EFFECT OF GRAPE SEED EXTRACT ON OXIDATIVE, COLOR AND SENSORY STABILITY OF PRE-COOKED, FROZEN, RE-HEATED BEEF SAUSAGE MODEL SYSTEM

BY

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THESIS

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ABSTRACT

The processing of meats at the factory level can trigger the onset of lipid oxidation, which can lead to meat quality deterioration. Warmed over flavor is an off-flavor, which is associated with oxidative deterioration in meat. To avoid or delay the auto-oxidation process in meat products, synthetic and natural antioxidants have been successfully used. Grape (*Vitis Vinifera*) is of special interest due to its high content of phenolic compounds. Grape seed extract sold commercially as a dietary supplement, has the potential to reduce lipid oxidation and WOF in cooked ground beef when added at 1%. The objective of study 1 was to compare the antioxidant activity of natural antioxidants including grape seed extract and some herbs belonging to the Lamiaciae family: rosemary (Rosmarinus Officinalis), sage (Salvia Officinalis) and oregano (Origanum Vulgare) with commercial synthetic antioxidants like BHT, BHA, propyl gallate and ascorbic acid using the ORAC assay. All sample solutions were prepared to contain 1.8 gm sample/10 ml solvent. The highest antioxidant activity was observed for the grape seed extract sample (359.75 μM TE), while the lowest was observed for BHA, propyl gallate and rosemary also showed higher antioxidant potential with ORAC values above 300 μmol TE/g. ORAC values obtained for ascorbic acid and Sage were between 250-300μ mol TE/g while lowest values were obtained for Butylated Hydroxytoluene (28.50 μM TE). Based on the high ORAC values obtained for grape seed extract, we can conclude that byproducts of the wine/grape industry have antioxidant potential comparable to or better than those present in synthetic counterparts. The objective of study 2 was to compare three levels of grape seed extract (GSE) to commonly used antioxidants in a pre-cooked, frozen, stored beef and pork sausage model system. Antioxidants added for comparison with control

included grape seed extract (100, 300, 500 ppm), ascorbic acid (AA, 100 ppm of fat) and propyl gallate (PG, 100 ppm of fat). Product was formed into rolls, frozen, sliced into patties, cooked on a flat griddle to 70C, overwrapped in PVC, and then frozen at –18C for 4 months. GSE- and PG-containing samples retained their fresh cooked beef odor and flavor longer ($p<0.05$) than controls during storage. Rancid odor and flavor scores of GSE-containing samples were lower ($p<0.05$) than those of controls after 4 months of storage. The L* value of all samples increased ($p<0.05$) during storage. Thiobarbituric acid reactive substances (TBARS) of the control and AA-containing samples increased ($p<0.05$); those of GSE-containing samples did not change significantly ($p>0.05$) over the storage period.

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Chapter 1. Introduction

1.1 General Introduction

Consumers are cooking less and eating out more (USA: Market profile, 2005). These factors have led to a dramatic increase in the consumption of ready-to-eat meat products. In addition to convenience, consumers also want a safe, nutritious, sensory experience when consuming meat products (Rojas & Brewer 2007).

Prior to cooking, meat lipids undergo autoxidation mediated through free radical reactions, ultimately producing products that can affect the flavor, odor and color. Intrinsic antioxidants including tocopherols, carnosine and antioxidant enzymes are capable of controlling oxidative reactions in the live animal and many muscle foods. However, meat processing reduces their activity. (Rojas and Brewer, 2007)

The processing of meats at factory level triggers the onset of lipid oxidation, which can lead to meat quality deterioration. Warmed over flavor is an off-flavor, which is associated with oxidative deterioration in meat. The process of warmed over flavor (WOF) development in meat products is attributed to the auto-oxidation of meat lipids forming hydroproxides. These hydroperoxides, via many different pathways decompose into a large number of volatile compounds (Gray et al., 1996). This is the primary cause of rancidity during frozen storage of meat and meat products (Channon and Trout, 2002). The biggest initiator of oxidation is cooking—it initiates the development of WOF. Cooked meat is susceptible to lipid oxidation and phospholipids are the primary contributors to lipid oxidation and warmed over flavor development ([Gandemer, 1999],

[Mottram, 1991] and [Pearson and Gray, 1983]). The shelf life and acceptability of processed, ready-to-eat uncured meats is limited because of the rapid onset of rancidity, denoted as WOF. This becomes evident during the refrigerated storage of cooked meat products within a few days (Vasundhara and Honikel, 1992).

To avoid or delay the auto-oxidation process in meat products, synthetic and natural antioxidants have been successfully used (McCarthy et al, 2001). Synthetic phenolic antioxidants such as BHA, BHT and TBHQ effectively inhibit WOF (Decker and Mei, 1996). There is an increasing interest in natural antioxidants because of the safety and toxicity problems of synthetic antioxidants (BHA, BHT and PG) that are commonly used in lipid containing/rich foods (Amarowicz et al, 2000; Ito et al, 1986; Van Esh, 1986).
The use of various natural antioxidants has been proven a safe and effective way to prevent warmed over flavor (Riznar et al, 2006; Nissen et al, 2004). Natural antioxidants are primarily plant phenolics that may occur in all parts of plants, such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt and Hudson, 1990). Plant phenolics are multifunctional and can act as reducing agents, free radical terminators, metal chelators and singlet oxygen quenchers (Mathew and Abraham, 2006). Grape (*Vitis Vinifera*) is of special interest due to its high content of phenolic compounds. Unlike skin, grape seed is rich in monomeric phenolic compounds such as catechin, epicatechin, and epicatechin-3-O-gallate, and in dimeric, trimeric and tetrameric procyanidins (Banon et al, 2007). Grape seed extract sold commercially as a dietary supplement, has a potential to reduce lipid oxidation and WOF in cooked ground beef when added at 1%. This antioxidant activity seems to be concentration dependant between 0.02% and 0.1%

(Rojas and Brewer, 2007). Grape seed extract ranges from 80 to 99% phenolics substances, particularly resveratrol. Resveratrol (trans-3, 4', 5-trihydroxystilbene), a phenolic compound produced primarily in the grapevine, is present in wines and various parts of the grape. It has strong antioxidant activity. When compared with other antioxidants, BHA > resveratrol > PG > tripolyphosphate > vanillin > phenol > BHT > alpha-tocopherol (Murcia and Martinez-Tome 2001). Resveratrol inhibits peroxidation in a concentration-dependent manner. It does not scavenge hydroxyl radical nor does it react with H_2O_2, making it an inefficient catalyst of subsequent oxidation (Murcia et al. 2001).

The objective of this study was to compare three levels of grape seed extract (GSE) to commonly used antioxidants in a pre-cooked, frozen, stored beef and pork sausage model system.

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Chapter 2. Literature Review

2.1 Lipid Oxidation

In fresh meat and meat products, color is a strong indicator of quality and an important visual cue involved in consumer perception of acceptable meat quality. Lipid oxidation is a major cause of muscle food deterioration (Ladikos & Lougovois, 1990). It has also been found that lipid oxidation can cause pathological changes in the mucous membrane of the alimentary tract, inhibit the activity of enzymes and increase the content of cholesterol and peroxides in blood serum thus potentially causing atherosclerosis.

A number of intrinsic properties and processing steps can predispose meat to lipid oxidation. Substrates necessary for this deteriorative reaction include unsaturated fatty acids, oxygen and chemical species that accelerate oxidation, e.g. iron (Kanner et al. 1988). All these substances are abundant in meat displayed aerobically or in high oxygen modified atmosphere packaging. Meats from non-ruminants contain relatively higher concentrations of unsaturated fatty acids within triacylglycerols (Enser et al., 1996). Meats from non-ruminants display more rapid lipid oxidation than that of ruminants (Tichivangana & Morrissey, 1985). Muscles that have greater proportion of red fibers are susceptible since they contain more iron and phospholipids than muscles that predominantly contain white fibers (Wood et al, 2004). Also, ground meat experiences greater amount of lipid oxidation than whole cuts because the grinding process incorporates oxygen, mixes the reactive components, and increases the surface area as a result of particle size reduction (Gray, Gomaa & Buckley, 1996). Lipid oxidation results in a wide range of secondary aldehyde products, which are predominantly n-alkanals, trans-2-alkenals, 4-hydroxy-trans-2-alkenals, and malondialdehyde (Esterbauer et al.,

1991). The presence of these lipid oxidation products is thought to alter Mb through covalent modification.

Myoglobin has been recognized, as a major catalyst for lipid oxidation in meat but its mode of action for catalyzing lipid oxidation is controversial. It is suggested that the interaction of metmyoglobin (MetMb) with hydrogen peroxide or lipid hydroperoxidases (LOOH) results in the formation of ferrylmyoglobin which can initiate free radical chain reactions (Min et al., 2010), (Chan et al., 1997), (Davies, 1990), (Egawa et al., 2000), (Kanner and Harel, 1985), (Min & Ahn, 2005) & (Rao et al., 1994). In addition to this, ferrylmyoglobin and MetMb can degrade LOOH to free radicals such as alkoxyl and peroxyl radicals, which can initiate and/or catalyze a series of propagation and termination steps in the free radical chain reactions of lipid oxidation (Frankel, 1987) & (Halliwell & Gutteridge, 1990). Some other researchers have limited the role of myoglobin as only a source of free ionic iron or haematin (Ahn ad Kim, 1998). The results from their studies indicated that free ionic iron and/or haematin released from myoglobin in the presence of hydrogen peroxide or lipid hydroperoxide, rather than ferrylmyoglobin, were the major catalysts for lipid oxidation in meat (Kanner et al., 1988) & (Puppo & Halliwell., 1988).

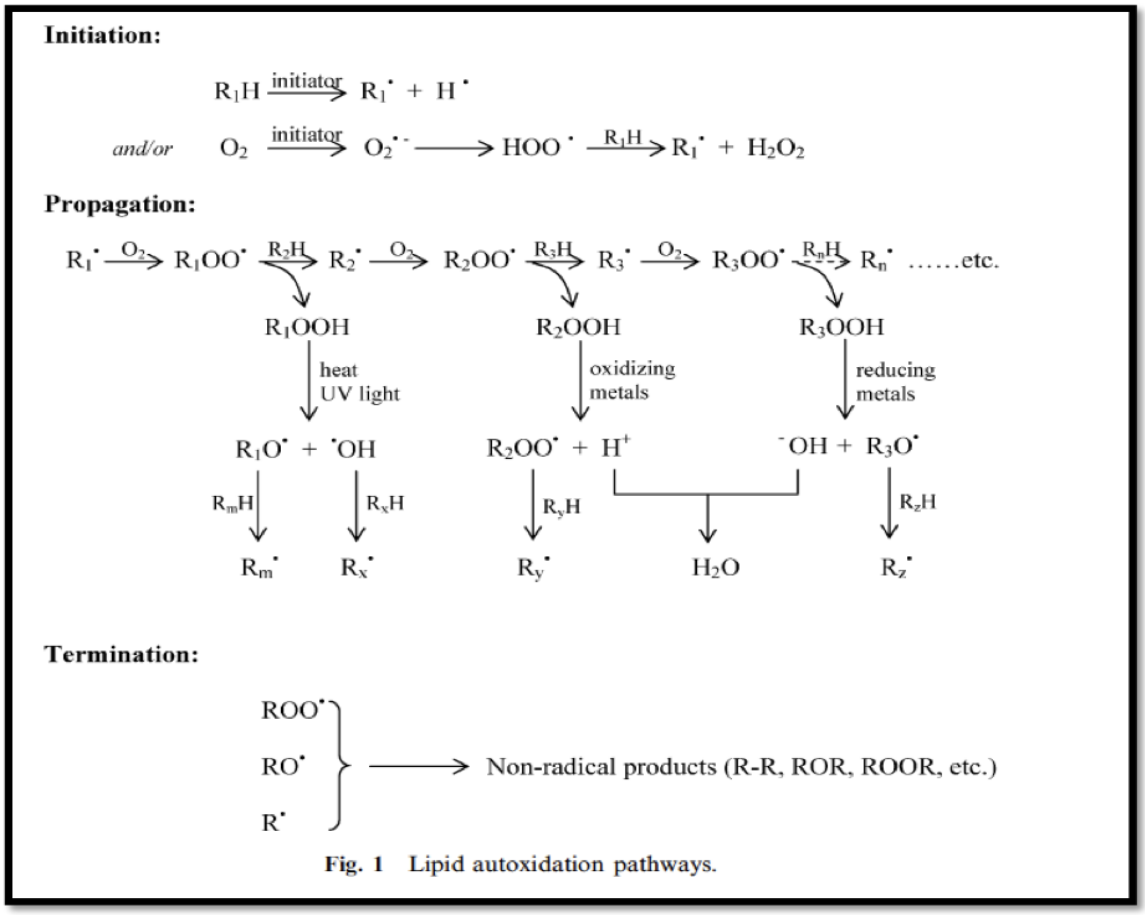
2.2 Mechanism of Lipid oxidation

Lipids are susceptible to oxidation in the presence of catalytic systems such as light, heat, enzymes, metals, metallo-proteins and microorganisms leading to complex processes of autoxidation, photooxidation, thermal or enzymatic oxidation most of which involve free

radicals and/or other reactive species as the intermediate. Autooxidation is the most common process among all and is defined as the spontaneous reaction of lipid with atmospheric oxygen through a chain reaction of free radicals. The process can be accelerated at higher temperatures as in thermal oxidation. Photooxidation involves excitation of a photosensitizer and energy transfer to lipid molecules or oxygen. Certain enzymes such as lipoxygenases can also catalyze oxidation. Unsaturated fatty acids are the major reactants affected by such reactions, whether they are present as free fatty acids, simple alkyl esters, acylglycerols or phospholipids.

It is widely accepted that lipid autoxidation occurs via a free radical chain mechanism that proceeds through three distinct stages of initiation, propagation and termination, leading to a series of complex chemical changes. In the presence of initiators like heat, light/ionizing radiation and metal ions/metalloproteins lose a hydrogen atom and produce free radicals. The lipid radicals then react with oxygen to form peroxy radicals that act as the chain carriers of the rapid progressing reaction by attacking a new lipid molecule. This reaction may repeat for several thousand times during propagation until no hydrogen source is available or the chain is interrupted e.g. by antioxidants. Therefore lipid oxidation is a self-propagating and self-accelerating process.

Figure 2.1 Lipid Autooxidation Pathways



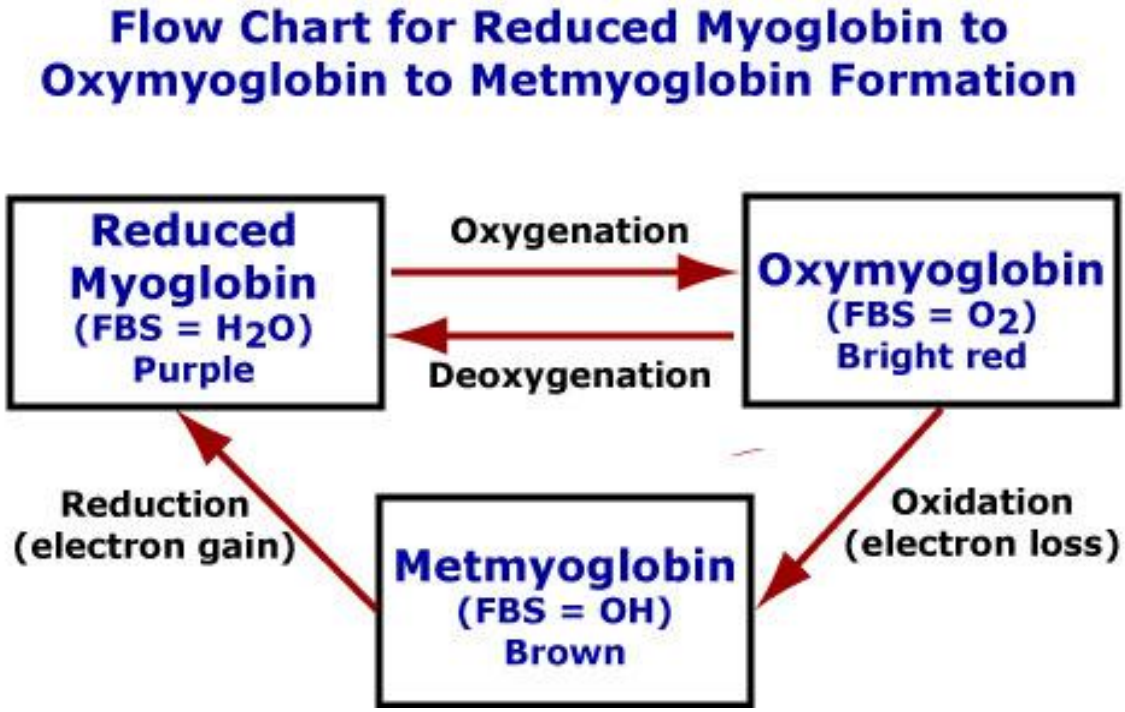
Source: Shahidi & Zhong, 2010

2.3 Oxidation of Myoglobin:

Myoglobin is the heme protein responsible for meat color. The role of heme proteins in general, and myoglobin specifically, in enhancing lipid oxidation has been studied extensively. Greater concentrations of iron and myoglobin are associated with greater rates of lipid oxidation (Faustman et al, 1992).

Meat discoloration occurs when the central iron atom within the heme group gets oxidized changing the red OxyMb to brownish MetMb. Oxymyoglobin is responsible for the red coloration in highly pigmented muscles such as beef and pink coloration in the less pigmented porcine muscles (Hayes et al., 2009). When ferrous heme iron oxidizes to its ferric form, oxygen is released and replaced by a water molecule. Many factors affect OxyMb oxidation (Faustman & Cassens, 1990) and (Renerre, 2000). The factors include temperature, pH, MetMb reducing activity, partial oxygen pressure and lipid oxidation. The oxidation of OxyMb is favored by higher temperatures (Brown & Mebine, 1969), lower pH values (Gotoh & Shikama, 1974) and the presence of non-heme iron (Allen & Cornforth, 2006). The rate of discoloration in meat is muscle specific. Muscles that contain greater relative proportions of red fibers, and thus more lipid and greater oxygen consumption rates, appear to discolor more quickly. MetMb reducing activity can happen enzymically or non-enzymically, and favors maintenance of ferrous forms of myoglobin in meat (Bekhit, Simmons & Faustman, 2005). Partial oxygen pressures in which a complete vacuum exists or in which oxygen saturation is attained, favor ferrous myoglobin forms. Low non-zero pO2 favors MetMb form (George & Stratman, 1952), (Ledward, 1970) & (Neill & Hastings, 1925).

Figure 2.2 Flow Chart for Reduced Myoglobin to Oxymyoglobin to Metmyoglobin Formation



http://meat.tamu.edu/colorflowchart.jpg

2.4 Lipid oxidation as a facilitator of myoglobin oxidation:

Many studies have reported that lipid oxidation enhances meat discoloration. Zakrys et al., (2008) recently investigated the quality parameters of beef that was packaged under 0%, 10%, 20%, 50% and 80% oxygen (20% CO2, balance nitrogen). Results showed that changes in OxyMb and a* values appeared to be driven by lipid oxidation and correlated strongly with TBARS. The phenomenon has been explained primarily on the reactivity of primary and secondary products derived from unsaturated fatty acids. Livestock supplementation with diets rich in poly unsaturated fatty acids leads to the meat obtained

from these animals being highly susceptible to lipid oxidation and discoloration (Nute et al, 2007) & (McKenna et al, 2005).

2.5 Assessment of Lipid Oxidation using TBA Assay: Oxidation of lipids is assessed by the TBA (Thiobarbituric acid) assay which is based on the reaction between TBA and MDA (malondialdehyde) and the production of a colored pigment, the concentration of which can be determined by measuring the absorbance at 532 nm. Some of the MDA is formed during the oxidation process; however, most of it is generated by the decomposition of lipid peroxides during the aid heat treatment of the assay (Guillen-Sans & Guzman-Chozas, 1998).

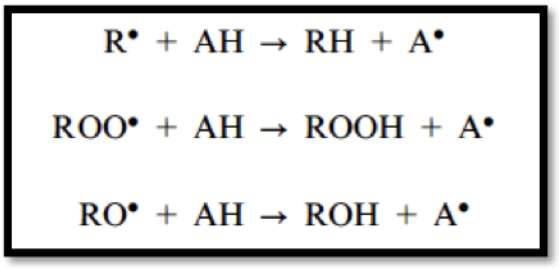
2.6 Role of Antioxidants in the prevention of Lipid Oxidation

Unsaturated lipids in meats are very susceptible to chemical attack by oxygen. The addition of antioxidants is a method of increasing shelf life, especially of lipids and lipid containing products. Antioxidants can interact with free radicals that start chain reactions to damage compounds. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by getting oxidized themselves (Yerlikaya & Gokoglu, 2010).

Antioxidants may exert their inhibitory effect against oxidation via different mechanisms and with varied activities. They may be broadly classified based on their mode of action into primary antioxidants which break the chain reaction of oxidation by scavenging free

radical intermediates, and secondary antioxidants, which prevent or retard oxidation by suppression of oxidation initiator or accelerators or regeneration of primary antioxidants. Primary antioxidants such as most phenolic compounds are able to neutralize free radicals by donating a hydrogen atom. Primary antioxidants can trap two lipid radicals by donating a hydrogen atom to one radical and receiving an electron from another radical to form stable non-radical products (Shahidi & Zhong, 2010).

Figure 2.3 Neutralization of Free Radicals by Primary Antioxidants



Source: Shahidi & Zhong, 2010

Secondary antioxidants prevent or retard oxidation by several mechanisms. They exert their inhibitory effect by suppressing the oxidation promoters, including metal ions singlet oxygen, prooxidative enzymes and other oxidants. Metal ions are known to act as catalysts of oxidation reaction producing free radicals through electron transfer. Metal chelators such as citric acid, phosphoric acid and ethylenediaminetertraacetic acid (EDTA) can decrease the pro-oxidant effect by forming a thermodynamically stable complex and reducing their redox potentials (Shahidi & Zhong, 2010)

2.6.1 Synthetic antioxidants:

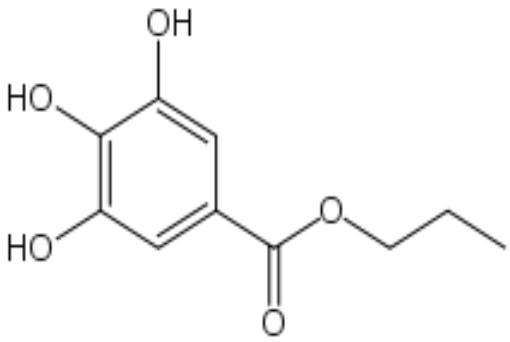
Butylated Hydroxyanisole (BHA): BHA is a phenolic antioxidant typically used as a food additive, particularly in fats and oils. In the European Union and USA, BHA is an authorized additive and also has a GRAS status in USA. A multitude of studies have been published studying the effects of BHA on metabolism and health in mammals (Iverson 1995; Whysner & Williams 1996; Williams et al., 1999). The properties appear contradictory as BHA has previously been classified as an antioxidant, a pro-oxidant, an anticarcinogen, a carcinogen and in models with known carcinogens both as a tumor promoter and a tumor inhibitor. The Joint FAO/WHO Expert Committee on Food Additives has evaluated the safety of BHA several times since 1961, and the latest acceptable daily intake has bee set at 0-0.5 mg kg^{-1} body weight (Petri et al., 2008).

Propyl Gallate: Propyl Gallate is the n-propyl ester of gallic acid (3,4,5-trihydroxybenzoic acid). It is soluble in ethanol, ethyl ether, oil, lard and aqueous solutions of polyethylene glycol (PEG) ethers of cetyl alcohol. It is only slightly soluble in water. Currently propyl gallate is being used as an antioxidant in a number of cosmetic products at maximum concentrations of 0.1%. It is generally recognized as safe antioxidant (GRAS) to protect fats, oils and fat containing foods from rancidity that results from the formation of peroxides (Becker & Lillian, 2007).

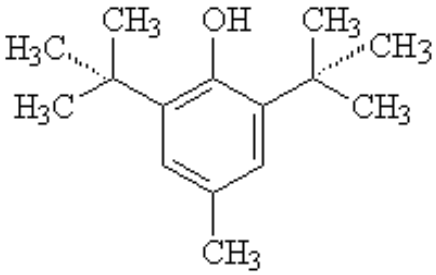
Butylated Hydroxy Toluene: It is chemically 2,6-di-tert-butyl-p-cresol (DBPC). It is a white crystalline solid with a faint characteristic odor. It is insoluble in water and propylene glycol, but it is freely soluble in alcohol. BHT is obtained by alkylation of p-

cresol with isobutene or by monobutylation of m-/p-cresol mixtures. BHT is used as a chemical antioxidant for food, cosmetics and pharmaceuticals much like BHA.

Figure 2.4 Structures of Common Synthetic Antioxidants

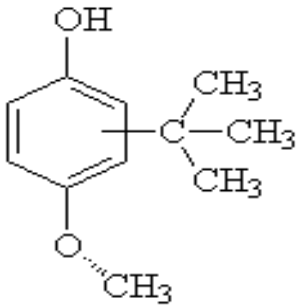


Propyl Gallate



BHT
butylated hydroxytoluene

Figure 2.4 (cont.)



BHA

butylated hydroxyanisole

2.6.2 Natural Antioxidants

Considering the possibility of undesirable influences of oxidized lipids on the human organism, it is of essential importance to minimize the content of products of lipid oxidation in food. In industrial processing, mainly synthetic antioxidants are used, in order to prolong the storage stability of food. Synthetic antioxidants like BHA ad BHT have been shown to exert carcinogenic effects in humans (Ames, B.M., 1983) & (Baardseth, P., 1989). There is a general trend toward replacing the use of synthetic antioxidants in food processing by the use of natural oxidation inhibitors or by the preferential use of ingredients that naturally possess antioxidant activity. Natural antioxidants are primarily plant phenolics that may occur in all parts of plants, such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt & Hudson, 1990). Plant phenolics are multifunctional and can act as reducing agents, free radical terminators, metal chelators and singlet oxygen quenchers (Mathew & Abraham, 2006).

The chemical structures of natural antioxidants are related to those of synthetic antioxidants; most phenolic antioxidants are pyrocatechol or pyrogallol derivatives, dihydrochromanols or flavonoids. Natural extracts such as rosehip, sage, citrus peel, sesame seed oil and grape have the same, and sometimes even better antioxidative characteristics (Tang et al., 2001) Natural plant extracts are applied to food particularly or in combinations (Luther et al., 2007; Alghazeer et al., 2008; Gokoglu & Yerikaya, 2008). Recently the antioxidant power and health benefits of grape seed extract have been demonstrated by scientific studies and this leads to its use as a dietary supplement and food additive (Nakamura et al., 2003).

Ascorbic acid: Ascorbic acid is a reducing agent that inhibits myoglobin oxidation and brown color development in beef (Wheeler et al., 1996). Ascorbic acid is widely used in the meat industry for its antioxidant properties. When used in cured meats, ascorbic acid can accelerate color development, inhibit nitrosamine formation, prevent oxidation and prevent color fading. Ascorbic acid is also used in fresh meat to prevent oxidation and color fading during storage. Ascorbic acid can prevent nitrosamine formation in cured meats by reducing nitrate to nitrogen oxide, which cannot react with the amines to form nitrosamines. Ascorbic acid also prevents oxidation of lipids and fats in both raw and cured meat products. Lipid oxidation causes the release of many lower weight molecules, which can impart off-flavors and rancid notes to meat. By reducing the oxygen in the environment, less oxygen is available to breakdown the lipids. The color of both fresh and cured meats is sensitive to decomposition caused by oxidation of the myoglobin in

the tissues. Ascorbic acids antioxidant action prevents the myoglobin from oxidizing to metmyoglobin, which has a brown color.

Grape Seed Extract: Grape (*Vitis vinifera*) is one of the world's largest fruit crops with an annual production of over 65 million metric tonnes. Most of the production has been processed to different products such as raisin, wine, vinegar, grape juices and different traditional products like grape concentrate in the food industry. Grape seeds are an important by product of the grape industry (Yemis et al., 2008). Grape seeds are rich in fiber (40%), lipid (16%), protein (11%) and complex phenols (7%) (Kim et al., 2006) Grape seed is rich in monomeric phenolic compounds like (+)–catechin and (-)-epicatechin-3-0-gallate, and dimeric, trimeric and tetrameric phenolic compounds (Jayaprakasha et al., 2001). Grape seeds are known to be a rich source of a number of phenolic compounds. Polyphenols in grape seeds are mainly flavonoids, including the monomeric flavan-3-ols catechin, epicatechin, gallocatehin, epigallocatechin and epicatechin 3-o-gallate and procyanidin dimmers, trimers and more highly polymerized procyanidins (Fuleki & Ricardo-da-silva, 1997). Catechin is usually the most important individual flavanol in both grape skins and seeds, although epicatechin is also usually well represented. Some grape varieties display similar levels of both monomers, or an even higher proportion of epicatechin (Chedea et al., in press) and (Gonzales-Manzano et al., 2004). Procyanidin B1 has been reported to be the main oligomer in skins (Mateus et al., 2001), whereas all C4-C8 procyanidin dimmers (i.e. B1-B4) are usually found in seeds, of which procyanidin B2 is normally the most abundant (Jordao et al., 2001).

Levels of galloyl flavan-3-ols are more important in seeds than in skins (Jordao et al., 2001).

Grape seed extract is known to be a powerful antioxidant that protects the body from premature aging, disease and decay. The pharmacological and nutraceutical benefits derived from grape seed polyphenols are because of their free radical scavenging capability. There are a number of studies reported that grape seed polyphenols reduce the risk of cancer and heart disease by inhibiting the oxidation of low density lipoprotein (Shi et al., 2003).

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Chapter 3. The comparison of Oxygen Radical Absorbance Capacity of Grape Seed Extract versus Oxygen Radical Absorbance Capacity of selected antioxidants

3.1 Abstract

To compare the antioxidant activity of natural antioxidants including grape seed extract and some herbs belonging to the Lamiaciae family: rosemary (Rosmarinus Officinalis), sage (Salvia Officinalis) and oregano (Origanum Vulgare) with commercial synthetic antioxidants like BHT, BHA, propyl gallate and ascorbic acid using the ORAC assay. All sample solutions were prepared to contain 1.8 gm sample/10 ml solvent. The highest antioxidant activity was observed for the grape seed extract sample (359.75 μM TE), while the lowest was observed for Butylated Hydroxyanisole, Propyl gallate and rosemary also showed higher antioxidant potential with ORAC values above 300 μmol TE/g. ORAC values obtained for Ascorbic acid and Sage were between 250-300μ mol TE/g while lowest values were obtained for Butylated Hydroxytoluene (28.50 μM TE). Based on the high ORAC values obtained for grape seed extract, we can conclude that byproducts of the wine/grape industry have antioxidant potential comparable or better than those present in synthetic counterparts.

3.2 Introduction

Reactive oxygen species such as hydroxyl (OH) and superoxide (O_2) radicals, hydrogen peroxide (H_2O_2) induce all forms of DNA damage including base modifications, breakage of the DNA and protein cross-links. These reactive species of oxygen as well as nitrogen can induce oxidative stress damage. Oxidative damage on cellular

macromolecules is implicated in the genesis of several diseases including cancer. The resultant oxidative damage on lipids generates lipid peroxides, which promotes the formation of additional free radicals in a type of chain reaction. Chemical compounds known to have free radical scavenging properties could effectively protect against this damage (Ajiboye, 2011). Among the many methods employed for controlling lipid oxidation, use of antioxidants is the most effective, convenient and economical means. Antioxidants are not only used in health-related areas due to their ability to protect the body but also by food manufacturers' world-wide to stabilize food lipids and thus prevent quality deterioration of the food product (Shahidi & Zhong, 2009). Antioxidants are compounds that, when present at low concentrations compard to that of an oxidizable substrate, markedly delay or prevent its oxidation. They do this by being oxidized themselves.

Antioxidants fitting into this definition include free radical scavengers, singlet oxygen quenchers, inactivators of peroxide and other ROS, metal ion chelators, and inhibitors of pro-oxidative enzymes among others. Antioxidants may be broadly classified into two subdivisions namely primary and secondary antioxidants based on their mode of action (Shahidi & Zhong, 2009). Antioxidant substances are widely distributed in plant materials, animal tissues and microorganisms. Antioxidants can be isolated as pure compounds from natural sources (natural antioxidants) or artificially synthesized (synthetic antioxidants). Natural and synthetic antioxidants have commonly been used in the meat industry in order to inhibit the development of oxidative reaction in meat products (Capitani et al, 2009).

The heightened interest in using natural ingredients in food is being driven by health conscious consumers due to the growing concerns about the toxicological safety of synthetic antioxidants. The goal is to replace these conventional synthetic antioxidants such as Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA) with natural anti-oxidative substances. (Trindade et al, 2010). The use of natural antioxidants has the advantage of being more acceptable to consumers as they are considered as "non-chemical". In addition, they do not require safety testing (Fasseus et al., 2008). A great deal of research has been done using natural antioxidants such as rosemary, sage, a-tocopherol and pine bark extract. (Barbut et al, 1985; Buckley et al, 1989; St Angelo et al, 1990; Stoick et al, 1991 & Ahn et al, 2002). Recently, grape seed extract is also gaining importance due to its potential as a powerful natural antioxidant.

The food industry is becoming increasingly interested in aromatic herbs, belonging mainly to the Lamiaceae family (which includes plants like sage, thyme, mint, lavender, basil etc.) due to the growing demand for healthier foods of natural origin. They are used not only for flavoring but also for their antioxidative properties, medicinal and antioxidative properties. The Lamiaciae family includes herbs such as rosemary, oregano, sage, Thyme, marjoram and basil. These herbs are found all over the world. (Hossain et al, 2010)

Rosemary (Rosemarinus Officinalis) is a common herb and household plant used all around the world for medicinal purposes. Results of many experiments have shown that

rosemary essential oil has antimicrobial (Prabuseenivasan, Jayakumar & Ignacimuthu, 2006), cognition improving (Moss et al, 2003) and some glucose level lowering properties therefore also used as a natural animal feed additive (Faixova & Faix, 2008)

Rosemary extract has been studied in detail for use as a natural antioxidant in meats (Rojas & Brewer, 2007; Nissen, 2004). It is characterized by two or three major components at fairly high concentrations (1,8-cineole, a-pinene, camphor & p-cimene) compared to other components present in trace amounts. It contains a high concentration of phenolic compounds (Leung, 1996). Rosemary contains monoterpenes (eteric oils), diterpene phenols (carnosic acid, carnosol, rosmanol, epirosmanol, isorosmanol, methyl carnosate), phenolic acids (rosmarinic acid), flavonols and triterpene acids (ursolic acid, oleanolic acid, butilinic acid) (Riznar et al, 2006). Oil soluble carnosic acid and rosmarinic acid in rosemary extract act to stabilize unsaturated fatty acids and ultimately delay their degradation. It is currently being used widely in the food market as a natural antioxidant (Bauman et al, 1999).

Oregano and sage belong to the same family as Rosemary; hence the method of inhibition of lipid oxidation is similar to that of rosemary (break the free radical chain reaction by donation of a hydrogen atom). The FDA has included Sage on its list of substances Generally Recognized As Safe (GRAS; CFR 182.20) for use as spices, and other natural seasonings and flavorings (Bouaziz et al, 2009). Sage contains a variety of volatile oils, flavonoids (including apigenin, diosmetin and luteolin) and phenolic acids including rosmarinic acid. The effectiveness of sage as a natural antioxidant in meat products has been demonstrated in raw and cooked beef patties (McGovern et al, 2007), refrigerated

stored porcine liver patties (Estevez, Ventanas & Cava, 2007), minced meatballs prepared from turkey meat (Karpinska et al., 2001) and frozen pork patties (McCarthy et al., 2001). Regarding oregano, dietary oregano oil supplementation appears to improve reproductive performance of sows (Allan & Bilkei, 2005), improve growth performance in pigs (Namkung et al., 2004) and broiler chickens (Giannenas et al., 2005), improve meat storage stability after slaughter in poultry, rabbits and sheep and protect against the negative effects of stress on chicken meat quality characteristics (Eileen et al., 2009).

Grape seeds are byproducts of the grape juice and wine industries. Grape seed extract is considered a powerful antioxidant that prevents premature ageing and disease. The seeds contain lipids, protein, carbohydrate and 5-8% polyphenols (depending on the variety of grapes; Chedea et al., 2009) Grape seed polyphenols contain flavan-3-ols such as catechins, epicatechin, gallocatechin, epigallocatechin and epicatechin. In addition, it also contains phenolic acid precursors (Gallic acid), procyanidin dimers, trimers and highly polymerized procyanidins (Chedea et al., 2009). Polyphenols are able to produce stable antioxidant radicals, which are unreactive to propagate the chain and compete with the lipid substrate (Chedea et al, 2009).

The objective of this study was to compare the antioxidant activity of natural antioxidants including grape seed extract and some herbs belonging to the Lamiaciae family: rosemary (Rosmarinus Officinalis), sage (Salvia Officinalis) and oregano (Origanum Vulgare) with commercial synthetic antioxidants like BHT, BHA, propyl gallate and ascorbic acid using the ORAC assay (Davalos et al., 2004; Prior et al., 2003)

3.3 Materials and Methods

3.3.1 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay is measures the antioxidant capacities of biological samples *in vitro* (Davalos et al., 2004). It accomplishes this by measuring the oxidative degradation of fluorescein, after it is mixed with 2,2'-azobis(2-amidino-propane)dihydrochloride (AAPH) which is an azo-initiator compound. These compounds produce the peroxyl radical that damage the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants help protect the fluorescein molecule from the degradative effects of azo-initiator compounds. The degree of protection of the fluorescent substance is quantified using a Fluorometer. The fluorescent intensity decreases as degradation proceeds, and is recorded every 2 min for a total of 120 min. The equipment used for the assay automatically measures and calculates the antioxidant capacity manufactured by Biotek, Roche Diagnostics.

3.3.2 Synthetic and natural antioxidants and chemicals

Grape seed extract (Finest Natural®, distributed by Walgreens Co., 200 Wilmot Rd, Deerfield IL 60015-4616), oregano (McCormick & Co. Inc. Hunt Valley, MD 21031-1100, USA), sage (McCormick & Co. Hunt Valley, MD, USA) and rosemary (McCormick Gourmet Collection, Hunt Valley, MD, USA) were sourced locally. Ascorbic acid, propyl gallate, Butylated Hydroxytoluene and Butylated Hydroxyanisole were sourced from Sigma-Aldrich, St Louis, MO, USA

3.3.3 Sample preparation

Dry samples (5 gm) were pulverized in a spice blender for 1 minute. After homogenizing, aliquots (see below) were weighed using an analytical balance (AG 135 Mettler Toledo, Switzerland). Water-soluble compounds (ascorbic acid, GSE, oregano, sage & rosemary) were extracted with water (procedure below). Liposoluble compounds (BHA, BHT & PG) were dissolved in ethanol (procedure below). Compounds (1.8 gram) were dissolved in 10 ml of either distilled water or ethanol (Capitani et al, 2009). Sage, oregano and rosemary were ground using, then weighed out (1.8 mg) and extracted with water. The ground spices were mixed with deionised water, and sonicated (Bransonic Ultrasonic Cleaner, 2510R-MTH, Bransonic Ultrasonic Corporation) in an ice water bath for 15 min. The mixture was shaken once again then centrifuged (Eppendorf centrifuge, 5417R) in 1.5 ml tubes at 12,000 x g for 2 minutes at 4^0C. The supernatant was used for analysis (Carlsen et al, 2010). Grape seed extract (1.8 mg) was mixed with de-ionized water and centrifuged in 1.5 ml tubes, vortexed for 10 seconds, and sonicated at room temperature for 10 minutes. The mixture was then centrifuged at 4^0C for 30 minutes at 26,000 x g. Supernatants were collected. All samples were prepared one day prior to analyses and stored at -20^0C.

3.3.4 Antioxidant capacity

The method for the ORAC was adapted from published methods (Davalos et al., 2004; Prior et al., 2003). Reagents solutions for fluorescein and azo initiator compound (AAPH) were first prepared using a 75mM phosphate buffer (pH 7.4). The samples BHT, BHA, propyl gallate, ascorbic acid, oregano, sage, rosemary and grape seed extract were

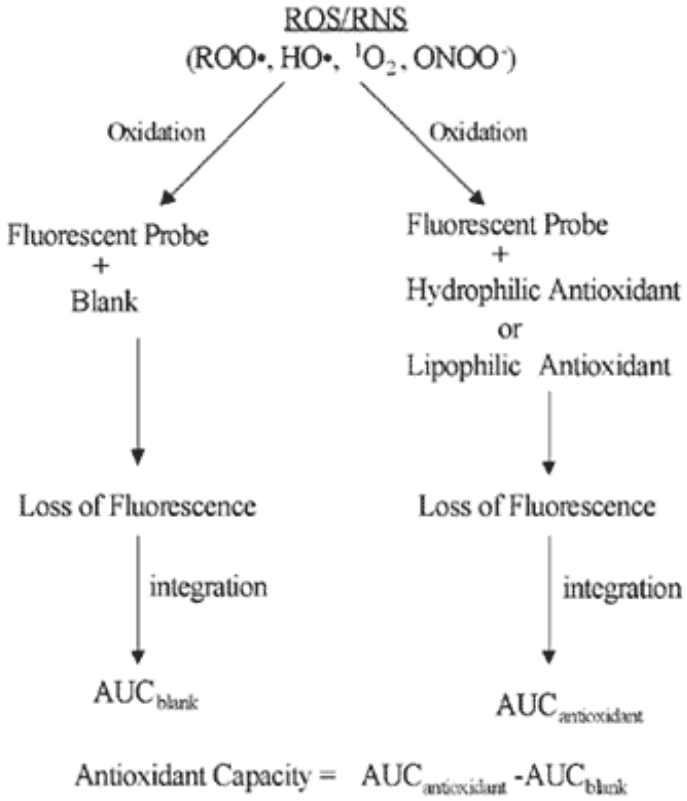
weighed out in triplicate. The Trolox standards dissolved in 75 mM phosphate buffer at concentrations ranging from 4 µM to 160 µM. A 75mM phosphate buffer blank was also measured in triplicate. For The blank, samples and standards (20µL) were added to a 96 well black-walled plate. Then 120 µL of fluorescein was added to each well and incubated for 15 minutes at 37^0C. After incubation, 60uL AAPH was added to each well. Fluorescence was determined immediately in a fluorescent FLx800tbi Synergy 2 multi-well plate reader, (Biotek, Winooski, VT), at 37^0C, sensitivity 60, every 2 min for 120 min with excitation at 485 nm and emission at 582 nm (Figure 3.1). The AAPH generates free radicals which react with the fluorescein to produce a non-fluorescent product. The loss of fluorescence is measured over time and the area is calculated using the following equation:

$$AUC = 0.5 + f_1/f_0 + f_1/f_0 + \ldots + 0.5(f_n/f_0)1$$

$$\text{Net AUC} = AUC_{sample} - AUC_{blank}$$

Where, AUC = Area under the curve, f_1 is the fluorescence of the first reading (2 min); f_0 is the fluorescence at time zero and f_n is the total number of fluorescence readings. Results are expressed as Trolox µM eq. using the standard curve y=0.148*x + 2.73, R=0.963.

Figure 3.1 Principle of the ORAC Assay (http://brunswicklabs.com)



The ORAC assay depends on the free radical damage to a fluorescent probe through the change in its fluorescence intensity. The change of fluorescence intensity is an index of the degree of free radical damage. In the presence of antioxidant, the inhibition of free radical damage by an antioxidant, which is reflected in the protection against the change of probe fluorescence in the ORAC assay, is a measure of its antioxidant capacity against the free radical. The uniqueness of the ORAC assay is that the reaction is driven to completion and the quantitation is achieved using "area under the curve" (AUC) (Figure 3.2). In particular, the AUC technique allows ORAC to combine both inhibition time and inhibition percentage of the free radical damage by the antioxidant into a single quantity.

The significance of ORAC is that the assays are performed *in vitro,* in a test tube, and therefore, do not determine the bioavailability within the body. A high ORAC value indicates that the tested sample possesses a high potency of antioxidant activity chemically.

Figure 3.2 Antioxidant activity of samples expressed as the net area under the curve (AUC); http://brunswicklabs.com**)**

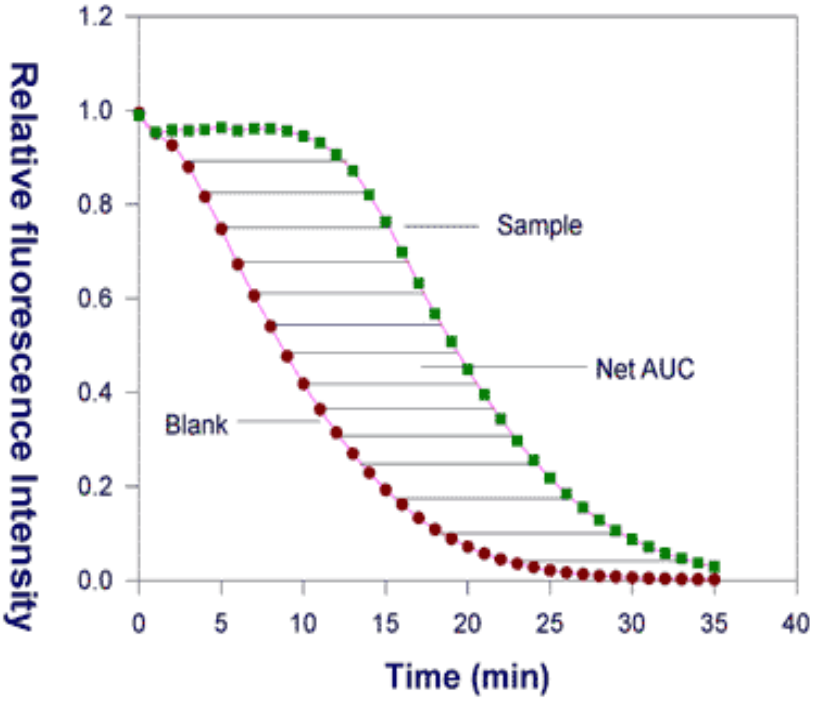
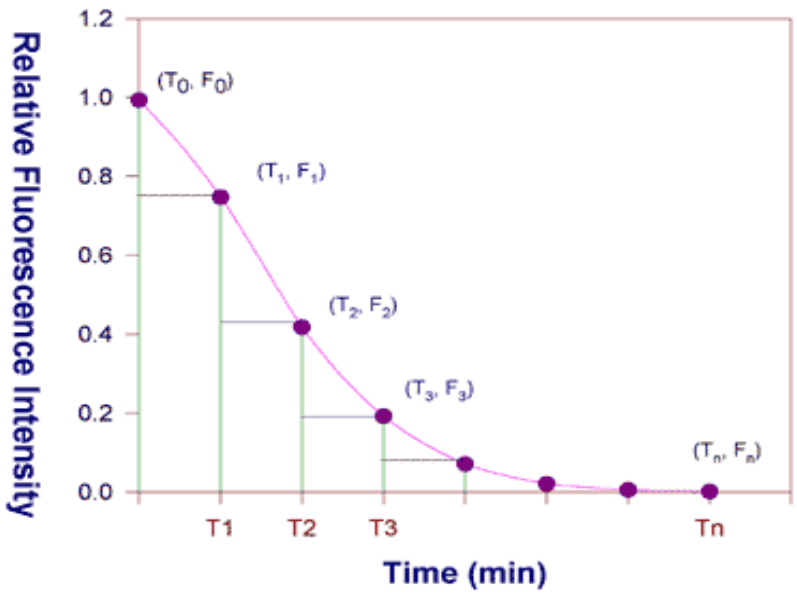


Figure 3.3 Calculation of ORAC values (http://brunswicklabs.com)



3.4 Results and Discussion

Table 3.1 shows the antioxidant activity of all the samples evaluated by the ORAC assay and expressed as Trolox Equivalents. The highest antioxidant activity was observed for the grape seed extract sample (359.75 µM TE), while the lowest was observed for Butylated Hydroxyanisole, Propyl gallate and rosemary also showed higher antioxidant potential with ORAC values above 300 µmol TE/g. ORAC values obtained for Ascorbic acid and Sage were between 250-300µ mol TE/g while lowest values were obtained for Butylated Hydroxytoluene (28.50 µM TE). Based on the high ORAC values obtained for grape seed extract, we can conclude that byproducts of the wine/grape industry have antioxidant potential comparable or better than those present in synthetic counterparts.

Table 3.1 Mean ORAC equivalents obtained for selected varieties of natural and synthetic antioxidants

Sample	Scientific name	Solvent	ORAC µmol TE/g
BHT	Butylated Hydroxytoluene	Ethanol	28.5
BHA	Butylated Hydroxyanisole	Ethanol	348.90
PG	Propyl Gallate	Ethanol	343.90
AA	Ascorbic Acid	Water	256.05
GSE	***Vitis Vinifera***	**Water**	**359.75**
ORG	*Origanum Vulgare*	Water	329.16
Sage	*Salvia Officinalis*	Water	267.56
Rosemary	*Rosemarinus officinalis*	Water	307.74

An inclusive evaluation of all possible antioxidant activity would require a combination of assay methods. Variations in activity of particular extracts are expected, and may be influenced by such factors as growth conditions, stability of the specific antioxidant components and variations in extractability. The cell material for this study was processed by a standard method, but it was not optimized to either preserve antioxidant activity or to individually optimize conditions for extraction from each source

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Chapter 4. Effect of grape seed extract on oxidative, color and sensory stability of a pre-cooked, frozen, re-heated beef sausage model system

4.1 Abstract

To compare grape seed extract (GSE) to common antioxidants in a pre-cooked, frozen, stored meat model system sausage was manufactured from lean beef (70%), pork fat (28%), and salt (2%). Antioxidants added for comparison with control included grape seed extract (100, 300, 500 ppm), ascorbic acid (AA, 100 ppm of fat) and propyl gallate (PG, 100 ppm of fat). Product was formed into rolls, frozen, sliced into patties, cooked on a flat griddle to 70C, overwrapped in PVC, and then frozen at –18C for 4 months. GSE- and PG-containing samples retained their fresh cooked beef odor and flavor longer ($p<0.05$) than controls during storage. Rancid odor and flavor scores of GSE-containing samples were lower ($p<0.05$) than those of controls after 4 months of storage. The L* value of all samples increased ($p<0.05$) during storage. Thiobarbituric acid reactive substances (TBARS) of the control and AA-containing samples increased ($p<0.05$); those of GSE-containing samples did not change significantly ($p>0.05$) over the storage period.

For details, please refer to:

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Chapter 5. Summary

Intrinsic antioxidants in meat like tocopherol or carnosine are capable of controlling oxidative reactions in a live animal. However, they are ultimately not effective in ready to eat meat products available in market. This is because they lose most of their effectiveness once animal is killed and muscle tissue is damaged during meat processing. Therefore the use of natural and synthetic antioxidants is essential to maintain quality of meat products and extend their shelf life. Natural and synthetic antioxidants do this by inhibiting lipid oxidation. Synthetic phenolic antioxidants like BHA, BHT and PG effectively inhibit warmed over flavor development. However due to the safety and toxicity concerns with synthetic antioxidants, there is an increased interest in application of natural antioxidants for inhibiting lipid oxidation in meats instead of synthetic antioxidants.

Grape seed extract is a polyphenol rich antioxidant that has shown potential of being a powerful antioxidant in meat products without adding any of its flavors to final product. A critical concern with grape seed however is that it also has potential of affecting the color of product when added at higher concentrations because it is rich purple colored due to the presence of many proanthocyanidins.

The goal of this research was to compare three levels of grape seed extract (100, 300 & 500 ppm) to commonly used antioxidants (BHA, BHT, PG, AA) in a precooked, frozen, stored beef model system and to also compare the oxygen radical absorbance capacity of

GSE with some other select antioxidants (sage, oregano, Rosemary, PG, BHT, BHA, AA). The beef sausage model system was manufactured using lean beef (70%), pork fat (28%), and salt (2%).

Results indicated that the grape seed extract was as efficient as PG in assisting the samples in retaining their fresh cooked beef odor. The rancid and grassy odor scores of samples containing 300 and 500 ppm GSE remained constant over the storage period. Rancid flavor scores for 300ppm and 500ppm GSE samples remained below 4 over the storage period, while those for control and ascorbic acid increased from 0 to about7 at the end of storage. One of main concern with GSE is the color it can transfer to final product because it is dark purple in color itself. GSE containing samples were dark in color at the beginning of the study. However with time, the lightness (L*) values of all samples increased during 4^{th} month of storage period. GSE was also efficient at maintaining redness (a*) values through the whole storage period. The TBAR values of GSE containing samples remained either fairly stable or decreased through 4 months of storage. GSE at lower concentrations (100 and 300 ppm) protected samples against lipid oxidation as well or better than propyl gallate. Based on all the sensory characteristics, it can be concluded that GSE at concentrations of 100 and 30 ppm were as efficient or more efficient at maintaining the quality of product through the period of storage. The polyphenols present in GSE prevent lipid oxidation by free radical scavenging and metal chelation. Future studies would be required to test the efficacy of GSE in other food systems.