 22 mg per L gallic acid as the standard:

$$\text{(FeCN)RAP (μM)value} = \frac{0\ to\ 2\ \min \Delta_{700\ nm} test\ sample}{0\ to\ 2\ \min \Delta_{700\ nm} standard} \times [(FeCN)RAP]_{std}$$

where $[(FeCN)RAP]_{std}$ was ≈ 1.29 μM gallic acid.

3.3.5 Free Radical Scavenging by 2,2-Diphenyl-1-Picrylhydrazyl

Three dilute concentrations of methanolic extracts (6.875, 8.25, and 9.625 mg antioxidant per liter solution) of natural antioxidants were evaluated in triplicate for antioxidant content by using a spectrophotometric assay described by Glavind (1963), with slight modification. Briefly, 1.0 mL of methanolic extract was added to 3.0 mL DPPH reagent (50 μM). The reaction mixture was vortexed and incubated in the dark at 25 ^{o}C. The absorbance of the mixtures was measured after 20 min at 517 nm against a reagent blank (pure methanol). The differences in absorbance between a test sample and a control (DPPH in MeOH) was considered as active. Results are expressed using ascorbic acid (0-20 mg L^{-1}) as a standard curve (Pearson's correlation coefficient: $r^2 = 0.9976$).

$$A_{517} = -0.387\ C + 0.545$$

where A is the Absorbance at 517 nm and C is the ascorbic acid equivalents.

3.3.6 Oxygen Radical Absorbance Capacity.

The ORAC assay was based on the procedure described by Engeseth *et al.* (2007). Briefly, free radical and indicator reagents were prepared in a 75 mM phosphate buffer (pH 7.0). Free radicals were produced by 12 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) using 70.3 nM fluorescein as an indicator. Trolox™ was used as a standard (0-4μM) and prepared in acetone:water:acetic acid (AWA). 10 mg antioxidant was dissolved in 100 mL ($^{w}/_{v}$) of 70:29.5:0.5 AWA. A portion of the solution (0.025 μL) was added to an eppendorf tube, where AWA was added to give a final volume of 1.0 mL.

The ORAC assay was performed on a fluorometer (BioTek FL600, BioTek Instruments Inc., Winooski, VT) using a 96-well black side with clear bottom plate (Corning Inc., Corning, NY). Each well contained 120 μL fluorescein, 20 μL of AWA (blank), Trolox ™ (standard

curve) or sample, and 60 μL of AAPH, added immediately prior to beginning measurement. For each run, one row consisted of a blank well followed by a Trolox™ standard curve of 1, 2, 3, and 4 μM Trolox™ (final concentration), repeated in reverse order and a second blank. Subsequent rows contained a similar symmetrically matched blank, 1 μM Trolox ™ (internal standard), and samples. Thus, each sample was measured in duplicate and values were averaged. Measurement was made at an emission wavelength of 515 nm and at an excitation wavelength of 493 nm every minute for 80 min. Results were expressed in μmol Trolox™ equivalents (TE) / L using the Trolox™ standard curve run with each group of samples.

3.4 Results and Discussion

3.4.1 Reducing Power as determined by Folin-Ciocalteu Reagent

Reducing capacity via reduction of FC reagent can be found in Figure 3.1. An increase in the absorption at 760 nm (visually, a dark blue color) is indicative of a high reducing potential, a positive marker of antioxidant potential. (Benzie & Strain, 1999). The absorbance was transformed in Gallic Acid Equivalents using a standard curve (see Appendix A, Figure A.2.3). Antioxidant potency was positively correlated to concentration in a linear fashion. Pomegranate showed the second highest reducing capacity of the antioxidants tested. These results are similar to those found by Okudu, *et al.* (1981) who found that ellagitaninns (the primary component of POMx) are potent reducers of copper, iron and chromium . However, these results contrast those found by Yoshida and others (1981) (those found in POMx) showed higher antioxidant activity than lower molecular mass polyphenols like gallic acid. The high reducing power of the plant extracts (pomegranate and grape seed) could be environmentally related. That is to say, the greater number of hydroxyl groups in the multi-ringed structures allow more deprotenation in the alkaline environment than the single-ringed structures of other phenolic-based antioxidants

(ascorbic acid and BHA). Proanthocyanidins and flavonoids (such as the 89% comprising grape seed extract) are known scavengers of reactive species and have the ability to chelate compounds with their *o*-diphenol group (Dixon *et al.,* 2005).

Dietary selenium showed no reductive capacity. While FCR is traditionally used to measure phenolic constituents, its basis is assumed to be the reduction of Mo(VI) to Mo(V). It is then expected for selenium to show little reductive capabilities. These results correlate to those of Scharff *et al.* (2000) who determined selenomethionine to be an inert compound with respect to hydrogen peroxide (a well-known, powerful oxidant). In this case, selenomethionine would act as an oxidizing agent and oxidation of the molybdate-complex that drives the FC reduction would not be observed. The results in Figure 3.1 also showed d,l-α-tocopherol to have low reducing ability at the concentrations measured (0-2.5 GAE). This corresponds well to the literature which attributes the antioxidant ability α-tocopherol to scavenging hydroxyl, alkoxyl and peroxyl radicals, as well as quenching singlet oxygen (Nicki, 1996; Gregory, 1996; Papas, 1999; Munnè-Bosch, 2005). Also, while vitamin E was dissolved in an organic solvent, the hydrophilic environment through which FCR is run leaves vitamin E unable to effectively compete with the other antioxidants.

3.4.2 Ferricyanide Reducing Antioxidant Capacity.

The spectrophotometric results for reducing capacity by the ferricyanide assay was transformed using the absorbance of gallic acid (22 mg per L) as a standard concentration. The results, found in Figure 3.2, were statistically similar to the results from FCR ($\rho = 0.9358$) (Appendix D, Table D.1.2). Reduction by ferricyanide corrected environmental limitations (by reacting at a neutral pH) as well as utilized a more biologically-relevant metal (high-spin

iron).The results are similar to those of the FCR assay ($\rho = 0.9358$), indicating mechanistic continuity among the antioxidants.

Figure 3.2 again displays the plant extracts having a higher reducing capacity than the synthetic antioxidant. The mechanistically similar principles of outer-sphere electron transfer likely account for the similarities by both metal reduction assays (Housecroft & Sharpe, 2005). Vitamin C was the only antioxidant to have shifted order in reductive capacity between the two assays. Tang *et al.* (2009) also noted the discrepancy regarding the high reducing ability of ascorbic acid in this assay (which shows disparity with the two SET assays). This may be explained in two ways: First, the solubility of ascorbic acid has been well documented (Shalmashi & Eliassi, 2008). Ascorbic acid is much more soluble in methanol (initial conditions of ferricyanide reduction) than water, and even less soluble in a co-solvent of water and methanol (conditions of FCR). This may be extrapolated when considering the effect of hydration status and structure of dihydroascorbate (oxidized ascorbic acid) at alkaline pH (Perone & Kretlow, 1966; Cioffi *et al*., 2000). Alkaline pH induces the hydrolysis of the lactone ring, causing an irreversible conversion (and therefore inactivity) to diketogulonate ion. When assayed at near-neutral conditions, ascorbic acid shows significantly higher reducing ability than most antioxidants at all concentrations, specifically the plant extracts. The chelating and reducing effects of ascorbic acid have been well documented (Fennema, 2008; Jagota & Dani, 1982). It is also likely that the catalytic metal has an effect, as Gregory (1996) noted that ascorbic acid is readily oxidized in the presence of ferric iron. Lastly, the large-caged chromogenic structure of the FC reagent (which would be able to dissipate electrical charge) is titanic compared to the smaller octahedral chromogenic reagent, ferricyanide. This, in addition to the limited reaction time during FCR, could inhibit ascorbic acid reduction of the M(IV) complex before

deactivation. The low activity of d,l-α-tocopherol was similar to the findings of Elmastas *et al.*(2006) who found same trend in Figure 3.2 between BHA and α-tocopherol in their study, likely due to the environmental conditions that select for hydrophilic antioxidants.

3.4.3 Free Radical Scavenging by 2,2-Diphenyl-1-Picrylhydrazyl

The range of concentrations used was limited by the parameters of the assay (i.e. amount of antioxidant and reaction time to reduce/decolorize the DPPH solution). Figure 3.3 shows that at all concentrations, each antioxidant was found to be significantly different. The results of DPPH scavenging were found to be similar to both FCR results ($\rho = 0.9268$) and ferricyanide reducing capacity ($\rho = 0.9404$) (Appendix D, Table D.1.2). The transformation of the data (Appendix A, Figure A.2.7) into equivalent units of ascorbic acid (AAE) (per Chen *et al.,* 2000; Molyneux, 2004) rather than percent inhibition or change in optical density gives results that are both informative and highly relevant. Authors have indicated that percent inhibition is misleading, while the use of change in optical density hard to relate Notably, gallic acid completely and immediately reduced the solution of DPPH; because of this, a direct comparison with the other antioxidants cannot be substantiated. Regardless, the results in Figures 2 and 3 were very similar to the references (gallic acid, α-tocopherol, ascorbic acid and butylated hydroxytoluene) used by Pfundstein (2010) who compared antioxidant activity of subunits of ellagitannins (the antioxidant-specific compounds in POMx) with results from DPPH, ORAC and FRAP. The results found in Figures 3.2 and 3.3 compared well with the DPPH and FRAP assays. The results in Figure 3.3 also correspond to those found by Okuda *et al.*(1989), who found higher DPPH scavenging by ellagitannins in comparison to ascorbic acid and α-tocopherol..

Ascorbic acid has been cited as highly reactive with DPPH, due to its two adjacent sites for hydrogen abstraction (Molyneux, 2004). The mechanism of this reaction is a single electron transfer from the antioxidant to DPPH, causing a decoloration of the solution at 517 nm. At the highest concentration (roughly 10 mg antioxidant per liter methanol), d,l-α-tocopherol measured low in reducing capacity (approximately 3 AA equivalents), despite the use of an organic solvent. BHA and grape seed extract showed nearly double the reducing power (approximately 6 AA equiv.) as vitamin E, a trend also reported by El-Baky *et al.* (2009) and Elmastas (2006), but differing from those of Hassanbaglou *et al*. (2012). Dietary selenium continued to show no reducing capabilities and remained identical to the blank solution (50 μM DPPH) after 20 min. The trends of reductive capacity via free radical scavenging found in Figure 3.3 again illustrated the antioxidant order found in the reduction of ferricyanide. At all concentrations, each antioxidant was found to be statistically different, retaining the same trends in reducing potential seen above. The plant extracts continued to show high reducing capacity at nearly 10 mg / L solution.

3.4.4 Oxygen Radical Absorbance Capacity.

The results of the ORAC assay are displayed in Table 3.1. The exclusion of d,l-α-tocopherol for the assay was the validated by Huang *et al.*(2002) as a result of substituting Trolox™ (a synthetic analog of vitamin E) for the standard curve. The substitution of Trolox™ is preferred both by relevance (Trolox™ contains the active phenolic group) and kinetic speed (Trolox reacts faster with AAPH than α-tocopherol).Often, authors find similarity between results from ORAC and results from other antioxidant assays: ABTS (Simonetti *et al.*, 1997; Frankel *et al.*, 1995), DPPH (De Beer *et al.*, 2003; Larrauri *et al.*, 1999), and lipid oxidation (Kondo *et al.*, 1999). In this study, ORAC (an HAT assay) showed no correlation to the SET

antioxidant assays: FCR (ρ = -0.4066), reducing capacity by ferricyanide (ρ = -0.0367) or scavenging by DPPH (ρ = -0.3440) (Appendix D, Table D.1.2). Only dietary selenium remained constant (showing no hydrogen atom donating capacity). Grape seed extract had three times the hydrogen atom donating ability (102 TE per 10 mg) as pomegranate extract (32 TE per 10 mg). Where BHA showed excellent hydrogen atom donating capacity (≈160 TE per 10 mg), gallic acid showed low H-atom donating capacity at 60 TE per 10 mg. The low values of gallic and ascorbic acid are similar to those found by Romero *et al.*(2010). The lack of h-atom donation by ascorbic acid is well known, and therefore it is not surprising that it had the lowest ORAC value. The low yet similar values of ascorbic and gallic acids are similar to those found by Romero *et al.* (2010). He and other authors (Apak, 2007) have indicated that both the type and number of side-chains on phenolic compounds impact a species antioxidant activity.

3.5 Conclusions

By comparing all four methods of measuring antioxidant activity, a more complete picture of dietary selenium, pomegranate and grape seed extract is obtained. While dietary selenium may show metabolic antioxidant activity, no assay indicated it can be used in a non-living system to retard oxidative deterioration. Pomegranate extract showed great potential as a reducing agent, but little potential as a hydrogen-donating species. Grape seed extract shows excellent effects in terms of reductive capacity and hydrogen-atom donation. Additionally, grape seed extract has been shown to reduce oxidative stress and extend the induction period of oxidation in multiple matrices (Jayaprakasha *et al*,2001; Rojas *et al.*,2006; Kulkarni *et al.*, 2011).

The measurement of antioxidant activity is complex, not only because of the nature of the antioxidant (hydrophilic vs. hydrophobic, phenolic vs. conjugated, etc.), but also because of the variety of mechanisms that can inhibit prooxidant species and delay the onset of oxidation.

Currently, some methods detect reduction capacity (a highly correlated parameter of antioxidation) while others detect free radical quenching. Knowing this, it is highly unlikely that a single approach to evaluating antioxidant activity is sufficient, making a multidimensional approach invaluable.

Also, because these assays utilize direct analysis techniques, caution should be taken when interpreting the results. As pointed out by multiple authors (Huang *et al.,* 2005; Apak 2007), these measurements are taken without respect to the complex nature of food and endogenous interfering agents. While these finding indicate promise, a multidimensional approach must be used in a model study to appropriately qualify whether these antioxidant extracts act in the desired nature.

3.6 References

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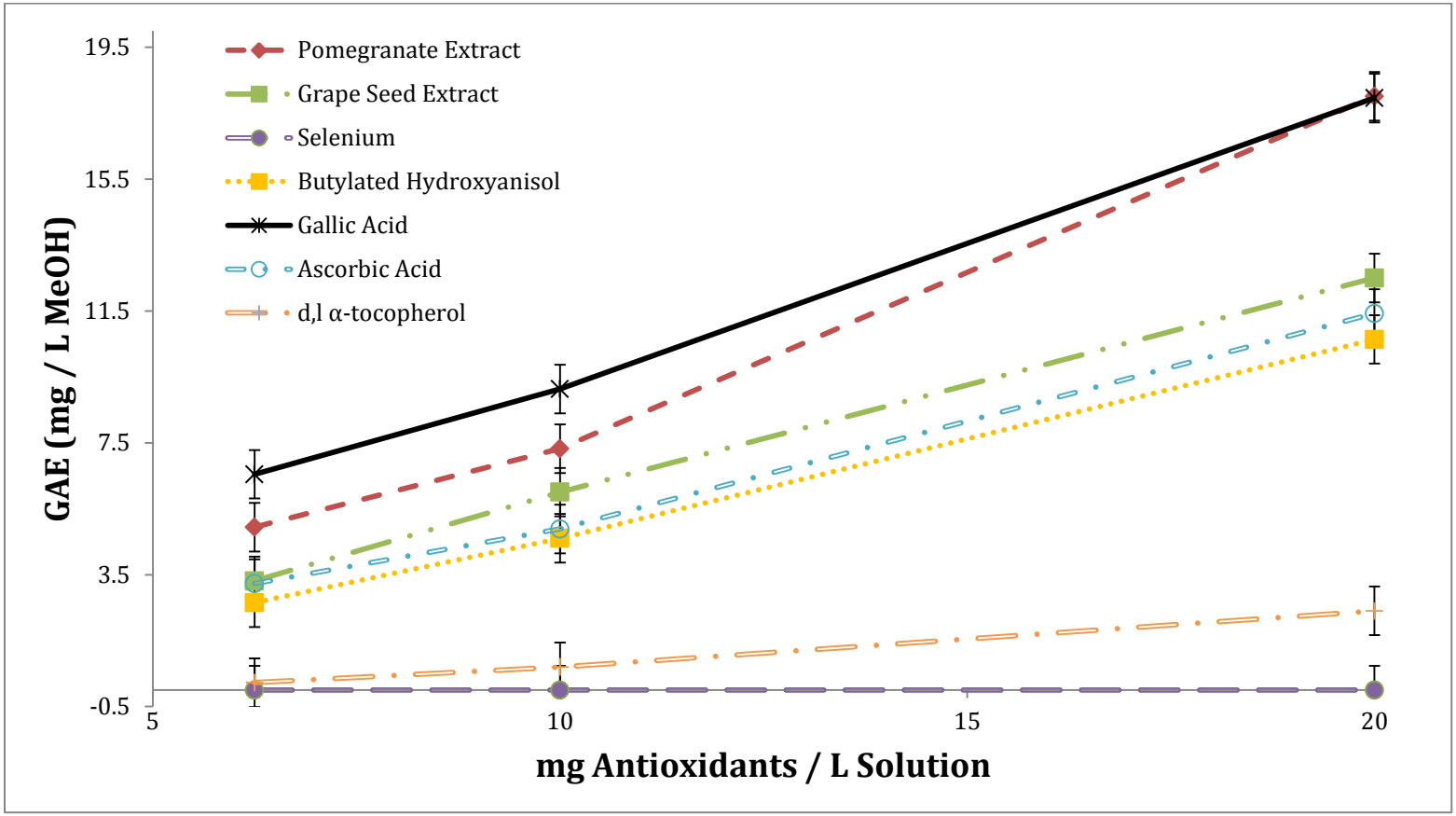
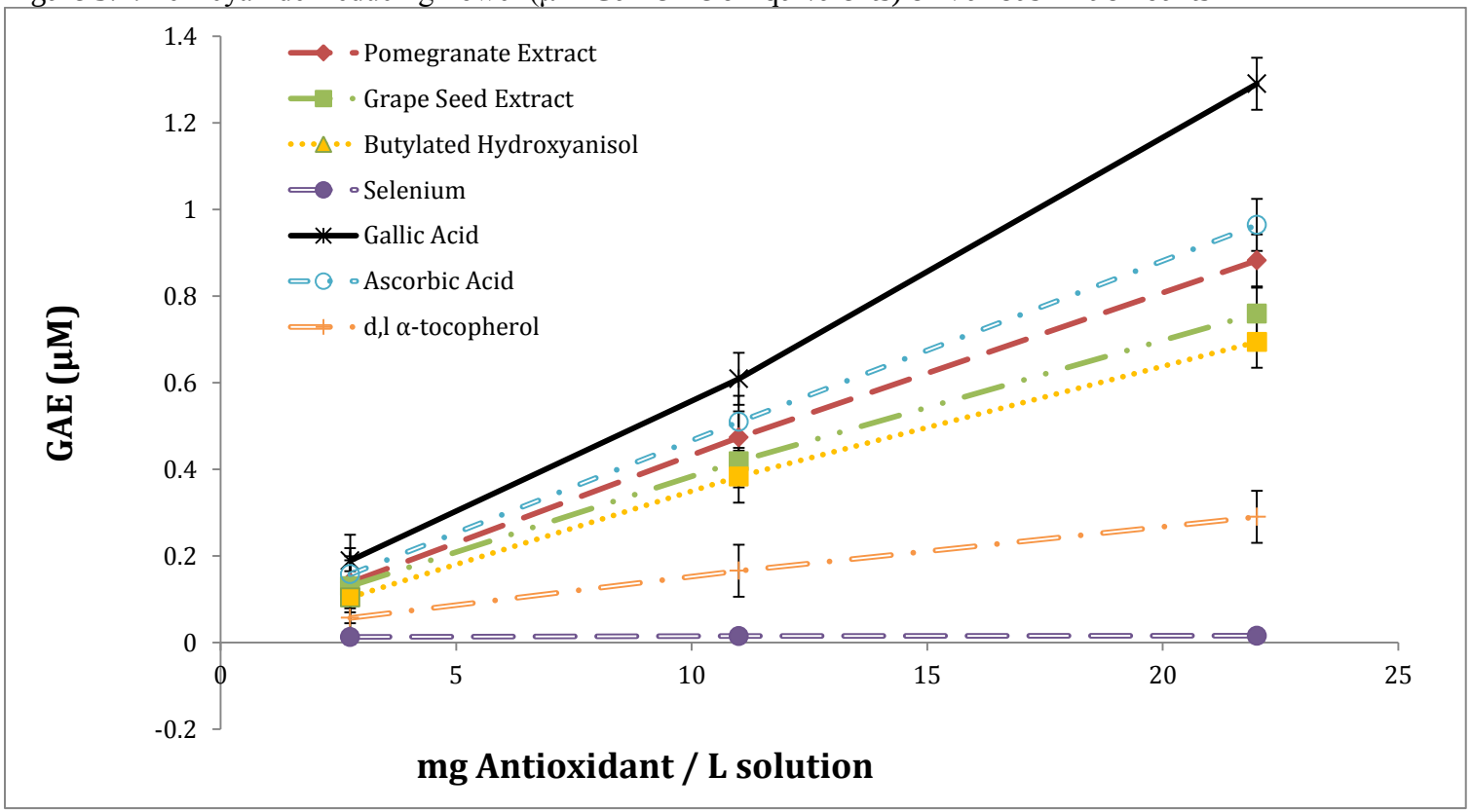
Figure 3.1: Gallic Acid Equivalents (mg L^{-1}) of Various Antioxidants at Increasing Concentrations[1]



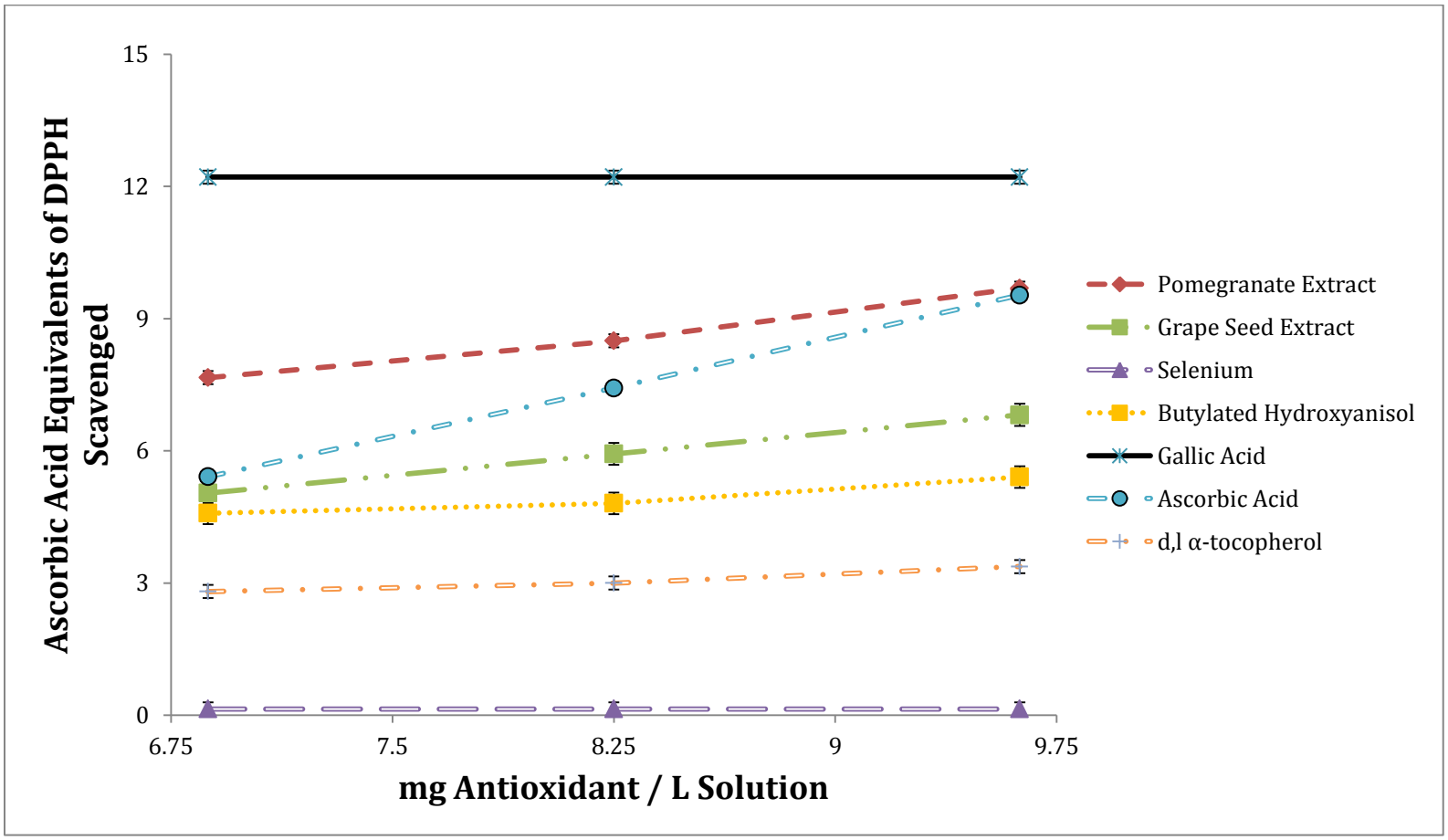
[1] Least square means of GAE (mg L^{-1})
Y error bars = standard error of the least square means

Figure 3.2: Ferricyanide Reducing Power (µM Gallic Acid Equivalents) of Various Antioxidants



[1] Least square means of µM GAE (mg L^{-1})
Y error bars = standard error of the least square means

Figure 3.3: Ascorbic Acid Equivalents (mg L^{-1}) of DPPH Free Radical Scavenging for Various Antioxidant at Increasing Concentration[1]



[1] Least square means of AA equivalents (mg L^{-1}).
Y error bars = standard error of the least square means

Table 3.1: Antioxidant Activity of Various Extracts as Determined by FCR, Ferricyanide, DPPH, & ORAC[1]

	Antioxidant Activity			
	FCR (GAE / 10 mg)	Ferricyanide (μM GAE / 11 mg)	DPPH (AAE / 9.7 mg)	ORAC (TE / 10 mg)
Pomagranate Extract	7.32 ± 1.3 [b]	0.43 ± 0.017 [bc]	9.74 ± 0.2 [b]	49.11 ± 2.7 [d]
Grape Seed Extract	6.01 ± 0.1 [c]	0.37 ± 0.014 [cd]	6.83 ± 0.3 [c]	101.71 ± 13.6 [b]
Dietary Selenium	0.02 ± 0.7 [e]	0.00 ± 0.004 [f]	0.11 ± 0.0 [f]	0.02 ± 0.0 [f]
Butylated Hydroxyanisole	4.64 ± 0.1 [d]	0.34 ± 0.016 [d]	5.49 ± 0.2 [d]	171.33 ± 27.1 [a]
Gallic Acid	10.03 ± 0.4 [a]	0.56 ± 0.016 [a]	12.22 ± 0.0 [a]	30.08 ± 3.8 [c]
Ascorbic Acid	4.91 ± 0.1[cd]	0.46 ± 0.082 [b]	9.51 ± 0.2[b]	14.71 ± 4.2 [e]
d,l-α-tocopherol	0.72 ± 0.3 [e]	0.12 ± 0.023 [e]	3.43 ± 0.1[e]	N/A

[1]Mean values and standard deviations not sharing letters in a row are significantly different ($p<0.0001$)

Chapter 4. Effect of Temperature and Concentration on Antioxidant Activity of Natural Antioxidants in Simplified Lipid Model Systems

4.1 Abstract

Pomegranate and grape seed extracts were evaluated as natural antioxidants in high lipid systems and compared to the effects of butylated hydroxyanisole (BHA, a commonly employed synthetic antioxidant) in two gelled, 27%-lipid matrices. The lipid in the first matrix was comprised of lard while the lipid in the second matrix was canola oil. Both matrices used food-grade gelatin, sodium stearoyl lactylate, and distilled water to create oil-in-water gelled patties. Hemoglobin was added as a naturally-occurring prooxidant. Both matrices were incubated at 30^{o}C and evaluated by chemical analysis (spectrophotometric evaluation of diene conjugation and TBARS) on days 0, 1, 2, 4, 7 and 9. The canola-oil treatment underwent descriptive analysis by a trained panel in addition to chemical analysis. In both systems, all analysis found BHA to be a more effective antioxidant than the natural antioxidants when used at the same concentration. Additionally, each antioxidant was found to be statistically different than the control (gelled patties with no added antioxidant).

4.2 Introduction

Because the definition of antioxidants is diverse, characterization of them in terms of substrate is important. For example, the same antioxidants used to retard oxidation of rubber can mechanistically vary greatly in comparison to those used for dietary purposes. Dietary antioxidants, such as selenium, can reduce endogenous metabolic bi-products (such as peroxides) through redox enzymes or cofactors to nonenzymatic antioxidants (oxidative enzyme inhibitors). Often, investigators have tried to compare antioxidant efficacies via direct analysis, a technique

that has been contested by many authors (Prior *et al*. 2005). The use of a model system is paramount to determining antioxidant activity in a food matrix. In the past, many authors have created matrices through ground meat systems (Rojas & Brewer, 2007), extracted oil from fish (King, Boyd & Sheldon, 1992; Fazel *et al.*, 2009), and β-carotene linoleate model systems (Jayaprakasha, Singh & Sakariah, 2000).

The use of a model system that is simple (does not contain many endogenous co-factors), oxidizes rapidly, and contains many ubiquitous polyunsaturated fatty acids is critical to aid in determining antioxidant efficacy. Pure canola oil and lard offer some characteristics, as shown in the review on natural antioxidant efficacy by Frankel (1993). As such, they were investigated to help determine their suitability as model systems. Lard is similar to ground beef in that it has low PUFA content (≈ 6%), but is still solid at room temperature. Alternatively, canola oil is the second most widely consumed oil in the United States, remains easy to qualify through gas chromatography, readily oxidizes, and has a large body of data quantifying its oxidation products (Broadbent & Pike, 2003).

Because the break down products of the autoxidation process differ from those of photooxidation and enzymatic oxidation, it is necessary to increase the thermal environment of each lipid model system. The employment of water (and consequently an emulsifier) is necessary when using phenolic compounds because of their high hydrophilicity. To maintain the stability of an oil-in-water emulsion, an inert binding agent such as a gum (Lee, Faemi, Hammond & White, 1995) or gelatin (Brewer, Peterson, Carr, McCusker, & Novakofski, 2005) should be employed. Gelatin can form thermo-reversible gels and is commonly used in meats between 1 and 5% (Gelatin Manufacturers Institute of America Inc., 1993).

4.3 Materials and Methods

Both the lard- and canola oil-model system experiments were designed as a randomized, complete block design with seven (lard) and four (canola oil) antioxidant treatments, six storage times and four replicates (two batches and two replicates / batch). Gelatin was food grade (J.T. Baker Chemical Co., Phillipsburg, N.J.; 3%, w/w). Sodium stearoyl lactylate was procured from Caravan Ingredients, Inc. (Lenexa, KS). Antioxidant treatments included a control (no antioxidant), 100 ppm pomegranate extract (POM), 100 ppm grape seed extract (GSE), 100 ppm dietary selenium or 100 ppm butylated hydroxyanisole (BHA) based on total weight of the batch. Lard was procured from a local supplier (Savoy, IL).

4.3.1 Anti- / Prooxidants

Five antioxidant treatments were evaluated: Pomegranate extract (POMx, *Punica granatum* L., Wonderful variety; Paramount Farms, CA, USA); Grape seed extract (GSE; (2007)(Gravinol Super TM, Kikkoman, Tokyo, Japan) contained 98% total flavanols (89% proanthocyanidins); Dietary selenium (Nature's Bounty, Bohemia, NY) was received commercially containing 200 μg organic selenium (of yeast origin) per tablet (Se; (2012)(Nature's Bounty, Bohemia, NY)); Butylated hydroxyanisole was reagent grade from Sigma-Aldrich Inc. (BHA; (2007) (St. Louis, MO, U.S.A.)). All crystalline or tablet antioxidants were ground using a mortar and pestle (pour lip diameter = 90 mm). Hemoglobin was reagent grade (Sigma, St. Louis, MO).

4.3.2 Lard-Model System Formation

Lard purchased locally (Morrell Snow Cap Lard, John Morrell & Co., Cincinnati, OH, USA; Savoy, IL) was heated to 80 ^{o}C for 5 min. After complete liquefaction, 7% ($^{w}/_{w}$) sodium stearoyl lactylate (Caravan Ingredients, Inc.; 9% w/w;) was added and mixed with a stir-bar for

10 minutes. Gelatin (J.T. Baker Chemical Co., Phillipsburg, N.J.; 3%, w/w), hemoglobin (Sigma, 0.02%), and antioxidant treatment (none, 100 ppm POM, 500 ppm POM, 1000 ppm POM, 100 ppm GSE, 100 ppm dietary selenium or 100 ppm BHA) were dry-mixed before being added to a portion, 27% ($^{w}/_{w}$) emulsified fat solution. The model was completed by adding boiling dH_2O (65% w/w). The mixture was mixed in a digital dual-range mixer (RW 20, IKA, Wilmington, NC, USA) for 1 minute at 2000 rpm before being segmented into 12.5 g aliquots in petri dishes (60 × 15 mm; Fisher Scientific, Pittsburgh, PA, U.S.A.). Product was stored in the dark at three different temperatures: 4, 30 or 50^{o}C, using either refrigeration or an incubation chamber (Isotemp oven 516G; Fisher Scientific, Pittsburgh, PA, U.S.A.). Analysis was performed on days 0, 1, 2, 4, 7, and 9. Patties resisted oxidation at refrigerated temperature (data not shown) and separated into a biphasic solution at 50 ^{o}C (data not shown). Chemical analyses, including thiobarbituric acid-reactive substances (TBARS) and conjugated dienes (CD), were conducted in triplicate on six replicate samples. Data were statistically analyzed.

4.3.3 Canola Oil-Model System Formation

Gelatin (J.T. Baker Chemical Co., Phillipsburg, N.J.; 4% w/w), sodium stearoyl lactylate (Caravan Ingredients, Inc.; 2% w/w) antioxidant treatment (none, 100 ppm POM, 100 ppm GSE, or 100 ppm BHA) and hemoglobin (Sigma; 0.02%), were dry mixed before being added to canola oil purchased locally (Meijer brand, Grand Rapids, MI). The model was completed by adding boiling dH_2O (66% w/w). The mixture was mixed (RW 20, IKA, Wilmington, NC, USA) for 2 minute at 2000 rpm before being segmented into 12.5 g aliquots in petri dishes (60 × 15 mm; Fisher Scientific, Pittsburgh, PA, U.S.A.). Product was stored in the dark at 30 ^{o}C in an incubation chamber (Isotemp oven 516G; Fisher Scientific, Pittsburgh, PA, U.S.A.). Sensory and chemical analysis was performed on day 0, 1, 2, 4, 7, and 9. Chemical analyses, including

thiobarbituric acid-reactive substances (TBARS) and conjugated dienes (CD), were conducted in triplicate on six replicate samples. Data were analyzed statistically.

4.3.4 Odor Evaluation of Canola Oil-Model System

Sensory testing was conducted at the University of Illinois (Urbana, IL) using nine panelists (4 male, 5 female) ages 23 to 65 (IRB protocol number 12848) experienced in oil odor evaluation. Training was performed during three thirty minute sessions under ambient lighting with approximately 30% relative humidity. Training included a fresh sample and two oxidized samples (no antioxidant and 100 ppm BHA). During training, a panel leader facilitated discussions of product characteristics. Panelists determined odor characteristics using a 15 cm semi-structured line scale (0 = none, 15 = extreme) (Appendix B, Figure B.1.1). Odor standards were determined by group consensus during 3 training sessions and assigned anchor values on the 15-cm scale. A complete list of terms, definitions, references and ratings can be found in (Appendix B, Table B.1.1). All references were prepared within 2 hours of evaluation and were served in lidded plastic soufflé cups (56.7 g; Solo Cup Company, Urbana, IL; Dart Container Corporation, Mason, MI).

Two replications were performed with evaluation occurring on days 0, 1, 2, 4, 7 and 9. Samples were macerated into 2.5 g aliquots and presented with a 3-digit random code in plastic cups with lids. The sensory panel evaluated two sets of four samples per session, in random order. Sensory data are reported in centimeters from the left end of the line scale.

4.3.5 Determination of Diene Conjugation

Conjugated dienes were determined as described by Juntachote, Berghofer, Siebendandl, and Bauer (2006). Briefly, a sample (0.5 g) was suspended in 5.0 mL of deionized water in a porcelain mortar (pour lip diameter = 90 mm). The sample was ground for 30 seconds until the

solution became homogeneous. A 0.5 mL aliquot of the suspension was mixed with 5.0 mL of extraction solution (3:2 hexane: isopropanol $^{v}/_{v}$) for 1 min using a touch mixer (Model 231, Fisher Scientific, Pittsburgh, PA, U.S.A.). After centrifugation at 2000 x g (Sorvall® RC-5B, Du Pont Company, Wilmington, DE, U.S.A.) for 5 min, the absorbance of the supernatant was determined at 233 nm (Lambda 950 UV/Vis/NIR spectrophotometer, Perkin Elmer, Inc.). The concentration of conjugated dienes was calculated using the molar extinction coefficient of 25,200 M^{-1} cm^{-1}. Results are expressed as mmol / kg sample. Hexane and isopropanol were reagent grade (Fisher Scientific, Pittsburgh, PA, U.S.A.)

4.3.6 Thiobarbituric Reactive Substances

Thiobarbituric reactive substances (TBARS) were determined as described by Miller (1998) based on the method developed by Witte, Krause, and Bailey (1970).with the following modifications: no antioxidants were added as the test units contained antioxidants. Absorbance was determined at 530 nm with a UV/Vis/NIR spectrophotometer (Lambda 950 UV/Vis/NIR spectrophotometer, Perkin Elmer, Inc.). Briefly, samples (5.0 g) were placed in a porcelain mortar (pour lip diameter = 90 mm). 45.5 mL of extraction solution containing 10% trichloroacetic acid in 0.02 M phosphoric acid (TCA / H_3PO_4) brought to 50 mL, was ground with a pestle for 1 min. The resulting mixture was filtered through Whatman No. 1 filter paper. One additional sample was spiked with 12 mL of 10 μM 1,1,3,3-tetraethoxypropane (TEP). Two 5 mL aliquots of each filtered sample were transferred into two separate screw-cap test tubes (15 × 200 mm). To one aliquot, 5.0 mL 0.02 M 2-thiobarbituric acid was added (test sample); to the second aliquot, 5.0 mL deionized water was added (sample blank). Test tubes were covered in Parafilm M, capped, inverted three times to mix and held in the dark for 18 hr. at room

temperature. Absorbance was determined spectrophotometrically, with sample blank absorbance subtracted from test sample readings.

A standard curve was derived based on the procedure described by Miller (1998). Aliquots (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 μL) of 25 μM of 1,1,3,3-tetraethoxy-propane (TEP) (Sigma-Aldrich, St. Louis, MO) were pipetted into 20 mL screw-cap test tubes, in duplicate. The total volume of each tube was 10 mL: 5 mL with TCA / H_3PO_4 solution and 5 mL of TBA reagent. The standard curve was constructed from absorbance versus concentration of malondialdehyde (as nmol MDA / mL) (MDA, St. Louis, MO). MDA recover was calculated and is expressed as a percentage. Concentration of TBARS was determined as μg MDA / g meat. It was assumed that recovery of TBARS was the same as for MDA. BHT crystalline, trichloroacetic acid crystal reagent and o-phosphoric acid were reagent grade (Sigma-Aldrich, St. Louis, MO, U.S.A.).

4.3.7 Statistical Analysis

Lard-model data were analyzed as a 7 (antioxidant treatment) × 6 (storage time) factorial design using the PROC MIXED procedure (SAS® 2012). Canola oil-model data were analyzed as a 4 (antioxidant treatment) × 6 (storage time) factorial design using the PROC MIXED procedure (SAS® 2012). TBARS and conjugated dienes main effects and interactions were considered significant at $p<0.05$. Sensory effects were considered significant at $p<0.10$. Separation of least square means was achieved using probability of difference, adjusted with the Tukey procedure for multiple comparisons.

4.4 Results and Discussion

4.4.1 Effect of Natural Antioxidants on Diene Conjugation

4.4.1.1 Effect of Natural Antioxidants on Diene Conjugation of Lard Model System

When held at 30 ^{o}C, the effect of storage time by antioxidant on diene conjugation (measured spectrophotometrically) was considered significant among the lard samples, as shown in Figures 4.1 and 4.2. This trend showed a small but definite increase, similar to those found by Chang *et al.* (1952). While all treatments increased over time, the synthetic antioxidant (BHA) and highest treatment of pomegranate extract (1000 ppm) showed the least increase in conjugated dienes from day 0 to day 9 (56% increase, BHA) and day 0 to day 4 (43% increase, POM_{1000} before beginning to decrease), respectively. This is indicative of high antioxidant capacity in these antioxidants at these levels. All antioxidants were significantly different than the control on day 9, however selenium nearly tripled in diene conjugation. These results indicate that selenium does not promote antioxidant activity (without cellular biotransformation), indicated by Tappel (1980). These results do not disagree with those of Tappel, however, Tappel incorporated dietary selenium using an oil carrier in rats to study pentane (a biological oxidation product). They do support the idea that selenium acts as an antioxidant *in vitro* (via selenium-glutathione peroxidase) rather than *in vivo*. When the amounts of each antioxidant were kept constant, BHA showed the lowest diene conjugation at 3.9 mmol / kg sample, while GSE had a 25% increase compared to BHA and POM which increased 59%. As expected, an increase in the amount of pomegranate extract used showed a decrease in the amount of oxidation product formation (in this case, diene conjugation).

4.4.1.2 Effect of Natural Antioxidants on Diene Conjugation of Canola Oil Model System

When held at 30 ^{o}C, all 4 antioxidants had significant effects on diene conjugation (measured spectrophotometrically) as shown in Figure 4.5. The measure of diene conjugation in the canola-oil system was drastically different than any previously seen. The pattern for all the antioxidants appeared bimodal with a trough appearing at day 4. It is possible that this is due to

linolenate oxidation occurring before linoleate oxidation (Wong, 1989). Notably, between day 7 and day 9, the matrix holding together the GSE and BHA models started to break down, causing separation of the hydrophilic and hydrophobic layers. The formation of diene conjugation did not appear significantly different from the control for the pomegranate and grape seed extract treatments on day 9, while the synthetic antioxidants did differ from the control (all of which were 69% greater in diene conjugation than the control).

4.4.2 Effect of Natural Antioxidants on TBARS

4.4.2.1 Effect of Natural Antioxidants on TBARS of the Lard Model System

TBARS values of the lard model system over 9 days of 30 ^{o}C storage are presented in Figure 4.3 and Figure 4.4. TBARS were affected by antioxidant, storage time and the interaction. With the exception of the control, TBARS remained unchanged until day 4 of the study, then increased over time. The results of the control were similar to those found in the pork model system used by Hernàndez-Hernàndez *et al.* (2009). 1000 ppm pomegranate extract was statistically similar to BHA, both showing the lowest values over time. In contrast, selenium and the control were not found to be statistically different by day 9, having the highest TBARS value recorded. Additionally, by day 9, 100 ppm pomegranate extract (while statistically different) showed similar TBARS values to the control, indicating that it is a much poorer antioxidant than BHA or GSE, when kept at the same concentration.

4.4.2.1 Effect of Natural Antioxidants on TBARS of the Canola Oil Model System

As is evident in Figure 4.6, the antioxidant treatment significantly affected TBARS values of the canola oil system over 9 days at 30 ^{o}C. The control was found to be statistically different starting on day 1, while none of the antioxidant treatments (POM, GSE, or BHA) were

determined to be statistically different from each other by day 9. These results (between the control and synthetic antioxidant) are similar to those found by Wanasundara & Shahidi (1994).

4.4.3 Effect of Natural Antioxidants on Sensory Attributes in a Canola Oil-Model System

No significant differences were detected in the intensities of the off-odor characteristics across the antioxidant treatments over time, but the control differed from the antioxidant treatments over time for all attributes (Figure 4.7). When the data were pooled over time, by antioxidant treatment panelists ranked all attributes as increasing over time (Figure 4.8). This indicates that these attributes are descriptive for oxidation of canola oil. Using hexanal as a reference for green was appropriate as many authors have found that the ratio of hexanal-to-nonanal (Morales, Rios & Aparicio, 1997) while many other authors note that an increase in hexanal in high-lipid matrices (especially beef) over time was positively correlated with sensory descriptors of oxidation (Brewer & Vega, 1995; Stetzer *et al.*, 2008; Teets & Were, 2008). The increase in oaty odor characteristics was also found by Shuh and Schieberle (2005), who determined that the odor active compound (2,4,6-nonatrienal) in oat flakes was also an active compound formed by the autoxidation of linolenic acid. These results also show similar results to the antioxidant activity of plant extracts in cooked patties reported by Nissen *et al.* (2004), who found an increase in painty (using linseed oil as a standard) which was highly correlated with TBARS, hexanal content, and a high score for the sensory term ‘rancid’. Malcomson *et al.* (1996) also showed that ‘painty’ was a useful indicator of consumer acceptance in canola oil over time (often correlated with the amount of pent-, hex- and heptanal found in oxidized canola oil). The ‘dairy’ attribute bears similarity to the breakdown products of PUFAs into compounds like nonadienes, which were shown by Morales, Rios & Aparicio (1997) to contribute greatly to the oxidation profile of linoleate 9-OOH degradation.

All samples shared significantly similar results until day 4. However, the drastic increase on day 7 of each attribute indicates that all antioxidants have the potential for controlling the negative sensory characteristics often associated with oxidation of canola oil.

4.5 Conclusion

Sensory analysis of the canola-oil containing samples positively indicated a difference in oxidation characteristics between all antioxidant treatments and the controls. However, the sensory study failed to differentiate between the antioxidants in terms of controlling oxidation. This could indicate no human preference in terms of antioxidant use, allowing manufacturers to use whichever antioxidant is most economically feasible. In regards to TBARS as a measure of antioxidant activity, BHA consistently gave lower oxidation values than the natural antioxidants when used at the same level. Grape seed extract showed statistically lower TBARS values than pomegranate extract in both the lard and the canola oil model systems, but higher values than BHA. A ten-fold increase in pomegranate extract concentration showed lower TBARS values than BHA in the lard-model system, but would need to be repeated in both systems to indicate that these levels aren't matrix-dependent.

4.6 References

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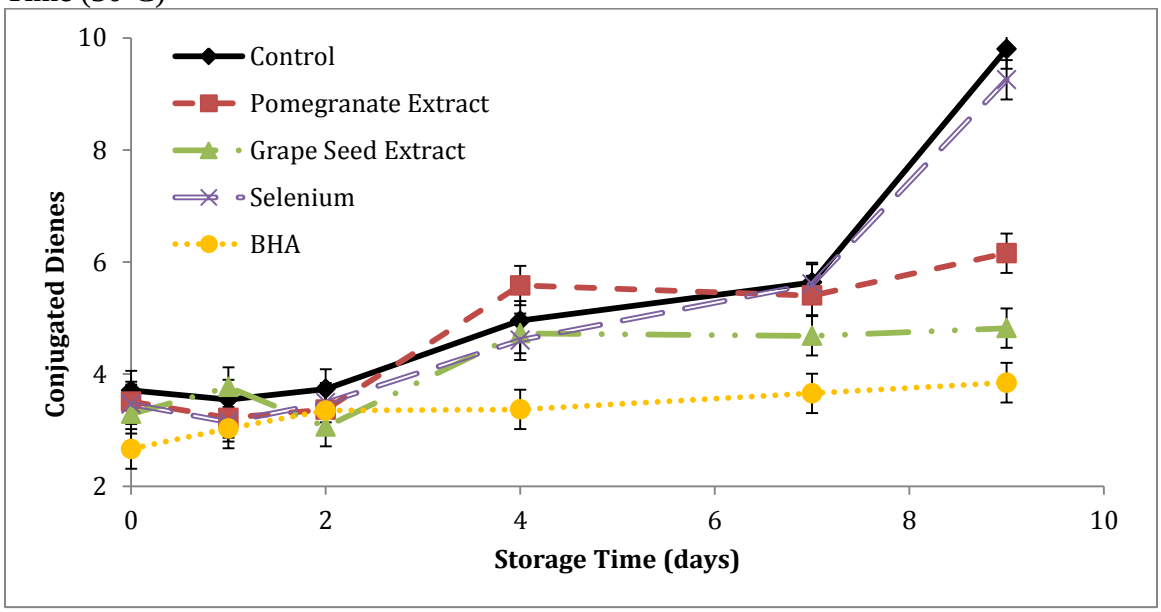
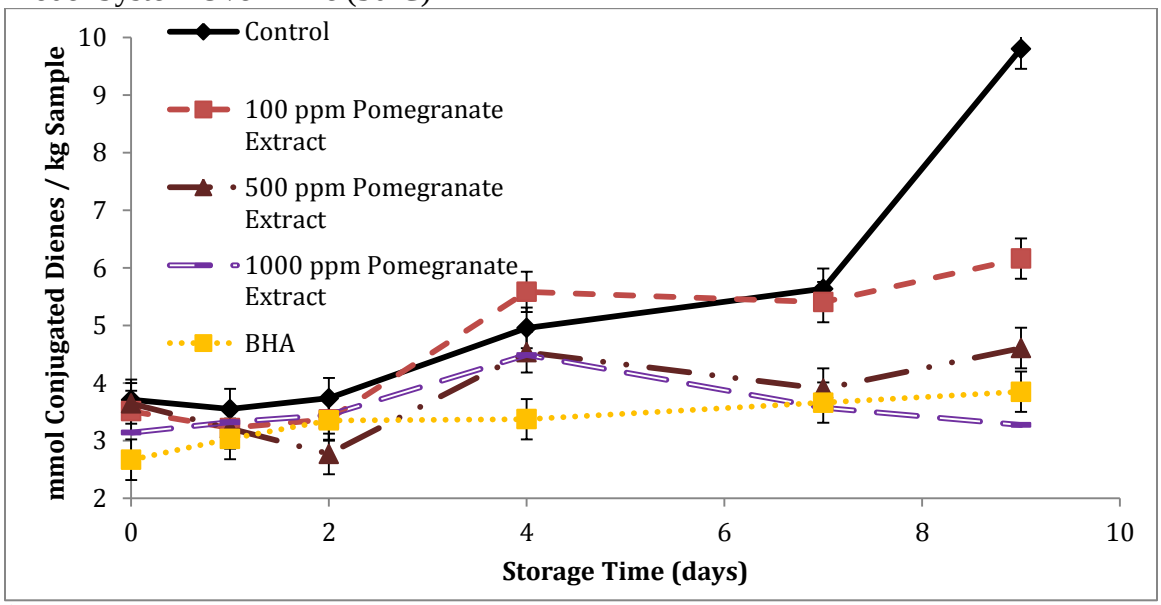
Figure 4.1: Effect of Antioxidants on Diene Conjugation[1] of Lard Model System Over Storage Time (30°C)



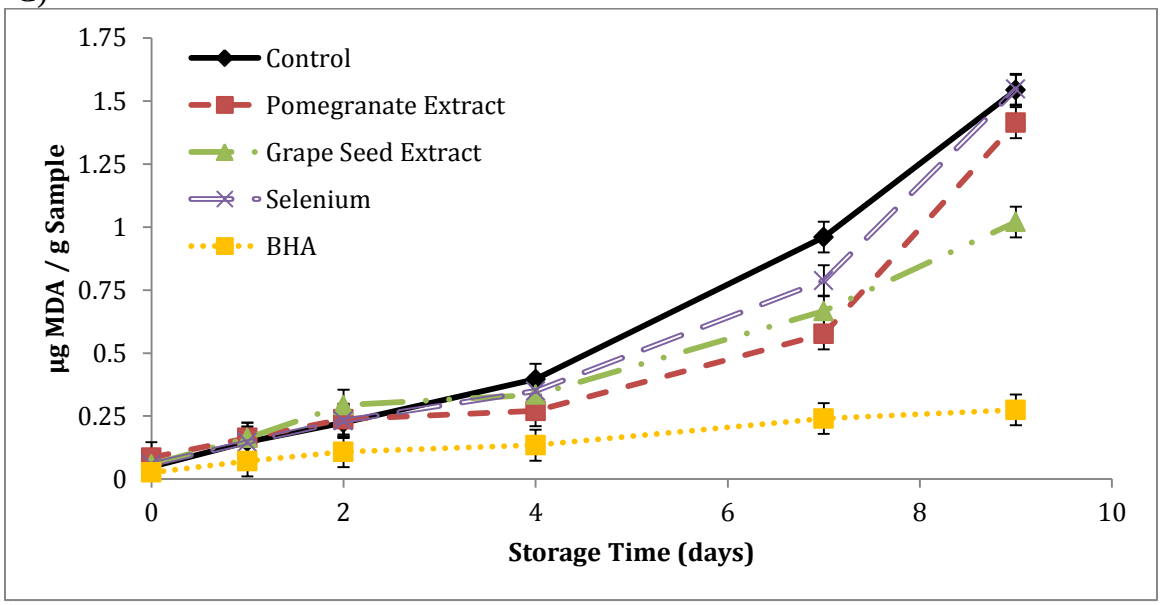
[1]Least Square Means (LSM) of conjugated dienes (mmol / kg sample)
Y Error bars = standard error of LSM

Figure 4.2: Effect of Increasing Level of Natural Antioxidant on Diene Conjugation[1] of Lard Model System Over Time (30°C)



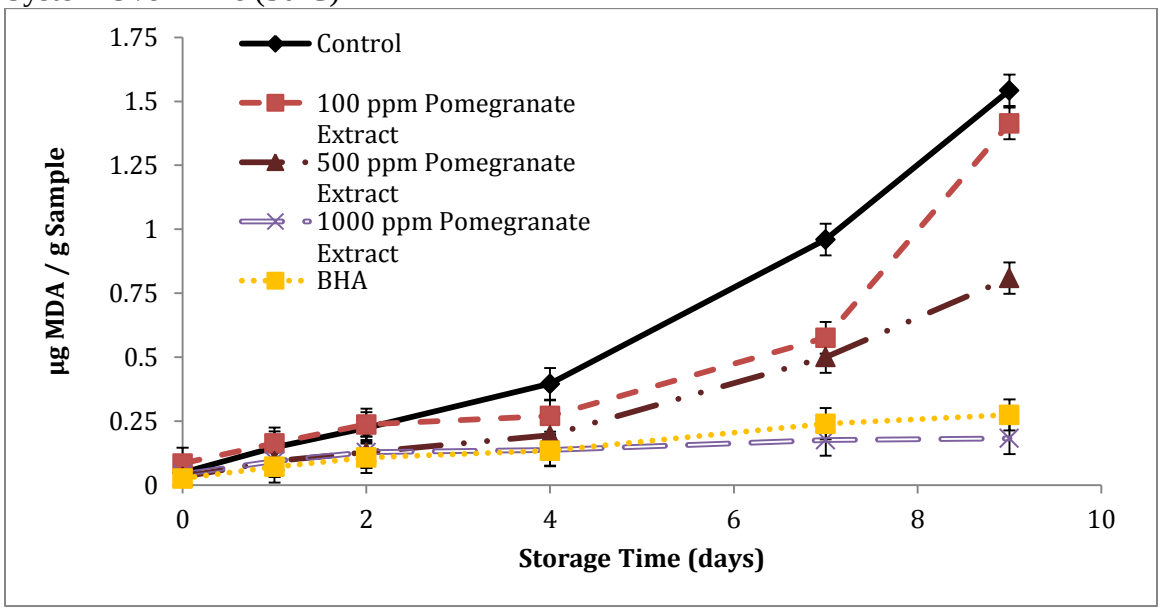
[1]Least Square Means (LSM) of conjugated dienes (mmol / kg sample)
Y Error bars = standard error of LSM

Figure 4.3: Effect of Natural Antioxidants on TBARS[1] of Lard Model System Over Time (30 °C)



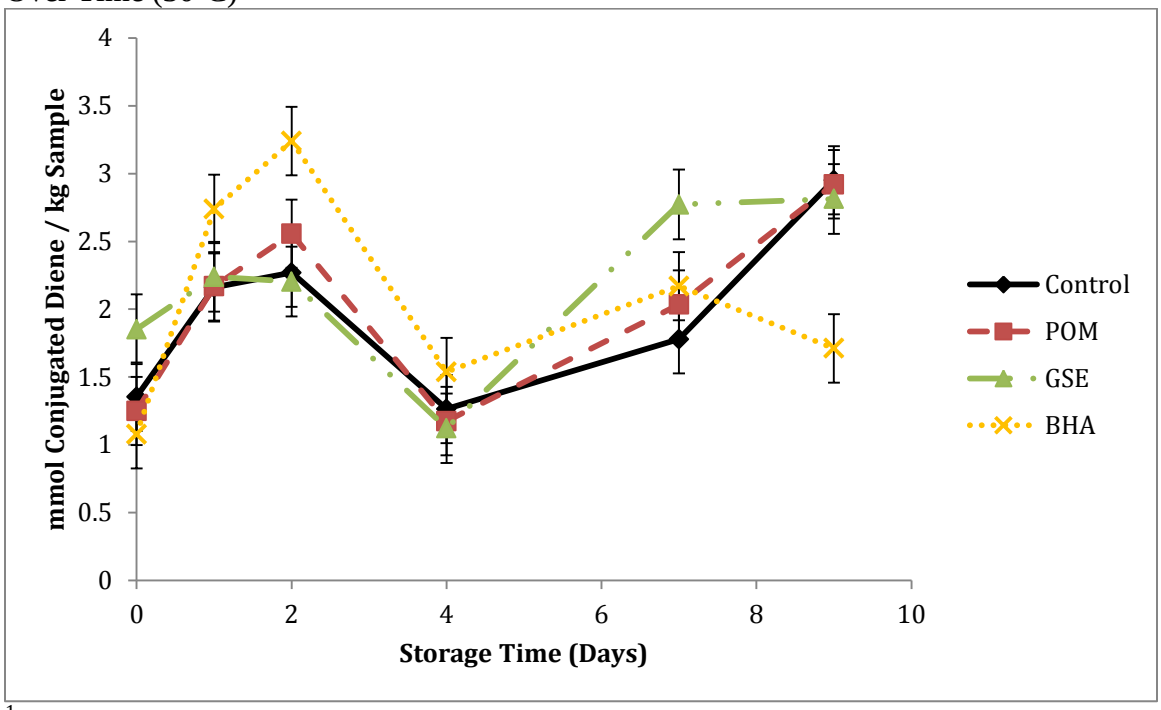
[1]Least Square Means (LSM) of TBARS (µg MDA / g sample)
Y Error bars = standard error of LSM

Figure 4.4: Effect of Increasing Level of Natural Antioxidant on TBARS[1] of Lard Model System Over Time (30°C)



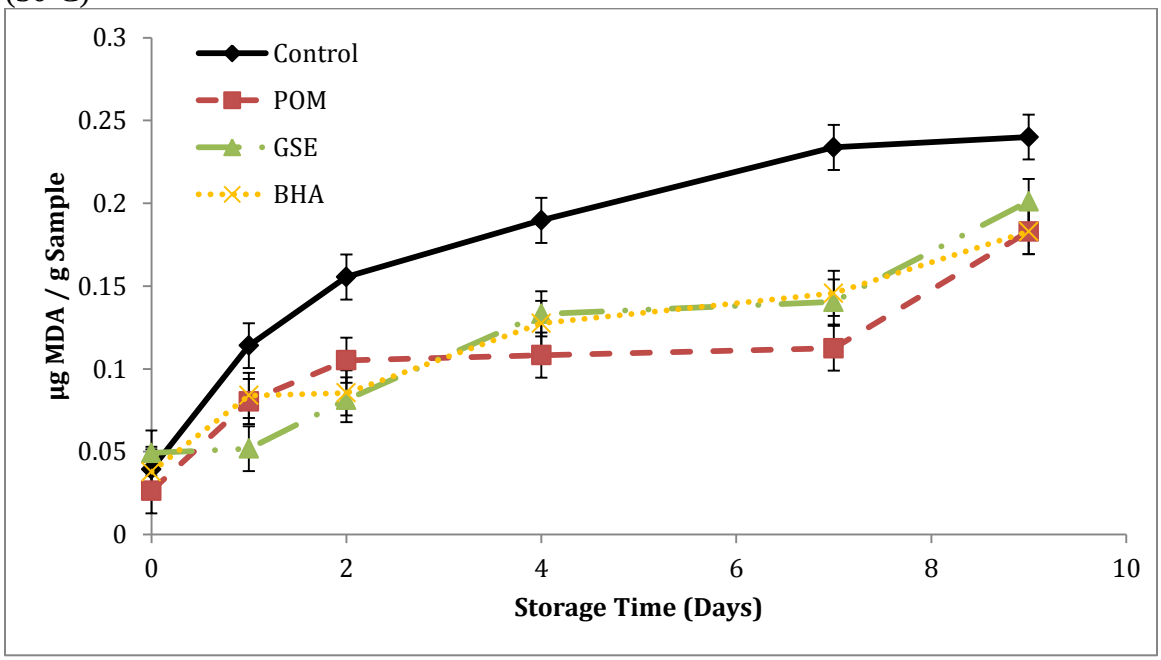
[1]Least Square Means (LSM) of TBARS (µg MDA / g sample)
Y Error bars = standard error of LSM

Figure 4.5: Effect of Natural Antioxidant on Diene Conjugation of Canola Oil Model System Over Time (30°C)



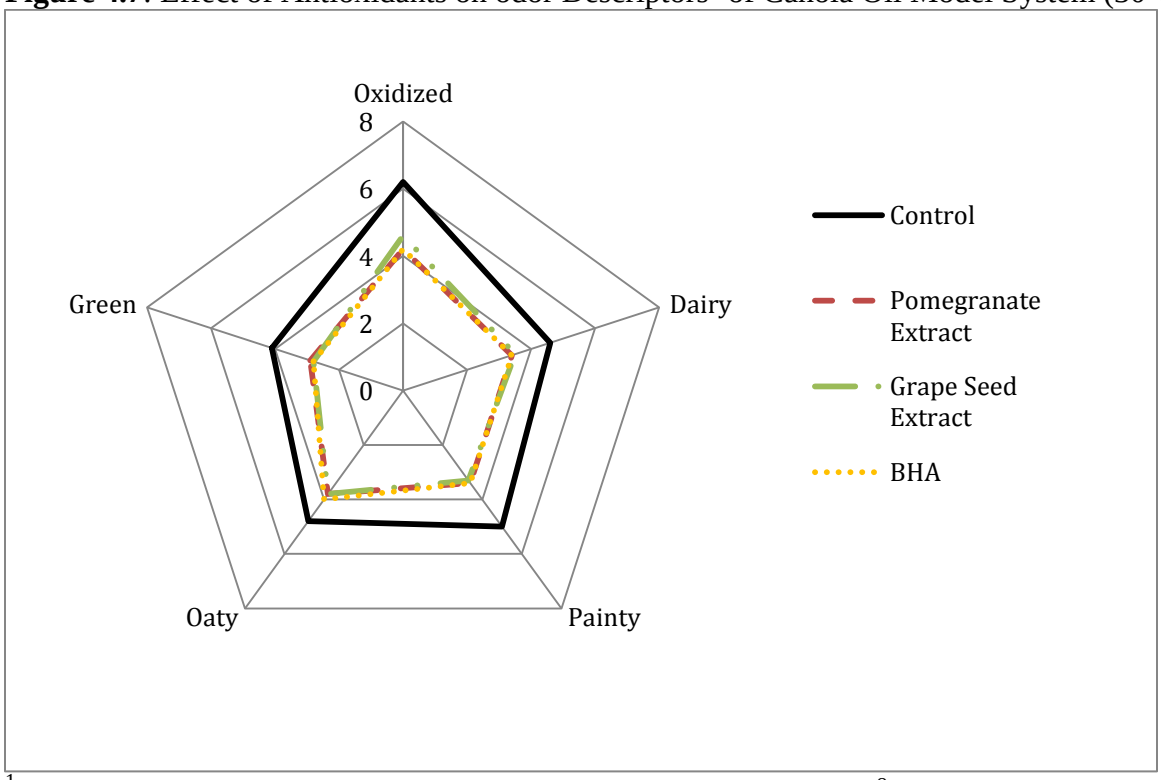
[1]Least Square Means (LSM) of conjugated dienes (mmol / kg sample)
Y Error bars = standard error of LSM

Figure 4.6: Effect of Natural Antioxidant on TBARS of Canola Oil Model System Over Time (30°C)



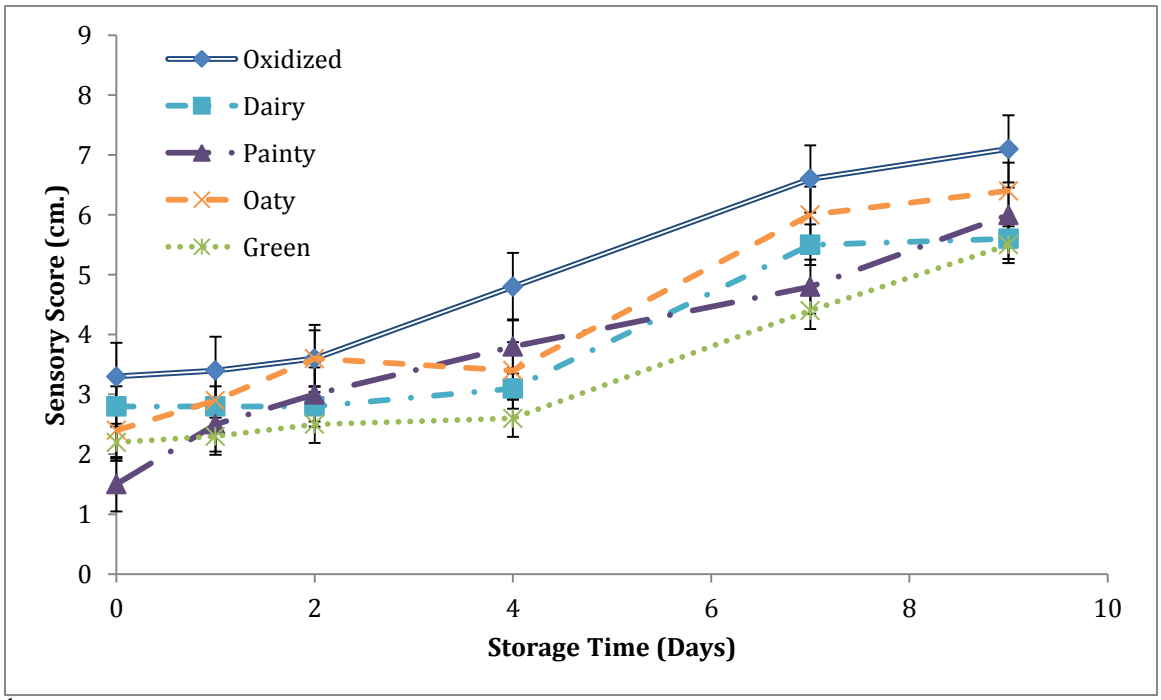
[1]Least Square Means (LSM) of TBARS (µg MDA / g sample)
Y Error bars = standard error of LSM

Figure 4.7: Effect of Antioxidants on odor Descriptors[1] of Canola Oil Model System (30°C)



[1] Least Square Means (LSM) of score value pooled over storage (30°C) time.

Figure 4.8: Effect of Time on Odor Descriptors of Canola Oil Model System (30°C)



[1]Least Square Means (LSM) of score values pooled over treatment.
Y Error Bars = standard error of LSM

Chapter 5: Effect of Irradiation, Antioxidant and Sodium Chloride Level on the Oxidation of High-Fat Ground Beef Patties

5.1 Abstract

To determine the effect of pomegranate extract on antioxidant activity, beef patties were subjected to multiple processing factors. Beef patties were treated with one of five antioxidants (control, pomegranate extract (POM), grape seed extract (GSE), dietary selenium and butylated hydroxyanisole (BHA)), one of two levels of sodium chloride (0.5% and 2%), and irradiated at 0 or 1.5 kGy. Samples were then evaluated for oxidative rancidity and antioxidant activity via instrumental measurement of color, thiobarbituric acid reactive substances (TBARS) and/or diene conjugation as analytical methods as well as evaluated by a trained sensory panel for descriptive analysis over 9 days at 2 ^{o}C. Oxidation (as measured from sensory and analytical measurements) increased as: irradiation dose increased, salt concentration increased and time increased. From TBARS values, BHA was found to be a statistically better antioxidant under all conditions (irradiation and salt content) than the natural antioxidants. Panelists were able to distinguish a difference (in terms of oxidation attributes) between patty samples while they were raw, but unable to distinguish a difference between patties after cooking.

5.2 Introduction

The advancement in shipping foods has allowed consumers to experience a plethora of cuisine regardless of seasonal limitations or growing-region selectivity. A caveat to increased access to food lies with pandemic concerns when animal products (such as ground beef) are infected with antibiotic resistant, pathogenic bacteria and shipped across the continent. Recently in late-2011, twenty individuals contracted an antibiotic-resistant strain of *Salmonella typhimurium* from contaminated beef (Rothchild, 2012). Because food borne illness causes loss

in the form of economic loss, reputational degradation, and loss in inventory, the food industry is consistently attempting to find effective ways to provide pathogen-free food to consumers. In terms of ground beef, one such method that has been evaluated is the process of irradiation. While controversial, the FDA has determined that moderate doses of irradiation (3.5 kGy for fresh, raw meat) are effective at eliminating pathogenic microbes (Brewer, 2009). In addition to microbial safety, Teets & Were (2008) have indicated that irradiation may assist in extending the induction period of lipid oxidation in regards to certain antioxidants such as flavonoids.

In 2006, 19 million pounds of livestock was processed in the U.S. (Haley, 2006). Oxidation quickly degrades high-fat, ground beef patties from a visual and odor/flavor related context. When meat is macerated, it becomes more susceptible to oxidative rancidity, color deterioration and pathogenic contamination. Visual interaction with meat products is the initial method of meat preference that consumers employ. Multiple authors (Glitsch, 2000; Rasvik, 1994) have indicated consumer preference towards bright red meat over dark or discolored meat. Secondary interactions, such development of off-odor and flavor compounds, also detract from purchase intent. These compounds are prevalent in macerated beef products because the process allows oxygen, a potent oxidizing agent, to interact with all aspects of the meat. Furthermore, the process of grinding meat allows endogenous oxidizing agents to come in contact with polyunsaturated lipids (PUFAs). PUFAs are readily oxidized (Rhee, 1988) and breakdown into highly odorous compounds often associated with rancidity (St. Angel *et al*., 1990).

To counteract the negative effects of oxidation, natural and synthetic antioxidants have been employed and studied at large. Butylated hydroxyanisole (BHA) has been widely used with other synthetic antioxidants at low doses. However, toxological effects and consumer preference (Rojas & Brewer, 2008; Formanek *et al.*, 2001) have indicated a trend towards consumer

preference for natural antioxidants. Previous studies have examined plants and herbs as natural antioxidants in raw meats (Chen, Jo, Lee & Ahn, 1999; Han & Rhee, 2005; Rojas & Brewer, 2008; Nicolade *et al.*,2006) and attributed antioxidant activity to the high amount of phenolic compounds these plants contain. Grape seed extract has been studied extensively in reducing lipid oxidation in ground beef when utilized at 1% (Ahn *et al*., 2007, Rojas & Brewer, 2008). To date, pomegranate has been sold commercially as an extract and while authors have studied the effects *in vivo* (Shakula *et al.*, 2008), none have evaluated the extract in a meat model system.

5.3 Materials and Methods

The first experiment was designed as a randomized complete block design with four antioxidant treatments, two irradiation levels, five storage times and four replicates (two batches and two replicates / batch). Antioxidant treatments included a control (no antioxidant), 100 ppm pomegranate extract ([POM], POMx, *Punica granatum* L., Wonderful variety; Paramount Farms, CA, USA), 100 ppm grape seed extract ([GSE], Gravinol Super™, Kikkoman, Tokyo, Japan), dietary selenium ([Se], Nature's Bounty™, Bohemia, NY, USA) and 100 ppm butylated hydroxyanisole ([BHA], Sigma, St. Louis, MO, USA) based on total weight of the batch. Ground beef was procured from a local supplier (Urbana, IL; 27% fat, as labeled). All meat purchased was mixed to form a uniform batch. Products were mixed by hand with the desired amount of salt (0.5% $^{w}/_{w}$, in experiment 1; 2% ($^{w}/_{w}$) in experiment 2) and antioxidants (none, POM, GSE, Se and BHA) at 100 ppm ($^{w}/_{w}$). Mixed beef was then placed on parchment paper and formed using a rolling pin and guide bars (0.375 cm). Samples were cut using a 5.5-cm (for physical or chemical analysis) or 7.5-cm (for sensory analysis) circular cookie cutter. Individual patties were laid on 11 × 14 cm. foam trays (Cryovac, Sealed Air Corporation, Elmwood Park,

NJ) and over-wrapped with commercial polyvinyl chloride (Multivac C500, Koch Supplies, Inc., Kansas City, MO) before being stored in the dark at 2 ^{o}C for up to 9 days.

5.3.1 Anti- / prooxidants

Five antioxidant treatments were evaluated: Pomegranate extract (POMx, *Punica granatum* L., Wonderful variety; Paramount Farms, CA, USA); Grape seed extract (GSE; (2007)(Gravinol Super TM, Kikkoman, Tokyo, Japan) contained 98% total flavanols (89% proanthocyanidins); Dietary selenium (Nature's Bounty, Bohemia, NY) was received commercially containing 200 μg organic selenium (of yeast origin) per tablet (Se; (2012) (Nature's Bounty, Bohemia, NY)); Butylated hydroxyanisole was reagent grade from Sigma-Aldrich Inc. (BHA; (2007) (St. Louis, MO, U.S.A.)). All crystalline or tablet antioxidants were ground using a mortar and pestle (pour lip diameter = 90 mm). Sodium Chloride was procured from U.S. Salt (Watkins Glen, NY, U.S.A.).

5.3.2 0.5%-Salt Ground Beef Patties

After patty formation, half of each variable of each variable condition were transported on ice to the University of Illinois Nuclear Radiation Facility (Urbana, IL) where they were exposed to 1.5 kGy of gamma-irradiation (Gammacell 220 Excell, MDS Nordion, Ottawa, ON, Canada, K2K 1×8). Samples were maintained at refrigeration temperature (4-8^{O}C) during irradiation. The cylindrical irradiation chamber was (diameter × height) 152 × 206 mm. Precise dose distribution in the irradiation chamber was measured using Gafchromic MD-55 (ISP Technologies Inc., Wayne, NJ) and Radiachromic FWT-60 film dosimeters (Far West Technology, Inc., Goleta, CA), which were calibrated by the National Institute of Standards and Technology. Samples were treated with dosages of 1.5 kGy. After irradiation, samples were stored at 2 $\pm$ 2 ^{o}C in the dark for 0, 2, 4, 7, and 9 days.

Sensory analyses were conducted in duplicate on patties made with 0.5% salt and antioxidant treatment (none, POM, GSE, or BHA). Panelists were trained using standards over 18 thirty-minute sessions and evaluation occurred in duplicate on days 0, 2, 4, 7, and 9. Physical analysis for 0.5%-salt patties was conducted on the same days as sensory analysis and included determination of color by a spectrocolorimeter (Konica Minolta Sensing Inc., Osaka, Japan). Chemical analyses included thiobarbituric acid-reactive substances (TBARS) conducted in triplicate on six replicate samples. Data were statistically analyzed as a 5 (days) × 2 (irradiation dose) × 4 (antioxidant) factorial design using SAS.

5.3.3 2%-Salt Ground Beef Patties

The second experiment was modeled after the first experiment, with the inclusion of dietary selenium as a fifth antioxidant treatment and an two-fold increase in sodium chloride content. Products were formed in an identical manner and stored in the dark at 2 ^{o}C for 9 days. Physical analysis of color was conducted on days 0, 1, 2, 4, 7 and 9 by spectrocolorimeter. Chemical analyses were conducted on the same days as physical analysis and included thiobarbituric acid-reactive substances (TBARS) and spectrophotometric determination of conjugated dienes. Both physical and chemical analyses were conducted in triplicate on three replicate samples. Data were statistically analyzed as a 6 (days) × 5 (antioxidant) factorial design using SAS.

5.3.4 Instrumental Color

Analytical color was determined on each evaluation day of each experiment using a Chroma Meter CR-400 reflectance spectrocolorimeter (Konica Minolta Sensing Inc., Osaka, Japan) standardized using a white standardization tile. Spectral curve was determined over the 400-700 nm range at 10 nm increments. L*, a*, and b* values were measured using Illuminant

D65 and a 10° observer (CIE, 1978). Hue angle ($[\tan^{-1}(b^*/a^*)] \times 53.6$) and Chroma ($\sqrt{(a^*)^2 + (b^*)^2}$) were also calculated (Minolta 1998). ΔE^*_{94} was determined using the following equation:

$$\Delta E^*_{94} = \sqrt{\frac{(\Delta L^*)^2}{K_L} + \left(\frac{\Delta C_{ab}{}^*}{1 + K_1 C_1{}^*}\right)^2 + \left(\frac{\Delta H_{ab}{}^*}{1 + K_2 C_1{}^*}\right)^2}$$

where

L_1^*, a_1^*, and b_1^* corresponded to a reference (the control)

$\Delta L^* = L_1^* - L_2^*$	$K_L = 1$
$\Delta a^* = a_1^* - a_2^*$	$K_1 = 0.045$
$\Delta b^* = b_1^* - b_2^*$	$K_2 = 0.015$
$C_1^* = \sqrt{(a_1{}^*)^2 + (b_1{}^*)^2}$	$C_2^* = \sqrt{(a_2{}^*)^2 + (b_2{}^*)^2}$
$\Delta C_{ab}^* = C_1^* - C_2^*$	$\Delta H_{ab}^* = \sqrt{(\Delta a^*)^2 + (\Delta b^*)^2 - (\Delta C_{ab}{}^*)^2}$

Significance was given as $\Delta E^*_{94} \approx 2.3$ or above which corresponded to the Just Noticeable Difference (JND) (Mahy, 1994).

5.3.5 Odor Evaluation of 0.5%-Salt Ground Beef Patties

Sensory testing was conducted at the University of Illinois (Urbana, IL) using ten panelists (3 male, 7 female) ages 20 to 65 (IRB protocol number 12849). Two panelists data were removed due to language barriers and incomplete understanding of attributes generated. Training was performed during nine one-hour sessions under ambient lighting with approximately 30% relative humidity. Training included a full replication of all variables. During training, a panel leader facilitated discussions of product characteristics. Using refrigerated raw and cooked patties, panelists determined odor characteristics based on 15 cm semi-structured line scale (0 = none, 15 = extreme) (Appendix B, Figures B.2.1 and B.2.2). Odor standards were determined by group consensus during 3 training sessions and served as anchor values for each respective standard during the remaining training sessions. A complete list of terms, definitions,

references and ratings can be found in Appendix B, Table B.2.2. All references were prepared within 2 hours of sample evaluation and were served in lidded plastic soufflé cups (56.7 g, Solo Cup Company, Urbana, IL; Dart Container Corporation, Mason, MI).

Two replications were performed with evaluation occurring on days 0, 2, 4, 7 and 9. Samples were randomized and presented in 4 oz. (113.4 g) plastic cups (Solo Cup Company, Urbana, IL; Dart Container Corporation, Mason, MI). The sensory panel evaluated two sets of five samples per session. Raw samples were evaluated before cooked samples, but cooked samples were evaluated in random order of treatment across a session. Sensory data are reported in centimeters from the left end of the line scale.

5.3.5.1 Patty Preparation for Sensory Analysis

Sensory testing only occurred on patties made with 0.5% salt. Raw and cooked patties were placed in a 40 ^{o}C incubator for 30 minutes before being served to panelists. Uncooked patties measured 5.5 cm.-diameter × 0.375 cm. in height (approximately 25 g). Uncooked patties were cut into fifths and placed into soufflé cups and labeled with 3-digit random code numbers.

Cooked samples were cooked to 70 ^{o}C on Farberware Open Hearth grills (Model 455N, Walter Kidde, Bronx, NY) at a setting of 3. The amount of time required to accomplish this temperature was determined in a previous study [data not shown] by inserting internal temperature monitoring using a copper Constantine copper fine-wire thermocouple (Model 5SC-TT-T-30-36, Omega Engineering, Inc., Stamford, Ct) connected to a 12-channel scanning thermocouple thermometer (Model 92000-00, Cole-Parmer Instrument Company, Vernon Hills, IL). Briefly, 7 cm-diameter patties were placed on the fryers for 2.25 minutes (the approximate half-time of the patties 70 ^{o}C end-point temperature) before being flipped, cooked for a total of

4.5 minutes. Cooked patties were blotted once before being cut into fifths and placed in plastic soufflé cups as previously described.

5.3.6 Determination of Diene Conjugation

Conjugated dienes were determined as described by Juntachote, Berghofer, Siebendandl, and Bauer (2006). Conjugated dienes were only determined on patties containing 2% salt ($^{w}/_{w}$). Briefly, a sample (0.5 g) was suspended in 5.0 mL of deionized water in a porcelain mortar (pour lip diameter = 90 mm). The sample was ground for 30 seconds until the solution became homogeneous. A 0.5 mL aliquot of the suspension was mixed with 5.0 mL of extraction solution (3:2 hexane:isopropanol ($^{v}/_{v}$)) for 1 min using a touch mixer (Model 231, Fisher Scientific, Pittsburgh, PA, U.S.A.). After centrifugation at 2000g (Sorvall® RC-5B, Du Pont Company, Wilmington, DE, U.S.A.) for 5 min, the absorbance of the supernatant (*n*-hexane) was determined at 233 nm (Lambda 950 UV/Vis/NIR spectrophotometer, Perkin Elmer, Inc., Waltham, MA, USA). The concentration of conjugated dienes was calculated using the molar extinction coefficient of 25,200 M^{-1} cm^{-1}. Hexane and isopropanol were reagent grade (Fisher Scientific, Pittsburgh, PA, U.S.A.)Results are expressed as mmol dienes / kg sample:

$$\frac{Abs_{233} \times \frac{1000\ mmol}{mol}}{25200\ \times (\frac{0.5_{sample}}{5.0_{water}} \times \frac{0.5_{aq}}{5.0_{hex:isoprop}} \times \frac{1000g}{kg})}$$

5.3.7 Thiobarbituric Reactive Substances (TBARS)

Thiobarbituric reactive substances (TBARS) were determined as described by Miller (1998) based on the method developed by Witte, Krause, and Bailey (1970) with the following modifications: no antioxidants were added as the test units contained antioxidants. Briefly, 5.0 g of patty samples were placed in a porcelain mortar (pour lip diameter = 90 mm). 45.5 mL of extraction solution containing 10% trichloroacetic acid in 0.02 M phosphoric acid (TCA /

H_3PO_4) brought to 50 mL, was ground with a pestle for 1 min. The resulting mixture was filtered through Whatman No. 1 filter paper. One additional sample was spiked with 12 mL of 10 μM 1,1,3,3-tetraethoxypropane (TEP). Two 5 mL aliquots of each filtered sample were transferred into two separate screw-cap test tubes (15 × 200 mm). To one aliquot, 5.0 mL 0.02 M 2-thiobarbituric acid was added (test sample); to the second aliquot, 5.0 mL deionized water was added (sample blank). Test tubes were covered in Parafilm M, capped, inverted three times to mix and held in the dark for 18 hr. at room temperature. Absorbance was determined at 533 nm with a UV/Vis/NIR spectrophotometer (Lambda 950 UV/Vis/NIR spectrophotometer, Perkin Elmer, Inc.) Absorbance was determined at 533 nm with a UV/Vis/NIR spectrophotometer (Lambda 950 UV/Vis/NIR spectrophotometer, Perkin Elmer, Inc.). The sample blank absorbance reading was then subtracted from test sample readings.

A standard curve was derived based on the procedure described by Miller (1998). Aliquots (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 μL) of 25 μM of 1,1,3,3-tetraethoxy-propane (TEP) (Sigma-Aldrich, St. Louis, MO) were pipetted into 20 mL screw-cap test tubes, in duplicate. The total volume of each tube was 10 mL: 5 mL with TCA / H_3PO_4 solution and 5 mL of TBA reagent. The standard curve was constructed from absorbance versus concentration of malondialdehyde (as nmol MDA / mL; Pearson's correlation coefficient: 0.9995) (MDA, St. Louis, MO).

$$Abs_{530} = 0.3396\ Conc_{MDA} + 0.0092$$

MDA recovery was calculated and is expressed as a percentage. Concentration of TBARS was determined as μg MDA / g meat. It was assumed that recovery of TBARS was the same as for MDA. BHT crystalline, trichloroacetic acid crystal reagent and *o*-phosphoric acid were reagent grade (Sigma-Aldrich, St. Louis, MO, U.S.A.).

$$\frac{\frac{nmol_{MDA}}{mL_{soln}} \times \frac{50ml_{soln}}{test\ tube} \times \frac{1\mu g}{1000ng} \times \frac{72.061ng}{nmol_{MDA}} \times}{\frac{5g_{sample}}{test\ tube}}$$

5.3.8 Statistical Analysis

Data from experiment 1 (patties with 0.5% salt) were analyzed as a 4 (antioxidant treatment) × 2 (irradiation dose treatment) × 5 (storage time) factorial design using the PROC MIXED procedure (SAS® 2012). TBARS and color parameters main effects and interactions were considered significant at $p<0.05$. Sensory characteristics were considered significant at $p<0.10$. Separation of least square means was achieved using probability of difference, adjusted with the Tukey procedure for multiple comparisons.

Data from experiment 2 (patties with 2% salt) were analyzed as a 5 (antioxidant treatment) × 6 (storage time) factorial design using the PROC MIXED procedure (SAS® 2012). Color parameters, TBARS, and conjugated dienes main effects and interactions were considered significant at $p<0.05$. Separation of least square means was achieved using probability of difference, adjusted with the Tukey procedure for multiple comparisons.

5.4 Results and Discussion

5.4.1 Effect of Natural Antioxidants on Color of Raw Beef Patties

5.4.1.1 Effect of Natural Antioxidants on Color on 0.5% -salt, Raw Patties Raw Beef Patties

The interaction between antioxidant over storage time was significant ($p<0.05$) for the L* and b* value for the raw patties (unirradiated, 0.5%- and 2.0%-salt). For all other values measured (a* value, chroma and hue angle), storage time was significant (Appendix C.1.1). This was expected as the ferrous myoglobin ($MbFe^{II}O_2$) is reduced to deoxymyoglobin. The spontaneous autoxidation of this critical pigment is an important parameter of meat quality. Because the appearance of food is the first stimulus consumers use to evaluate products,

prevention of deleterious changes is important. The oxidation of myoglobin to metmyoglobin causes meat to lose its bright red color and turn dullish gray. Consumers positively associate this color change with nutrient loss, off-odor and off-flavor development and loss of freshness. Multiple factors affect the rate of oxidation: pH (post-mortem lactic acid production being of particular importance), partial pressure of O_2, activity of metmyoglobin reductase, temperature, addition of salt, and light (Moller & Skibsted, 2006). This visual form of oxidation is indicative of lipid oxidation, as the redox cycling of iron is well known to initiate peroxidation.

In irradiated patties, the interaction between the antioxidants over storage time was significant for the L*, b* and chroma values. Irradiated patties were significantly different in color than non-irradiated patties, as expected (Oslon, 1998; Kim *et al.*, 2002). Satterlee *et al.* (1972) suggests that this is due to the reduction of the myoglobin (MbO_2 before irradiation) being converted to metmyoglobin. The Hunter (1952) system developed to denote lightness (L*), redness-to-greenness (a*) and yellowness-to-blueness (b*) can be manipulated to indicate hue angle (intermediate colors between a* and b*) and chroma (degree of saturation). In theory, every aspect of color can be determined through a spectrocolorimeter; however, food matrices often suffer from irregularity in shape, continuity and surface texture, which is extremely apparent in meat (Pomeranz & Meloan, 2001). It is well known that no single measure (L*, a*, or b*) or transformation of the Hunter values (chroma or hue angle) of color can accurately account for consumer perception. The instrumental values most currently used to assess meat color are a* and chroma (McCarthy *et al*. 2001).

In addition to being overly simple, instruments are often too specific in their differentiation of color at nanometer wavelengths. This is apparent when distinguishing between Figures 5.1 and 5.2, where distinguishing significance between L*, a* and b* is mostly a trial in

patience and attention span. Human sensory panels using paint tiles, such as those described by Nicolade *et al.* (2006) have proved superior, but time-consuming and costly. In an attempt to find a balance between instrumental values and the propensity of human sight towards certain wavelengths (those in the yellow/green range), the International Center for Illuminance (CIE, 1995) has developed a distance metric called ΔE to address perceptual non-conformities while simultaneously utilizing the L*, a*, and b* color space (CIE, 1995). Through multiple revisions, the CIE has addressed factors that affect visual perception, such as the MacAdam ellipses. These changes can be addressed as such:

> 1986 - Addition of an induction of a lightness-to-chroma factor (2:1), where hue is a constant defined as 1.
>
> 1994 - Addition of weighting factors to give lightness, chroma and hue different proportional weights in terms of perception.
>
> 2000 - Additional weights to correct for the relationship between chroma and hue.

By using the reference color of a fresh meat patty (control) on Day 0, a ΔE can be given to each sample. Using the just noticeable difference (JND) value of 2.3 reported by Mahy (1994), it is possible to determine one aspect when the oxidation of myoglobin becomes significantly altered. For the values given in Table 5.2, it becomes apparent that only BHA maintain a similar visual color as the control in unirradiated samples, a trend noted by Stout, Muthukamarappan, & Julson (2000). The result of GSE having no effect on color changes in beef patties over time was expected (Rojas & Brewer, 2007). In Table 5.3, 1.5 kGy the treated samples appear to maintain similarity to the irradiated control through day 2, and pomegranate has an added effect of maintaining the same color as the control through day 4.

5.4.1.2 2%-salt Patties

When salt content was increased, greater oxidation was induced (Figure 5.4). It should be noted that every sample remained similar in color to the control through day 2, and only samples using pomegranate as an antioxidant showed a just noticeable difference from the control by day 4. By day 7, all samples were noticeably different from the control in terms of color, indicating that antioxidant effects on metmyoglobin formation had altered the color. As Hultin (1980) and Srinivisan (1996) have pointed out, oxidation of meat pigments is due to free radicals and likely a predecessor to lipid oxidation. Because of this, measurement of pigment oxidation is an integral parameter of study for meat-muscle oxidation.

5.4.2 Effect of Natural Antioxidants and Irradiation on Odor Descriptors of Beef Patties

Table 5.1 Antioxidant Formulation and Code

Antioxidant	Amount	Code based on Irradiation Dose	
		0 kGy	1.5 kGy
Control	0 ppm	CON	CON_{IRR}
Pomegranate Extract	100 ppm	POM	POM_{IRR}
Grape Seed Extract	100 ppm	GSE	GSE_{IRR}
Butylated Hydroxyanisole	100 ppm	BHA	BHA_{IRR}

Significant differences occurred in the intensities of the off-odor characteristics (Figure 5.3) in terms of antioxidant treatment (sweet, wet cardboard), and over storage time (raw, sweet, wet cardboard and rancid) but not over the interaction between storage time and antioxidant for each odor descriptor. These findings reaffirm the relationship (fresh meat odor giving way to sweetness and finally rancidity over 10 days) given by Nissen *et al*. (2004) in their

comprehensive evaluation of plant extracts (including grape skin) as methods of controlling oxidation.

The statistically significant ($p \leq 0.10$) difference in antioxidant treatments was found between the CON and POM_{IRR} patties in terms of developing sweetness throughout the storage time. The attribute of wet cardboard developed the most differences among antioxidant treatments, with high correlation being found between the raw, irradiated patties and raw, unirradiated patties. The CON_{IRR} and POM_{IRR} treatments were each statistically higher than all unirradiated treatments (at $p \leq 0.10$). Additionally, the POM treatment was found statistically lower than the GSE_{IRR}, and BHA_{IRR} treatments (a result opposite to those of Stout, Muthukamarappan, & Julson (2000) who found BHA and irradiation improved color and flavor of irradiated beef). POM_{IRR} additionally showed a lower value relative to CON in terms of wet cardboard over time. Wet cardboard is an attribute that can be associated with 'musty' and 'sweaty'. Such attributes have been a known indicator of ozone (Brewer, 2009) and oxidation (Chen *et al.*, 1999; Ahn *et al.*, 1999b, 1998a; Jo & Ahn, 2000) which is linked to the irradiation of meat products (Jo & Ahn, 2000).

These results confirm previous experiments using beef as a model system which indicated that grape seed extract (Rojas & Brewer, 2007) and BHA (Wettasinghe & Shahidi, 1999) have the potential for controlling some of the negative sensory characteristics associated with oxidation of meat over time. Certainly, no product can control oxidation products indefinitely.

With the exception of 'sulfury', all attributes increased over time. Panelists found a statistical decrease in terms of the raw attribute immediately from day 0 to day 2 but could only detect a decrease again between day 7 and day 9. Conversely, panelists noticed a statistical

increase between day 0 and day 4 in both wet cardboard and rancid. Both wet cardboard and rancid have been used as indicators of oxidation previously (Rojas & Brewer, 2007). The last notable difference was in the term sweet, in which a notable increase developed between day 0 and statistically on day 7. This has also been noted by Nissen *et al.* (2006) in addition to a decrease in meatiness over time. While Thakur & Sing (1994) and Mottram (1998) found sulfury to be byproduct of irradiation processing, this study found it was not an indicator of oxidation over time, antioxidant treatment or processing treatment.

5.4.2.2 Effect of Natural Antioxidants on Odor Descriptors of Cooked Beef Patties

The effect of cooking altered panelist perception of oxidation in the beef patties (Figure 5.4). After cooking, there was no significant difference due to antioxidant or irradiation. This result is not surprising due to the unfolding of protein during heating which leads to an increase in hydrophobic groups (Sydow, 1975). Brewer & Vega (1995) as well as other authors (Aria *et al.*,1970) supported and indicated that exposed hydrophilic groups bind and inactivate the odor properties of aldehydes (such as hexanal, a potent off-odor of lipid oxidation). Additionally, prominent flavor components, such as hexanal, are driven off during heating).A difference due to storage time appeared only in wet cardboard and rancid attributes and only between day 0 and day 7. This is encouraging as it suggests the effect of cooking beef patties eradicates perception of irradiation and added antioxidants up to a week, when kept at refrigerated temperature.

5.4.3 Effect of Natural Antioxidants on Lipid Oxidation of Raw Beef Patties

5.4.3.1 TBARS of 0.5%-salt Patties

The TBARS values of raw, unirradiated (Figure 5.5) and raw, irradiated (Figure 5.6) beef patties with 0.5% salt and 100 ppm antioxidants over 9 days of refrigerated storage were significant due to antioxidant and storage time, but not the interaction between the two. Overall,

the TBARS values did not change during the first 4 days of storage for beef patties utilizing an antioxidant treatment (regardless of irradiation treatment). Only the control (?? The ones without antioxidants?) of the irradiated beef patties showed a significant difference on day 4. Between day 4 and day 7, there was a significant difference between all samples. This could indicate a level of acceptance for raw patties, regardless of processing treatment (i.e. irradiation vs. non-irradiated beef patties). For raw, unirradiated beef patties, only those patties containing BHA as an antioxidant showed a significantly lower level of oxidation when comparing day 9 to day 0 Where the control showed an 88% increase in TBARS, those patties containing BHA showed only a 70% increase. This differed statistically to those patties treated with pomegranate and grape seed extract (increasing 83% and 84%, respectively). In irradiated patties, this difference was more prominent. Antioxidant treatments involving BHA_{IRR} and GSE_{IRR} showed a significant difference from the CON_{IRR} and POM_{IRR} (63% and 26% compared to 89% and 87% increases). An interesting observation was made in patties containing BHA_{IRR} and GSE_{IRR} in TBARS on Day 7. The steep decline in TBARS noted may indicate that either irradiation at 1.5 kGy was enough to deactivate the antioxidant, or the high level of irradiation created a cascade of oxidation products that burdened the antioxidant so that it was less effective after 7 days.

5.4.3.2 TBARS and Conjugated Dienes of 2%-Salt Patties

The increase of sodium chloride on antioxidant efficacy in unirradiated beef patties (as measured by chemical analysis) offers similar results to those found in lower-salt patties. The results of both assays of the control were similar of Srinivasan *et al.* (1996) in oxidation of surimi-like beef heart. When considering the unirradiated, raw beef patties, it remains clear that BHA serves as the best antioxidant in retarding oxidation of raw beef (Figure 5.5, 5.7). In Figure 5.7, pomegranate and grape seed extracts both appeared to significantly retard oxidation induced

by salt up to day 7, but started to fail (indicated by association with TBARS values similar to the control) by day 9. In looking at salt-induced oxidation, multiple authors reported a prooxidative effect at 2% ($^{w}/_{w}$) salt in cooked beef patties (Chen, 1984; Han and Rhee, 2005) and cooked turkey patties (King & Earl, 1988) after 6 days (Han and Rhee, 2005) of refrigerated storage. Multiple mechanisms have been proposed as to how sodium chloride acts as a prooxidant, from disruption of membrane structural integrity (Rhee, 1999) to acceleration of metmyoglobin to inducing the formation of free iron ions naturally found in meat (Kanner *et al.*, 1991).

5.5 Conclusions

Butylated hydroxyanisole (BHA) exhibited the best antioxidant activity in ground beef under each processing condition (both irradiated and non-irradiated beef during refrigerated storage), as was demonstrated by the lower TBARS values, lower levels of diene conjugation and off odors associated with rancid, wet cardboard and sweet. BHA did not alter the instrumental color measures of ΔE^{*}_{94} past day 2 in non-irradiated, 0.5%-salt beef patties. BHA, a synthetic antioxidant, still shows the most potential at reducing autoxidation and maintaining the shelf life in high-fat, ground beef patties.

5.6 References

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Table 5.2: Effects of Antioxidants over time on ΔE^*_{94}[1] for 0.5% salt, Irradiated Beef Patties

	Storage Time					
Treatment	**Day 0**	**Day 2**	**Day 4**	**Day 7**	**Day 9**	**SEM**
CON_{IRR}	0.00	**2.53**	**5.28**	**4.90**	**6.48**	**0.664**
POM_{IRR}	1.62	1.31	0.09	**3.92**	**7.11**	
GSE_{IRR}	**3.04**	2.02	**3.90**	**4.70**	**5.27**	
BHA_{IRR}	0.96	1.77	**6.44**	**5.82**	**5.32**	

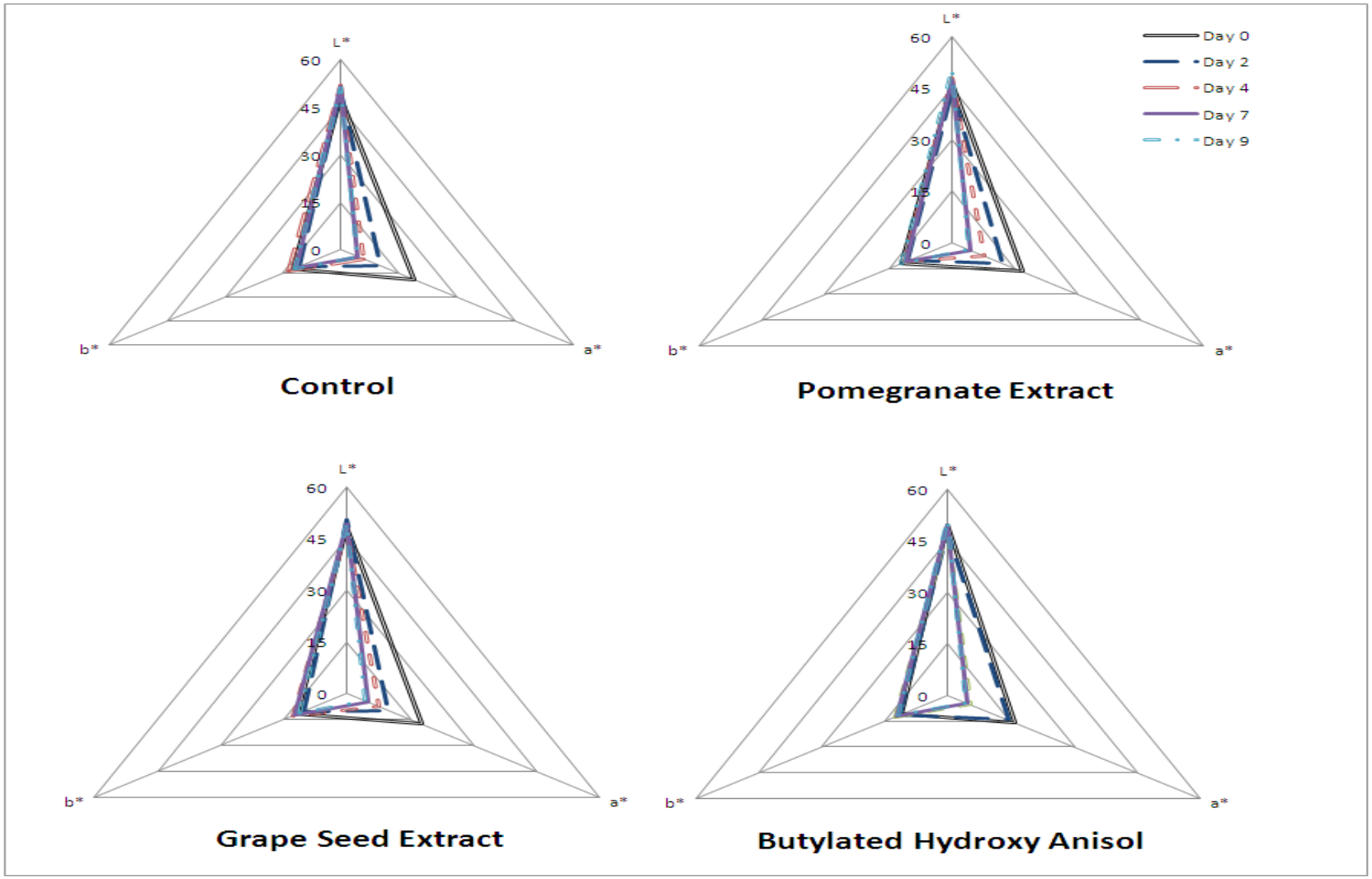
[1]Means within a column that are bolded are significantly different than the Control on Day 0.

Table 5.3: Effects of Antioxidants over time on ΔE^*_{94}[1] for 0.5%-Salt, Non-Irradiated Beef Patties

	Storage Time					
Treatment	**Day 0**	**Day 2**	**Day 4**	**Day 7**	**Day 9**	**SEM**
Control	0.00	**3.86**	**8.76**	**8.46**	**8.58**	**0.720**
Pomegranate Extract	1.68	**4.15**	**4.79**	**7.33**	**8.61**	
Grape Seed Extract	0.41	**3.97**	**6.25**	**7.89**	**7.78**	
BHA	1.42	1.56	**7.68**	**7.99**	**8.15**	

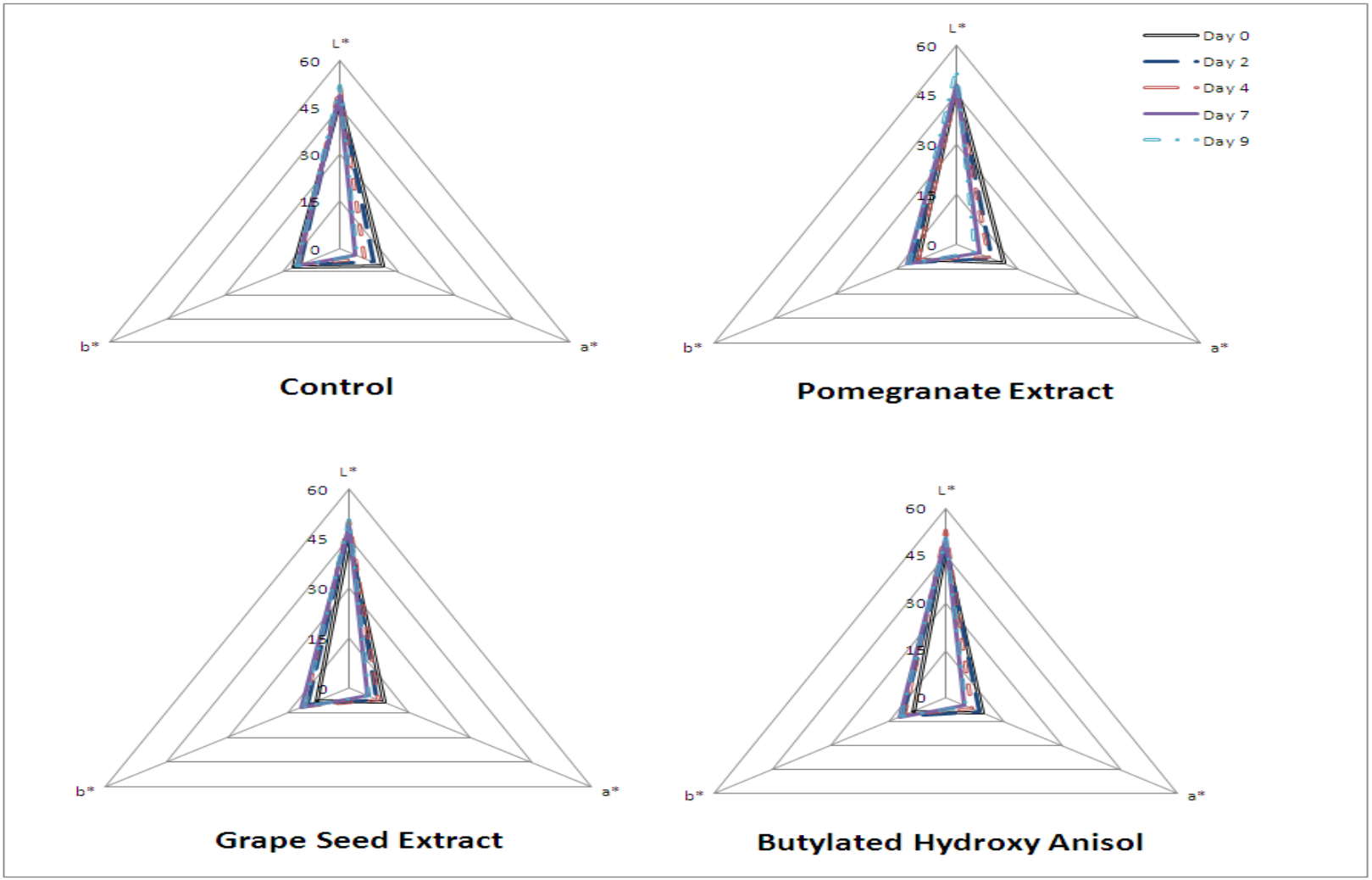
[1]Means within a column that are bolded are significantly different than the Control on Day 0.

Figure 5.1: L*, a* and b* [1] Values of 0.5%-Salt Ground Beef Patties Over Time



[1] Least Square Means (LSM) of score value pooled over storage (4°C) time.

Figure 5.2: L*, a* and b* [1] Values of *Irradiated* 0.5%-Salt Ground Beef Patties Over Time



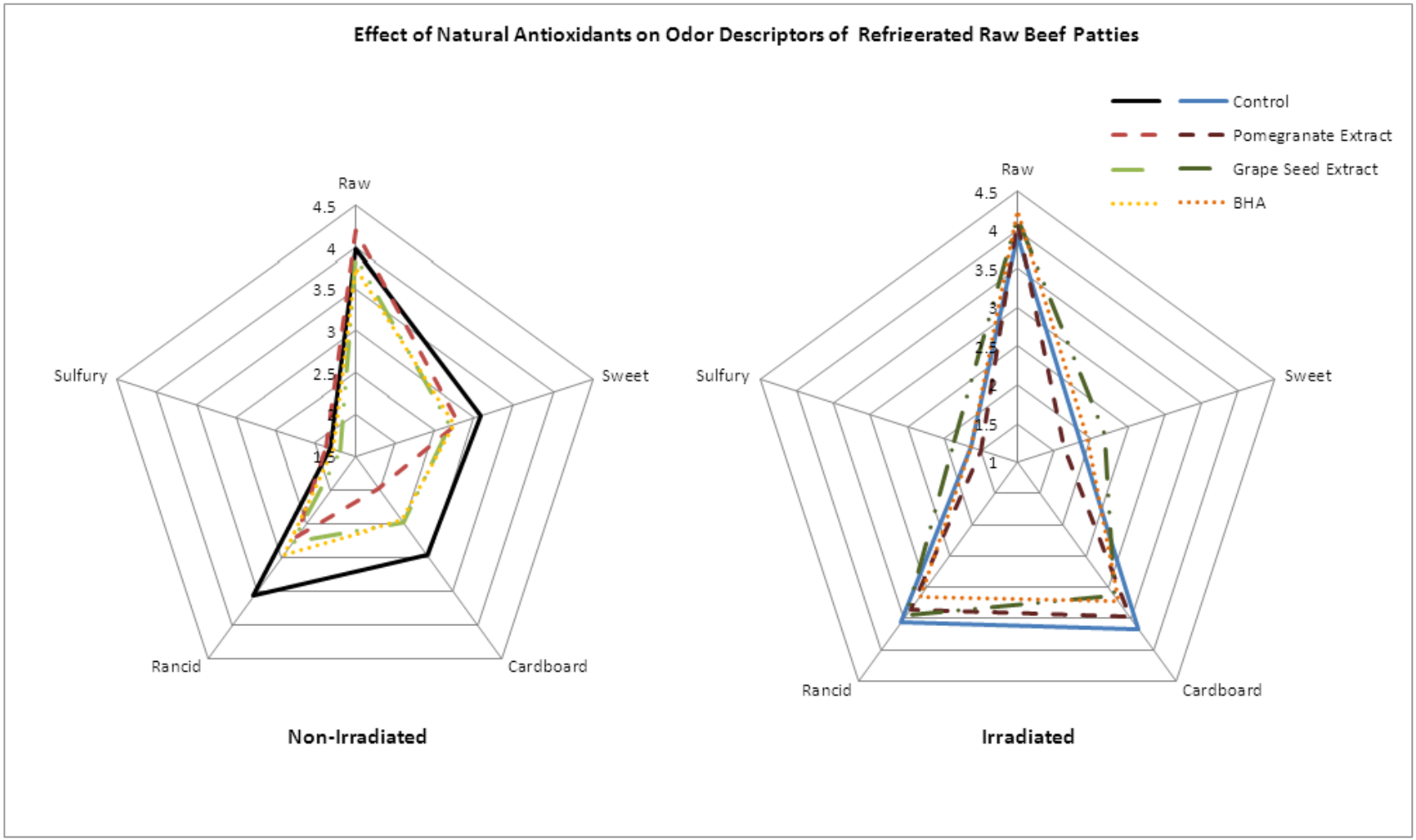
[1] Least Square Means (LSM) of score value pooled over storage (4°C) time.

Table 5.4: Effects of Antioxidants over Time on ΔE^*_{94}[1] for 2%-Salt Ground Beef Patties

	Storage Time						
Treatment	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 9**	**SEM**
Control	0.00	1.15	1.52	2.27	***4.58***	***6.17***	***0.446***
Pomegranate Extract	1.39	1.46	2.09	***2.36***	***3.05***	***4.51***	
Grape Seed Extract	0.76	0.61	1.74	1.52	***3.22***	***4.55***	
Dietary Selenium	0.80	1.55	1.28	1.87	***3.79***	***4.46***	
BHA	0.91	1.03	1.29	1.22	***2.85***	***3.14***	

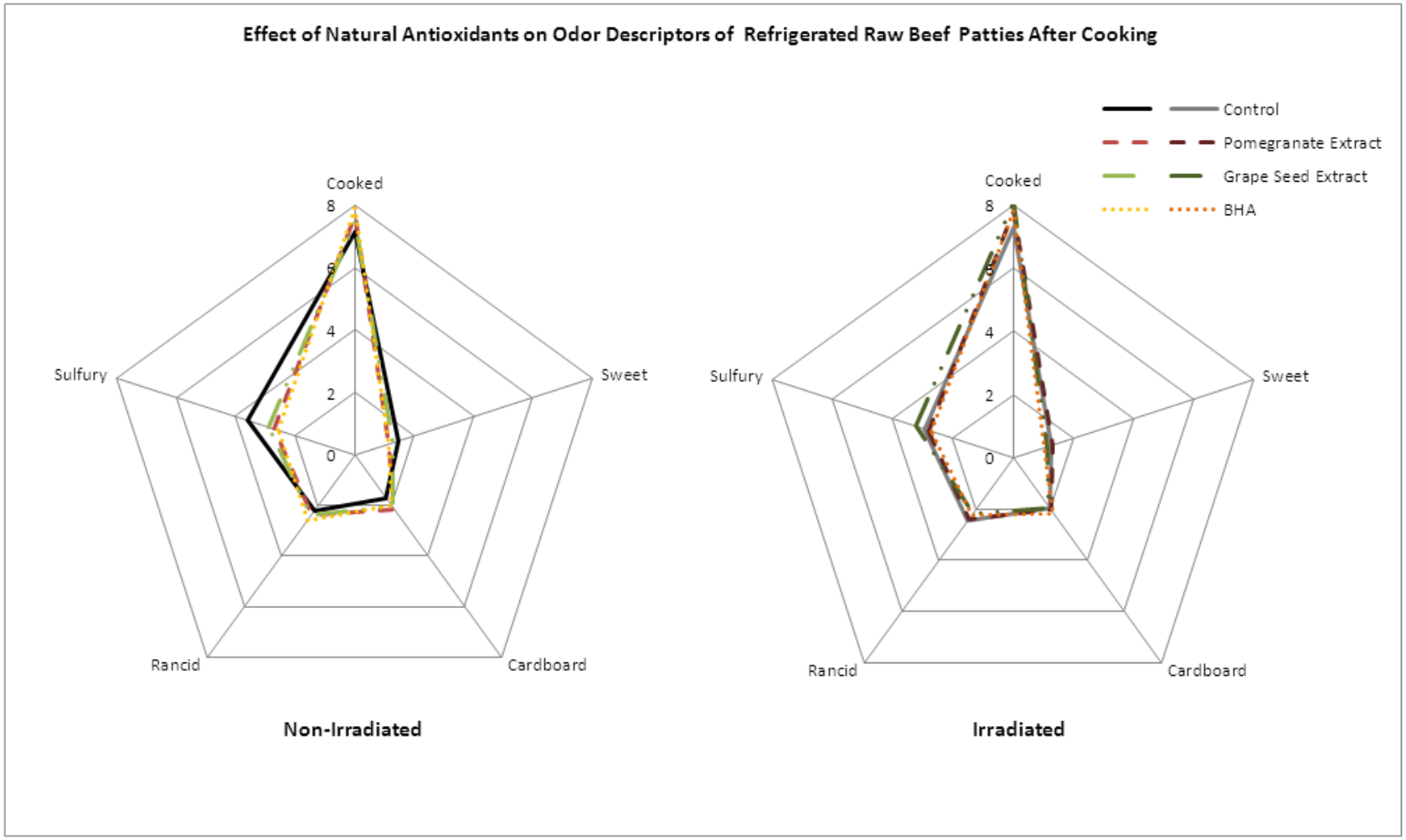
[1]Means within a column that are bolded are significantly different than the Control on Day 0.

Figure 5.3: Effect of Natural Antioxidants and Irradiation on Odor Descriptors[1] of Refrigerated Raw Beef Patties



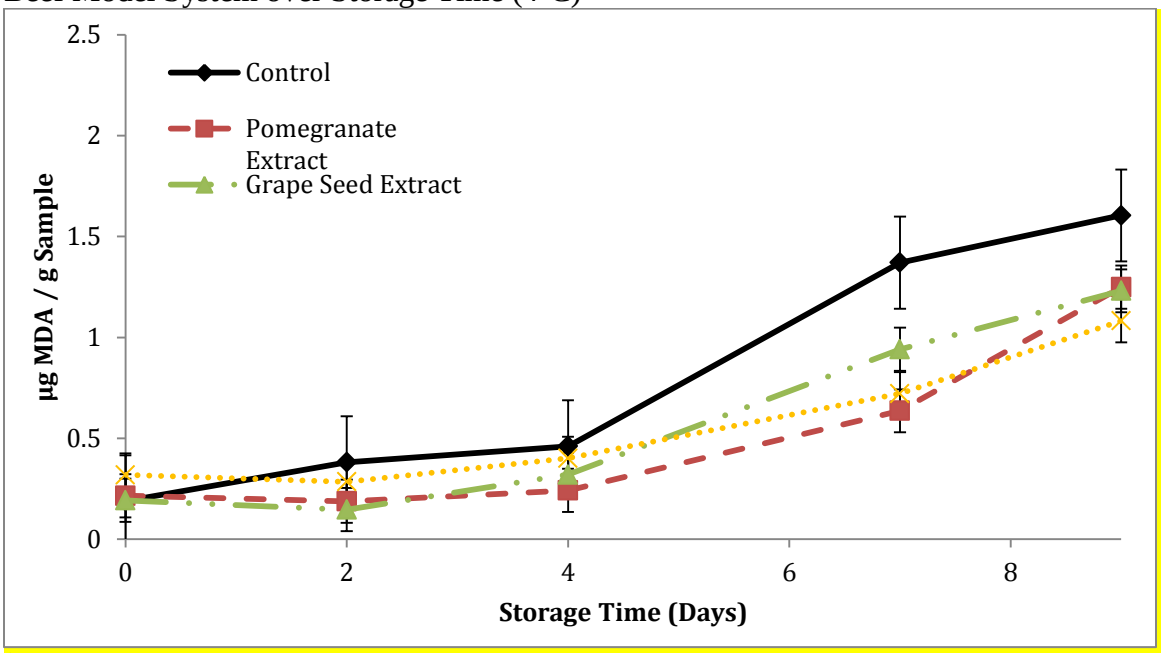
[1] Least Square Means (LSM) of score value pooled over storage (4°C) time.

Figure 5.4: Effect of Natural Antioxidants and Irradiation on Odor Descriptors[1] of Refrigerated Raw Beef Patties After Cooking



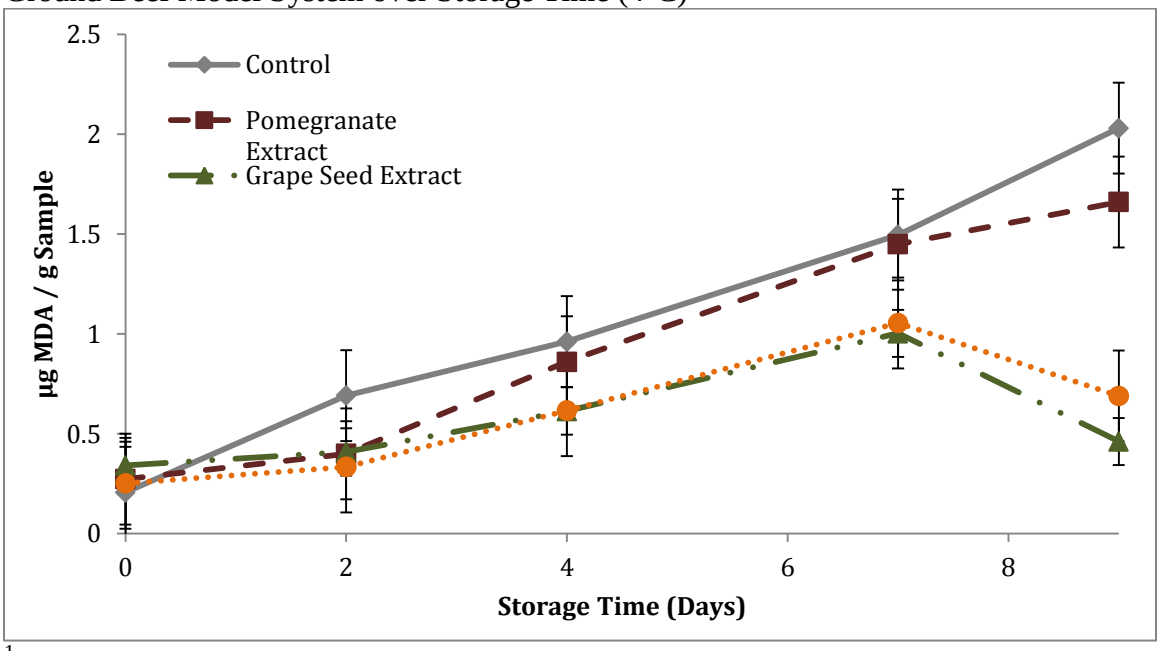
[1] Least Square Means (LSM) of score value pooled over storage (4°C) time.

Figure 5.5 Comparative Effect of Antioxidants[1] on TBARS of Raw, 0.5%-Salt Ground Beef Model System over Storage Time (4°C)



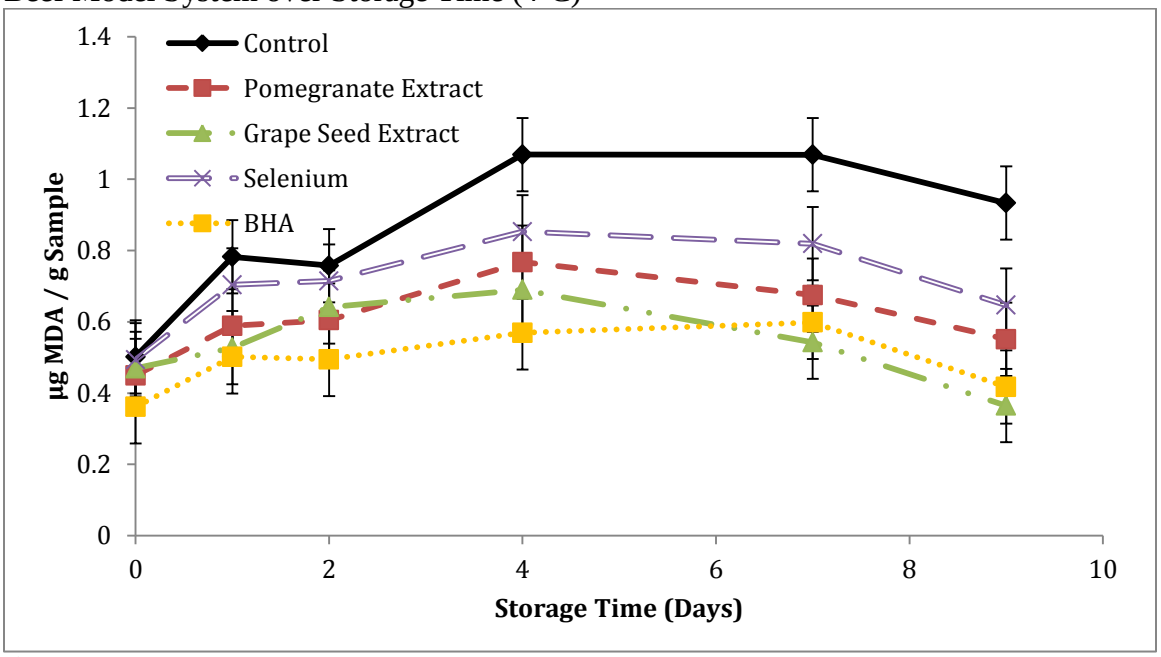
[1]Least Square Means (LSM) of TBARS (µg MDA / g sample)
Y Error bars = standard error of LSM

Figure 5.6 Comparative Effect of Antioxidants[1] on TBARS of Raw, *Irradiated* 0.5%-salt Ground Beef Model System over Storage Time (4°C)



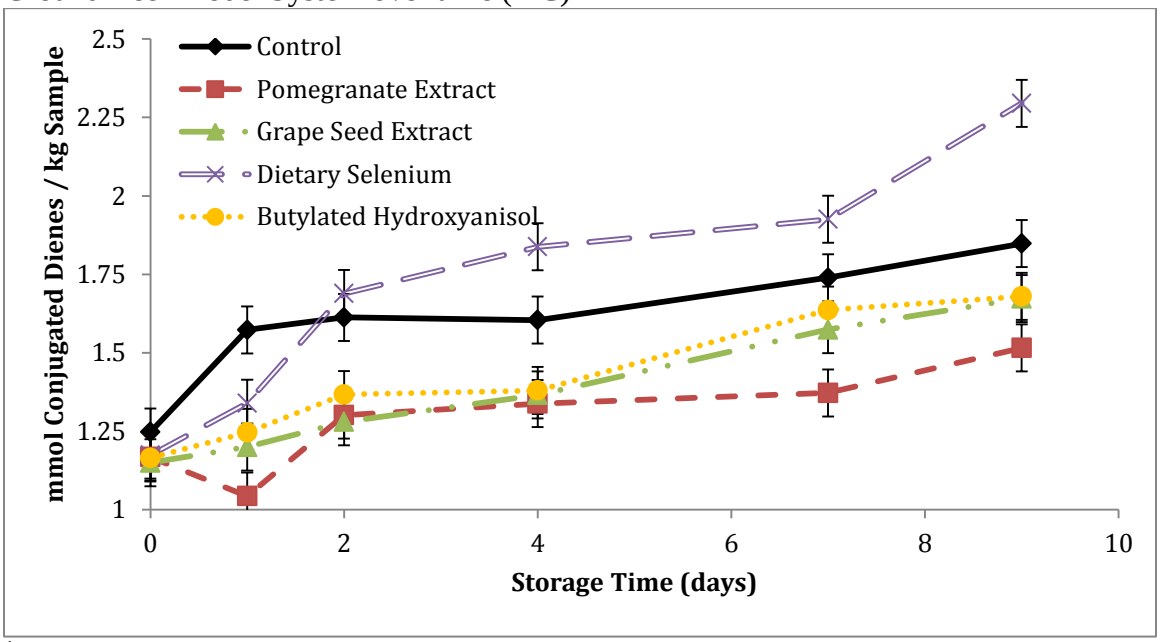
[1]Least Square Means (LSM) of TBARS (µg MDA / g sample)
Y Error bars = standard error of LSM

Figure 5.7 Comparative Effect of Antioxidants on TBARS[1] of 2%-Salt, Raw Ground Beef Model System over Storage Time (4°C)



[1]Least Square Means (LSM) of TBARS (µg MDA / g sample)
Y Error bars = standard error of LSM

Figure 5.8: Comparative Effect of Antioxidants on Conjugated Dienes[1] of 2% NaCl Raw Ground Beef Model System over time (4°C)



[1]Least Square Means (LSM) of diene conjugation (mmol / kg sample)
Y Error bars = standard error of LSM

Chapter 6: Conclusions

Reducing lipid autoxidation in foods is a priority in maintaining quality attributes, nutritional status, and consumer acceptability. The food industry has discovered multiple avenues for controlling oxidation. Physical environmental control, elimination of activity of endogenous enzymes, addition of hydrogen-donating compounds, and the addition of reducing agents are all commonly employed methods. Because the chemical nature of each antioxidant is different (e.g. hydrophilicity/solubility, number and placement of hydroxyl groups, reductive potential), the nature of the antioxidant and matrix must be known to determine effective systems for controlling oxidation. The consumer-driven preference towards natural, plant-based antioxidants over conventional synthetically-produced antioxidants becomes an economical concern when antioxidants (at the same weight-by-weight basis) do not work in similar context.

Four experiments were conducted to evaluate the effectiveness of pomegranate extract as an antioxidant in comparison to grape seed extract (an effective natural antioxidant) and butylated hydroxyanisole (an effective synthetic antioxidant). When directly analyzed through multiple well-known techniques, pomegranate extract was found to be an exceptional antioxidant, performing better than both the well-known synthetic and natural antioxidants. However, direct analysis is flawed through an over-abundance of antioxidant, synthetically-generated free radical compounds, and a chemical matrix that bears little similarity to standard food systems.

All three antioxidants were then evaluated in a gelled, emulsified lard model system, using three concentrations of pomegranate extract (100 ppm, 500 ppm, and 1000 ppm) compared to 100 ppm of BHA and grape seed extract. At ten times the

concentration of the other antioxidants, POM extract finally extended the induction period of oxidation beyond that of BHA, while at five times the concentration it exhibited the antioxidant capacity of grape seed extract. At the same concentration of BHA and GSE in a gelled, emulsified canola-oil model, it showed lower antioxidant capacity than the well-known antioxidants, but was still distinguishable from the control in chemical assays. A sensory study, however, indicated that all three antioxidants were comparable in retarding oxidation.

In the final study, all three antioxidants were compared in a ground beef model system. The meat model system contains more endogenous oxidation species, but is representative of an actual food product. Chemical assays indicated that pomegranate extract was not as efficient at reducing autoxidation, salt-induced oxidation or irradiation-induced oxidation when compared to GSE and BHA. On the other hand, a sensory study indicated that while each antioxidant had slightly different characteristics, all were more effective at controlling salt-induced and autoxidation when compared to a control. No antioxidant was effective at extending the induction period of oxidation in the meat samples after being irradiated. Additionally, the effect of cooking minimized the off-odor effect of both irradiation- and salt-induced oxidation characteristics.

In summary, pomegranate extract has the potential to extend the induction period of oxidation (reducing off-odor and flavor characteristics), but only if incorporated at higher levels than antioxidants which are currently employed (i.e. BHA). While the relationship between pomegranate extract, grape seed extract and BHA appeared independent of the food matrix, it is necessary to understand the nature of the matrix because each matrix will have individual pro- and antioxidant constituents.

Appendix A: Experimental Design
Figure A.1.1. Lard Model System Flow Diagram

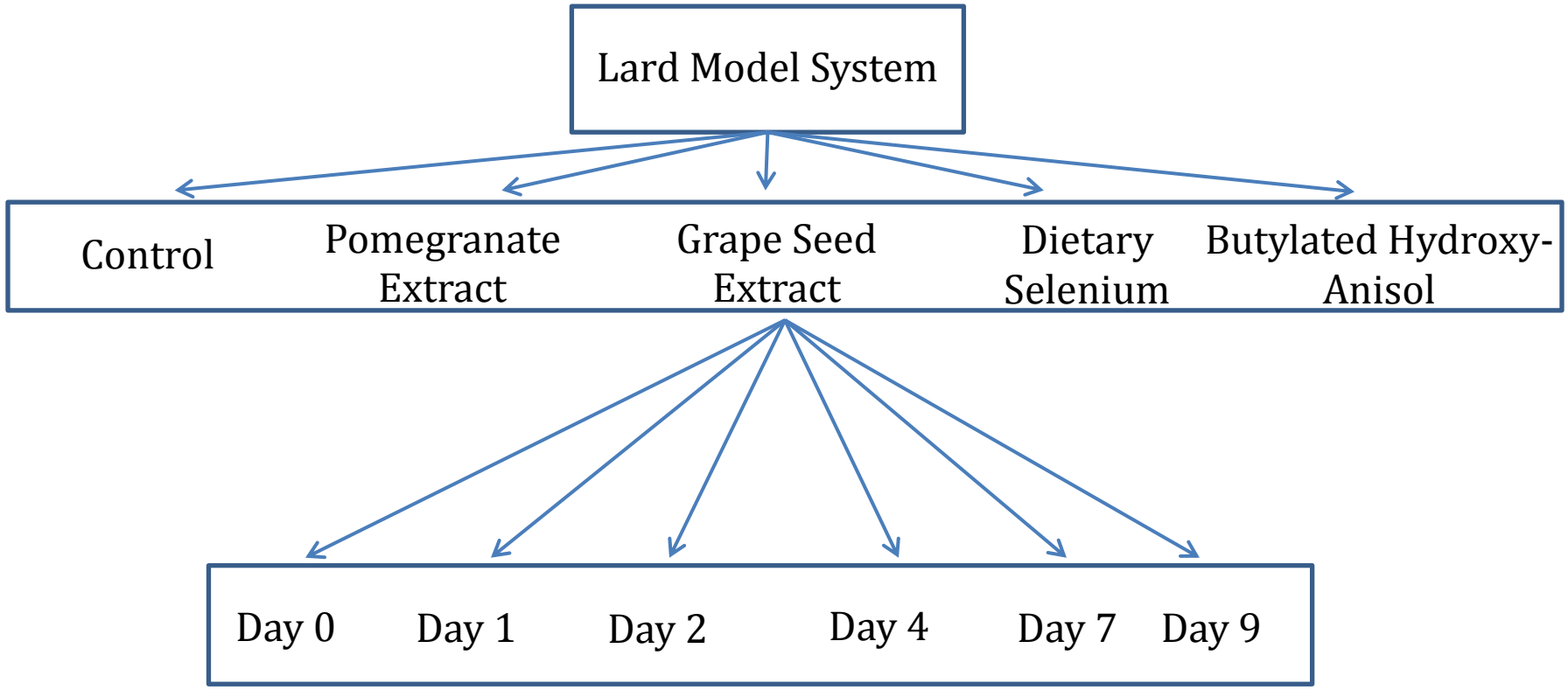


Figure A.1.2.: Canola Oil Model System Experimental Design

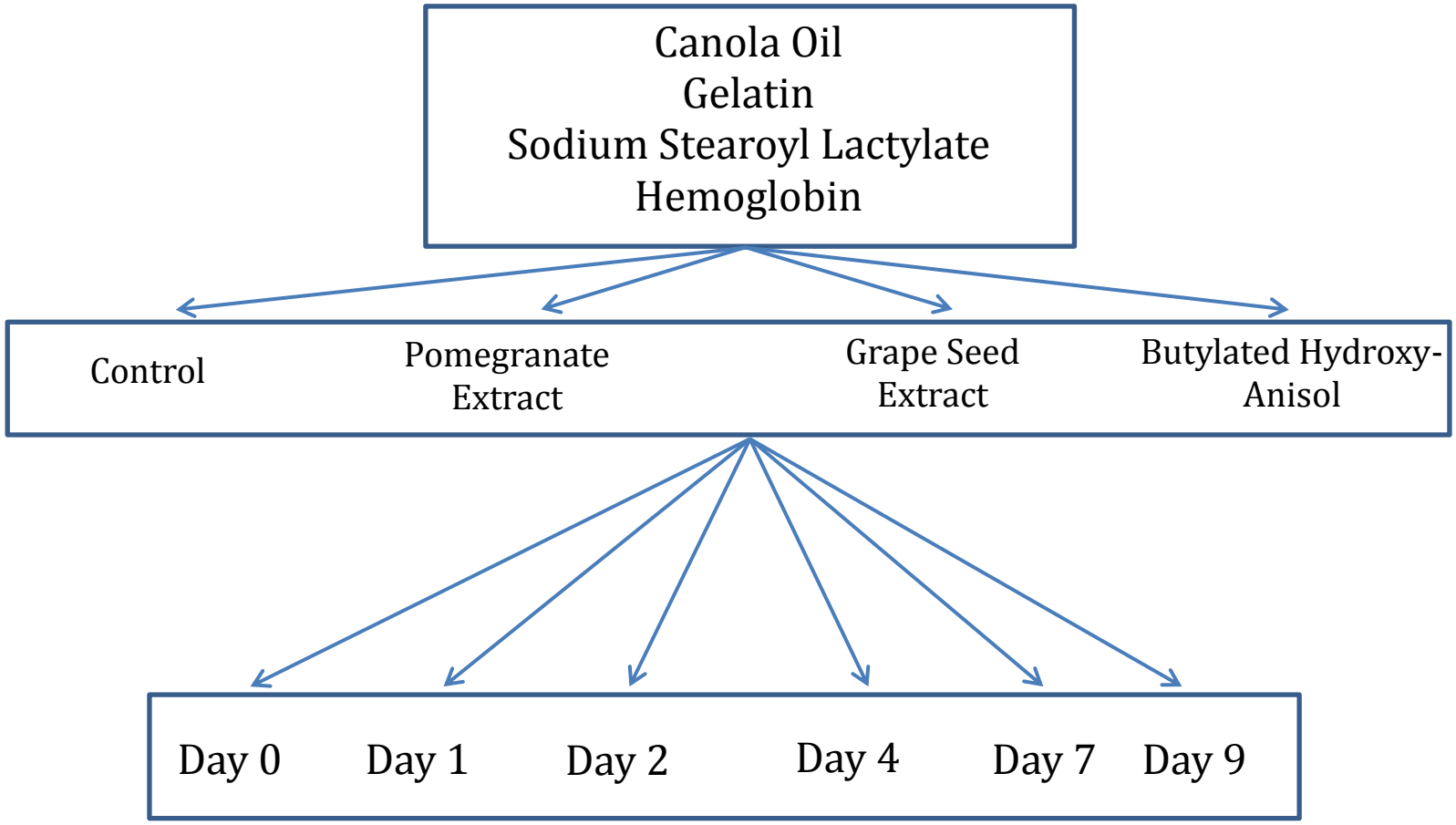


Figure A.1.3 Ground Beef Model System Flow Diagram

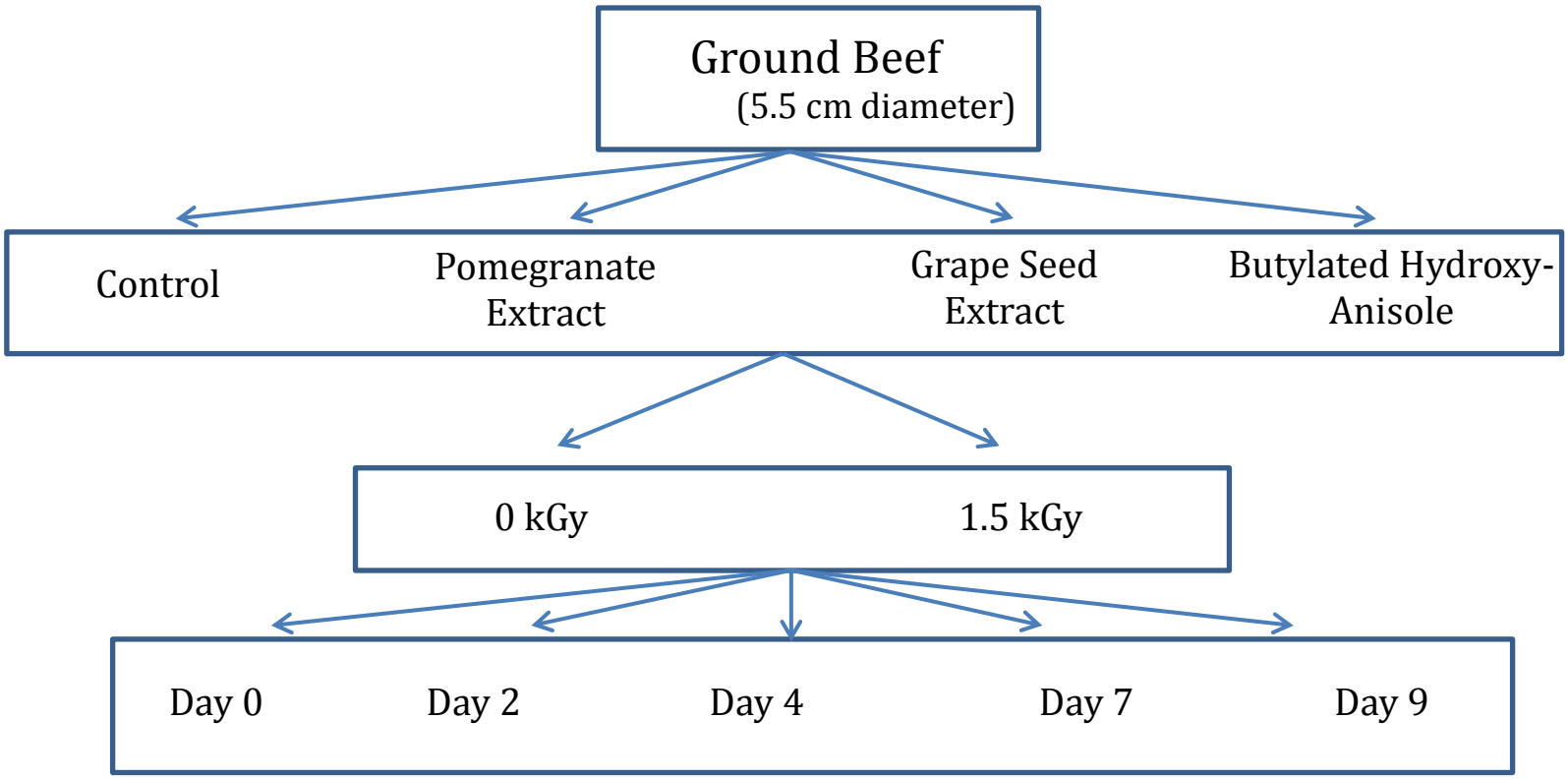


Figure A.2.1 Total Phenolics Determined by Folin-Ciocalteu Reagent

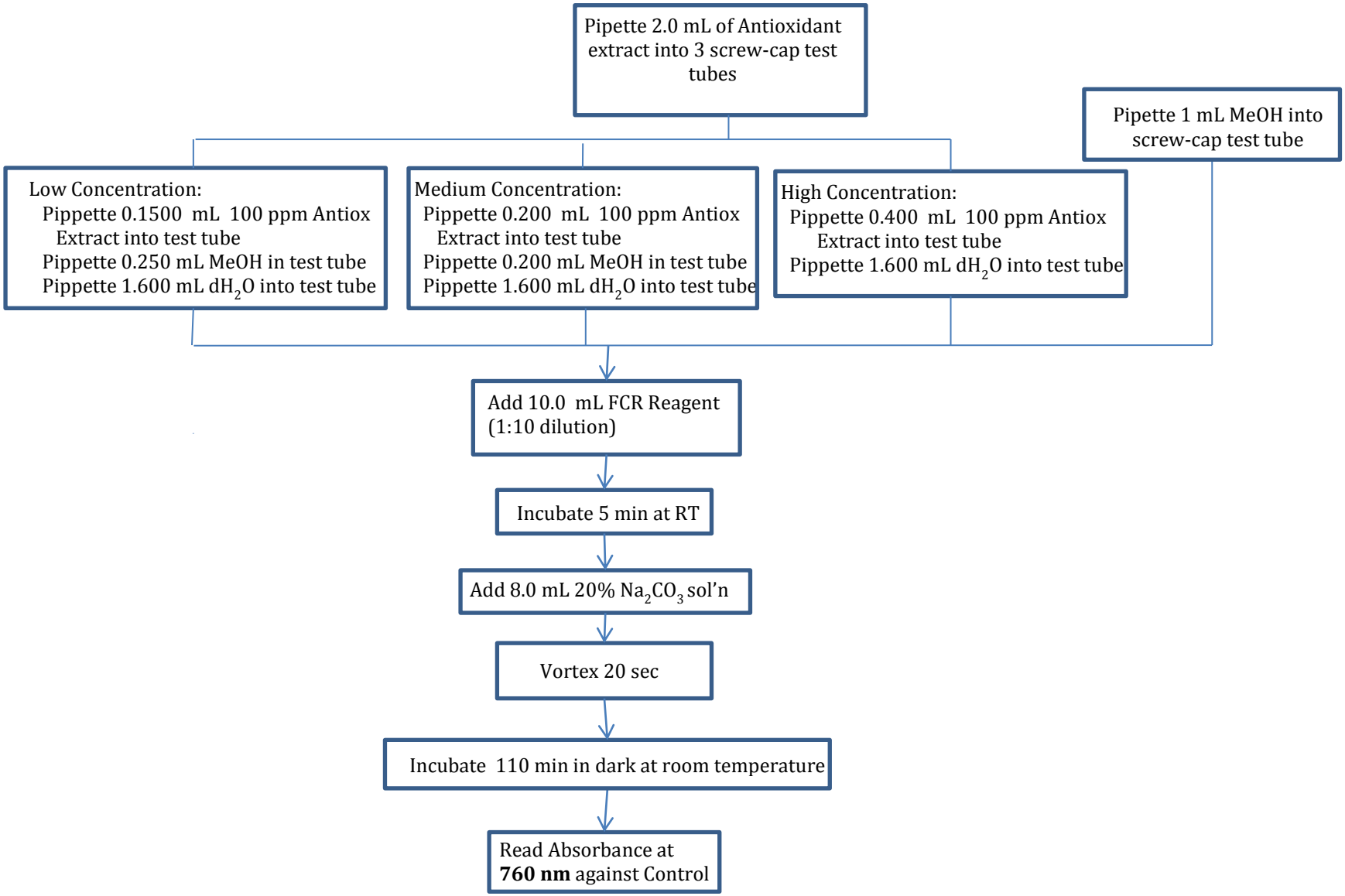


Figure A.2.2 FCR Standard Curve of Gallic Acid

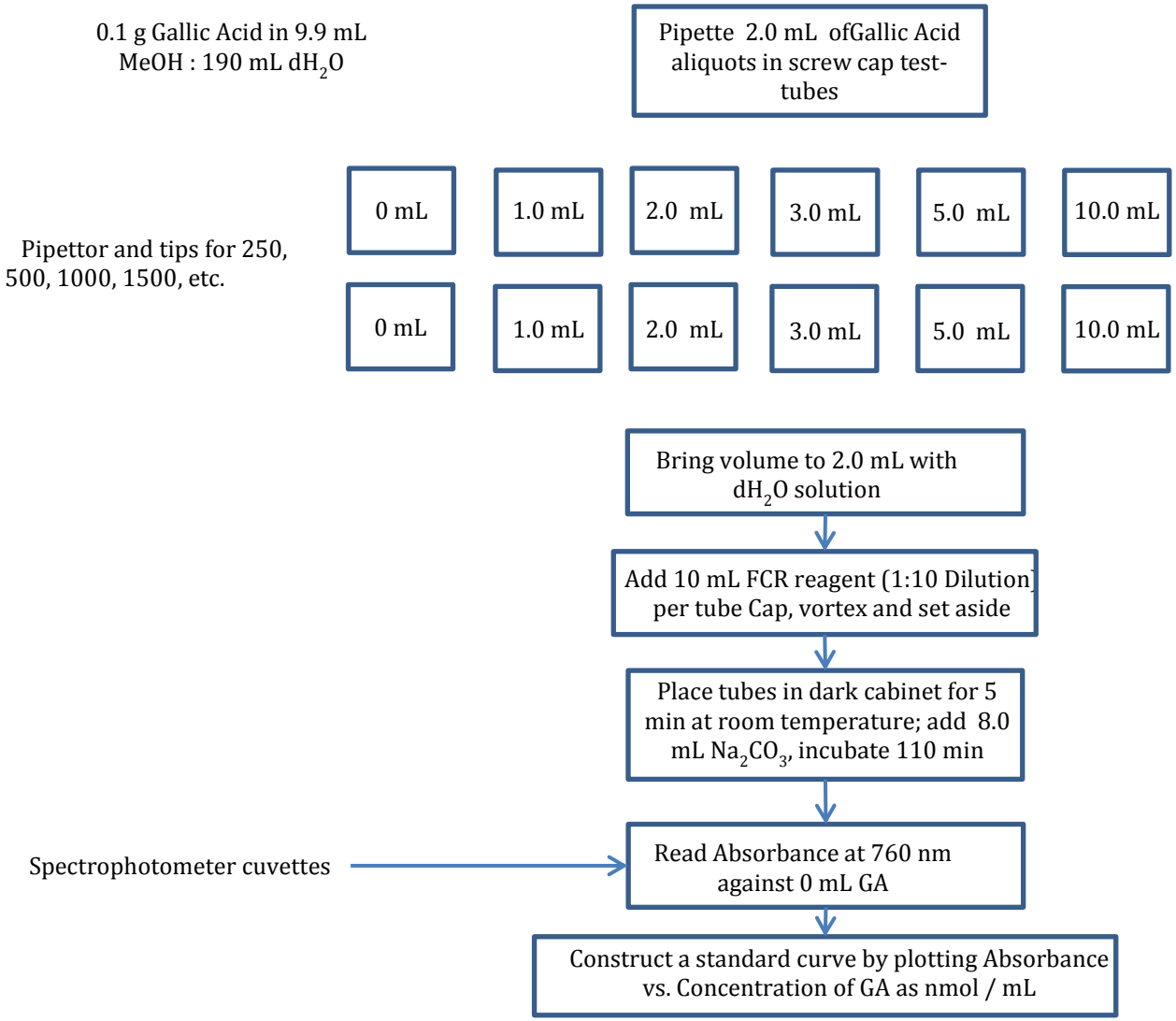


Table A.2.1 Gallic Acid Standard Curve

Gallic Acid (mg / L MeOH)	Absorbance (A_{760})		
0	0.0812	0.0803	0.0811
1	0.1821	0.1771	0.1843
2	0.2799	0.2741	0.2706
3	0.3748	0.3700	0.3751
5	0.5534	0.5448	0.5406
10	1.0136	0.9814	0.9922

Figure A.2.3 Gallic Acid Standard Curve

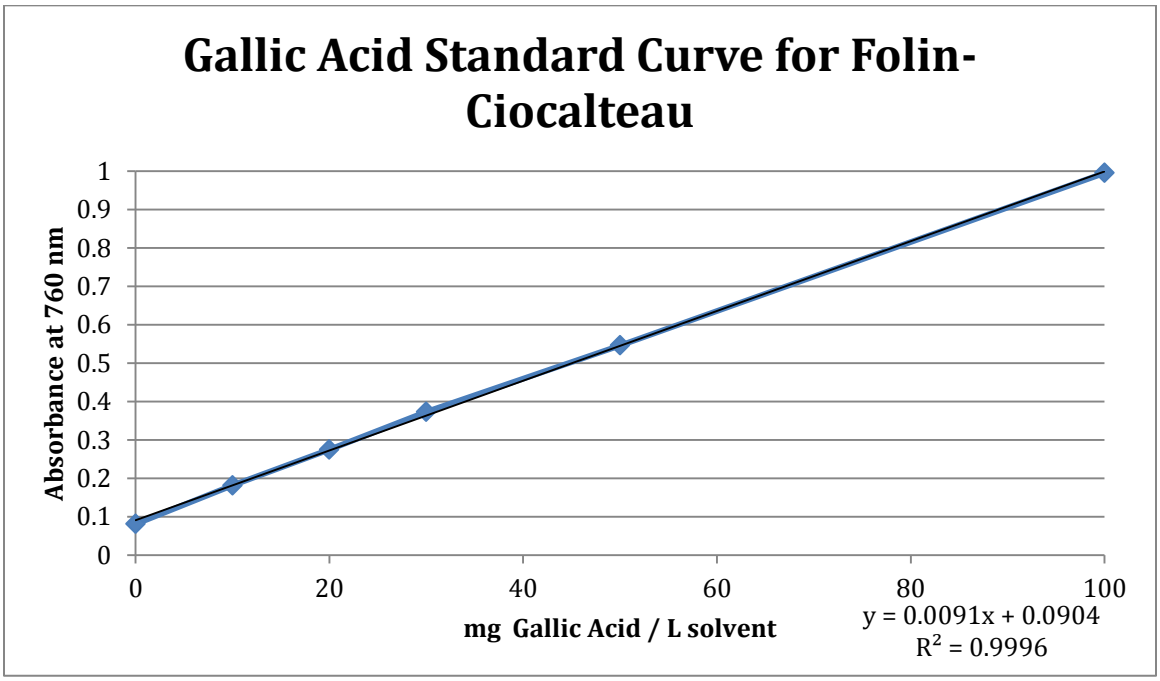


Figure A.2.4 Ferricyanide Reducing Antioxidant Power

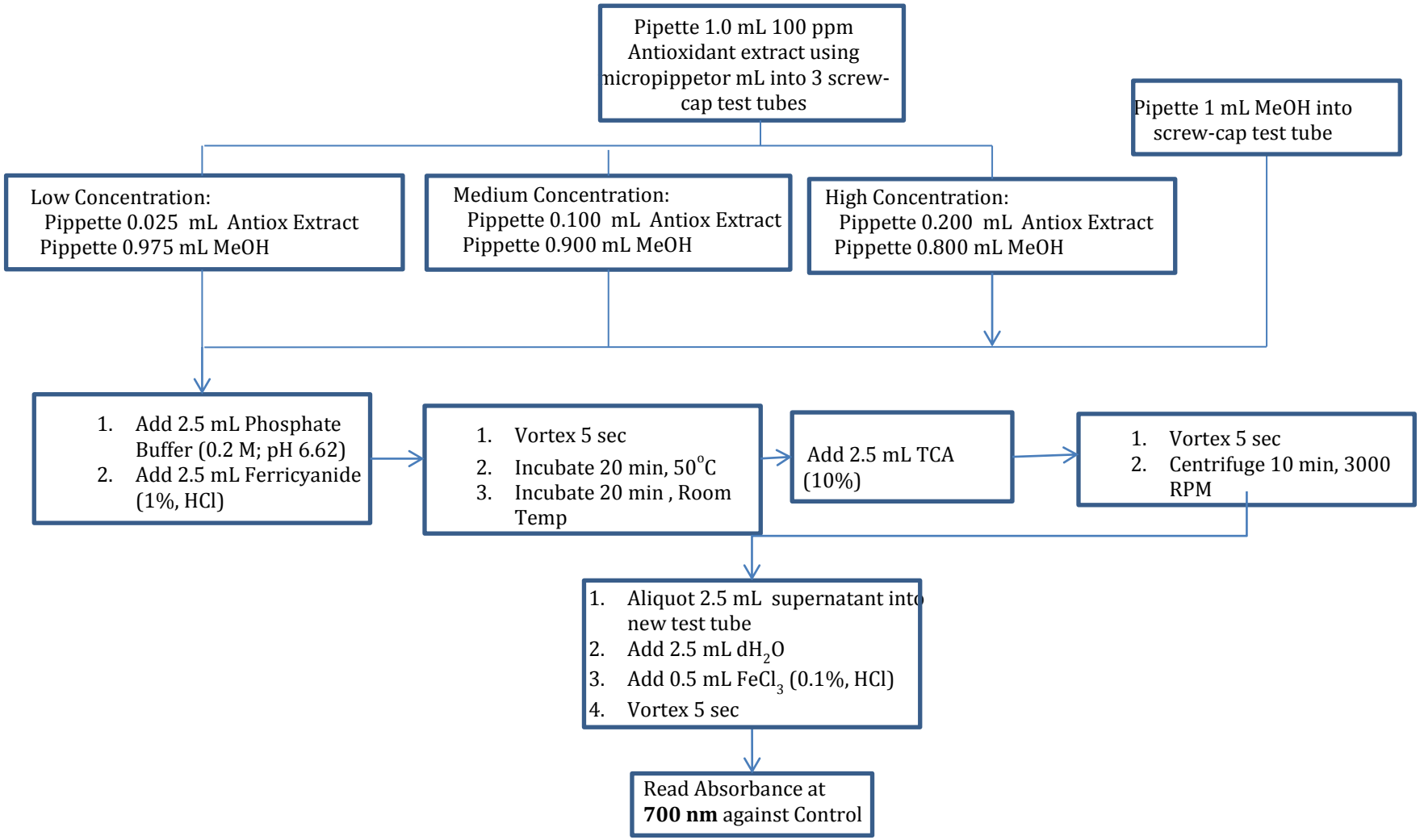


Figure A.2.5 Free Radical Scavenging by DPPH

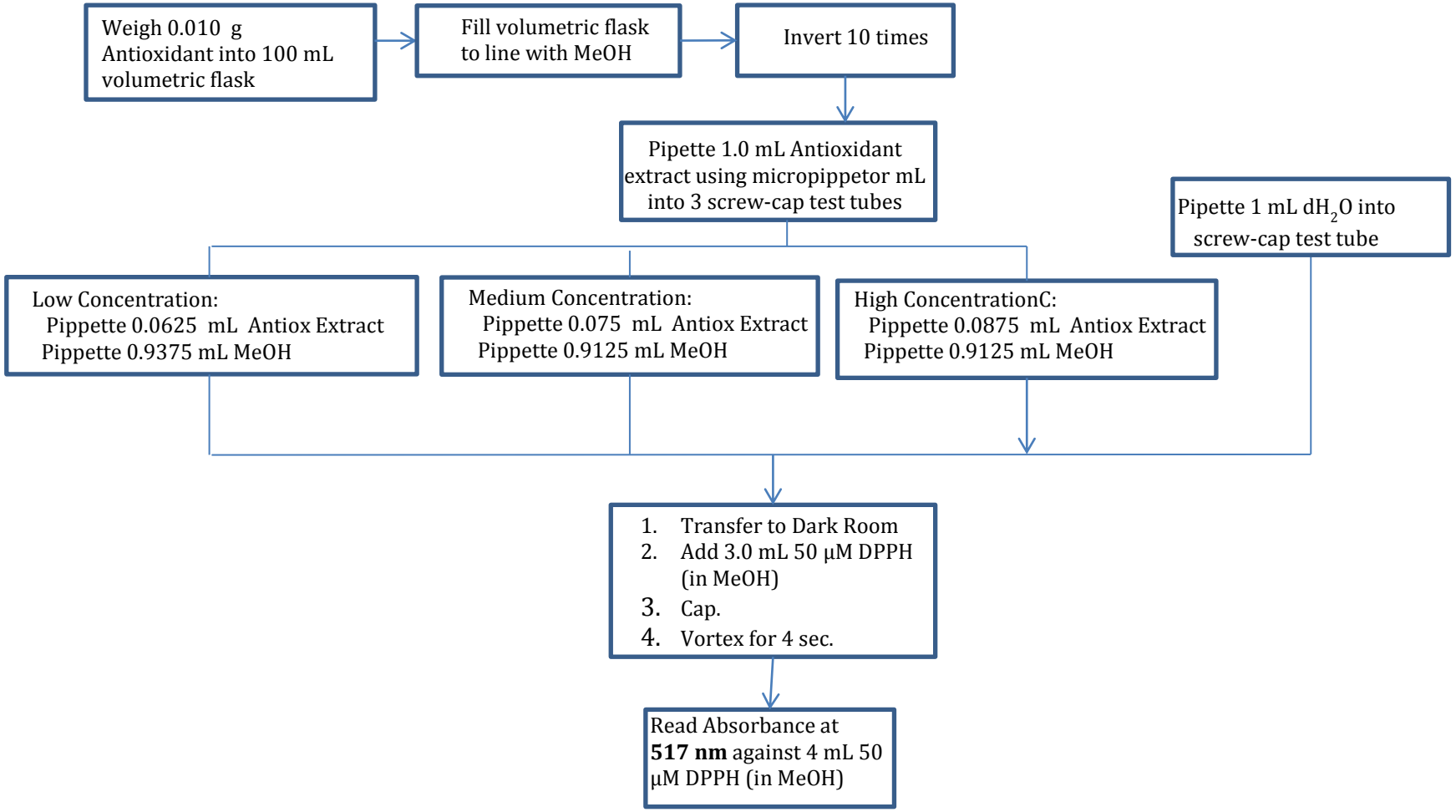


Figure A.2.6 DPPH Standard Curve of Ascorbic Acid

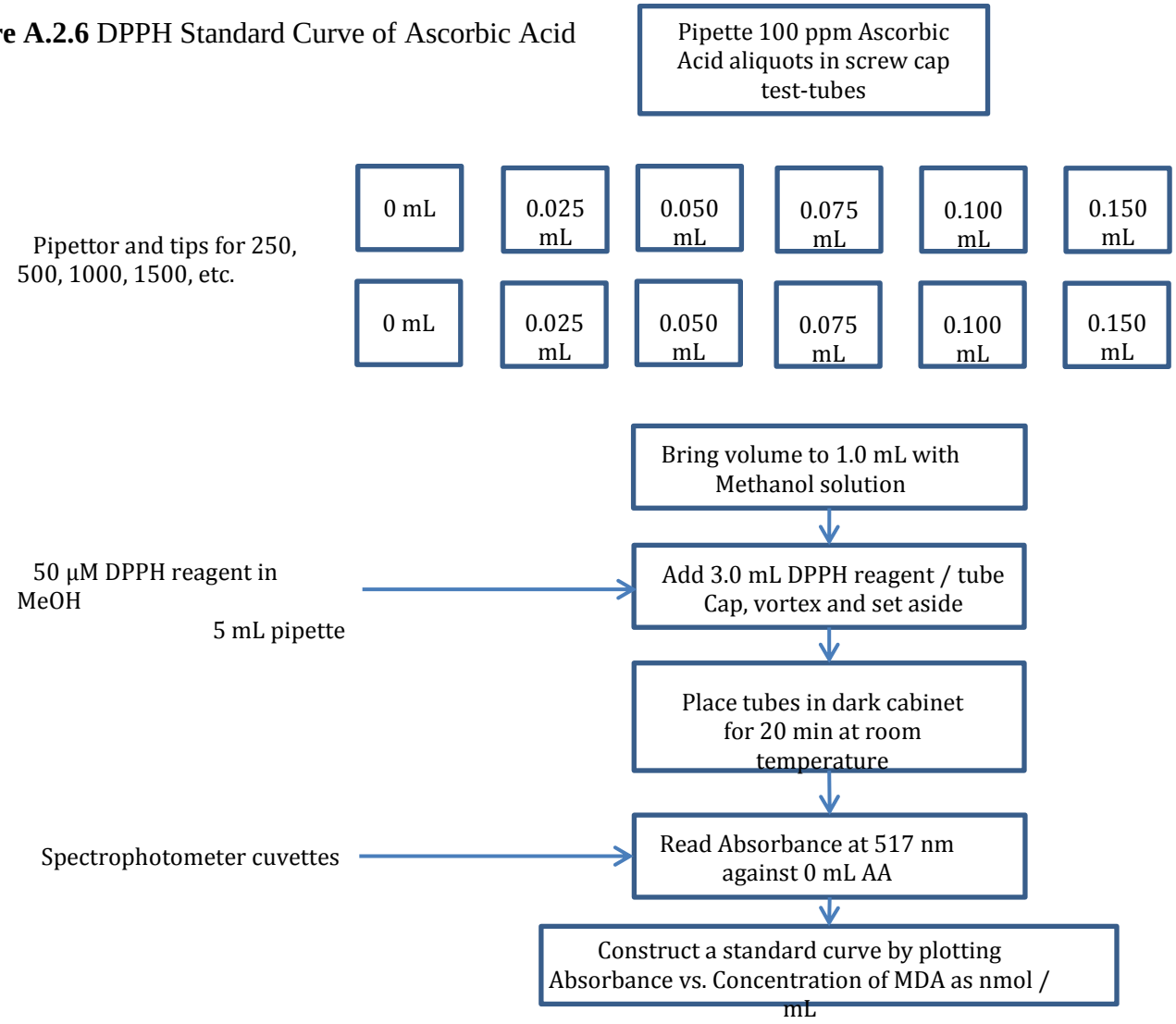


Table A.2.2 DPPH Standard Curve

Ascorbic Acid (mg / L MeOH)	Absorbance (A_{517})		
0	0.5387	0.5389	0.5388
2.5	0.4434	0.4522	0.4492
5	0.3616	0.3676	0.359
7.5	0.2637	0.2508	
10	0.1551	0.1478	0.1485
15	0.0739	0.0697	0.0708
20	0.0687	0.0687	0.0661

Figure A.2.7 Ascorbic Acid Standard Curve for Free Radical Scavenging by DPPH

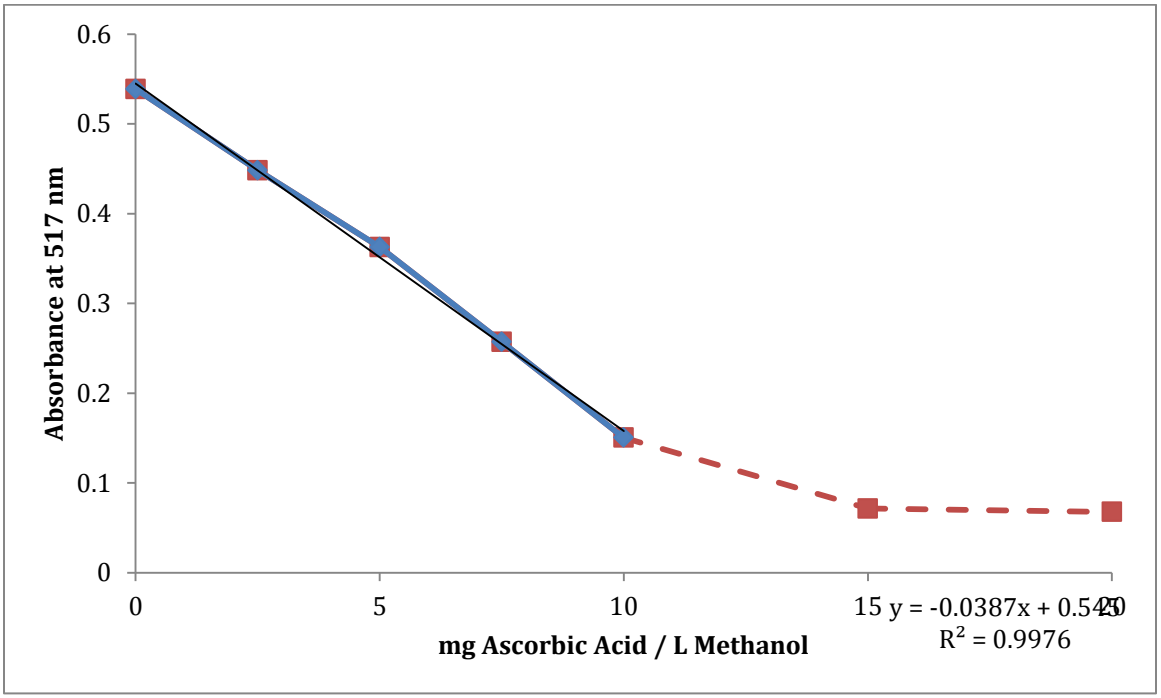


Figure A.2.8 Conjugated-Dienes Extraction Procedure

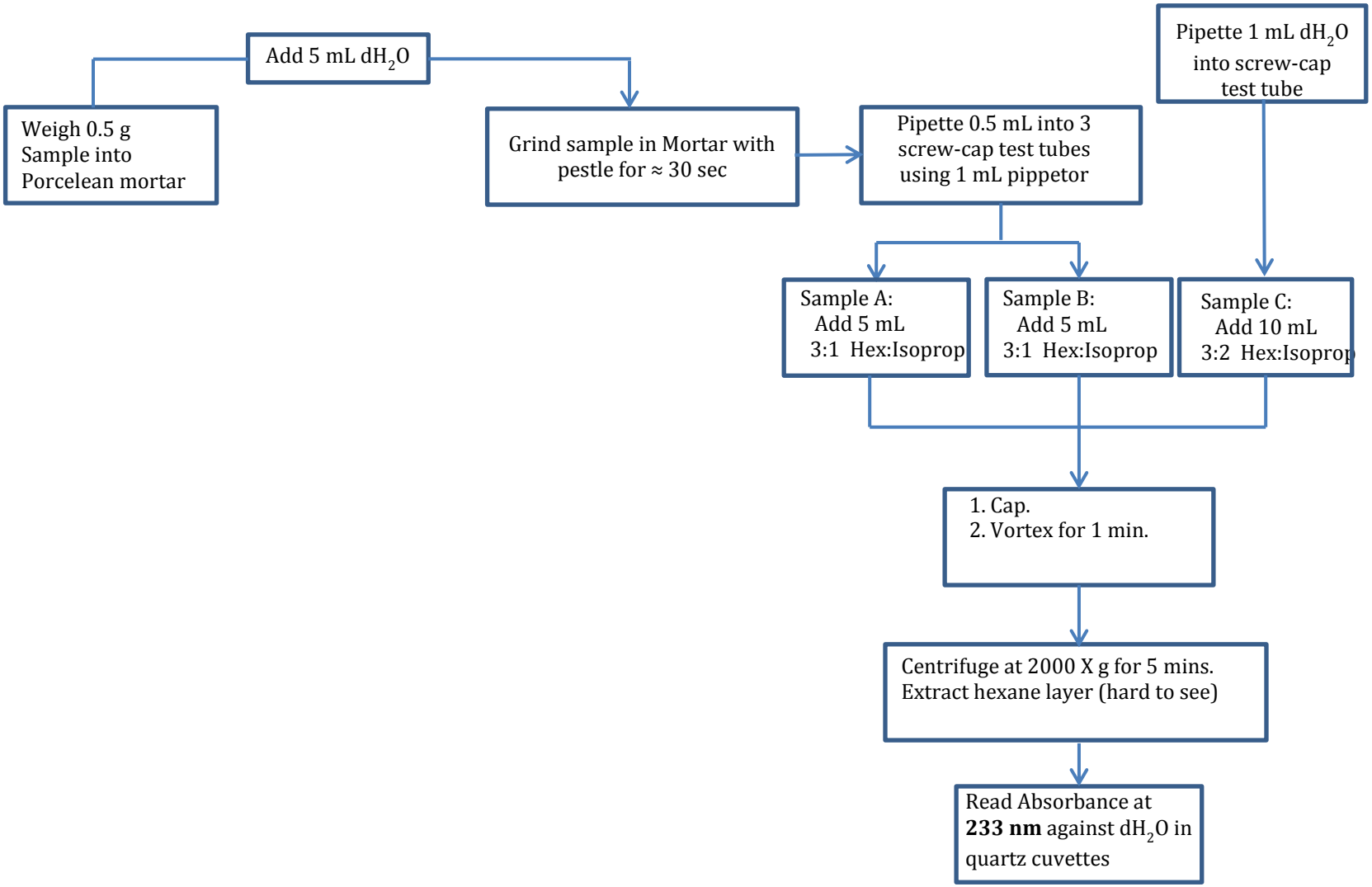


Figure A.2.9 TBARS Extraction Procedure

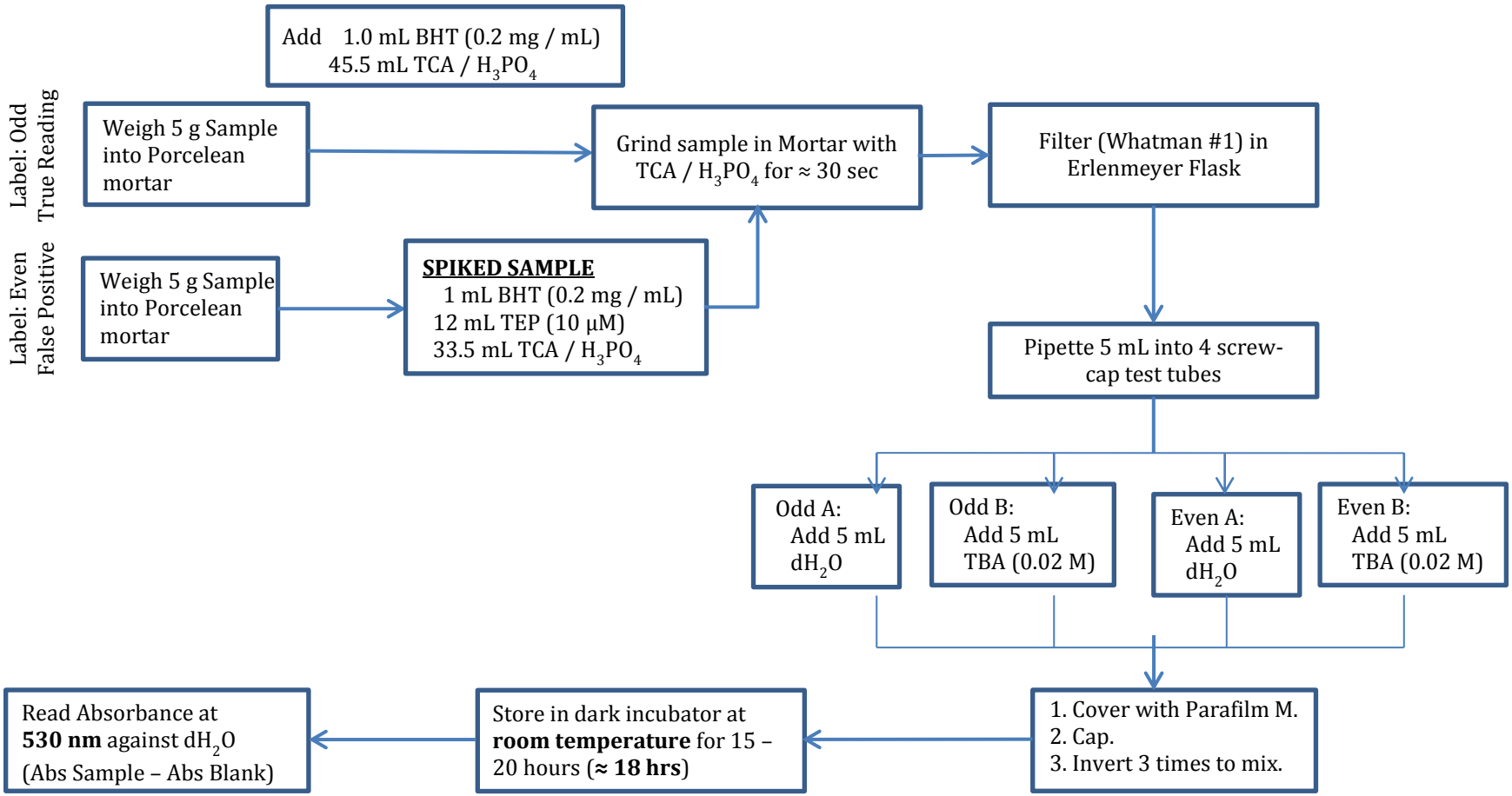


Figure A.2.10 TBARS Standard Curve

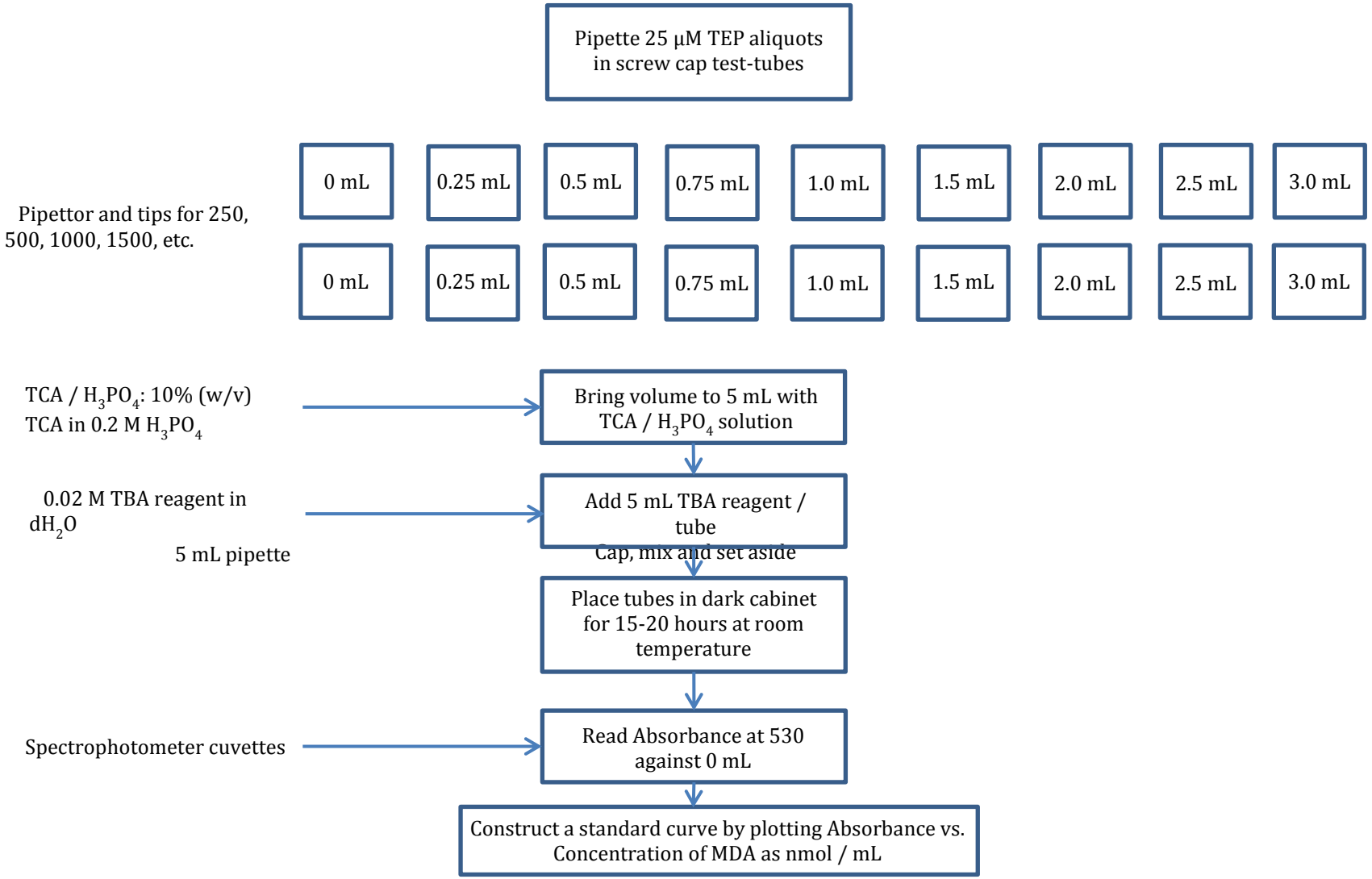
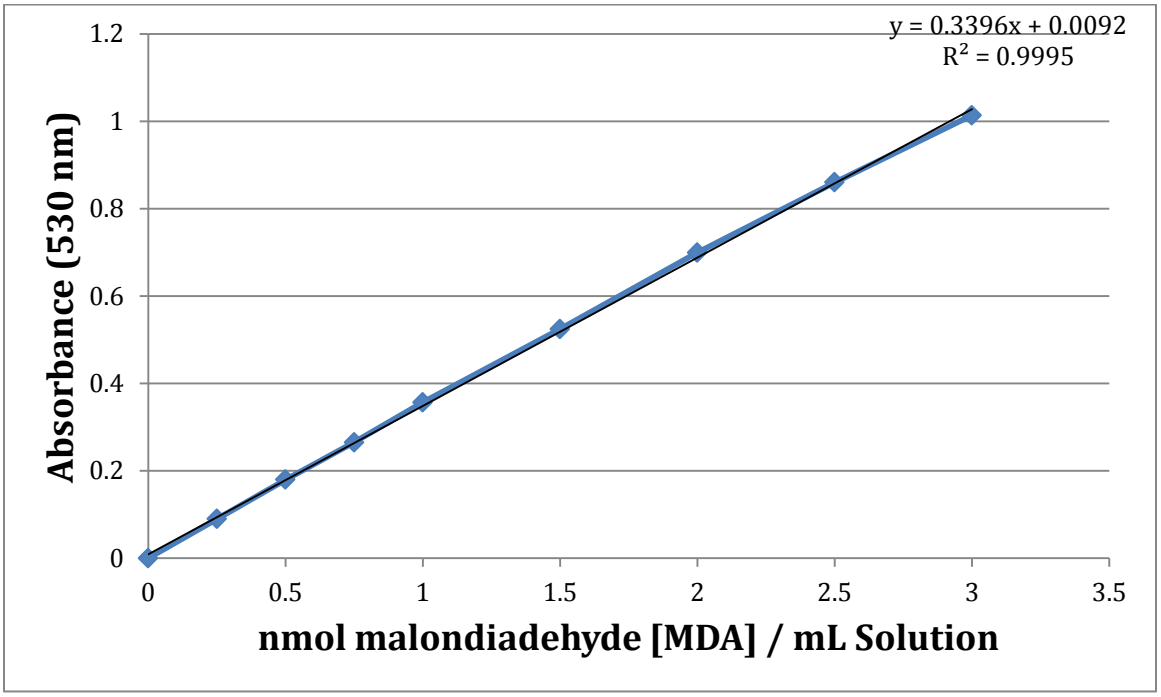


Table A.2.3 TBARs Standard Curve for Meat Patties

TEP (mL)	MDA nmol / tube	MDA nmol / mL	Absorbance (A_{530}) Adjusted	
0	0	0	0.0652	
0.25	6.25	1.25	0.1549	0.0897
0.5	12.5	2.5	0.2449	0.1797
0.75	18.75	3.75	0.3303	0.2651
1.0	25	5	0.4218	0.3566
1.5	37.5	7.5	0.5895	0.5243
2.0	50	10	0.7645	0.6993
2.5	62.5	15	0.9254	0.8602
3.0	75	20	1.0788	1.0135

Figure A.2.11 Malondialdehyde Standard Curve for TBARS

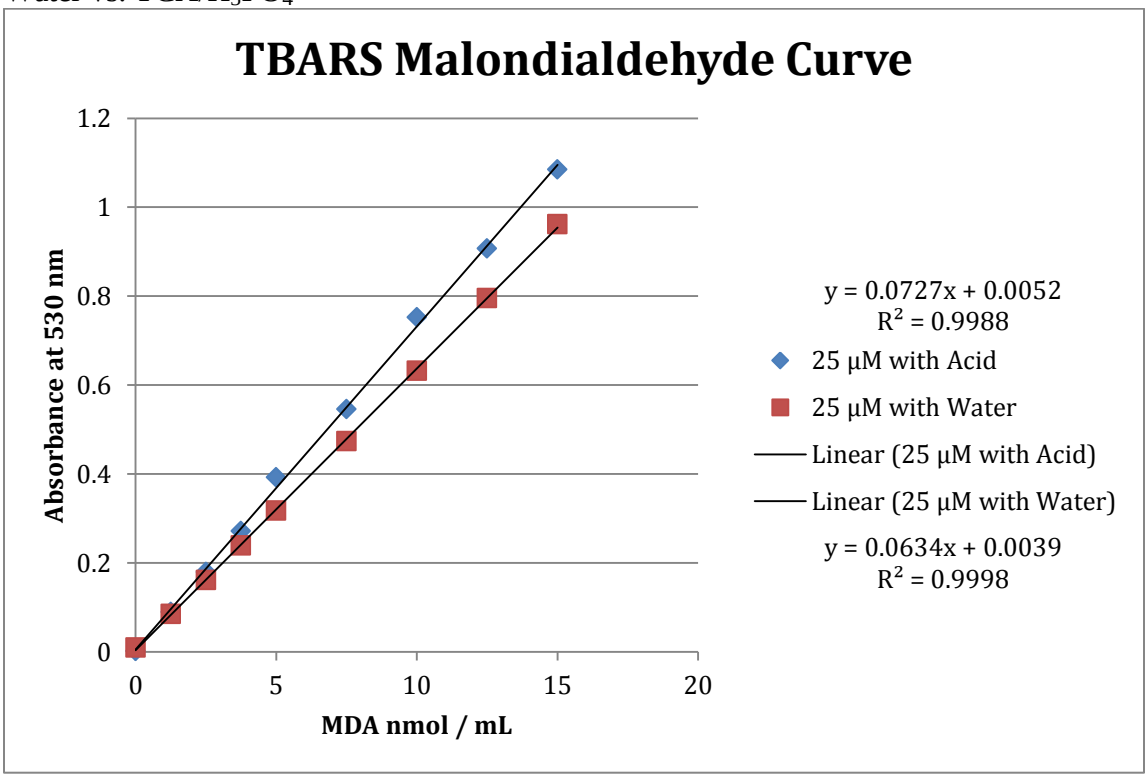


$$\frac{\mathbf{X}\ \text{nmol MDA}}{\text{mL}} \times \frac{50\ \text{mL}}{\text{tube}} \times \frac{1\ \mu\text{mol}}{1000\ \text{nmol}} \times \frac{72.061\ \mu\text{g MDA}}{\mu\text{mol MDA}} \times \frac{\text{tube}}{\mathbf{Y}\ \text{g sample}} = \boldsymbol{Z}\ \frac{\mu g\ MDA}{g\ Sample}\ or\ \boldsymbol{Z}\ \frac{mg\ MDA}{kg\ Sample}$$

Table A.2.4 TBARs Standard Curve for Meat Patties in Water vs. TCA/H_3PO_4

TBARS Standard Curve			
TEP (mL)	**MDA nmol / mL**	**Absorbance (530 nm)**	
		Acid	**Water**
0.00	0	0.0022	0.0102
0.25	1.25	0.0894	0.0853
0.50	2.5	0.1796	0.1616
0.75	3.75	0.2721	0.2395
1.00	5	0.393	0.3179
1.50	7.5	0.5457	0.4738
2.00	10	0.7528	0.6323
2.50	12.5	0.9069	0.7959
3.00	15	1.0852	0.962

Figure A.2.12 Solvatochromic shift for TBARs Standard Curve for Meat Patties using Water vs. TCA/H_3PO_4



A.3.1 Solutions for TBARS Test

Table A.3.1 TBARs Solutions to be prepared

#	Solution	ppm	Frequency	Storage Conditions
1	0.02 M TEP, mL	500	Weekly	Overwrapped in Aluminum foil at 4°C
2	25 μM TEP, mL	100	For Curve	
3	10 μM TEP, mL	100	Daily	at 4°C
4	0.02 M TBA, mL	250	Daily	at 4°C
5	BHT 0.2 mg/mL, mL	100	Weekly	Overwrapped in Aluminum foil at 4°C
6	TCA / H_3PO_4, mL	2 ☒ 1000	Weekly	at 4°C

1. 0.02 M TEP – Stock solution Procedure

Weigh **2.27 g** (in electronic balance) in a 100 mL beaker
Dissolve with deionized water (dH_2O)
Pour the solution into a 500 mL glass volumetric flask
Bring the volume up to solution using dH_2O

Table A.3.2 Preparation of 500 mL of 0.02 M TEP

Calculations to estimate the amount of TEP		
TEP to be used :	Malondialdehyde bis(diethyl acetate) 97%	
Molecular weight:	220.31	
g in 0.02moles:	220.31 × 0.02 =	4.41
Correction for 97%:	4.41 ÷ 0.97 =	4.54
g per 1000 mL:		4.54
g per 500 mL:	4.52 ÷ 2.00 =	2.27

2. 25 μM TEP Procedure

Measure 125 μL with a micropipetter in a 100 mL glass volumetric flask
Dissolve with d H_2O
Bring volume up to solution using dH_2O

Table A.3.3 Preparation of 100 mL of 25 μM TEP

Calculations to estimate the amount of TEP	
TEP to be used :	Malondialdehyde bis(diethyl acetate) 97%
Molecular weight:	220.31
Stock solution	0.02 M TEP
V × 20×10^3 μM:	25 μM × 100 mL
V, mL:	(25 × 100) ÷ (20×10^3)
V, mL:	0.125

3. 10 μM TEP Procedure

Measure 50 μL with a micropipetter in a 100 mL glass volumetric flask
Dissolve with d H_2O
Bring volume up to solution using dH_2O

Table A.3.4 Preparation of 100 mL of 10 μM TEP

Calculations to estimate the amount of TEP	
TEP to be used :	Malondialdehyde bis(diethyl acetate) 97%
Molecular weight:	220.31
Stock solution	0.02 M TEP
V × 20×10^3 μM:	10 μM × 100 mL
V, mL:	(10 × 100) ÷ (20×10^3)
V, mL:	0.05

4. 0.02 M TBA (Thiobarbituric Acid) – Procedure

Weigh 0.735 g (in electronic balance) in a 100 mL beaker
Dissolve with deionized water (dH_2O)
Pour the solution into a 250 mL glass volumetric flask
Bring the volume up to solution using dH_2O

Table A.3.5 Preparation of 250 mL of 0.02 M TBA

Calculations to estimate the amount of TBA		
TBA to be used :	Thiobarbituric Acid, minimum 98%	
Molecular weight:	144.1	
g in 0.02moles:	144.1 × 0.02 =	2.88
Correction for 98%:	2.88 ÷ 0.98 =	2.94
g per 250 mL:	2.94 ÷ 4.00 =	0.735

5. BHT 0.2 mg / mL Procedure

Weigh 20 mg (0.02 g in electronic balance) in a 100 mL beaker
Dissolve with deionized water (dH_2O)
Pour the solution into a 100 mL glass volumetric flask
Bring the volume up to solution using dH_2O

Table A.3.6 Preparation of 100 mL of 0.2 mg/mL BHT

Calculations to estimate the amount of BHT		
Concentration Required:	0.2 mg per mL	
Mg required per 100 mL:	100 × 0.2	20

6. TCA / H_3PO_4: 10% (w/v) TCA in 0.2 M H_3PO_4 Procedure

The order of the procedure is very important for safety reasons

Pour 700 mL dH_2O into a 1000 mL glass beaker
Weigh 23.06 g of o-Phosphoric Acid (85%) in a 100 mL beaker
Place the two beakers into the hood
Pour the Phosphoric acid into the dH_2O. WORK INSIDE THE HOOD.
Once the acid is dissolved, pour the acid solution into a 1000 mL glass volumetric flask
Bring up to volume using dH_2O
Weigh 1000 g of Trichloroacetic acid (TCA) in a 500 mL glass beaker
Place the 500 mL beaker with the TCA into the hood
Pour approximately 500 mL of the 0.2 M H_3PO_4into the 1000 mL glass beaker
Place part of the 100 g of TCA into the glass beaker with 0.2 M H_3PO_4
Mix until the TCA begins to dissolve
Continue until all the TCA has been dissolved
Pour the TCA / H_3PO_4 mixture into a 1000 mL glass volumetric flask
Bring the solution to volume using dH_2O

Table A.3.7 Preparation of 1 L of 0.2 M TCA / H_3PO_4

Calculations to estimate the amount of Phosphoric acid		
$H_3PO_4$to be used :	o-Phosphoric Acid, 85%	
Molecular weight:	98	
g in 0.02moles:	98.00 × 0.02 =	19.6
Correction for 85%:	19.6 ÷ 0.85 =	23.06
g per 1000 mL:	32.1 ÷ 1.00 =	23.06

Amount of Fe in Myoglobin

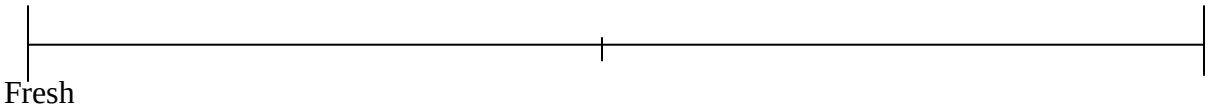
$$\frac{1\ mg\ Fe}{25\ g\ ground\ beef} \times \frac{1\ g}{1000\ mg} \times \frac{1\ mol\ Fe}{55.845\ g\ Fe} \times \frac{6.022 \times 10^{23}\ atoms\ Fe}{1\ mol\ Fe} \times \frac{1\ molecule\ Hbg}{4\ atoms\ Fe} \times \frac{1\ mol\ Hgb}{6.022e23\ molecules\ Hgb} \times \frac{65706\ g\ Hgb}{1\ mol\ Hgb} = 0.29414 \text{ g / 25 g sample}$$

Appendix B: Sensory Testing
Figure B.1.1 Canola Oil-Model System Scorecard

Odor Evaluation of Oil Patties

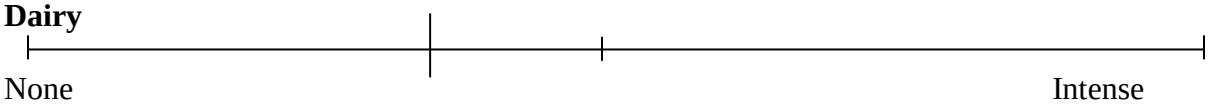
Sample # _____ **Panelist (Initials) _____**

Oxidized



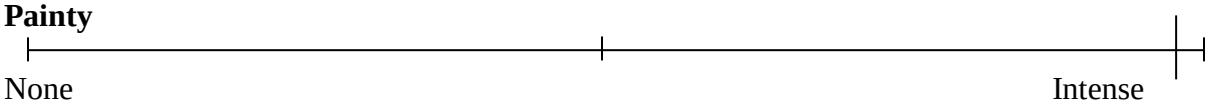
Fresh
Oxidized

Dairy



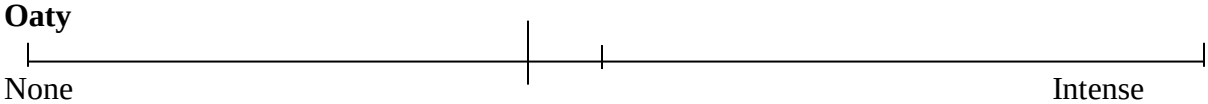
None Intense

Painty



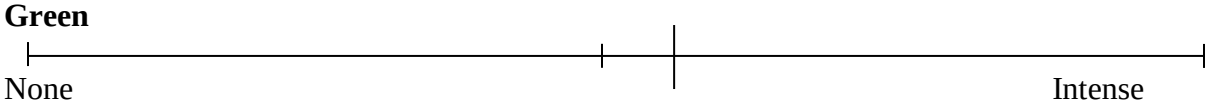
None Intense

Oaty



None Intense

Green



None Intense

Table B.1.1 Canola Oil-Model System Terms and Definitions

Attribute	Reference & Definition	Location on Scale
Fresh	1.0 g Fresh Canola Oil in a 2 oz. cup with lid *The odor associated with 8 month old Canola Oil, stored in the absence of light at ambient temperature.*	0.0 cm
Oxidized	0.5 g Oxidized Canola Oil *The odor associated with 8 month old Canola Oil, stored in the absence of light at ambient temperature.*	15.0 cm
Dairy	1 mL of a solution (1 mL of Crème Fraiche in 75 mL dH_2O) in a 2 oz. cup with lid *The odor associated with the dairy characteristics of a dilute solution of Crème Fraiche.*	5.2 cm
Painty	2 mg Linseed Oil covered by 5.0 mL dH_2O in a 2 oz. cup with lid. *The odor associated with a dilute solution of paint.*	14.6 cm
Oaty	0.05 g Quaker™ oatmeal + 5.0 mL boiling dH2O, immediately capped. *The odor associated with freshly prepared oatmeal.*	6.7 cm
Green	2 ppm hexanal solution (0.1 mL of $Henxanal_{EtOH}$ / 100 mL dH_2O) in lidded 2 oz. cup *The odor associated with fresh cut grass that has been sun-dried.*	7.7 cm

Table B.2.2 Effects of Antioxidants on Odor Descriptors in Canola Oil-Gelled Model System

Variables		Day 0	Day 1	Day 2	Day 4	Day 7	Day 9	SEM
Oxidized	**Control**	3.4 ^{ghij}	3.9 ^{fg}	3.2 ^{hij}	4.6 ^{ef}	11.6 ^{a}	10.3 ^{b}	0.5625
	Pomegranate Extract	3.0 ^{hij}	3.1 ^{hij}	3.7 ^{fgh}	3.7 ^{fgh}	5.5 ^{d}	6.5 ^{c}	
	Grape Seed Extract	3.5 ^{ghij}	3.6 ^{ghi}	3.7 ^{fgh}	5.4 ^{d}	5.0 ^{de}	6.4 ^{c}	
	BHA	3.5 ^{ghij}	3.2 ^{hij}	3.7 ^{fgh}	5.4 ^{d}	4.2 ^{f}	5.2 ^{d}	
Dairy	**Control**	3.1 ^{h}	3.2 ^{h}	2.6 ^{ij}	3.0 ^{h}	9.8 ^{a}	6.1 ^{b}	0.3381
	Pomegranate Extract	2.6 ^{ij}	3.1 ^{h}	3.0 ^{h}	2.7 ^{h}	4.3 ^{de}	5.6 ^{c}	
	Grape Seed Extract	2.6 ^{ij}	2.3 ^{j}	2.5 ^{i}	3.0 ^{h}	4.0 ^{ef}	6.2 ^{b}	
	BHA	3.0 ^{h}	2.6 ^{ij}	3.1 ^{h}	3.7 ^{fg}	3.8 ^{fg}	4.4 ^{d}	
Painty	**Control**	4.1 ^{d}	2.9 ^{fgh}	2.8 ^{gh}	2.9 ^{fg}	9.5 ^{a}	8.0 ^{b}	0.4530
	Pomegranate Extract	3.1 ^{fgh}	2.6 ^{hi}	3.1 ^{fgh}	2.1 ^{i}	4.0 ^{d}	5.5 ^{c}	
	Grape Seed Extract	3.9 ^{e}	2.3 ^{i}	3.0 ^{fgh}	2.9 ^{fg}	2.6 ^{hi}	5.1 ^{c}	
	BHA	3.4 ^{ef}	2.1 ^{i}	3.1 ^{fgh}	3.2 ^{fg}	3.0 ^{fgh}	5.6 ^{c}	
Oaty	**Control**	2.3 ^{nop}	3.0 ^{ijkl}	3.3 ^{hijk}	3.6 ^{fgh}	9.7 ^{a}	7.2 ^{b}	0.4709
	Pomegranate Extract	2.0 ^{p}	2.8 ^{jklmn}	3.4 ^{ghi}	2.6 ^{lmno}	5.5 ^{d}	6.3 ^{c}	
	Grape Seed Extract	2.4 ^{mnop}	2.8 ^{jklmn}	3.9 ^{fg}	3.8 ^{fgh}	4.0 ^{f}	6.1 ^{c}	
	BHA	2.9 ^{jklmn}	2.8 ^{jklmn}	3.8 ^{fgh}	3.7 ^{fgh}	4.9 ^{e}	6.0 ^{c}	
Green	**Control**	2.4 ^{fg}	2.3 ^{gh}	2.7 ^{ef}	2.1 ^{ghi}	8.5 ^{a}	6.7 ^{b}	0.3094
	Pomegranate Extract	1.9 ^{ij}	2.7 ^{ef}	2.2 ^{ghi}	1.7 ^{j}	4.1 ^{d}	5.2 ^{c}	
	Grape Seed Extract	2.1 ^{ghi}	2.0 ^{hij}	2.9 ^{e}	2.0 ^{hij}	2.7 ^{ef}	5.3 ^{c}	
	BHA	2.4 ^{fg}	2.2 ^{ghi}	2.3 ^{gh}	2.2 ^{ghi}	2.4 ^{fg}	5.2 ^{c}	

Figure B.2.1 0.5%-Salt *Raw* Beef Patty Scorecard

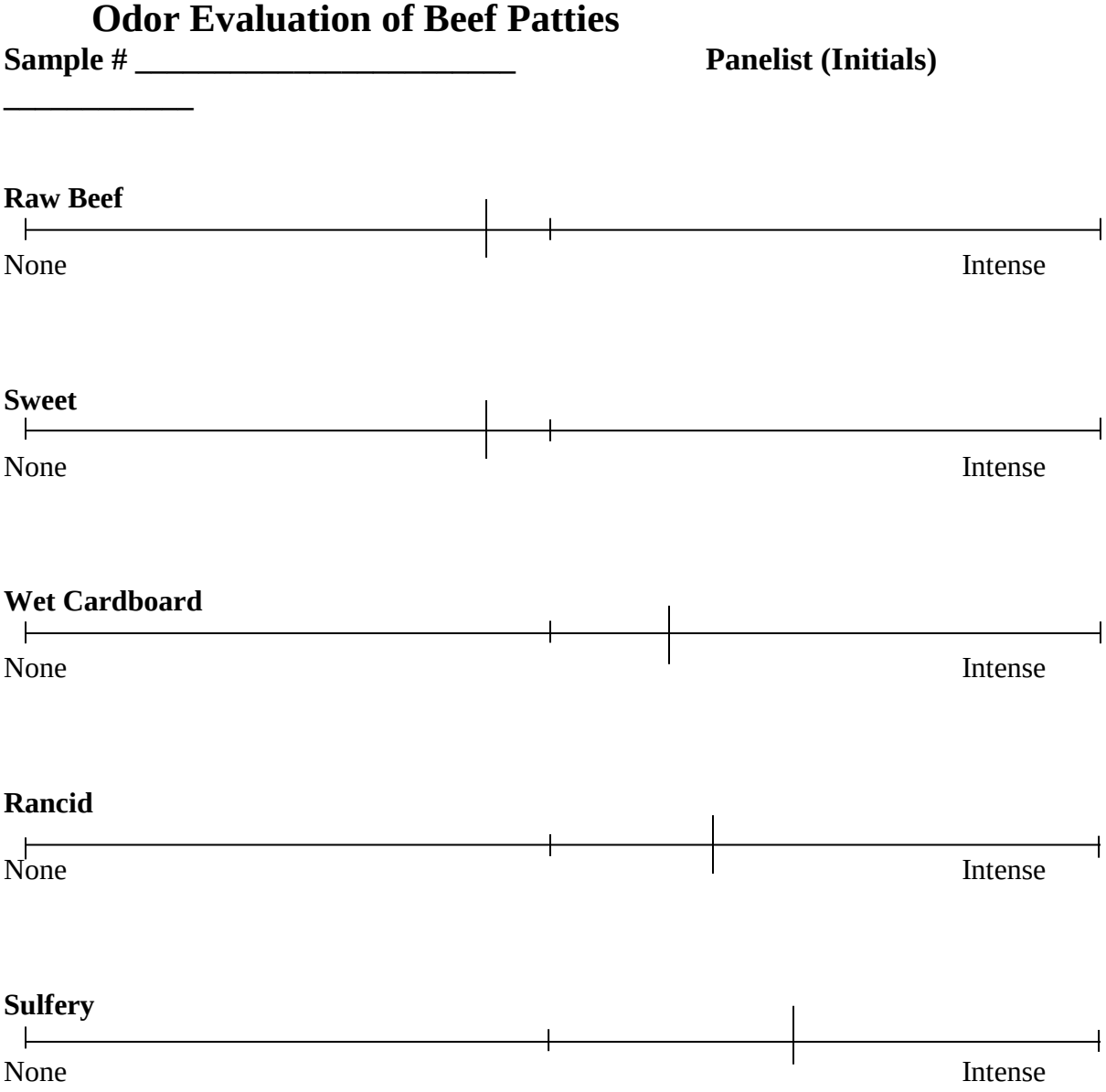


Figure B.2.2 0.5%-Salt *Cooked* Beef Patty Scorecard

Odor Evaluation of Beef Patties

Sample # _____ **Panelist (Initials) _____**

Cooked Beef

None Intense

Sweet

None Intense

Wet Cardboard

None Intense

Rancid

None Intense

Sulfery

None Intense

Table B.2.1 0.5%-salt Beef Patty Model System Terms and Definitions

Attribute	Reference & Definition	Location on Scale
Raw Beef	0.5 g Frozen, raw beef warmed to 40^{o}C *The odor associated with fresh ground beef.*	6.4 cm
Cooked Beef	0.5g of fresh beef cooked for 4 min 30 sec, placed in a 2 oz. cup with lid *The odor associated with freshly cooked beef patties.*	6.6 cm
Sweet	1 mL of a solution (1.0 g of Hershey's ™ Carmel in 10 mL dH_2O) in a 2 oz. cup with lid *The odor associated with caramelized sugar.*	6.6 cm
Wet Cardboard	0.5 cm^2 piece of cardboard wetted with 1 drop of dH_2O in a Nalgene squeeze bottle. *The odor associated with wet cardboard.*	9.2 cm
Rancid	0.5 g of lard heated to 80^{o}C in a 2 oz. cup with lid *The odor associated with the lard.*	7.5 cm
Sulfury	0.25 g of the yolk from a hard-boiled egg. *The odor associated with the cooked yolk of an egg.*	11.0 cm

Table B.2.2 Effects of Antioxidants on Odor Descriptors over Time in 0.5%-Salt, Non-Irradiated Raw Beef Patties

Variables		Day 0	Day 2	Day 4	Day 7	Day 9	SEM
Raw Beef	**Control**	5.7 ^{a}	3.3 ^{b}	2.8 ^{b}	5.0 ^{a}	2.1 ^{c}	0.3975
	Pomegranate Extract	5.1 ^{a}	4.2 ^{b}	3.6 ^{c}	4.2 ^{b}	4.4 ^{b}	
	Grape Seed Extract	4.8 ^{a}	3.2 ^{c}	4.2 ^{ab}	4.1 $^{b\ c}$	3.1 ^{c}	
	BHA	4.3 ^{a}	3.8 ^{bc}	4.1 ^{ab}	3.3 ^{c}	3.5 ^{c}	
Sweet	**Control**	3.3 $^{a\ a}$	2.6 $^{b\ a}$	3.5 $^{a\ a}$	3.2 $^{a\ ab}$	3.2 $^{a\ ab}$	0.4521
	Pomegranate Extract	2.3 $^{b\ bc}$	2.6 $^{b\ a}$	3.5 $^{a\ a}$	3.8 $^{a\ a}$	1.4 $^{c\ c}$	
	Grape Seed Extract	2.5 $^{a\ b}$	2.7 $^{a\ a}$	3.1 $^{a\ a}$	2.5 $^{a\ b}$	2.5 $^{a\ b}$	
	BHA	1.6 $^{b\ c}$	2.8 $^{a\ a}$	2.9 $^{a\ a}$	3.2 $^{a\ ab}$	3.4 $^{a\ a}$	
Wet Cardboard	**Control**	1.7 $^{c\ a}$	3.3 $^{b\ a}$	2.8 $^{b\ ab}$	4.2 $^{a\ a}$	2.9 $^{b\ a}$	0.2684
	Pomegranate Extract	1.0 $^{c\ b}$	1.0 $^{c\ b}$	2.3 $^{b\ b}$	3.4 $^{a\ b}$	2.3 $^{b\ b}$	
	Grape Seed Extract	1.2 $^{c\ ab}$	3.0 $^{b\ b}$	2.8 $^{b\ ab}$	3.8 $^{a\ ab}$	3.3 $^{ab\ a}$	
	BHA	0.7 $^{c\ b}$	1.1 $^{c\ b}$	3.3 $^{b\ a}$	4.4 $^{a\ a}$	3.2 $^{b\ a}$	
Rancid	**Control**	2.1 ^{d}	3.4 ^{c}	3.7 ^{bc}	4.5 ^{a}	4.1 ^{ab}	0.4012
	Pomegranate Extract	1.3 ^{d}	2.7 ^{bc}	2.2 ^{c}	4.4 ^{a}	2.9 ^{b}	
	Grape Seed Extract	1.5 ^{c}	2.1 ^{b}	3.3 $^{a.}$	3.3 ^{a}	3.4 ^{a}	
	BHA	0.9 ^{c}	2.2 ^{b}	3.8 ^{a}	4.0 ^{a}	4.1 ^{a}	
Sulfury	**Control**	1.1	2.2	1.8	2.1	2.2	0.3726
	Pomegranate Extract	1.6	2.3	1.8	2.1	2.2	
	Grape Seed Extract	1.6	1.6	1.7	1.8	1.8	
	BHA	1.5	1.5	2.1	1.9	2.0	

Table B.2.3 Effects of Antioxidants on Odor Descriptors over Time in 0.5%-Salt, Irradiated Raw Beef Patties

Variables		Day 0	Day 2	Day 4	Day 7	Day 9	SEM
Raw Beef	Control	4.2 ^{a}	4.4 ^{a}	4.0 ^{a}	4.0 ^{a}	2.7 ^{b}	0.3975
	Pomegranate Extract	5.0 ^{a}	3.5 ^{c}	3.8 ^{bc}	4.4 ^{ab}	3.3 ^{c}	
	Grape Seed Extract	5.1 ^{a}	4.2 ^{b}	3.6 ^{bc}	4.4 ^{b}	3.5 ^{c}	
	BHA	4.7 ^{ab}	4.1 ^{b}	4.7 ^{ab}	4.8 ^{a}	2.9 ^{c}	
Sweet	Control	1.3 ^{b} **a**	2.2 ^{a} **a**	2.0 ^{a} **b**	2.2 ^{a} **b**	1.4 ^{b} **ab**	0.4521
	Pomegranate Extract	1.0 ^{c} **a**	2.1 ^{a} **ab**	1.7 ^{ab} **b**	2.0 ^{a} **b**	1.2 ^{bc} **b**	
	Grape Seed Extract	1.6 ^{b} **a**	1.4 ^{b} **b**	3.0 ^{a} **a**	3.2 ^{a} **a**	1.6 ^{b} **ab**	
	BHA	0.9 ^{c} **a**	1.9 ^{b} **ab**	1.7 ^{b} **b**	3.0 ^{a} **a**	2.0 ^{b} **a**	
Wet Cardboard	Control	3.1 ^{c} **a**	2.9 ^{c} **a**	4.6 ^{a} **a**	3.8 ^{b} **a**	3.7 ^{b} **ab**	0.2684
	Pomegranate Extract	2.8 ^{c} **a**	3.0 ^{c} **a**	3.7 ^{b} **b**	3.7 ^{b} **a**	4.4 ^{a} **a**	
	Grape Seed Extract	2.6 ^{b} **a**	3.0 ^{ab} **a**	3.2 ^{ab} **b**	2.9 ^{b} **b**	3.5 ^{a} **b**	
	BHA	2.9 ^{c} **a**	2.7 ^{c} **a**	3.5 ^{b} **b**	3.2 ^{bc} **ab**	4.1 ^{a} **ab**	
Rancid	Control	3.7 ^{a}	2.4 ^{b}	3.6 ^{a}	3.8 ^{a}	4.0 ^{a}	0.4012
	Pomegranate Extract	2.3 ^{c}	2.8 ^{c}	3.5 ^{b}	3.6 ^{b}	4.7 ^{a}	
	Grape Seed Extract	2.7 ^{c}	2.6 ^{c}	3.9 ^{b}	4.6 ^{a}	3.4 ^{b}	
	BHA	2.7 ^{c}	2.8 ^{c}	2.9 ^{bc}	3.3 ^{b}	4.3 ^{a}	
Sulfury	Control	2.6	1.1	1.7	1.3	1.4	0.3726
	Pomegranate Extract	1.4	1.8	1.5	1.8	0.8	
	Grape Seed Extract	2.1	1.0	2.3	2.2	1.8	
	BHA	1.6	1.5	1.8	1.7	1.9	

Table B.2.4 Effects of Antioxidants on Odor Descriptors over Time in 0.5%-Salt, Non-Irradiated Cooked Beef Patties

Variables		Day 0	Day 2	Day 4	Day 7	Day 9	SEM
Cooked Beef	**Control**	7.4	7.9	6.9	7.1	6.8	0.4108
	Pomegranate Extract	7.3	7.5	7.6	7.3	8.1	
	Grape Seed Extract	8.1	7.1	7.7	6.5	6.9	
	BHA	9.1	8.2	8.6	7.2	7.0	
Sweet	**Control**	1.5	1.6	1.3	1.3	1.4	0.1147
	Pomegranate Extract	1.0	1.3	1.5	1.0	1.5	
	Grape Seed Extract	1.6	1.3	0.7	1.7	1.0	
	BHA	1.2	0.8	0.8	1.5	1.1	
Wet Cardboard	**Control**	1.1 ^{b}	1.5 ^{b}	1.6 ^{b}	2.4 ^{a}	2.3 ^{a}	0.1988
	Pomegranate Extract	1.6 ^{c}	2.7 ^{a}	1.5 ^{c}	2.2 ^{b}	1.9 ^{bc}	
	Grape Seed Extract	1.7 ^{c}	1.2 ^{d}	2.2 ^{b}	2.9 ^{a}	1.8 ^{bc}	
	BHA	1.2 $^{d\ c}$	1.7 ^{b}	1.8 ^{b}	2.8 ^{a}	2.1 ^{b}	
Rancid	**Control**	2.3 ^{a}	1.5 ^{b}	1.8 ^{b}	2.7 ^{a}	2.4 ^{a}	0.3634
	Pomegranate Extract	1.7 ^{c}	2.4 ^{b}	1.7 ^{c}	3.0 ^{a}	2.7 ^{ab}	
	Grape Seed Extract	1.8 ^{b}	1.8 ^{b}	2.0 ^{b}	3.6 ^{a}	3.1 ^{a}	
	BHA	1.5 ^{c}	2.2 ^{ab}	1.8 ^{bc}	2.2 ^{ab}	2.5 ^{a}	
Sulfury	**Control**	2.8	3.0	6.6	2.8	1.9	0.7258
	Pomegranate Extract	2.5	3.6	2.3	3.4	2.8	
	Grape Seed Extract	2.9	2.4	2.9	3.8	3.2	
	BHA	3.3	3.4	3.5	3.2	2.4	

Table B.2.5 Effects of Antioxidants on Odor Descriptors over Time in 0.5%-Salt, Irradiated Cooked Beef Patties

Variables		Day 0	Day 2	Day 4	Day 7	Day 9	SEM
Cooked Beef	**Control**	7.5	8.1	8.5	7.1	7.1	0.4108
	Pomegranate Extract	8.2	7.0	8.9	7.4	8.1	
	Grape Seed Extract	8.1	7.8	7.4	7.9	8.5	
	BHA	8.8	7.1	8.1	7.8	7.1	
Sweet	**Control**	0.9	1.1	1.1	1.1	1.6	0.1147
	Pomegranate Extract	1.1	1.1	0.9	1.2	1.3	
	Grape Seed Extract	1.0	1.6	1.2	1.4	1.6	
	BHA	0.7	0.9	0.9	1.5	1.1	
Wet Cardboard	**Control**	1.7 ^{b}	1.8 ^{b}	2.4 ^{a}	2.5 ^{a}	2.5 ^{a}	0.1988
	Pomegranate Extract	1.3 ^{c}	1.3 ^{c}	1.8 ^{b}	3.0 ^{a}	3.1 ^{a}	
	Grape Seed Extract	1.0 ^{c}	2.6 ^{a}	1.7 ^{b}	2.8 ^{a}	1.5 ^{d}	
	BHA	1.4 ^{c}	2.1 ^{b}	2.5 ^{ab}	2.9 ^{a}	1.5 ^{c}	
Rancid	**Control**	2.0 ^{c}	2.0 ^{c}	2.5 ^{ab}	2.8 ^{a}	2.2 ^{bc}	0.3634
	Pomegranate Extract	1.8 ^{c}	2.6 ^{b}	1.9 ^{c}	3.2 ^{a}	2.7 ^{ab}	
	Grape Seed Extract	1.9 ^{c}	2.5 ^{b}	1.9 ^{c}	3.0 ^{a}	1.9 ^{c}	
	BHA	1.4 ^{c}	2.3 ^{ab}	2.2 ^{b}	2.7 ^{a}	2.1 ^{b}	
Sulfury	**Control**	3.5	2.5	2.7	2.5	2.4	0.7258
	Pomegranate Extract	2.7	2.0	2.5	2.8	3.0	
	Grape Seed Extract	3.7	2.9	2.2	2.5	3.0	
	BHA	3.5	2.8	2.3	2.8	2.2	

Appendix C: Spectrocolorimetric Data

Table C.1.1 Effects of Antioxidants Over Time on L*, a* and b* values for 0.5%-Salt, Non-Irradiated Beef Patties

Variables		Antioxidants				SEM
		Control	Pomegranate Extract	Grape Seed Extract	BHA	
L*	Day 0	47.1 $^{\Theta}$	47.1 $^{\Theta}$	44. 2 $^{\Omega}$	46.8 $^{\Theta}$	Antioxidant*Storage
	Day 2	49.5 $^{\Delta EZH}$	47.6 $^{H\Theta}$	48.2 $^{ZH\Theta}$	48.2 $^{H\Theta}$	5.4152
	Day 4	51.6 $^{\alpha\beta\Delta}$	46.7 $^{\Theta}$	50.4 $^{\beta\Delta EZ}$	53.0 $^{\alpha}$	
	Day 7	48.9 $^{EZH\Theta}$	47.8 $^{H\Theta}$	48.0 $^{H\Theta}$	50.7 $^{\beta\Delta E}$	
	Day 9	51.9 $^{\alpha\beta}$	52.6 $^{\alpha\beta}$	50.9 $^{\alpha\beta\Delta E}$	50.7 $^{\beta\Delta E}$	
a*	Day 0	11.2 ^{a} **a**	11.5 ^{a} **a**	8.8 ^{a} **b**	9.4 ^{a} **b**	Antioxidant Storage
	Day 2	8.7 ^{b} **a**	8.6 ^{b} **a**	7.1 ^{b} **b**	8.4 ^{b} **a**	0.5384 0.4268
	Day 4	6.2 ^{c} **b**	7.8 ^{c} **a**	7.2 ^{b} **a**	6.4 ^{c} **b**	
	Day 7	4.1 ^{d} **b**	5.5 ^{d} **a**	4.6 ^{c} **b**	4.6 ^{d} **b**	
	Day 9	4.3 ^{d} **b**	4.5 ^{e} **ab**	5.1 ^{c} **a**	5.1 ^{d} **a**	
b*	Day 0	12.0 $^{\alpha\beta}$	9.5 $^{\Theta I}$	7.9 $^{\Omega}$	8.6 $^{I\Omega}$	Antioxidant*Storage
	Day 2	10.6 ^{EZH}	11.2 $^{\alpha\beta\Delta Z}$	10.2 $^{ZH\Theta}$	11.3 $^{\alpha\beta\Delta E}$	1.2906
	Day 4	10.9 $^{\Delta EZ}$	9.6 $^{H\Theta I}$	11.0 $^{\beta\Delta EZ}$	11.0 $^{\beta\Delta EZ}$	
	Day 7	11.3 $^{\alpha\beta\Delta E}$	12.1 $^{\alpha}$	11.8 $^{\alpha\beta\Delta}$	12.2 $^{\alpha}$	
	Day 9	11.3 $^{\alpha\beta\Delta E}$	11.9 $^{\alpha\beta\Delta}$	11.0 $^{\beta\Delta E}$	11. 5 $^{\alpha\beta\Delta E}$	

Table C.1.2 Effects of Antioxidants Over Time on Hue Angle and Chroma for 0.5%-Salt, Non-Irradiated Beef Patties

Variables		Antioxidants				SEM	
		Control	Pomegranate Extract	Grape Seed Extract	BHA		
Hue Angle	Day 0	33.5 ^{e} **b**	35.3 ^{e} **a**	33.7 ^{e} **b**	34.7 ^{d} **ab**	Antioxidant	Storage
	Day 2	47.3 ^{d} **a**	40.5 ^{d} **b**	46.5 ^{d} **a**	39.0 ^{c} **c**	1.2412	0.9813
	Day 4	66.9 ^{c} **a**	54.6 ^{c} **c**	60.0 ^{c} **b**	67.5 ^{b} **a**		
	Day 7	69.7 ^{b} **a**	68.3 ^{b} **b**	68.5 ^{b} **ab**	69.4 ^{a} **ab**		
	Day 9	70.3 ^{a} **ab**	71.7 ^{a} **a**	69.7 ^{a} **b**	69.5 ^{a} **b**		
Chroma	Day 0	16.5 ^{a} **a**	15.0 ^{a} **b**	11.9 ^{d} **d**	12.7 ^{b} **c**	Antioxidant	Storage
	Day 2	13.8 ^{b} **a**	14.5 ^{a} **a**	12.6 ^{bc} **b**	14.4 ^{a} **a**	0.4528	0.3589
	Day 4	12.7 ^{c} **b**	12.5 ^{c} **b**	13.2 ^{a} **a**	13.0 ^{b} **ab**		
	Day 7	12.0 ^{d} **b**	13.3 ^{b} **a**	12.7 ^{ab} **ab**	13.1 ^{b} **a**		
	Day 9	12.1 ^{d} **a**	12.7 ^{bc} **a**	12.1 ^{cd} **a**	12.6 ^{b} **a**		

Table C.1.3 Effects and Interactions of Antioxidants on Storage Color Attributes of 0.5%-Salt, Non-Irradiated Beef Patties

Variable		p-value
L*	Antioxidant	<0.0001
	Storage	0.0042
	Antioxidant * Storage	0.0106
a*	Antioxidant	0.9605
	Storage	< 0.0001
	Antioxidant * Storage	0.2732
b*	Antioxidant	0.0664
	Storage	< 0.0001
	Antioxidant * Storage	0.0348
Hue Angle	Antioxidant	0.4363
	Storage	< 0.0001
	Antioxidant * Storage	0.1595
Chroma	Antioxidant	0.9037
	Storage	< 0.0001
	Antioxidant * Storage	0.3360

Table C.2.1 Effects of Antioxidants Over Time on L*, a* and b* values for 0.5%-Salt, Irradiated Beef Patties

Variables		Antioxidants				SEM
		Control	Pomegranate Extract	Grape Seed Extract	BHA	
L*	Day 0	47.1 ^{d} **a**	47.1 ^{cd} **a**	44.2 ^{d} **b**	46.8 ^{d} **a**	Antioxidant*Storage
	Day 2	49.5 ^{b} **a**	47.6 ^{bc} **b**	48.2 ^{c} **b**	48.2 ^{c} **b**	6.1790
	Day 4	51.6 ^{a} **b**	46.7 ^{d} **d**	50.4 ^{a} **c**	53.0 ^{a} **a**	
	Day 7	48.9 ^{c} **b**	47.8 ^{b} **c**	48.9 ^{b} **b**	50.7 ^{b} **a**	
	Day 9	51.9 ^{a} **b**	52.6 ^{a} **a**	50.9 ^{a} **c**	50.7 ^{b} **c**	
a*	Day 0	11.2 ^{a} **a**	11.5 ^{a} **a**	8.8 ^{a} **b**	9.4 ^{a} **b**	Antioxidant Storage
	Day 2	8.7 ^{b} **a**	8.6 ^{b} **a**	7.1 ^{b} **b**	8.4 ^{b} **a**	0.5384 0.4256
	Day 4	6.2 ^{c} **b**	7.8 ^{c} **a**	7.1 ^{b} **a**	6.4 ^{c} **b**	
	Day 7	4.1 ^{d} **b**	5.5 ^{d} **a**	4.6 ^{c} **b**	4.6 ^{e} **b**	
	Day 9	4.3 ^{d} **b**	4.5 ^{e} **b**	5.0 ^{c} **a**	5.1 ^{d} **a**	
b*	Day 0	12.0 ^{a} **a**	9.5 ^{c} **b**	7.9 ^{d} **d**	8.6 ^{d} **c**	Antioxidant*Storage
	Day 2	10.6 ^{c} **b**	11.2 ^{b} **a**	10.2 ^{c} **b**	11.3 ^{bc} **a**	0.7674
	Day 4	10.9 ^{bc} **a**	9.6 ^{c} **b**	11.0 ^{b} **a**	11.0 ^{c} **a**	
	Day 7	11.3 ^{b} **b**	12.1 ^{a} **a**	11.8 ^{a} **a**	12.2 ^{a} **a**	
	Day 9	11.3 ^{b} **bc**	11.9 ^{a} **a**	11.0 ^{b} **c**	11.5 ^{b} **ab**	

Table C.2.2 Effects of Antioxidants Over Time on Hue Angle and Chroma for 0.5%-Salt, Irradiated Beef Patties

Variables		Antioxidants				SEM	
		Control	Pomegranate Extract	Grape Seed Extract	BHA		
Hue Angle	Day 0	43.8 ^{e} **a**	37.0 ^{e} **d**	39.7 ^{d} **c**	40.3 ^{e} **b**	Antioxidant	Storage
	Day 2	47.7 ^{d} **c**	50.3 ^{d} **b**	53.3 ^{c} **a**	50.7 ^{d} **b**	1.2412	0.9813
	Day 4	56.9 ^{c} **a**	48.6 ^{c} **c**	53.3 ^{c} **b**	56.0 ^{c} **a**		
	Day 7	65.7 a a	61.3 ^{b} **c**	64.3 ^{a} **b**	64.9 ^{a} **ab**		
	Day 9	64.5 ^{b} **a**	64.6 ^{a} **a**	61.2 ^{b} **b**	61.8 ^{b} **b**		
Chroma	Day 0	16.48 ^{a} **a**	14.97 ^{a} **b**	11.86 ^{c} **d**	12.87 ^{b} **c**	Antioxidant*Storage	
	Day 2	13.76 ^{b} **b**	14.52 ^{a} **a**	12.6 ^{bc} **c**	14.35 ^{a} **ab**	1.4959	
	Day 4	12.74 ^{c} **ab**	12.53 ^{c} **b**	13.23 ^{a} **a**	12.96 ^{b} **ab**		
	Day 7	12.01 ^{d} **b**	13.32 ^{b} **a**	12.69 ^{b} **a**	13.06 ^{b} **a**		
	Day 9	12.07 ^{d} **b**	12.71 ^{c} **a**	12.14 ^{c} **ab**	12.58 ^{b} **ab**		

Table C.2.3 Affects and Interactions of Antioxidants on Storage Color Attributes of 0.5%-Salt, Irradiated Beef Patties

Variable		p-value
L*	Antioxidant	0.0272
	Storage	<0.0001
	Antioxidant * Storage	0.0230
a*	Antioxidant	0.1758
	Storage	< 0.0001
	Antioxidant * Storage	0.4787
b*	Antioxidant	0.0048
	Storage	< 0.0001
	Antioxidant * Storage	<0.0001
Hue Angle	Antioxidant	0.2909
	Storage	< 0.0001
	Antioxidant * Storage	0.6717
Chroma	Antioxidant	0.0044
	Storage	< 0.0001
	Antioxidant * Storage	<0.0001

Table C.3.1 Effects of Antioxidants Over Time on L*, a* and b* values for 2%-Salt Beef Patties

Variables		Antioxidants					SEM
		Control	Pomegranate Extract	Grape Seed Extract	Dietary Selenium	BHA	
L*	Day 0	50.1 ^{a} **a**	48.7 ^{b} **abc**	49.7 ^{a} **bc**	48.8 ^{b} **d**	49.2 ^{b} **c**	0.3320
	Day 1	48.9 ^{b} **c**	48.3 ^{b} **bcd**	48.8 ^{b} **d**	50.8 ^{a} **a**	50.4 ^{a} **a**	
	Day 2	48.9 ^{c} **c**	48.8 ^{c} **ab**	50.8 ^{a} **a**	49.8 ^{b} **c**	50.5 ^{a} **a**	
	Day 4	49.7 ^{a} **ab**	48.2 ^{c} **cd**	48.6 ^{bc} **d**	49.1 ^{ab} **d**	49.4 ^{a} **bc**	
	Day 7	49.4 ^{bc} **bc**	49.1 ^{c} **ab**	50.2 ^{a} **b**	50.4 ^{a} **ab**	49.8 ^{ab} **b**	
	Day 9	49.5 ^{b} **bc**	48.1 ^{c} **d**	49.4 ^{b} **c**	50.2 ^{a} **bc**	49.5 ^{b} **bc**	
a*	Day 0	13.0 ^{c} **a**	13.3 ^{bc} **a**	13.8 ^{ab} **a**	13.8 ^{ab} **a**	13.9 ^{a} **a**	0.3347
	Day 1	11.6 ^{d} **b**	12.3 ^{c} **b**	12.6 ^{bc} **b**	12.6 ^{bc} **b**	12.9 ^{a} **b**	
	Day 2	11.4 ^{ab} **b**	11.3 ^{ab} **c**	11.5 ^{ab} **c**	11.7 ^{a} **c**	11.1 ^{b} **c**	
	Day 4	7.6 ^{d} **c**	8.9 ^{c} **d**	11.1 ^{a} **c**	8.9 ^{c} **d**	9.6 ^{b} **d**	
	Day 7	4.7 ^{c} **d**	6.7 ^{a} **e**	6.4 ^{a} **d**	5.6 ^{b} **e**	6.9 ^{a} **e**	
	Day 9	2.7 ^{d} **e**	5.1 ^{b} **f**	4.4 ^{c} **e**	4.3 ^{c} **f**	6.6 ^{a} **f**	
b*	Day 0	13.8 ^{b} **a**	13.8 ^{b} **a**	14.2 ^{a} **a**	14.2 ^{a} **a**	13.9 ^{ab} **a**	0.1624
	Day 1	13.6 ^{b} **a**	13.8 ^{ab} **a**	14.1 ^{a} **a**	13.5 ^{b} **b**	13.7 ^{b} **a**	
	Day 2	13.6 ^{a} **a**	13.0 ^{b} **b**	12.8 ^{b} **c**	12.6 ^{c} **c**	12.6 ^{c} **c**	
	Day 4	13.1 ^{b} **b**	13.2 ^{b} **b**	13.6 ^{a} **b**	13.6 ^{a} **b**	13.3 ^{ab} **b**	
	Day 7	13.5 ^{a} **ab**	13.1 ^{b} **b**	13.4 ^{ab} **b**	13.3 ^{ab} **b**	13.3 ^{ab} **b**	
	Day 9	13.6 ^{a} **a**	13.1 ^{bc} **b**	12.9 ^{cd} **c**	12.7 ^{d} **c**	13.4 ^{ab} **b**	

Table C.3.2 Effects of Antioxidants Over Time on Hue Angle and Chroma for 2%-Salt Beef Patties

Variables		Antioxidants					SEM
		Control	Pomegranate Extract	Grape Seed Extract	Dietary Selenium	BHA	
Hue Angle	Day 0	43.8 $^{e\ a}$	43.2 $^{f\ ab}$	43.0 $^{e\ abc}$	42.9 $^{f\ bc}$	42.1 $^{f\ c}$	1.0403
	Day 1	46.7 $^{d\ a}$	45.3 $^{e\ b}$	45.2 $^{d\ b}$	44.1 $^{e\ c}$	43.7 $^{e\ d}$	
	Day 2	47.7 $^{d\ a}$	46.6 $^{d\ b}$	45.4 $^{d\ c}$	45.4 $^{d\ c}$	46.1 $^{d\ bc}$	
	Day 4	56.3 $^{c\ a}$	52.9 $^{c\ c}$	47.6 $^{c\ e}$	53.4 $^{c\ b}$	51.7 $^{c\ d}$	
	Day 7	66 $^{b\ a}$	59.6 $^{b\ e}$	60.3 $^{b\ c}$	63.4 $^{b\ b}$	59.2 $^{b\ d}$	
	Day 9	73.4 $^{a\ a}$	65.2 $^{a\ c}$	66.4 $^{a\ b}$	66.9 $^{a\ b}$	60 $^{a\ d}$	
Chroma	Day 0	18.95 $^{a\ b}$	19.18 $^{a\ b}$	19.77 $^{a\ a}$	19.84 $^{a\ a}$	19.63 $^{a\ a}$	0.2711
	Day 1	17.93 $^{b\ c}$	18.5 $^{b\ b}$	18.94 $^{b\ a}$	18.49 $^{b\ b}$	18.77 $^{b\ ab}$	
	Day 2	17.9 $^{b\ a}$	17.28 $^{c\ b}$	17.24 $^{c\ b}$	17.22 $^{c\ b}$	16.90 $^{c\ b}$	
	Day 4	15.26 $^{c\ d}$	16.11 $^{d\ c}$	17.55 $^{c\ a}$	16.59 $^{d\ b}$	16.68 $^{c\ b}$	
	Day 7	14.36 $^{d\ c}$	15.05 $^{e\ ab}$	14.84 $^{d\ ab}$	14.74 $^{e\ bc}$	15.25 $^{d\ a}$	
	Day 9	13.96 $^{e\ c}$	14.44 $^{f\ b}$	13.67 $^{e\ c}$	13.61 $^{f\ c}$	15.02 $^{d\ a}$	

For $\mu^{\alpha\beta}$ where α indicates a difference at P≤0.05 between rows and β indicates a difference at P≤0.05 between columns

Table C.3.3 Affects and Interactions of Antioxidants in Storage Color Attributes of 2%-Salt Ground Beef Patties

Variable		p-value
L*	Antioxidant	0.0355
	Storage	0.5861
	Antioxidant * Storage	0.9184
a*	Antioxidant	0.0053
	Storage	< 0.0001
	Antioxidant * Storage	0.8048
b*	Antioxidant	0.6457
	Storage	< 0.0001
	Antioxidant * Storage	0.5814
Hue Angle	Antioxidant	0.0036
	Storage	< 0.0001
	Antioxidant * Storage	0.7850
Chroma	Antioxidant	0.1938
	Storage	< 0.0001
	Antioxidant * Storage	0.6316

Appendix D: Spectrophotometric Data

Table D.1.1 Comparison of Antioxidant Assays on Various Natural Antioxidants

Antioxidant	**Folin-Ciocalteu Reagent** (Gallic Acid Equivalents)			**Ferricyanide Reducing Power** (μM Gallic Acid Equivalents)			**DPPH Scavenging** (Ascorbic Acid Equivalents)			**ORAC** (Trolox Equivalence)
Concentration:	6.25 mg	10 mg	20 mg	2.75 mg	11 mg	22 mg	6.9 mg	8.25 mg	9.6 mg	10 mg
Pom. Extract	4.9 [b]	7.3 [b]	18.0 [a]	0.14 [b]	0.43 [bc]	0.87 [c]	7.7 [b]	8.5 [b]	9.7 [b]	49.1 ± 2.7 [d]
Grape Seed Extract	3.3 [c]	6.0 [c]	12.5 [b]	0.13 [bc]	0.37 [cd]	0.75 [d]	5.0 [d]	5.9 [d]	6.8 [d]	101.7 ± 13.6 [b]
Dietary Selenium	0.0 [f]	0.0 [e]	0.0 [f]	0.05 [e]	0.02 [f]	0.02 [g]	0.1 [g]	0.1 [g]	0.1 [g]	0.0 ± 0.0 [f]
BHA	2.7 [d]	4.6 [d]	10.6 [d]	0.10 [c]	0.34 [d]	0.68 [e]	4.6 [e]	4.8 [e]	5.4 [e]	171.3 ± 27.1 [a]
Gallic Acid	6.5 [a]	9.1 [a]	18.0 [a]	0.19 [a]	0.56 [a]	1.27 [a]	12.2 [a]	12.2 [a]	12.2 [a]	30.0 ± 3.8 [c]
Ascorbic Acid	3.3 [c]	4.9 [cd]	11.4 [c]	0.16 [b]	0.46 [b]	0.95 [b]	5.4 [c]	7.4 [c]	9.5 [c]	14.7 ± 4.2 [e]
d,l α-tocopherol	0.2 [e]	0.7 [e]	2.4 [e]	0.06 [d]	0.12 [e]	0.29 [f]	2.8 [f]	3.0 [f]	3.4 [f]	*N/A*
SEM	0.112	0.681	0.736	0.015	0.031	0.057	0.017	0.028	0.015	

Table D.1.2 Pearson Correlation Coefficients

	Folin-Ciocalteu Reagent	**Reduction by Ferricyanide**	**Scavenging by DPPH**	**O_2 Radical Abs. Cap.**
Folin-Ciocalteu Reagent	**1.00000**	0.93582 *p < 0.0001*	0.92684 *p < 0.0001*	-0.40658 *p < 0.0674*
Reduction by Ferricyanide	0.93582 *p < 0.0001*	**1.00000**	0.94004 *p < 0.0001*	-0.35669 *p < 0.1125*
Scavenging by DPPH	0.92684 *p < 0.0001*	0.94004 *p < 0.0001*	**1.00000**	-0.34404 *p < 0.1267*
O_2 Radical Abs. Cap.	-0.40658 *p < 0.0674*	-0.35669 *p < 0.1125*	-0.34404 *p < 0.1267*	1**.00000**

Table D.2.1 Effect of Natural Antioxidants on Diene Conjugation of Gelled Lard Model System at 30°C

	Conjugated Dienes (mmol Conjugated Diene / kg Lard Model)					
	Storage Time					
Antioxidant	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 9**
Control	3.71 *cdef*	3.55 *def*	3.74 *cdef*	4.96 bcde	5.64 *bc*	9.80 *a*
Pomegranate Extract	3.51 *def*	3.22 *ef*	3.37 *ef*	5.58 bc	5.40 *bcd*	6.16 *b*
Grape Seed Extract	3.29 *ef*	3.77 *cdef*	3.06 *ef*	4.73 *bcde*	4.68 *bcde*	4.82 *bcde*
Dietary Selenium	3.47 *def*	3.15 *ef*	3.49 *def*	4.61 *bcdef*	5.61 *bc*	9.25 *a*
Butylated Hydroxyanisol	3.38 *ef*	3.669 *cdef*	3.35 *ef*	3.85 *cdef*	3.03 *ef*	2.67 *f*

P-value		**SEM**	3.1151
	Antioxidant	< 0.0001	
	Storage	< 0.0001	
	Antioxidant * Storage	0.0004	

Table D.2.2 Effect of Natural Antioxidants on TBARS of Gelled Lard Model System at 30°C

	TBARS (µg Malondialdehyde / g Lard Model)					
	Storage Time					
Antioxidant	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 9**
Control	0.05 *no*	0.15 *jklm*	0.22 *hijk*	0.40 f	0.96 *c*	1.54 *a*
Pomegranate Extract	0.09 *lmno*	0.16 *jkl*	0.24 *hij*	0.27 ghi	0.58 *e*	1.41 *b*
Grape Seed Extract	0.06 *mno*	0.16 *jkl*	0.29 *ghi*	0.34 *fgh*	0.67 *e*	1.02 *c*
Dietary Selenium	0.06 *mno*	0.15 *jklmn*	0.23 *ijk*	0.35 fg	0.79 *d*	1.55 *a*
Butylated Hydroxyanisol	0.03 *o*	0.07 *lmno*	0.11 *lmno*	0.14 *klmn*	0.24 *hij*	0.28 *ghi*

P-value			**SEM**	0.0980
	Antioxidant	< 0.0001		
	Storage	< 0.0001		
	Antiox. * Storage	< 0.0001		

Table D.2.3 Effect of Natural Antioxidants on Diene Conjugation of Gelled Lard Model System at 30°C

		Conjugated Dienes (mmol Conjugated Diene / kg Lard Model)					
		Storage Time					
	Antioxidant	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 9**
	Control	3.71 *defgh*	3.55 *efgh*	3.74 *defgh*	4.96 bcde	5.64 *bc*	9.80 *a*
100 ppm		3.51 *efgh*	3.22 *efgh*	3.37 *efgh*	5.58 bc	5.40 *bcd*	6.16 *b*
500 ppm	**Pomegranate Extract**	3.66 *defgh*	3.21 *efgh*	2.77 *h*	4.53 *bcdefg*	3.90 *cdefgh*	4.61 *bcdefg*
1000 ppm		3.14 *fgh*	3.32 *efgh*	3.44 *efgh*	4.84 bcdef	3.57 *efgh*	3.27 *efgh*
	Butylated Hydroxyanisol	3.38 *efgh*	3.66 *defgh*	3.35 *efgh*	3.85 *cdefgh*	3.03 *gh*	2.67 *h*

P-value		**SEM**	2.5712
	Antioxidant	< 0.0001	
	Storage	< 0.0001	
	Antiox. * Storage	< 0.0001	

Table D.2.4 Effect of Natural Antioxidants on TBARS of Gelled Lard Model System

		TBARS (µg Malondialdehyde / g Lard Model)					
		Storage Time					
	Antioxidant	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 9**
	Control	0.05 *nop*	0.15 *ijklm*	0.22 *ghij*	0.40 f	0.96 *c*	1.54 *a*
100 ppm	**Pomegranate Extract**	0.09 *lmnop*	0.16 *ijklm*	0.24 *ghi*	0.27 gh	0.58 *e*	1.41 *b*
500 ppm		0.03 *op*	0.09 *lmnop*	0.13 *jklmno*	0.20 *ghik*	0.50 *e*	0.81 *d*
1000 ppm		0.04 *nop*	0.10 *klmnop*	0.12 *ijklmnop*	0.15 ijklm	0.17 *ijklm*	0.18 *hijkl*
	Butylated Hydroxyanisol	0.03 *p*	0.07 *mnop*	0.11 *klmnop*	0.14 *jklmn*	0.24 *ghi*	0.28 *g*
P-value						**SEM**	0.0980
	Antioxidant	< 0.0001					
	Storage	< 0.0001					
Storage	Antiox. *	< 0.0001					

Table D.2.5 Effect of Natural Antioxidants on Diene Conjugation of Canola Oil Model System at 30 °C

	Conjugated Dienes (mmol Conjugated Diene / kg Oil Model)					
	Storage Time					
Antioxidant	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 9**
Control	1.35 ^{ij}	2.16 ^{e}	2.27 ^{e}	1.26 ^{jk}	1.78 ^{gh}	2.95 ^{b}
Pomegranate Extract	1.25 ^{ijk}	2.17 ^{e}	2.56 ^{d}	1.18 ^{jk}	2.03 ^{ef}	2.92 ^{b}
Grape Seed Extract	1.85 ^{fg}	2.24 ^{e}	2.20 ^{e}	1.12 ^{jk}	2.77 ^{bcd}	2.81 ^{bc}
Butylated Hydroxyanisol	1.08 ^{efk}	2.74 ^{bcd}	3.24 ^{a}	1.54 ^{hi}	2.17 ^{e}	1.71 ^{gh}
P-value					**SEM**	0.2523
Antioxidant		0.5438				
Storage		< 0.0001				
Antioxidant * Storage		0.0024				

Table D.2.6 Effect of Natural Antioxidants on TBARS of Canola Oil Model System at 30 °C

	TBARS (µg Malondialdehyde / g Oil Model)					
	Storage Time					
Antioxidant	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 9**
Control	0.04 ^{jk}	0.11 $^{g\ h}$	0.16 ^{d}	0.19 ^{bc}	0.23 ^{a}	0.24 ^{a}
Pomegranate Extract	0.03 ^{k}	0.08 ^{i}	0.11 ^{h}	0.11 ^{h}	0.11 ^{gh}	0.18 ^{c}
Grape Seed Extract	0.05 ^{j}	0.05 ^{j}	0.08 ^{i}	0.13 ^{efg}	0.14 ^{ef}	0.20 ^{b}
Butylated Hydroxyanisol	0.04 ^{jk}	0.08 ^{i}	0.09 ^{i}	0.13 ^{fg}	.15 ^{de}	0.18 ^{c}
P-value					**SEM**	0.0136
Antioxidant		< 0.0001				
Storage		< 0.0001				
Antioxidant * Storage		0.0373				

Table D.3.1 Effect of Natural Antioxidants on TBARS of 2%-Salt Ground Beef Patties

	TBARS (µg Malondialdehyde / g Ground Beef Patty)						
	Storage Time						SEM
Antioxidant	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**Day 9**	
Control	0.50 ^{b}	0.78 ^{ab}	0.76 ^{ab}	1.07 ^{a} **a**	1.07 ^{a} **a**	0.93 ^{a} **a**	0.0727
Pomegranate Extract	0.45 ^{b}	0.59 ^{ab}	0.61 ^{ab}	0.77 ^{a} **ab**	0.68 ^{ab} **bc**	0.55 ^{ab} **b**	0.0727
Grape Seed Extract	0.47 ^{ab}	0.53 ^{ab}	0.64 ^{ab}	0.69 ^{a} **b**	0.54 ^{a} **c**	0.37 ^{b} **b**	0.0727
Dietary Selenium	0.49 ^{b}	0.70 ^{ab}	0.72 ^{ab}	0.85 ^{a} **ab**	0.82 ^{a} **ab**	0.65 ^{ab} **ab**	0.0727
Butylated Hydroxyanisol	0.36	0.50	0.50	0.57 **b**	0.60 **bc**	0.42 **b**	0.0727
SEM	0.0796	0.0796	0.0796	0.0796	0.0796	0.0796	

P-value

Antioxidant	0.0170
Storage	0.0238
Antiox. * Storage	0.1330