SHELF LIFE EXTENSION OF SEED BUTTER MADE WITH SESAME, SUNFLOWER AND PUMPKIN SEEDS

THESIS

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By

Yung-Hsin Chien, B.S.
Graduate Program in Food Science and Technology

The Ohio State University

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Master's Examination Committee:

Dr. Melvin Pascall, Advisor

Dr. Hua Wang

Dr. Farnaz Maleky
Abstract

This study investigated the effects of natural antimicrobials, antioxidants and stabilizers on the microbiological, chemical and physical properties of seed butter made with sesame, sunflower and pumpkin seeds.

The first part of this thesis (Chapter 2) investigated the antimicrobial effects of grape seed extract (GSE) and cinnamaldehyde against *Salmonella enterica* and *Listeria innocua* in the seed butter. The seed butter samples added were 5, 10 and 15% GSE and 0.1, 1.0 and 1.5% cinnamaldehyde, respectively. *S. enterica* and *L. innocua* were inoculated into the seed butter and the samples stored at 25°C. The population of *S. enterica* and *L. innocua* were enumerated after 0, 1, 3, 7 and 9 days of storage. The results showed that GSE at 10 and 15% significantly (*p*<0.05) reduced both *S. enterica* and *L. innocua* after one day of storage when compared with the control, which was without added antimicrobial agents. Cinnamaldehyde also showed significant (*p*<0.05) effects against *S. enterica*, but *L. innocua* appeared resistance to cinnamaldehyde. The reduction of *L. innocua* in cinnamaldehyde fortified seed butter was not significant (*p*>0.05) when compared with the control. In conclusion, GSE was an effective antimicrobial agent against *S. enterica* and *L. innocua*, but cinnamaldehyde was not able to reduce *L. innocua* in the seed butter.
The second part of this thesis (Chapter 3) investigated the stabilizing effects of hydrogenated rapeseed oil (HRO) and palm kernel oil (PKO) on the texture and oil separation properties of the seed butter. One, 2 and 3% HRO and PKO were added to the seed butter, respectively, and the samples were stored at 25°C. The texture of the seed butter was analyzed after 0, 7, 14, 21, 28, 35, 42 and 49 days of storage. The oil separation was determined after 0, 1, 3, 5, 7, 14, 21, 28, 35 and 42 days of storage. The results showed that after 28 days, HRO at 2 and 3% significantly (p<0.05) prevented an increase in hardness in the seed butter samples when compared with the control, which was without adding stabilizers. For preventing oil separation, 3% HRO stabilized seed butter showed significantly (p<0.05) less oil separation when compared with the control after three days of storage at 25°C. When compared with the control, PKO showed significant (p<0.05) effects on stabilizing the hardness of the seed after 35 days of storage, but it did not have significant (p>0.05) effects on maintaining the cohesiveness and adhesiveness. For preventing oil separation, PKO showed insignificant (p>0.05) effects on stabilizing the oil in the seed butter at 25°C. In conclusion, HRO was an effective stabilizer by preventing excessive hardness and oil separation in the seed butter, but PKO was found to be ineffective as a stabilizer.

The third part of this thesis (Chapter 4) investigated the antioxidant ability of α-tocopherol in seed butter oil. For testing the antioxidant ability of α-tocopherol, 100, 400, 700 ppm α-tocopherols were added to oil extracted from the seed butter. The seed butter oil in this study contained 235.62 – 279.88 ppm α-tocopherol. These prepared samples were stored at 30°C and 50°C in the dark. The peroxide value (PV), p-anisidine value
(pAV) and totox value (TV) were determined after 0, 4, 7, 14, 20 and 31 days of storage. The results showed that temperature significantly (p<0.05) influenced the PV, pAV and TV of the seed butter oil. The addition of α-tocopherol did not show significant (p>0.05) antioxidant ability when compared with the control. However, the TV in seed butter oil with addition of 400 ppm α-tocopherol was significantly higher than the control stored at 30°C on day 31 of storage. Furthermore, at 50°C, the TVs of 400 and 700 ppm α-tocopherol fortified seed butter oils were significantly higher than the control after 31 days of storage. This result showed that α-tocopherol increased the rate of lipid oxidation instead of performing an antioxidant function in the seed butter oil.
Dedication

To my family and Dr. Yi-Chung Fu
Acknowledgments

I would like to thank my advisor Dr. Melvin Pascall for his patience, guidance, and support throughout this graduate study. I appreciate the opportunity he gave me to pursue a Master’s degree at The Ohio State University. I would also like to thank Dr. Hua Wang and Dr. Farnaz Maleky for their help and for being my committees members. I want to thank Dr. Jaesung Lee for his assistance and guidance during my research.

My gratitude also goes to my lab mates and all my friends in this field. Their knowledge and experiences helped me to complete this work. I sincerely thank them for all their patience, encouragement and interest.

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Vita

October 11 1990.............................................Born – Keelung City, Taiwan

2008 – 2012.............................................B.S. Food Science and Technology

National Chung Hsing University, Taiwan

Fields of Study

Major Field: Food Science and Technology
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CHAPTER 1

LITERATURE REVIEW

1.1. Seed butter processing

Seed butter is a semi-perishable food made mainly from pulverized edible seeds, sweeteners, stabilizers and salt (sodium chloride). Its principle processing method include roasting, grinding, chilling and tempering (MacDonald, Galloway, & Kakuda, 1985). Roasting, one of the main procedures in seed butter processing, aims to give the product its optimal color and desirable flavor (MacDonald et al., 1985). The seeds can be roasted by either batch or continuous methods. The benefits of the batch method include separately roasting variable seeds and efficiently removing moisture. On the other hand, the continuous method can provide saving in labor cost, produce uniformly roasted seeds, be smoother in operations and can reduce spillage of the seeds (Woodroof, 1983). During roasting the seeds are sent through an oven at 160°C for 10 to 40 minutes (MacDonald et al., 1985). Grinding, the easiest and the most critical step, reduces the size of the seeds and provides the finished product with a uniform consistency. In the commercial production of seed butter, the process includes milling, grinding and homogenization. Generally, the milling process consists of two linked attrition mills that gradually grind...
the seeds into a creamy butter. For the second milling, the clearance between plates is usually between 0.003 to 0.032 inches. To prevent the formation of a burnt flavor the temperature should not be higher than 82°C. Grinding also occurs as a two-stage process designed to achieve a fine paste in a step-by-step manner. A large number of blades (156-210 blades) and high speeds (6,000 – 12,000 rpm) are applied in this process. Homogenization is seen as a finishing process which gives the seed butter a fine and creamy texture.

Before the seed butter is filled into containers, chilling is an important process that aims to reduce the temperature of the finished product to 32 – 43°C, depending on the stabilizer that is used. Tempering is the last step, and it is storing the finished seed butter up to 72 hours at 10 – 38°C without disturbance until the proper crystallization of the fat is formed. This is a general step that helps to stabilize the product (MacDonald et al., 1985; Woodroof, 1983).

1.2. Seed butter spoilage

During storage and distribution, one or more quality attributes of food products would decline due to conditions such as temperature, humidity, oxygen and light. Spoilage may make foods unacceptable or harmful to consumers. The main reasons of food spoilage are chemical, physical and microbial changes (Man & Jones, 1994). These changes in seed butter will be discussed in the following sections.
1.2.1. Microbial spoilage

Microorganisms are everywhere, even in extreme environments. Many factors can influence microbial growth and survival. These are categorized as intrinsic and extrinsic factors. Intrinsic factors include nutrients, pH value, redox potential ($E_h$) and water activity. Nutrients in foods provide compounds that microorganisms cannot synthesize on their own but are needed as sources of energy for growth. pH significantly influences the activity and stability of macromolecules like enzymes and further affects the metabolism of microorganisms. Generally, the optimal pH ranges for bacteria, yeasts and filamentous fungi are 6.0 – 8.0, 4.5 – 6.0 and 3.5 – 4.0, respectively. Redox potential ($E_h$) is the tendency to accept or donate electrons in an oxidation – reduction (redox) reaction. Obligate or strict aerobic bacteria tend to grow in high $E_h$ environments while obligate anaerobes prefer low or negative $E_h$ environments. The water activity ($a_w$) of a substrate that contains microorganisms makes the movement of water either enter or leave the cell’s cytoplasm. When $a_w$ is below 0.6, although most microorganisms will stop growing, some may adapt to the environment and survive (Adams & Moss, 2008). According to Beuchat et al. (2013), some foodborne pathogens are viable in low-$a_w$ food for months to years.

Extrinsic factors include relative humidity, temperature and gaseous atmosphere. Relative humidity is interrelated with water activity, and it is the measure of water activity of the gaseous phase. When a food product with low water activity is stored in a higher relative humidity environment, the water would migrate into the food and increase its water activity. On the other hand, the water in the food would be lost when the relative
humidity is lower than the water activity of the food. Microorganisms can grow at a wide range of temperature from about -8 to 100°C in liquid water at atmospheric pressures. They can be categorized into four groups as thermophiles, mesophiles, psychrophiles and psychrotrophs with optimal growth temperature range of 55 – 75°C, 30 – 40°C, 12 – 15°C, and 25 – 30°C, respectively (Adams & Moss, 2008).

Microbial spoilage can occur in the form of surface slime or colonies, texture degradation, and gas forming, pigment changes, off-odors and flavors (Adams & Moss, 2008). Also, some foodborne pathogens produce toxic compounds and can cause human diseases. Prior to seed butter processing, the seeds can be contaminated if held in inappropriate storage conditions. For instance, fungi may contaminate the seeds and produce mycotoxins such as aflatoxin, the most toxic mycotoxin associated with seeds. This can cause aflatoxicosis or aflatoxin poisoning, with symptoms that include gastrointestinal problems and liver lesions (Chang, Sreedharan, & Schneider, 2013). In poor sanitary environments, foodborne pathogens can also cross-contaminate processed seed butter. As a low-a_w food, seed butter does not support the growth of microorganisms, but some of them can remain viable for months or even years (Beuchat et al., 2013; Podolak, Enache, Stone, Black, & Elliott, 2010). *Clostridium botulism, Salmonella* and *Listeria* have been known to be viable in peanut butter (Burnett, Gehm, Weissinger, & Beuchat, 2000; He, Guo, Yang, Tortorello, & Zhang, 2011; Kenney & Beuchat, 2004; Park, Oh, & Kang, 2008; Podolak et al., 2010; Sheppard et al., 2012).
1.2.1.1. Selected microorganisms

**Salmonella**

*Salmonella* infection is usually called salmonellosis, and would cause diarrhea, fever, abdominal cramps, and even death without proper clinical treatment (Scallan et al., 2011). *Salmonella* is a gram-negative, non-sporeforming rod and is a facultative anaerobe. It can grow at temperatures ranging from 5 to 47°C with an optimum at 37°C. The minimum water activity ($a_w$) for *Salmonella* to grow is around 0.93, but they can adapt to low water activity and survive in intermediate moisture foods (Adams & Moss, 2008).

For *Salmonella* contamination in peanut butter, several studies have shown it can occur. Park et al. (2008) also found that *Salmonella* was viable in peanut butter at 22°C for 2 weeks. Furthermore, He et al. (2011) showed that *Salmonella* only dropped 3.2 – 5.0 log CFU/g from 8 log CFU/g in peanut butter at 25°C after one month. With this ability of the organism to survive in low-water activity foods, outbreaks caused by *Salmonella* contaminated peanut butter was first reported in Australia in 1996 (Scheil, Dalton, Murray, & Wilson, 1998). In the U.S., the first outbreak caused by *Salmonella* in peanut butter occurred in 2006, and at least 628 consumers from 47 states were infected (CDC, 2007). One year later, another outbreak occurred and 529 consumers from 43 states were infected by *Salmonella* in peanut butter and peanut butter crackers (CDC, 2009). In addition to these widespread outbreaks occurred in 2012 and 41 people from 20 states were infected (Macdonald et al., 2013). In 2014, six more consumers from 5 states were also infected (CDC, 2014). Not only was peanut butter the substrate, sesame paste
was also contaminated by *Salmonella* in 2013 and 16 consumers from 5 states were infected (CDC, 2013). Most of the outbreaks were presumed to be caused by insufficient hygiene, cross-contamination, processing or storage at inadequate locations, contaminated equipment, and contamination by personnel (Podolak et al., 2010). Therefore, it appears that GMPs may be a necessary hurdle to prevent seed butter from being contaminated by *Salmonella* (Chang et al., 2013).

*Listeria*

*Listeria* is a gram positive, facultative anaerobe, non-sporeforming bacteria. Six species have been recognized, with *Listeria monocytogenes* being the main pathogenic species (Jay et al., 2005; Adams and Moss, 2008). *L. monocytogenes* seldom infects non-pregnant healthy people, but it is lethal in individuals with neoplasm, AIDS, alcoholism, diabetes, cardiovascular disease, renal transplant, or corticosteroid therapy (Jay et al., 2005). *L. monocytogenes* is widely distributed in the environment, and it is able to grow on most non-acid foods. This provides it many opportunities to contaminate food products through the food chain. Previous studies have shown that it could survived in low-water activity foods such as peanut butter at 22°C for one year (Kenney and Beuchat, 2004; Koseki et al, 2015). Therefore, *Listeria* is a potent pathogen contaminating oilseed spreads and infect the consumers in the future.
1.2.2. Lipid Oxidation

Edible oils in seed butter can be oxidized during processing, storage and distribution via autoxidation and photosensitized oxidation. Autoxidation is the process in which triplet oxygen (\(^3\text{O}_2\)) reacts with the radical form of acylglycerols in oils and produces lipid hydroperoxides with conjugated dienes. This reaction can be catalyzed by free fatty acids (FFAs), mono- and diacylglycerols, metals, and thermally oxidized compounds (Choe & Min, 2006). This type of lipid oxidation includes three steps - initiation, propagation and termination:

Initiation: \(\text{RH} \rightarrow \text{R}^· + \text{H}^·\)
Propagation: \(\text{R}^· + ^3\text{O}_2 \rightarrow \text{ROO}^·\)
\(\text{ROO}^· + \text{RH} \rightarrow \text{ROOH} + \text{R}^·\)
Termination: \(\text{ROO}^· + \text{R}^· \rightarrow \text{ROOR}\)
\(\text{R}^· + \text{R}^· \rightarrow \text{RR}\)

\(\text{R}: \text{lipid alkyl}\) \hspace{1cm} (Choe & Min, 2006)

In photosensitized oxidation, sensitizers such as chlorophylls are excited to the triplet state with light, then they may react with hydrogen or an electron and produce radicals (type I). In other pathways, the sensitizers react with \(^3\text{O}_2\) and produce superoxide anion, or transfer the energy to \(^3\text{O}_2\) and form \(^1\text{O}_2\) (type II) (Fig. 1.1). Depending on the sensitizer types, substrates, concentration of substrates and oxygen, the rate of the type I or II reactions would be varied. \(^1\text{O}_2\) can directly react with double bonds in edible oils to produce conjugated and non-conjugated hydroperoxides without forming alkyl radicals (Choe & Min, 2006). Lipid hydroperoxides are stable at room temperature, but at high temperatures they easily decompose to alkoxy radicals then form aldehydes, ketones acids, esters, alcohols, and short-chain carbonyls (Choe & Min, 2006) that most are responsible for off-odors and flavors.
Seed butter contains 40 – 45% oil in general, of which 75 – 80% are made with unsaturated fatty acids (USDA. 1984; USDA, 2009; USDA, 2010). This makes the oil of seed butter susceptible to oxidation during storage (St Angelo, 1996). Therefore, lipid oxidation is a major cause of off-flavor and rancidity in these intermediate moisture foods. The compounds in oxidized oils, especially those in the early oxidation stages, have very low threshold values and give the seed butter unacceptable odors (Labuza & Dugan, 2009). Lipid oxidation also reduces the nutritional quality of the product and could produce toxic compounds (Frankel, 1991).

1.2.3. Texture

Texture is one of the main qualities of food products. The qualities are appearance, flavor, texture and nutrition. Texture is perceived by the senses directly, so it is extremely
important for a food product to fulfill consumers’ enjoyment (Bourne, 2002). During storage, impropriate environments may degrade the texture of foods. When the relative humidity is low, vegetables would lose crispness and increase the rate of senescence reactions, and some foods like dried fruits, candies, cakes and breads would lose moisture and harden in texture. On the contrary, high humidity could cause the texture of potato chips, dried or fried snacks to become too soft. This also makes some crystalline foods such as salt, sugar, dried meal and dried drink mixes get sticky and become caked. Temperature fluctuation can lead to the melting of fats in candies and formulated foods. Also, it can cause moisture loss and changes to the crystallization structure of frozen foods. For instance, ice cream would become sandy to the taste, and meats may get freezer burn if they lose too much moisture at freezing temperatures. Furthermore, temperature fluctuations could influence the stability of the emulsion state in foods such as mayonnaise, margarine, salad dressing or dessert toppings (Labuza, 1982; Man & Jones, 1994).

The characteristics of texture are widely diverse depending on the types of foods and the human demand on the these foods (Bourne, 2002). For food such as spreads, factors such as hardness, cohesiveness and adhesiveness are major texture properties considered by consumers and manufacturers (Radočaj, Dimić, Diosady, & Vujasinović, 2011; Resurreccion, 2002; Totlani & Chinnan, 2007). Moreover, texture is sometimes more important than flavor and color (Di Monaco, Giancone, Cavella, & Masi, 2008). Hardness is related to spreadability and could be affected if oil separation occurs in seed butter. Hardness is generally used to describe the quality of the spread. For instance,
spreadability decreases when the hardness increases (Radočaj, Dimić, & Vujasinović, 2012). Labuza and Schmidl (1985) recommended that the seed butter should be removed from retail trade if nutrient value, flavor, color, and firmness are no longer acceptable to consumers.

1.3. Methods to extend the shelf life of seed butter

1.3.1. Antimicrobial agents

Antimicrobial agents are, “substances used to preserve food by preventing the growth of microorganisms and subsequent spoilage, including fungistatic, mold and rope inhibitors” as defined by the U.S. Food and Drug Administration (FDA; 21CFR 170.3(o)(2)). Extending shelf life and maintaining the quality through the limitation of microbial growth are the major functions of antimicrobial agents (Davidson, Sofos, & Branen, 2005). They can be microbicidal and kill target microorganisms or they can be microbiostatic and inhibit their growth (Adams & Moss, 2008). Antimicrobial agents may be classified as synthetic and natural types (Table 1.1). With increasing demand of reducing food additives, manufacturers have started to explore the use of natural alternatives or combinations of natural and synthetic compounds to replace synthetic antimicrobial agents (Davidson et al., 2005). Natural antimicrobial agents can be obtained from animals, microorganisms and plants (Davidson et al., 2005). Extracted oils and isolated compounds of plants, herbs and spices contain large number of antimicrobial compounds with the ability to inhibit various types of bacteria, yeasts and molds. Table 1.2 has a list of highly recognized plants, herbs, and spices that have been reported as
sources of antimicrobial agents. In the list, cinnamon and clove exhibit strong inhibitory effects, and allspice performed medium inhibitory effects. To choose the proper antimicrobial agent, several points must be considered. These are: (1) the target microorganism, (2) physical properties of the antimicrobial agent such as polarity and boiling point, as examples (3) reaction with the food ingredients, to include the influence and activity of selected antimicrobial agent and the possible formation of undesirable flavors, odors and colors, (4) toxicity, and (5) sensory effects.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Microbial Target</th>
<th>Primary Food Applications</th>
<th>CFR Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid, acetates, diacetates, dehydroacetic acid</td>
<td>Yeasts, bacteria</td>
<td>Baked goods, condiments, confections, dairy products, fats/oils, meats, sauces</td>
<td>184.1005, 182.6197, 184.1754, 184.1185, 184.1721, 172.130</td>
</tr>
<tr>
<td>Benzoic acid, benzoates</td>
<td>Yeasts, molds</td>
<td>Beverages, fruit products, margarine</td>
<td>184.1021, 184.1733</td>
</tr>
<tr>
<td>Dimethyl dicarbonate</td>
<td>Yeasts</td>
<td>Beverages</td>
<td>172.133</td>
</tr>
<tr>
<td>Lactic acid, lactates</td>
<td>Bacteria</td>
<td>Meats, fermented foods</td>
<td>184.1061, 184.1207, 184.1639, 184.1768</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Bacteria</td>
<td>Meats</td>
<td>GRAS Notice No. GRN 000067</td>
</tr>
<tr>
<td>Lysozyme</td>
<td><em>Clostridium botulinum</em>, other bacteria</td>
<td>Cheese, casings for frankfurters, cooked meat, and poultry products</td>
<td>184.1550; GRAS Notice N. GRN 000064</td>
</tr>
<tr>
<td>Natamycin</td>
<td>Molds</td>
<td>Cheese</td>
<td>172.155</td>
</tr>
<tr>
<td>Nisin</td>
<td><em>Clostridium botulinum</em>, other bacteria</td>
<td>Cheese, casings for frankfurters, cooked meat, and poultry products</td>
<td>174.1538; GRAS Notice No. GRN 000065</td>
</tr>
<tr>
<td>Nitrite, nitrate</td>
<td><em>Clostridium botulinum</em></td>
<td>Cured meats</td>
<td>172.160, 172.170, 172.175, 172.177</td>
</tr>
<tr>
<td>Parabens (alkyl esters (propyl, methyl, heptyl) of (p)-hydroxybenzoic acid)</td>
<td>Yeasts, molds, bacteria (Gram positive)</td>
<td>Beverages, baked goods, syrups, dry sausage</td>
<td>184.1490, 184.1670, 172.145</td>
</tr>
<tr>
<td>Propionic acid, propionates</td>
<td>Molds</td>
<td>Bakery products, dairy products</td>
<td>184.1081, 184.1221, 184.1784</td>
</tr>
<tr>
<td>Sorbic acid, sorbates</td>
<td>Yeasts, molds, bacteria</td>
<td>Most foods, beverages, wines</td>
<td>182.3089, 182.3225, 182.3640, 182.3795</td>
</tr>
<tr>
<td>Sulfites</td>
<td>Yeasts, molds</td>
<td>Fruits, fruit products, potato products, wines</td>
<td>Various</td>
</tr>
</tbody>
</table>

Table 1.1. Traditional or regulatory (U.S. Food and Drug Administration) food antimicrobials. (Davidson et al., 2005).
<table>
<thead>
<tr>
<th>Plant (scientific Name)</th>
<th>Major Components (in descending order)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allspice (<em>Pimenta dioica</em>)</td>
<td>Eugenol, methyl ether cineol</td>
</tr>
<tr>
<td>Basil (<em>Ocimum basilicum</em>)</td>
<td>d-linalool, methyl chavicol, eugenol, cineol, geraniol</td>
</tr>
<tr>
<td>Black pepper (<em>Piper nigrum</em>)</td>
<td>Monoterpenes, sesquiterpenes</td>
</tr>
<tr>
<td>Bay (<em>Laurus nobilis</em>)</td>
<td>Cineol, 1-linalool, eugenol, geraniol</td>
</tr>
<tr>
<td>Caraway seed (<em>Carum carvi</em>)</td>
<td>Carvone, limonene</td>
</tr>
<tr>
<td>Celery seed (<em>Apium graveolens</em>)</td>
<td>d-limonene</td>
</tr>
<tr>
<td>Cinnamon (<em>Cinnamomum zeylanicum</em>)</td>
<td>Cinnamic aldehyde, l-linalool, p-cymene, eugenol</td>
</tr>
<tr>
<td>Clove (<em>Syzygium aromaticum</em>)</td>
<td>Eugenol, cariofilene</td>
</tr>
<tr>
<td>Coriander (<em>Coriandum sativum</em>)</td>
<td>d-linalool, d-α-pinene, β-pinene</td>
</tr>
<tr>
<td>Cumin (<em>Cuminum cyminum</em>)</td>
<td>Cuminaldehyde, p-cymene</td>
</tr>
<tr>
<td>Fennel (<em>Foeniculum vulgare</em>)</td>
<td>Anethole</td>
</tr>
<tr>
<td>Galic (<em>Allium sativum</em>)</td>
<td>Diallyl disulfide, diethyl sulfide, diallyl trisulfide, allicin</td>
</tr>
<tr>
<td>Lemongrass (<em>Cymbopogon citratus</em>)</td>
<td>Citral, geraniol</td>
</tr>
<tr>
<td>Marjoram (<em>Origanum majorana</em>)</td>
<td>Linalool, cineol, methyl chavicol, eugenol, terpineol</td>
</tr>
<tr>
<td>Mstard (<em>Brassica hirta B. juncea, B. nigra</em>)</td>
<td>Allyl-isothiocyanate</td>
</tr>
<tr>
<td>Onion (<em>Allium cepa</em>)</td>
<td>d-n-propyl disulfide, methyl-n-propyl disulfide</td>
</tr>
<tr>
<td>Oregano (<em>Origanum vulgare</em>)</td>
<td>thymol, carvacrol, α-pinene, bornyl acetate</td>
</tr>
<tr>
<td>Parsley (<em>Petroselinum crispum</em>)</td>
<td>α-pinene, fenol-eter-apioi</td>
</tr>
<tr>
<td>Rosemary (<em>Rosmarinus officinalis</em>)</td>
<td>Borneol, cineol, camphor, α-pinene, bornyl acetate</td>
</tr>
<tr>
<td>Sage (<em>Salvia officinalis</em>)</td>
<td>Thujone, cineol, borneol, thymol, eugenol</td>
</tr>
<tr>
<td>Tarragon (<em>Artemisia dracunculus</em>)</td>
<td>Methyl chavicol, anethole</td>
</tr>
<tr>
<td>Thyme (<em>Thymus vulgaris</em>)</td>
<td>thymol, carvacrol, 1-linalool, geraniol, p-cymene</td>
</tr>
<tr>
<td>Vanilla (<em>Vanilla planifolia, V. pompona, V. tahilensis</em>)</td>
<td>Vanillin, canilllic, p-hydroxbenzoic, p-coumaric acids</td>
</tr>
</tbody>
</table>

Table 1.2. Selected plants and their major antimicrobial compounds. (Davidson et al., 2005).
1.3.1.1. Selected antimicrobial agents

Grape seed extract

Grape seed extract (GSE) is a derivative of grape seeds, agro-industrial waste from wine, grape juice and other food processing industries. Grape seed extract contains about 500 – 600 mg/g total phenolic compounds (Baydar, Sagdic, Ozkan, & Cetin, 2006) with flavan-3-ol such as (+)-catechin and (-)-epicatechin (Fig. 1.2) that have been shown to have antimicrobial activities (Perumalla & Hettiarachchy, 2011). Catechin inhibits microorganisms by deteriorating the bilayer membrane and breaking down the cell structure and function, eventually killing the cell. Epicatechin may perform its inhibitory effect by changing the osmotic pressure of the cell, then destructing the cytoplasmic membrane and making the cell component leak till death (Perumalla & Hettiarachchy, 2011). Studies have reported that GSE performed significant inhibitory effects against Campylobacter spp., Salmonella enteric ser. Enteritidis, S. enterica ser. Typhimurium, Staphylococcus aureus, and Listeria monocytogenes (Al-Habib, Al-Saleh, Safer, & Afzal, 2010; Baydar et al., 2006; Rhodes, Mitchell, Wilson, & Melton, 2006; Silván et al., 2013). Moreover, the antimicrobial activity of GSE is not influenced by pH, and the active phenolic compound is non-pigmented (Rhodes et al., 2006). These benefits would significantly increase the applicability of GSE in seed butter. However, there are few studies about GSE being used as a natural antibacterial agent in seed butter.
Cinnamaldehyde

Cinnamaldehyde is widely used in food products as a flavoring agent, and it is the main and active compound in cinnamon oil. Due to consumers’ increasing concerns about food safety, shelf life extension and food quality improvement, the use of essential oil extracts as natural antimicrobial agents meets a marketing need (Inouye, Takizawa, & Yamaguchi, 2001; Lu, Ding, Ye, & Ding, 2011). Cinnamaldehyde can be directly added to food product during processing, or could be added to the packaging material (de Oliveira, Brugnera, do Nascimento, Batista, & Piccoli, 2012; Jo et al., 2015; Ravishankar et al., 2010; Ravishankar et al., 2012). Cinnamaldehyde has shown effectiveness against *Salmonella enterica* ser. Typhimurium, *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli* O157:H7 (de Oliveira et al., 2012; Kim, Park, & Park, 2004; Nostro et al., 2012; Ravishankar et al., 2012; Zhou et al., 2006). The most important feature of cinnamaldehyde as a potential antimicrobial agent is its hydrophobicity. This means it can attack bacteria cell membrane and mitochondria, destructing the cell structure and making them more permeable (Knobloch et al., 1986; Sikkema et al., 1994). This hydrophobicity of cinnamaldehyde makes it a suitable natural antimicrobial agent in seed butter, which is rich in edible oils.
1.3.2 Antioxidants

Antioxidants are usually used at low concentrations to delay lipid oxidation (Choe & Min, 2009). Based on the function, antioxidants can be classified as primary, synergists, and secondary antioxidants (Fig. 1.4). Primary antioxidants stop free radical chain reactions by donating electrons to free radicals. Synergistic antioxidants can be classified as oxygen scavengers and chelators that donate hydrogen to the phenoxy radical and react with free oxygen. Secondary antioxidants decompose lipid peroxides into stable end products (Maga & Tu, 1995). Based on their sources, antioxidants are classified as either natural or synthetic. Natural antioxidants are preferred by consumer because of the perception of their safety. For instance, α-tocopherol was tolerated even at doses of 2000 mg/kg bodyweight in rabbits, 5000 mg/kg in rats, and 50,000 mg/kg in mice. On the other hand, synthetic antioxidants could be toxic at high concentrations in foods (Maga & Tu, 1995). But they could withstand frying and baking at high temperatures while most natural antioxidants cannot (Branen, Davidson, Salminen, & Thorngate, 2002). For oilseed spreads, non-chemical methods including vacuum, nitrogen and carbon dioxide, and packaging are usual ways to prevent the lipid oxidation and extend the shelf life of seed butters. However, although manufacturers can remove most oxygen from the headspace of the jar, it is almost impossible to eliminate all the oxygen blended in the
seed butter matrix during processing. Therefore, antioxidants are in order to effectively delay the lipid oxidation.

Figure 1.4. Categorization of food antioxidants. (Maga & Tu, 1995).
1.3.2.1. Selected antioxidant

α-tocopherol

Tocopherols (vitamin E) are popular lipid-soluble and GRAS natural antioxidants used in the food industry. They can be classified into four main groups (α-, β-, γ- and δ-) (Yanishlieva, Gordon, & Pokorný, 2001; Shintani, Cheng, & DellaPenna, 2002). The major mechanism by which tocopherols protect lipids from oxidation is scavenging the free radicals to prevent primary oxidation and chain reactions (Gordon, 1990; Holownia, Erickson, Chinnan, & Eitenmiller, 2001; Yanishlieva et al., 2001; Choe & Min, 2005). Kamal-Eldin (2006) reported that α- and γ-tocopherols showed stronger antioxidant activity when compared with other tocopherols. Furthermore, γ-tocopherol performed better than α-tocopherol at high concentrations but a became a worse antioxidant at low concentrations (Fuster, Lampi, Hopia, & Kamal-Eldin, 1998). This means that less α-tocopherol is needed when compared with γ-tocopherols in order to achieve optimal antioxidant activity (Seppanen, Song, & Saari Csallany, 2010). Also, α-tocopherol may act as a prooxidant that catalyzes lipid oxidation at high concentrations (Frankel, 2014).

Figure 1.5. Structure of α-, β-, γ- and δ-tocopherol.
1.3.3. Stabilizers

Stabilizers are used to prevent oil separation and maintain the texture of oilseed spreads (Aryana, Resurreccion, Chinnan, & Beuchat, 2003). They are usually partially hydrogenated vegetable oil, mono-, di-, or tri-glycerides of vegetable oils or their combinations (Woodroof, 1983). The main mechanism of stabilizers is in forming a stable crystallization network to stabilize the structure of these products. Alpha (α), Beta prime (β’), and Beta (β) are the three main crystallization forms of triacylglycerols (Fig. 1.6). In a triacylglycerol mixture, β’ structure is the major crystallization form, but the β structure is the major form in pure triacylglycerols. The order of stability and melting point is β > β’ > α structure (Flöter & Duijn, 2006). Oils used as stabilizers usually have a high solid fat content, high melting points (>25°C) and the ability to form a stable crystallization network structure in the matrix (Woodroof, 1983). However, due to health concerns, consumers are becoming apprehensive about foods containing hydrogenated oil. Therefore, stabilizers such as palm oil with low long-chain saturated fatty acids and low trans fatty acids are becoming a potential option for oilseed spreads manufacturers (Hinds, Chinnan, & Beuchat, 1994).
1.3.3.1. Selected Stabilizers

**Hydrogenated vegetable oil**

Hydrogenation significantly reduces levels of polyunsaturated fatty acids (PUFA), changes the melting point, and manipulates the crystallization properties of vegetable oils. Hydrogenation is done to modify the melting point and improve the oxidative stability of vegetable oils. Cottonseed oil, corn oil and rapeseed oil are usually hydrogenated to meet specific properties for use in the food industry (Dowd, 2011; Flöter & Duijn, 2006; Moreau, 2011; Przybylski, 2011). According to Totlani & Chinnan (2007), Fix-X™, a blend of fully hydrogenated cottonseed and rapeseed oil containing 33
– 37% C22:0 (behenic acid), was able to adequately stabilize peanut butter at 1.0 – 2.0% and 35°C for three months. Therefore, the properties of hydrogenated vegetable oil can be able to retain the texture, prevent oil separation, and extend the shelf life of the seed butter.

**Palm oil**

Palm oil is widely used in cooking oils, margarine, spreads, shortening and confectionery (Pande, Akoh, & Lai, 2012). It is extracted from the oil palm fruits. Generally, palm oil is obtained from the pulp, and palm kernel oil is extracted from the kernel of oil palm. The differences between them are the composition of fatty acids. Palm oil contains 50% saturated fatty acids, and palm kernel oil contains nearly 85% saturated fatty acids (Pande et al., 2012). The main characteristics of palm oil are its high solid fat content, high oxidative stability, high and low melting triacylglycerols (TAGs), constant supply, competitive price, slow crystallization properties, structural hardness, and its tendency to recrystallize, a fact that works to increase its applicability in food products (Pande et al., 2012). Studies have shown the potential of palm oil as a natural stabilizer in seed and nut spreads (Aryana, 2000; Hinds et al., 1994). As a stabilizer in peanut butter, palm oil was estimated to prevent oil separation at 21 – 24°C for more than one year (Hinds et al., 1994). Palm oil was also reported to improve the oil holding capacity (OHC) of peanut butter (Aryana et al., 2003), which again shows its potential to prevent oil separation in seed butter. However, the effect of palm oil as a stabilizer may be lost
with incorrect processing, because palm oils contain a high content of (6 – 7%) glycerides that have anti-crystallization properties (Hasenhuettl & Hartel, 2008).

1.4. Methods to test physical properties of seed butter

1.4.1. Texture profile analysis

The determination of texture profile analysis (TPA) is done by compressing and decompressing the samples on a flat plate twice by a probe or a platen attached to a drive system. This method imitates the chewing action of the teeth. Figure 1.7 shows a TPA curve generated by the Instron Universal Testing Machine during a test. Hardness 1 is the peak force in the first bite, and it is used to describe hardness of the sample or the resistance to the applied force. The ratio of Area 2 to Area 1 is defined as cohesiveness. It is the structural integrity of the molecular structure of the material and is reflective of its ability to withstand the compressive force. In sensory science, cohesiveness is the energy needed to break or pulverize the food for swallowing (Hinds, 2003). Area 3 in the first bite is the adhesiveness, and it is the area under the negative part of the first bite. This is the work required to pull the compression probe or plate from the sample. In sensory science, adhesiveness is the energy required to remove the food adhering to the teeth and palate (Gills & Resurreccion, 2000). Gumminess was defined as the product of hardness 1 x (Area 2/Area 1). Chewiness was defined as the product of gumminess x springiness. Both gumminess and chewiness are the secondary parameters of cohesiveness. Chewiness is used for solid foods, but gumminess is used for semi-solid foods. However, TPA is able to measure hardness, cohesiveness, adhesiveness, fracturability, stringiness,
springiness, gumminess and chewiness of a food sample. These texture parameters performed good correlations with sensory attributes (Bourne, 2002).

Figure 1.7. A generalized TPA curve from the UTM. (Bourne, 2002).

1.5. Methods to test oxidative status of seed butter

1.5.1. Primary oxidation

1.5.1.1. Peroxide value

Hydroperoxides are produced in the initial stages of lipid oxidation, and then decompose to volatile or non-volatile secondary oxidation products during the later
stages of the process. The rate of hydroperoxides formation is much higher than its decomposition in the initial stages, but this reverses during the later stages. Therefore, the peroxide value is an indicator of primary lipid oxidation (Shahidi & Zhong, 2005).

The Iodometric Titration Method can be used to determine the peroxide value of an oil sample. This method is based on the theory that hydroperoxides are known to oxidize iodine ions (I\(^-\)). In this process, saturated potassium iodide added to the solution reacts with the peroxide in the oil sample, and iodine is liberated. Using starch as an endpoint indicator, the solution is titrated with standard sodium thiosulfate. From this, the PV concentration is calculated and the rate of primary oxidation determined (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002).

The Ferric Ion Complexes Method can also be used to determine the peroxide value and thus the primary oxidation of an oil sample. This method is based on the oxidation of ferrous ion (Fe\(^{2+}\)) to ferric ion (Fe\(^{3+}\)) by hydroperoxides produced during oxidation of a lipid based product in an acidic medium. During the reaction the ferric ions are complexed to ferric thiocyanate by potassium thiocyanate or xylenol orange and determined at 500 – 510 nm or 550 – 610 nm, respectively, by use of a UV-Vis spectrophotometer (Eymard & Genot, 2003).

The use of Fourier transform infrared spectroscopy (FTIR) can provide another option to test for lipid oxidation in selected foods. This method is based on the ability of infrared spectroscopy to quantify the presence of hydroperoxides by measuring their characteristic O-H stretching absorbance band. In this test, the intensity of the signals produced during the test is proportional to the concentration of hydroperoxides in the test
sample. It is a simple and rapid way to investigate the oxidation status of an oil in a food sample (Sedman, Voort, Ismail, & Maes, 1998).

1.5.1.2. Conjugated dienes and trienes

During the primary oxidation stages of an edible oil, both autooxidation and photosensitized oxidation can produce conjugated dienes and trienes (Choe & Min, 2006). Conjugated dienes exhibit a high absorbance at 234 nm, and conjugated trienes absorb at 268 nm. Increasing absorbance reflects the primary oxidation status of lipids. This method has high correlation with PV but shows less specificity and sensitivity because the absorbance may be influenced by other compounds having the same absorbing regions (Shahidi & Zhong, 2005).

1.5.2. Secondary oxidation

As was mentioned previously, hydroperoxides are produced during primary oxidation, and they are capable of decomposing to aldehydes, ketones, acids, esters, alcohols, and short-chain carbonyls as secondary products (Choe & Min, 2006). To determine total oxidation status of a sample, primary and secondary oxidation products must be quantified. To measure secondary oxidation, the following methods could be used.
1.5.2.1. Thiobarbituric acid (TBA) test

The TBA test is a common method used to determine secondary oxidation in lipids. This method is based on the fact that polyunsaturated fatty acids degrade and produce malonaldehyde (MA), and this compound can react with thiobarbituric acid (TBA) to form a pink MA-TBA complex having strong absorbance at 530 – 535 nm. However, alkenals and alkadienals also react with TBA and have absorbance at the same wavelength region. Therefore the term thiobarbituric acid reactive substances (TBARS) is used instead of MA (Shahidi & Zhong, 2005). The absorbance is then calculated as TBA value to represent the secondary oxidation status of a sample. Despite its limitations, the TBA test is widely used to measure secondary oxidation in lipid based foods.

1.5.2.2. p-Anisidine value (pAV)

In secondary oxidation, hydroperoxides decompose to aldehydes, ketones, alcohols and short-chain hydrocarbons (Choe & Min, 2006). Aldehydes are the target compounds in the p-anisidine value test, especially 2-alkenals and 2,4-alkadienals. Aldehydes in the lipids react with the p-anisidine reagent under acid conditions and form yellowish products absorbing at 350 nm. This method well correlates with the quantity of total volatile compounds. And it is highly correlated with sensory scores and PV. However, pAV is limited for comparing single oil type because the initial pAV varies among oil sources (Doleschall, Recseg, Kemény, & Kovári, 2003).
1.5.2.3. Totox value (TV)

Totox value is the combination of peroxide value and \( p \)-anisidine value:

\[
\text{Totox value} = 2\text{PV} + \text{pAV} \quad \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots 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CHAPTER 2

ANTIBACTERIAL EFFECTS OF GRAPE SEED EXTRACT AND CINNAMALDEHYDE AGAINST SALMONELLA ENTERICA AND LISTERIA INNOCUA IN SEED BUTTER

2.1. Abstract

Foodborne illness has been a problem for a long time. Outbreaks caused by oilseed spreads contaminated with foodborne pathogens have threatened hundreds of people’s lives in the U.S. within recent times (CDC, 2007; CDC, 2009; CDC, 2013; CDC, 2014). This study investigated the antimicrobial effects of grape seed extract (GSE) and cinnamaldehyde against *Salmonella enterica* and *Listeria innocua* in seed butter. The seed butter was made of sesame, sunflower and pumpkin seeds by grinding them at 1500 rpm for mixing and 3000 rpm for fine grinding. For testing the antimicrobial abilities of GSE and cinnamaldehyde, 5, 10 and 15% GSE and 0.1, 1.0 and 1.5% cinnamaldehyde were added to the seed butter, respectively. *S. enterica* and *L. innocua* were inoculated into the seed butter and the samples were stored at 25°C. The population of *S. enterica* and *L. innocua* were enumerated after 0, 1, 3, 5, 7 and 9 days of storage. The results showed that GSE at 10 and 15% significantly (p<0.05) reduced both *S. enterica* and *L. innocua*.
*innocua* after one day of storage when compared with the control, which was without added antimicrobial agents. Cinnamaldehyde also showed significant (p<0.05) effect against *S. enterica*, but *L. innocua* appeared more resistance. The reduction of *L. innocua* in cinnamaldehyde fortified seed butter was not significant (p>0.05) when compared with the control. In conclusion, GSE was an effective antimicrobial agent against *S. enterica* and *L. innocua*, but cinnamaldehyde was not able to reduce *L. innocua* in the seed butter.

### 2.2. Introduction

Seed butters are spreadable products made from milling seeds into a paste. Pumpkin, sunflower and sesame seeds, and soybean are common ingredients used for making seed butters. Similar products can be made from nuts, including almond, cashew, hazelnut, macadamia, peanut, pecan, pistachio, and walnut (Mangels, 2001). The major difference between a nut and a seed is that most nuts are composed of a hard outer shell that encloses a seed. Seed butters are usually good sources of protein, and they also contain other nutrients such as copper, folic acid, potassium, tocopherols, and zinc (Mangels, 2001). They are consumed in variable ways, but spreading on a toast is the most common way of eating seed butter. Used as toppings on crackers or dipping for vegetables are other ways to consume seed butters. Furthermore, they can be added to bakery products such as cookies, for example (Shakerardekan, Karim, Ghazali, & Chin, 2013).

“The shelf life of a product is the length of time it can remain on a store shelf without adverse consumer reaction.” (Labuza, 1982) This depends on the quality, food safety, and nutritional value of the product (Labuza, 1982). Microbial contamination is a
main factor causing food spoilage and foodborne illness. In the U.S., the first outbreak caused by *Salmonella* in peanut butter occurred in 2006, and at least 628 consumers from 47 states were infected (CDC, 2007). One year later, another outbreak occurred and 529 consumers from 43 states were infected by *Salmonella* in peanut butter and peanut butter crackers (CDC, 2009). In addition to these, widespread outbreaks occurred in 2012 and 41 people from 20 states were infected (Macdonald et al., 2013). In 2014, six more consumers from 5 states were also infected (CDC, 2014). Not only was peanut butter the substrate, sesame paste was also contaminated by *Salmonella* in 2013 and 16 consumers from 5 states were infected (CDC, 2013).

Seed butters can be contaminated as a result of poor sanitization practices, inappropriate equipment design, and improper ingredient control. Although vegetative microbial pathogens poorly survive in low-water activity foods such as seed butters, studies have reported that some microorganisms are able to tolerate these conditions for a period of time and could cause food safety problems (Podolak et al., 2010). However, even though most manufacturers strictly follow Good Manufacturing Practice (GMP), they cannot guarantee the sterility of processed seed butters. Therefore, antimicrobial agents can be added to seed butters as a hurdle to increase food safety and better guarantee the expectation of longer shelf life. The objective of this study was to determine the effect of natural antibacterial agents against *Salmonella* and *Listeria* pre-inoculated in seed butter.
2.3. Materials and Methods

2.3.1. Experimental design

The experimental design for the microbiological analysis of the seed butter samples is shown in Figure 2.1. This includes one control, two types of antibacterial agents (GSE and cinnamaldehyde) at three concentration levels. These concentration levels were 0.1, 1 and 1.5% (w/w) of cinnamaldehyde, and 5, 10 and 15% (w/w) of GSE. Each treatment was replicated three times. The storage temperature was 25°C to simulate the way that consumers usually store these products. For all experiments, the storage time points were 0, 1, 3, 5, 7 and 9 days to determine the effects of cinnamaldehyde and GSE (as natural antimicrobial agents) on the growth and survival of S. enterica and L. innocua.
Figure 2.1. Experimental design for the effect of antimicrobials in the seed butter.
2.3.2. Seed butter production

The seed butter was prepared using the formulation shown in Table 2.1.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>% in seed butter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pumpkin seed&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.87</td>
</tr>
<tr>
<td>Sunflower seed&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.10</td>
</tr>
<tr>
<td>Sesame seed paste (Tahini)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.14</td>
</tr>
<tr>
<td>Honey&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.14</td>
</tr>
<tr>
<td>Salt (sodium chloride)</td>
<td>2.75</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pumpkin and sunflower seeds were purchased from King Nut Co. Inc. (Solon, OH)

<sup>b</sup>Sesame seed paste (Tahini) was purchased from International Golden Foods, Inc. (Benservile, IL)

<sup>c</sup>Honey was purchased from Golden Food Service (Columbus, OH)

Table 2.1. Ingredients and formulation.

The ingredients and antimicrobial agents were ground in two stages. In the first stage, the ingredients were blended and ground at 1500 rpm and 50°C in a Stephen UMC 5 electronic mixer (Stephen Food Service Equipment GmbH, Halen, Germany) for 2 minutes. In the second stage, the ingredients were ground at 3000 rpm and 50°C for 16 minutes. At the end of this process, the finished seed butter was poured into propylene containers and cooled to ambient room temperature (23 ± 1°C) before further sample preparation and storage.

2.3.3. Culture preparation

*Salmonella enterica* (ATCC 53647) and *Listeria innocua* (ATCC 33090) were used for this experiment. The stock culture of each microorganism was prepared by transferring a loopful of the *S. enterica* and *L. innocua* into 30 mL of Trypticase soy broth (TSB) (Difco, Becton Dickinison, Sparks, MD) and incubating the cultures at 37°C
for 24 hours. Each broth was centrifuged (Kendro Laboratory Products, Sorvall RC 5C Plus, Newtown, CT) at 6,000 rpm and 4°C for 10 minutes. The supernatant was decanted and the suspension was resuspended in 30 mL 0.85% sterile saline (pH 6.5). The concentrations of *S. enterica* and *L. innocua* were approximately $10^7 - 10^8$ Colony Forming Units (CFU)/mL, quantified before each replicate.

### 2.3.4. Sample preparation

For each analysis 20g aliquots of seed butter with 0, 5, 10 and 15% GSE and 0, 0.1, 1 and 1.5% cinnamaldehyde were placed in sterile stomacher bags (240mL) (Fisher Scientific, Pittsburgh, PA), and were inoculated with 200 µl of *S. enterica* ($10^7 - 10^8$ CFU/mL) or *L. innocua* ($10^7 - 10^8$ CFU/mL), respectively. The samples were homogenized in the stomacher for 2 minutes at room temperature (23 ± 1°C). The sterile stomacher bags with pre-inoculated seed butter were sealed and stored at 25°C for up to 9 days.

### 2.3.5. Total plate count (TPC)

Prior to inoculation of the seed butter samples, both the inocula were analyzed to determine the microbial populations (CFU/mL) by serially diluting (1:10) in 0.85% sterile saline and pour plated (1mL in duplicate) with tryptic soy agar (TSA). All plates were incubated at 37°C for 48 hours then counted.

The inoculated seed butters stored for 1, 3, 5, 7, and 9 days at 25°C were analyzed for populations of *S. enterica* and *L. innocua*. To each stomacher bag, 50mL aliquots of
0.85% sterile saline were added, and the mixtures were homogenized in the stomacher for two minutes. Homogenized samples were serially diluted (1:10) in 0.85% sterile saline then pour plated with TSA. The plates were incubated at 37°C for 48 hours before examining for presumptive *S. enterica* and *L. innocua* colonies.

2.3.6. Statistical analysis

This was conducted by using JMP 10 (SAS Institute Inc., Cary, NC) for one-way Analysis of Variance (ANOVA) with Tukey-Kramer test to determine the significant effects of different levels of antimicrobial agents in the seed butter during storage with a 95% confidential interval. The Tukey-Kramer test analyzed significant differences between individual treatments. The statistical analysis was used to compare the inhibitory effects of different levels of cinnamaldehyde and GSE against *S. enterica* and *L. innocua* in the seed butter.

2.4. Results

2.4.1. Antimicrobial activity of grape seed extract in seed butter

The inhibitory activities of the GSE incorporated seed butter are shown in Figures 2.2 and 2.3. Compared with the control, *S. enterica* counts significantly (p<0.05) decreased after one day of storage in 10 and 15% GSE seed butters, but there was no significant (p>0.05) difference in the *S. enterica* counts between 10 and 15% GSE seed butters. However, 5% GSE seed butter showed an insignificant (p>0.05) reduction in *S. enterica* when compared with the control. In the 10 and 15% GSE seed butters, *S.
*S. enterica* was reduced by 1.34 and 1.59 log CFU/g after one day of storage, respectively. After three days of storage, *S. enterica* reductions of 1.03, 1.64 and 1.97 log CFU/g were recorded for the 5, 10 and 15% GSE seed butters, respectively. After nine days of exposure, the reductions of *S. enterica* were 1.64, 2.23 and 2.46 log CFU/g in 5, 10 and 15% GSE seed butters, respectively. The results indicated that 10 and 15% GSE effectively reduced *S. enterica* in the seed butter.

For the antimicrobial activity of GSE against *L. innocua*, after one day of incubation, the reductions were significantly (p<0.05) higher in the 10 and 15% GSE seed butters when compared with the control and the 5% GSE seed butter. Reduction in the 5% GSE seed butter had no significant (p>0.05) difference when compared with the control. There was a significant (p<0.05) difference in *L. innocua* reduction between the 10 and 15% seed butters after five days of storage. *L. innocua* counts were reduced by 2.57 and 3.15 log CFU/g in 10 and 15% GSE seed butters after one day of storage, respectively. After nine days of storage, the *L. innocua* counts were reduced by 0.83, 2.92 and 3.55 log CFU/g in the 5, 10 and 15% GSE seed butters, respectively.
Figure 2.2. Inhibitory effect of grape seed extract (GSE) against *S. enterica* at 25°C.

Figure 2.3. Inhibitory effect of grape seed extract (GSE) against *L. innocua* at 25°C.
2.4.2. Antimicrobial activity of cinnamaldehyde in seed butter

The antimicrobial efficacy of the cinnamaldehyde in seed butter was investigated against *S. enterica* and *L. innocua*. In this experiment, one control was tested. This was seed butter without added antimicrobial agents. Figures 2.4 and 2.5 summarize the results obtained. Reduction in the *S. enterica* counts had no significant (p>0.05) difference between each treatment until the fifth day of storage. After five days of exposure, the reduction in *S. enterica* in 1.5% cinnamaldehyde seed butter (2.26 log CFU/g) was significantly (p<0.05) higher than the reduction in other seed butter samples. After seven days of storage, the 1 and 1.5% cinnamaldehyde seed butters significantly (p<0.05) reduced *S. enterica* counts by 1.86 and 2.59 log CFU/g when compared with the control and the 0.1% cinnamaldehyde seed butter. There were significant (p<0.05) reductions in *S. enterica* between the 1.0 and 1.5% cinnamaldehyde seed butters. On the other hand, Figure 2.5 shows that seed butter with 0.1, 1.0, 1.5% cinnamaldehyde did not significantly (p>0.05) reduce *L. innocua* after nine days of storage at 25°C when compared with the control.
Figure 2.4. Inhibitory effect of cinnamaldehyde against *S. enterica* at 25°C.

Figure 2.5. Inhibitory effect of cinnamaldehyde against *L. innocua* 25°C.
2.5. Discussion

In this study, GSE showed strong antimicrobial effects against *S. enterica* and *L. innocua* in the seed butter. The antimicrobial ability of GSE was supported by Baydar et al. (2006) that the inhibitory effects of GSE against *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium at 1, 2.5, 5 and 10% at 37°C. Also, Over, Hettiarachchy, Johnson, & Davis (2009) reported that 20 and 40 mg/mL GSE in brain heart infusion (BHI) broth effectively reduced *S. enterica* ser. Typhimurium by 1.22 and 3.20 log CFU/mL after 24 hours at 37°C, respectively; This present study also demonstrated that GSE exhibited antimicrobial properties against *L. innocua* in the seed butter. A similar result was reported by Rhodes et al. (2006), when they stated that the antimicrobial ability of GSE caused *L. monocytogenes* to be reduced from 6 – 7 log CFU/mL to a non-detectable level within 10 minutes at 20°C. However, compared with the previous studies (Over et al., 2009; Rhodes et al., 2006) that demonstrated the antimicrobial effect of GSE against bacteria in nutrient media, this present study indicated the antimicrobial ability of GSE against *S. enterica* and *L. innocua* in a specific and more complicated food system, seed butter, which contains fatty acids, proteins, carbohydrates and other minerals. In other words, the results showed a more realistic reaction between the antimicrobial agents and the foodborne pathogen.

For the determination of the antimicrobial effects of cinnamaldehyde, the results showed that it was able to reduce *S. enterica* in the seed butter. Ravishankar et al. (2010) also reported the antimicrobial effects of 0.1% cinnamaldehyde in phosphate buffered saline (PBS) solution against *S. enterica* ser. Typhimurium, *S. enterica* ser. Enteritidis
and *S. newport* from 5.0 log CFU/mL to undetectable levels in only one hour at 37°C. On the contrary, *L. innocua* showed significant resistance to cinnamaldehyde. Not only in the seed butter, this resistance was also mentioned in a previous study conducted by Ravishankar et al. (2012). This study was about the antilisterial effects of 0.5, 1.5 and 3.0% cinnamaldehyde incorporated into edible films. *L. monocytogenes* also showed resistance to cinnamaldehyde and showed a non-significant (p>0.05) reduction when compared with the control. de Oliveira et al. (2012) also reported that biofilm of *L. monocytogenes* on a stainless wall was resistant to cinnamaldehyde. This result indicated that cinnamaldehyde may not be an appropriate antimicrobial agent in the seed butter because it could not reduce *L. innocua* effectively.

In the control (natural seed butter), *S. enterica* was able to survive at 25°C for up to 17 days and was merely reduction by 1.34 log CFU/g. This is not shown in the figures. A similar result was reported by Park et al. (2008) when they tested *S. enterica* ser. Tennesse viable in peanut butter at 22°C for 14 days. This bacteria strain was reduced by 0.34 – 1.27 log CFU/g after seven days of storage depending on the formula of selected peanut butter. Burnett et al. (2000) also showed that *S. enterica* was able to survive in peanut butter at a count of 5.68 log CFU/g at 21°C for 24 weeks, and the population only reduced by 2.28 – 3.95 after 14 days. *L. innocua* also showed the ability to survive in natural seed butter samples with a 0.19 log CFU/mL reduction after at least 17 days at 25°C. Kenney and Beuchat (2004) also reported that *L. monocytogenes* was able to survive and be reduced from 4.42 to 0.62 log CFU/mL in peanut butter at 20°C for 24
weeks. This means that adding antimicrobial agents to seed butter is a necessary hurdle in the avoidance of foodborne illness, and the results showed that it could be achieved.

2.6. Conclusion

The results of this study showed that GSE is an effective natural antimicrobial agent against both *S. enterica* and *L. innocua* in the seed butter at 25°C. Also, cinnamaldehyde can effectively inhibit *S. enterica* at 25°C. However, *L. innocua* is relatively resistant to cinnamaldehyde in seed butter. Therefore, GSE is a better antimicrobial agent when compared with cinnamaldehyde in seed butter.

2.7. References


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CHAPTER 3

STABILIZING EFFECTS OF HYDROGENATED RAPESEED OIL AND PALM KERNEL OIL ON THE TEXTURE AND OIL SEPARATION OF SEED BUTTER MADE WITH SESAME, SUNFLOWER AND PUMPKIN SEEDS

3.1. Abstract

This study investigated the stabilizing effects of hydrogenated rapeseed oil (HRO) and palm kernel oil (PKO) on retaining the texture and preventing oil separation in seed butter. The seed butter was made of sesame, sunflower and pumpkin seeds by homogenizing them at 1500 rpm for mixing and 3000 rpm for fine grinding. To determine the stabilizing abilities of HRO and PKO, 1, 2 and 3% HRO and PKO were added to the seed butter, respectively. The stabilized seed butter samples were stored at 25°C. The hardness, cohesiveness and adhesiveness of the samples were measured using a texture analyzer after storage at 0, 7, 14, 21, 28, 35, 42 and 49 days. The oil separation in each sample was measured after 0, 1, 3, 5, 7, 14, 21, 28, 35 and 42 days of storage. The results showed that after four weeks of storage, HRO at 2 and 3% significantly (p<0.05) effected the hardness of the seed butter samples when compared with the control, which was without adding stabilizers. The results also showed that the 3% HRO stabilized seed
butter had significantly (p<0.05) less oil separation than the control after three days of storage at 25°C. Compared with the control, PKO significantly (p<0.05) influenced the hardness of the seed after 35 days of storage, but it did not have significant (p>0.05) effects on maintaining the cohesiveness and adhesiveness. For preventing oil separation, PKO had no significant (p>0.05) effect on the stability of the oil in the seed butter at 25°C. In conclusion, HRO was shown to be a better stabilizer than PKO.

3.2. Introduction

Seed butter is a low-moisture semi-perishable product made of edible seeds, sweeteners, stabilizers and salt (sodium chloride). Roasting, grinding, chilling and tempering are the major processing steps of this product (MacDonald et al., 1985). The common ingredients used for producing seed butter are pumpkin, sunflower and sesame seeds, and soybean. Seed butter has grown to be a popular product in the market, being an alternative to margarine (Olga Radočaj, Dimić, & Vujasinović, 2012). Seed butter can be consumed by spreading on a toast, topping on crackers or dipping with vegetables. It also can be combined in bakery or confectionery products, for example (Shakerardekani et al., 2013). Furthermore, seed butter can be mixed with milk, sugar or some spices to make a seed butter punch, such as soy butter punch, as example.

The shelf life of seed butter depends on the quality, food safety, and nutritional value of the product (Labuza, 1982). The physical properties of seed butter can change during storage. Oil separation is a major problem that could occur in high oil content products. During storage at room and or elevated temperatures, seed butter could separate
into two layers. When this occurs, the seed oil rises to the surface and a dry layer of solid seed particles will deposit at the bottom (Aryana et al., 2003). This separated oil can contaminate the packaging material and affect the appearance of the product (Ereifej, Rababah, & Al-Rababah, 2005). It also increases the exposure of the seed oil to air. As time progresses, the solid phase tends to become progressively harder while the seed oil separates, and this phenomenon makes such seed butters unappealing to consumers. This problem could be solved by the addition of hydrogenated vegetable oils or other stabilizers. For health concerns, consumers are increasingly choosing food products without hydrogenated oils, so a more acceptable substitute ingredient is necessary for seed butters (Mozaffarian, Aro, & Willett, 2009).

Palm oil is widely used in cooking oil, margarine, spreads, shortening and confectionery (Pande et al., 2012). It is extracted from the oil palm fruit. The main characteristics of palm oil are high solid fat content, high oxidative stability, high and low melting triacylglycerols (TAGs), constant supply, competitive price, slow crystallization properties, structural hardness, and a tendency to recrystallize. This makes palm oil a useful functional additive to many food products (Pande et al., 2012). Studies have shown the potential of palm oil as a natural stabilizer in seed and nut spreads (Aryana, 2000; Hinds et al., 1994). As a stabilizer in peanut butter, palm oil was estimated to prevent oil separation at 21 – 24°C for more than one year (Hinds et al., 1994). Aryana et al. (2003) also reported that palm oil improved the oil holding capacity (OHC) of peanut butter, which again shows its potential to prevent oil separation and maintain the texture of seed butter.
To extend the shelf life of a seed butter, the physical properties of the product should be controlled during storage. Thus, the objective of this study was to determine the effects of hydrogenated rapeseed oil (HRO) and palm kernel oil (PKO) on retaining the texture and preventing oil separation in the seed butter.

3.3. Materials and Methods

3.3.1. Experimental design

The experimental design for the physical analysis of the seed butter samples is shown in Figure 3.1. Seed butter samples with two types of stabilizers at three concentration levels and a control were studied. These concentration levels were 1, 2 and 3% (w/w) of PKO and HRO, respectively. Each treatment was replicated three times. The storage temperature was 25°C. For the oil separation part of the study, the time points were 0, 1, 3, 5, 7, 14, 21, 28, 35 and 42 days. For the texture profile analysis, the time points were 0, 7, 14, 21, 28, 35, 42 and 49 days. These experiments were done to determine the effects of PKO and HRO on the texture and oil separation of the seed butter samples.
Figure 3.1. Experimental design for physical analysis of seed butter treated with various stabilizers.
3.3.2. Seed butter production

The seed butter was prepared using the formulation shown in Table 2.1. The ingredients with different concentrations of PKO (Dr. Adorable Inc., Malaysia) and HRO (Palsgaard® 6111, Palsgaard, NJ) were ground in two stages. In the first stage, the ingredients were blended and ground at 1500 rpm and 50°C in a Stephen UMC 5 electronic mixer (Stephen Food Service Equipment GmbH, Halen, Germany) for 2 minutes. In the second stage, the same ingredients were ground at 3000 rpm and 50°C for 16 minutes. At the end of this process, the finished seed butter was poured into propylene containers and cooled to ambient room temperature (23 ± 1°C) before further sample preparation and storage.

3.3.3. Texture profile analysis

This experiment was to determine the effects of HRO and PKO on texture properties of the seed butter samples during storage at different temperatures. For the texture profile analysis, a Texture Analyzer TA-XT2 (Texture Technologies Corp and by Stable Micro Systems, Ltd., Hamilton, MA) was used to measure the hardness, cohesiveness and adhesiveness of the seed butter samples. The method was modified from the one reported by Radočaj, Dimić, & Vujasinović (2012). The Texture Analyzer was fitted with a 5kg load cell. The resulting force-time curves were generated to calculate the hardness, adhesiveness and cohesiveness of the seed butter samples during storage at 25°C. Figure 1.7 shows the force-time curve.
A cone-shaped acrylic probe (40°) was used for all measurements, and each sample (150g) was tested in the seed butter container (cylinder shape: 45 mm depth and 36 mm diameter) without stirring. The pre-test speed was 2 mm/s and the test speed was 1 mm/s. The target depth was 42 mm. The trigger load was 4.0 g, and the data rate was 100 points/s. After each test, the probe automatically returned to the original position for testing the next sample. The instrumental texture attributes are described in Table 3.1.

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness</td>
<td>The peak force required to compress the sample between the seed butter and the cone-shaped probe</td>
</tr>
<tr>
<td>Adhesiveness</td>
<td>The work to pull from the cone-shaped probe away the surface of a food product.</td>
</tr>
<tr>
<td>Cohesiveness</td>
<td>The strength of internal bonds in the seed butter matrix</td>
</tr>
</tbody>
</table>

Table 3.1. Instrumental texture attribute.

3.3.4. Oil separation

This experiment was conducted to determine the effects of HRO and PKO on inhibiting oil separation in the seed butter. The method used was modified from the one reported by Ereifej, Rababah, & Al-Rababah (2005). For each test, 50 grams of the seed butter with different concentrations of HRO and PKO were loaded in a plastic cup (100 mL capacity), and covered with a perforated aluminum foil (1.5625 hole/cm²). The cup was inverted and placed in a petri dish with 5 filter papers (Schleicher & Schuell No. 589) designed to absorb the oil separated from the seed butter (Fig. 3.2). The weight of
the petri dish with absorbed oil on the filter papers was measured during storage. This was done to determine the percentage of oil separation after 0, 1, 3, 5, 7, 14, 21, 28, 35 and 42 days of storage. This oil separation test was evaluated at 25°C. The percent of oil separation was calculated as follows:

\[
\text{Oil Separation (\%)} = \left( \frac{B - A}{M} \right) \times 100 \quad \ldots \ldots \ldots \ldots \ldots \ldots (3.2)
\]

where \( A \) is the original weight (g) of filter papers and the petri dish before the test, \( B \) is the weight (g) of filter papers and the petri dish with separated oil, and \( M \) is the weight (g) of seed butter sample at the start of the test.

![Figure 3.2. Perforated aluminum foil and oil separation kit.](image)

**3.3.5. Statistical analysis**

This was conducted by using the JMP 10 statistical software (SAS Institute Inc., Cary, NC) for one-way Analysis of Variance (ANOVA) and Tukey-Kramer testing to
determine the significant effects of different concentrations of HRO and PKO on the physical properties of the seed butter during storage, with a 95% confidence interval. The Tukey-Kramer test analyzed significant differences between the individual treatments. The statistical analysis was used to compare the effects of different concentrations of HRO and PKO on retaining the hardness, adhesiveness and cohesiveness, and preventing oil separation in the seed butter stored at 25°C.

3.4. Results

3.4.1. Texture profile analysis

The effects of HRO and PKO on the hardness of the seed butter samples are presented in Figures 3.3 and 3.4. After 35 days of storage, the seed butter samples stabilized with PKO were significantly (p<0.05) lower in harness than the control. However, there were no significant (p>0.05) differences in the effect of PKO concentrations on the hardness of the seed butter. The peak hardness of the control was on day 42 of storage, then it dropped by 439.2 gram force on day 49 of storage.

In Figure 3.4, the seed butter containing 3% HRO was significantly (p<0.05) higher in hardness than all the other samples on day zero of storage. The 1% HRO stabilized seed butter was significantly (p<0.05) lower in hardness than the control after 35 days of storage. After 42 days of storage, 2% and 3% HRO stabilized seed butters had significantly (p<0.05) lower hardness than the control. Seed butter stabilized with 2% HRO had no significant (p>0.05) difference in the hardness with 3% stabilized samples.
The seed butter stabilized with 3% HRO had significantly less harness than the PKO stabilized seed butter samples after 28 days of storage. After 35 days of storage, the seed butter stabilized with 2% HRO was significantly lower in hardness than the PKO stabilized seed butter.

Figure 3.3. Harness of the seed butters stabilized with PKO.
Figure 3.5 shows the effects of HRO on the adhesiveness of the seed butter. On day zero the adhesiveness of 2 and 3% HRO stabilized seed butters were significantly (p<0.05) higher than those of the other seed butter samples. The control and 1% HRO stabilized seed butters significantly (p<0.05) increased in the adhesiveness after seven days of storage. However, HRO did not show significant (p>0.05) effects on adhesiveness of the seed butter samples when compared with the control. The results showed that even though not having a significant effect on adhesiveness of the seed butter, the 3% HRO had the greatest effect on reducing it.

Figure 3.6 shows the effects of PKO on the adhesiveness of the seed butters. On day zero, the seed butter samples containing PKO had no significant (p<0.05) effect on the adhesiveness when compared with the control. All seed butter samples significantly (p<0.05) increased in adhesiveness after seven days of storage. However, PKO did not
show significant (p>0.05) effects on adhesiveness in the seed butter samples when compared with the control.

Figure 3.5. Adhesiveness of the seed butters stabilized with HRO.
Figure 3.6. Adhesiveness of the seed butter stabilized with PKO.

The effects of HRO on the cohesiveness are shown in Figure 3.7. On day zero, HRO had no significances (p>0.05) on cohesiveness of the seed butter when compared with the control. From 7 to 49 days of storage, the cohesiveness of all the seed butters dropped and remained at 0.15 – 0.23 g·s. The results showed insignificant (p>0.05) effects of HRO on cohesiveness of the seed butter samples.

The effects of PKO on the cohesiveness were shown in Figure 3.8. On day zero, the seed butter samples had no significant (p>0.05) difference in cohesiveness when compare with the control. From 7 to 49 days of storage, the cohesiveness of all the seed butters dropped and remained at 0.15 – 0.23 g·s. The results showed the PKO had no significant (p>0.05) effects on cohesiveness of the seed butter samples.
Figure 3.7. Cohesiveness of the seed butters stabilized with HRO.

Figure 3.8. Cohesiveness of the seed butter stabilized with PKO.
3.4.2. Oil separation

Figure 3.9 shows the effects of PKO on preventing oil separation in the seed butter samples. The oil separation in the control and PKO stabilized seed butters increased to 9.52 – 10.73% after seven days of storage, then the rate decreased. After 42 days of storage, PKO did not significantly (p>0.05) prevent oil separation in the seed butter when compared with the control. Oil separation in the control and 1, 2, 3% PKO stabilized seed butters were 11.09, 12.03, 12.35 and 13.06% on day 42 of storage, respectively.

The effects of 1, 2 and 3% HRO on preventing oil separation in the seed butter samples are shown in Figure 3.10. After three days of storage, the oil separation in the 3% HRO stabilized seed butter was significantly (p<0.05) lower than the control and the 1 and 2% HRO stabilized samples. The rates of oil separation in the control and HRO stabilized seed butters decreased after five days of storage. Seed butters stabilized with 1 and 2% HRO had no significant (p>0.05) effects on oil separation when compared with the control. After 42 days of storage at 25°C, the oil separation in the control and 1, 2 and 3% HRO stabilized seed butters were 11.09, 9.86, 9.56 and 6.82%, respectively.
Figure 3.9. Oil separation of the seed butters stabilized with PKO.

Figure 3.10. Oil separation of the seed butters stabilized with HRO.
3.5. Discussion

The hardness of all the seed butter samples could increase within storage time, and this could be caused by the migration of the oil toward the surface and the gravitational settling of solid particles at the bottom (Totlani & Chinnan, 2007). If this occurs, it will negatively affect the quality of the product. In this study, 3% HRO maintained the harness at an almost constant level at 25°C for at least 49 days. The results also showed that the stabilizing effect of HRO increased within increasing concentrations. On the other hand, PKO did not slow the same stabilizing effects on preventing the excessive hardness of the seed butter. These results agreed with a similar study reported by Aryana et al. (2003) when palm oil stabilized peanut butter stored at 21°C and 30°C insignificantly (p>0.05) prevented hardening when compared with the unstabilized peanut butter samples. Peanut butter stabilized with Fix-X™ (hydrogenated rapeseed and cottonseed oil) was significantly (p<0.05) softer than unstabilized and palm oil stabilized peanut butters at 21 and 30°C up to 23 weeks.

The adhesiveness of all the seed butter samples could increase within storage time. If this occurs, the seed butter gets sticky and unacceptable to consumers. Generally, lower adhesiveness is demanded characteristic of oilseed spreads. However, the seed butter stabilized with 3% HRO had the lowest adhesiveness during storage. The results agreed with the study reported by Aryana et al. (2003) that Fix-X™ effectively prevented the increasing of adhesives in peanut butter, otherwise palm oil had no effect on the characteristic. In this study, the results showed that HRO and PKO had no effect on this
characteristic in the seed butter. This result was agreed with the study reported by Aryana et al. (2003).

HRO showed a strong stabilizing ability against the migration of oil toward the surface of the samples. Totlani & Chinnan (2007) also reported the stabilizing effect of hydrogenated vegetable oil. No oil separation was observed in peanut butter stabilized with 1.0, 1.5 and 2.0% Fix-X™ at 35°C after 90 days of storage. On the contrary, the results in this study showed that PKO did not prevent oil separation in the seed butter. Aryana et al (2003) reported a similar result in peanut butter. In Aryana et al. study, Fix-X™ stabilized peanut butters showed less oil separation when compared with palm oil stabilized peanut butters at 30°C.

In summary, HRO provided a stable crystallization network structure for the seed butter matrix to maintain the hardness and withstand the migration of oil toward the surface (Smith, Bhaggan, Talbot, & Van Malssen, 2011). According to Berger (1998), PKO only contains 17% solid fat content at 25°C, and this characteristic may be the reason why PKO was ineffective as a stabilizer to form a stable crystalline network structure in the seed butter.

3.6. Conclusion

In this study, HRO showed positive effects on retaining hardness of the seed butter up to 49 days, but it did not have effects on the cohesiveness and adhesiveness. HRO showed as an effective stabilizer forming stable crystallization network structure and preventing the oil separation in seed butter up to 42 days of storage at 25°C. Compared
with HRO, PKO showed less stabilizing ability on preventing excessive hardness of the seed butter. PKO did not perform effects on maintaining cohesiveness and adhesiveness of the seed butters, either. For oil separation, the results showed that PKO was not an effective stabilizer to form a stable crystallization network structure in the seed butter.

3.7. References


4.1. Abstract

This study investigated the antioxidant effect of α-tocopherol inhibiting or delaying the lipid oxidation in seed butter oil. The seed butter was made of sesame, sunflower and pumpkin seeds by grinding them at 1500 rpm for mixing and 3000 rpm for find grinding. The seed butter oil was extracted by centrifuging the seed butter at 6000 rpm for 20 minutes. For testing the antioxidant ability of α-tocopherol, 100, 400, 700 ppm α-tocopherols were added in the seed butter oils. The seed butter oil naturally contained 235.62 – 279.88 ppm of α-tocopherol. These samples were stored at 30°C and 50°C in the dark. The peroxide value (PV), p-anisidine value (pAV) and totox value (TV) were determined after 0, 4, 7, 14, 20 and 31 days of storage. The results showed that temperature significantly (p<0.05) influenced the PV, pAV and TV of the seed butter oil. The addition of α-tocopherol did not show significant (p>0.05) antioxidant ability in the seed butter oil when compared with the control. However, at 50°C, 400 and 700 ppm α-tocopherol fortified seed butter oils were significantly higher in TV than the control on
day 31 of storage. This result showed that α-tocopherol may act as a prooxidant in the seed butter oil other than providing antioxidant effects.

4.2. Introduction

Seed butter is a product made with seeds, sweeteners, stabilizers and salt. The seeds is usually roasted then milled with other ingredients. After milled into fine paste, the seed butter is tempered to form a crystalline network structure against oil separation (MacDonald et al., 1985). Pumpkin, sunflower and sesame seeds, and soybean are the common ingredients used for making seed butters. Seed butters are good sources of protein, and they also contain other nutrients such as copper, folic acid, potassium, tocopherols, and zinc (Mangels, 2001).

Seed butter usually contains a high ratio of edible oils, so lipid oxidation is a critical point to consider if an attempt is made to extend its shelf life. Lipids can be oxidized when exposed to light, moisture, prooxidants or heat, and the ensuing reactions can cause off-flavors which could be unacceptable to consumers. Lipid oxidation can also affect the palatability, nutritional quality, and toxicity of the seed butters (Choe & Min, 2006). Vacuum and modified atmosphere packaging are common non-chemical methods used to prevent or limit lipid oxidation. However, oxygen could be blended in during processing and cause lipid oxidation in seed butters.

To extend the shelf life of seed butter, the lipid oxidation should be control during storage. Thus the objective of this study is to determine the effect of α-tocopherol as a
natural antioxidant in preventing or delaying lipid oxidation in the seed butter made with sesame, sunflower and pumpkin seeds.

4.3. Materials and Methods

4.3.1. Experimental design

The experimental design for the lipid oxidation analysis for the seed butter samples is shown in Figure 4.1. This includes one control and α-tocopherol at three concentrations. These concentrations were 100, 400 and 700 ppm (w/v). Each treatment was duplicated. The storage temperatures were 30 and 50°C. The lipid oxidation status of seed butter oils was determined after 0, 4, 7, 14, 20 and 31 days of storage.
Figure 4.1. Experimental design for lipid oxidation analysis of α-tocopherol as a natural antioxidant in the seed butter.
4.3.2. Seed butter production

The seed butter was prepared using the formulation shown in Table 2.1. The ingredients were ground in two stages. In the first stage, the ingredients were blended and grounded at 1500 rpm and 50°C in a Stephen UMC 5 electronic mixer (Stephen Food Service Equipment GmbH, Halen, Germany) for 2 minutes. In the second stage, the ingredients were ground at 3000 rpm and 50°C for 16 minutes. At the end of this process, the finished seed butter was poured into propylene containers and cooled to ambient room temperature (23 ± 1°C) before further sample preparation and storage.

4.3.3. Sample preparation

The oil samples was prepared by centrifuging the seed butters at 6000 rpm and 20°C for 10 minutes, then supernatant was transferred to flasks. α-Tocopherol was added in the oil samples and well homogenized with a stirrer bar at low speed for ten minutes. These homogenized oil samples were then stored in the dark at 30 and 50°C for further analysis.

4.3.4. Peroxide value

This test was done to measure the rate of lipid oxidation occurring in the samples. The Peroxide Value test measures hydroperoxides formed during the primary oxidation process. This method was modified from the AOCS Cd 8b-90 Method by the use of an automatic titrator DL70ES (Mettler-Toledo International Inc., Columbus, OH). For this test, a 0.5 gram aliquot of extracted oil was weighed and placed into a beaker. Then, a 25 mL aliquot of 3:2 (v/v) mixture of ACS grade glacial acetic acid (GFS Chemicals Inc.,
Columbus, OH) and ACS grade isooctane (Thermo Fisher Scientific, Fair Lawn, NJ) was added to the beaker. A 0.25 mL aliquot of freshly prepared saturated potassium iodide solution was added, and the beaker was shaken for one minute. Then, a 15 ml quantity of deionized water was immediately added. The solution was then titrated with 0.01N sodium thiosulfate solution with constant and vigorous agitation until the yellow iodine color almost disappeared. The PV (milliequivalents peroxide/ 1000g oil) was calculated as follows:

\[
PV = \frac{(S - B) \times N \times 1000}{m} \quad (4.1)
\]

where S is the volume of titrant (mL), B is the volume of blank (mL), m is the quantity of oil (g), and N is the normality of sodium thiosulfate (Na₂S₂O₃).

4.3.5. \textit{p}-Anisidine value

This test was done to measure the amount of aldehydes in the seed butter oil samples. Aldehydes are the products of secondary lipid oxidation process. This method was from AOCS Cd 18-90 Method by the use of UV-2401 PC UV-Vis recording spectrophotometer (Shimadzu Co., Columbia, MD). For this test, one gram oil was weighed in a 25mL volumetric flask then diluted with HPLC grade isooctane (Fisher Scientific, Fair Lawn, NJ). This solution was loaded to a 10mm glass cuvette and the absorbance (Ab) was measured at 350nm. Isooctane in the reference cuvette is used as blank. Then the solution was pipetted 5mL into a test tube with Teflon™ - lined screw
cap, and 5mL isoctane was pipetted to another test tube. Exactly 1mL $p$-anisidine reagent was added to both tubes mixed well. The $p$-anisidine reagent was prepared by dissolving 0.25g $p$-anisidine (Sigma Aldrich, St. Louis, MO) in 100mL ACS glacial acetic acid (Fisher Scientific, Fair Lawn, NJ). After 10 minutes, the absorbance (As) of the sample solution reacted with $p$-anisidine reagent in the first tube was measured at 350nm, and the solution from the second tube was used as the blank. The $p$-anisidine value (pAV) was calculated as follows:

$$pAV = \frac{25 \times (1.2As - Ab)}{m}$$

where As is the absorbance of the oil solution reacted with the $p$-anisidine reagent, Ab is the absorbance of the oil solution, and m is quantity of the oil (g).

**4.3.6. Totox value**

Totox value (TV) is a measure of total lipid oxidation of fats and oils. It combines PV and pAV. The TV was calculated as follows:

$$TV = 2 \times PV + pAV$$

(4.3)
4.3.7. High performance liquid chromatography (HPLC) analysis

4.3.7.1. Sample preparation

The experiment was designed to determine the concentration of α-tocopherol in the natural seed butter. The oil in the seed butter was obtained by centrifuging the seed butter samples at 6000 rpm and 20°C for 10 minutes, then dissolving the extracted oil with 100% HPLC grade hexane (Thermo Fisher Scientific, Fair Lawn, NJ). The ratio of oil and hexane is 1:1. This solution was filtered through a 0.45 µm nylon membrane, then injected into the HPLC for α-tocopherol quantification. The concentration of α-tocopherol in the oil of seed butter was 235.62 – 279.88 ppm.

4.3.7.2. HPLC Analysis

The HPLC analysis was done to determine the concentration of α-tocopherol in oil extracted from the seed butter samples using a method reported by Tsai, Chen, & Pan (2003). The α-Tocopherol concentrations were quantified using reverse-phase HPLC. Filtered solution (10 µL) were injected into an Agilent Technologies 1100 series HPLC system (Santa Clara, CA) equipped with a G1311A quaternary pump, G1322A degasser, G1313A ALS auto-sampler, and fitted with a G1315B diode array detector (Santa Clara, CA). An Econex Sphere 5 C_{18} column with 250mm x 4.6mm dimension and 5µm particle size (Torrance, CA) was used to carry out an isocratic separation at room temperature (23 ± 1°C) with 100% HPLC grade methanol as the mobile phase at a flow rate of 1 mL/min. The detecting wavelength was 292 nm. Chromatograms were analyzed using the OpenLAB software provided with the instrument (Santa Clara, CA). The concentration of
α-tocopherol was determined from two standard curves that were prepared with pure α-tocopherol in the range of 7.50 – 240.08 µg/mL and 162.54 – 676.97 µg/mL, respectively (Fig. 4.2). Depending on the α-tocopherol concentration in the samples, the parts of the standard curve that overlapped the samples were used for quantification calculations. Those curves were selected because they demonstrated good R values.
Figure 4.2. The HPLC standard curves with linear equation for α-tocopherol: (A) low concentration (7.50 – 240.08 µg/mL); (B) high concentration (162.54 – 676.97 µg/mL).
4.4. Results

4.4.1. Peroxide value

The PV test is a measure of the concentration of peroxides formed during primary lipid oxidation, and it is widely used to determine the oxidative status of lipid based foods. Tables 4.1 and 4.2 show PV of the control and α-tocopherol fortified seed butter oils. The control was without added α-tocopherol, but it originally contained 235.62 – 279.88 ppm α-tocopherol. The initial PV of the seed butter oil was 36.04 meq O₂/kg oil. After 31 days of storage, the PVs for the control and 100, 400, 700 ppm α-tocopherol fortified seed butter oils increased to 61.39, 67.52, 68.50 and 71.52 meq O₂/kg oil at 30°C, respectively. At 50°C, the PVs for the control and 100, 400, 700 ppm α-tocopherol fortified seed butter oil rose up to 138.88, 144.70, 152.16 and 153.93 meq O₂/kg after 31 days of storage, respectively. The results showed that temperature was a significant (p<0.05) factor influencing the PV of the seed butter oil. However, α-tocopherol did not have significant (p>0.05) effects on the PV of seed butter oil when compared with the control.
### Table 4.1. Peroxide values of the seed butter oil stored for 31 days at 30°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Storage time (day)</th>
<th>0</th>
<th>4</th>
<th>7</th>
<th>14</th>
<th>20</th>
<th>31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>36.04 ± 1.00</td>
<td>44.58 ± 0.16</td>
<td>42.36 ± 0.31</td>
<td>49.94 ± 1.18</td>
<td>53.61 ± 0.65</td>
<td>61.39 ± 1.63</td>
</tr>
<tr>
<td>100 ppm</td>
<td></td>
<td>36.04 ± 1.02</td>
<td>45.88 ± 1.92</td>
<td>45.77 ± 0.25</td>
<td>52.28 ± 0.40</td>
<td>58.54 ± 4.49</td>
<td>67.52 ± 4.60</td>
</tr>
<tr>
<td>400 ppm</td>
<td></td>
<td>36.04 ± 1.04</td>
<td>44.72 ± 0.68</td>
<td>44.31 ± 0.25</td>
<td>51.59 ± 0.80</td>
<td>56.35 ± 0.28</td>
<td>68.50 ± 0.49</td>
</tr>
<tr>
<td>700 ppm</td>
<td></td>
<td>36.04 ± 1.06</td>
<td>43.28 ± 1.25</td>
<td>43.41 ± 0.45</td>
<td>51.98 ± 1.86</td>
<td>56.07 ± 2.56</td>
<td>71.52 ± 2.95</td>
</tr>
</tbody>
</table>

### Table 4.2. Peroxide values of the seed butter oil stored for 31 days at 50°C.

<table>
<thead>
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<th>Sample</th>
<th>Storage time (day)</th>
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<th>14</th>
<th>20</th>
<th>31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>36.04 ± 1.01</td>
<td>52.64 ± 1.58</td>
<td>60.77 ± 2.62</td>
<td>84.11 ± 1.14</td>
<td>109.40 ± 0.38</td>
<td>138.88 ± 3.82</td>
</tr>
<tr>
<td>100 ppm</td>
<td></td>
<td>36.04 ± 1.03</td>
<td>53.86 ± 0.14</td>
<td>61.40 ± 0.21</td>
<td>81.57 ± 0.2</td>
<td>112.80 ± 0.98</td>
<td>144.70 ± 0.80</td>
</tr>
<tr>
<td>400 ppm</td>
<td></td>
<td>36.04 ± 1.05</td>
<td>50.95 ± 0.29</td>
<td>60.53 ± 0.54</td>
<td>82.80 ± 0.80</td>
<td>112.73 ± 1.37</td>
<td>152.16 ± 0.44</td>
</tr>
<tr>
<td>700 ppm</td>
<td></td>
<td>36.04 ± 1.07</td>
<td>49.64 ± 1.25</td>
<td>60.99 ± 0.45</td>
<td>85.42 ± 1.86</td>
<td>112.07 ± 2.56</td>
<td>153.93 ± 2.95</td>
</tr>
</tbody>
</table>
4.4.2. *p*-Anisidine value

The pAV test is a measure of the amount of aldehydes, principally 2-alkenals and 2,4-dienals, in the seed butter oil. Aldehydes are secondary lipid oxidation products. The results were shown in Tables 4.3 and 4.4. The initial pAV of seed butter oil was 4.70. After 31 days of storage at 30°C, the pAVs for the control and 100, 400, 700 ppm α-tocopherol fortified seed butter oil changed to 5.31, 4.28, 4.79 and 4.77. At 50°C, the pAVs for the control and 100, 400, 700 ppm increased to 9.12, 8.34, 7.59 and 7.18. The results showed that temperature significantly (p<0.05) influenced the pAV of the seed butter oil. However, α-tocopherol did not show significant (p>0.05) effects on the pAV of the seed butter oil when compared with control at 30°C and 50°C.
### p-Anisidine value

<table>
<thead>
<tr>
<th>Sample</th>
<th>0</th>
<th>4</th>
<th>7</th>
<th>14</th>
<th>20</th>
<th>31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.70 ± 0.00</td>
<td>5.00 ± 0.21</td>
<td>5.17 ± 0.10</td>
<td>5.10 ± 0.10</td>
<td>6.22 ± 1.25</td>
<td>5.31 ± 0.14</td>
</tr>
<tr>
<td>100 ppm</td>
<td>4.70 ± 0.00</td>
<td>3.97 ± 0.75</td>
<td>4.14 ± 1.26</td>
<td>4.30 ± 1.20</td>
<td>5.12 ± 2.26</td>
<td>4.28 ± 1.34</td>
</tr>
<tr>
<td>400 ppm</td>
<td>4.70 ± 0.00</td>
<td>4.58 ± 0.00</td>
<td>4.32 ± 0.41</td>
<td>5.02 ± 0.45</td>
<td>5.25 ± 1.41</td>
<td>4.79 ± 0.78</td>
</tr>
<tr>
<td>700 ppm</td>
<td>4.70 ± 0.00</td>
<td>4.66 ± 1.05</td>
<td>4.61 ± 1.15</td>
<td>4.84 ± 1.28</td>
<td>5.18 ± 1.73</td>
<td>4.77 ± 1.32</td>
</tr>
</tbody>
</table>

Table 4.3. *p*-Anisidine value of the seed butter oil stored for 31 days at 30°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>0</th>
<th>4</th>
<th>7</th>
<th>14</th>
<th>20</th>
<th>31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.70 ± 0.00</td>
<td>4.78 ± 0.59</td>
<td>4.86 ± 0.55</td>
<td>5.60 ± 0.38</td>
<td>6.12 ± 0.24</td>
<td>9.12 ± 0.56</td>
</tr>
<tr>
<td>100 ppm</td>
<td>4.70 ± 0.00</td>
<td>4.45 ± 0.79</td>
<td>4.55 ± 1.12</td>
<td>4.95 ± 1.26</td>
<td>5.39 ± 0.61</td>
<td>8.34 ± 1.26</td>
</tr>
<tr>
<td>400 ppm</td>
<td>4.70 ± 0.00</td>
<td>4.33 ± 1.09</td>
<td>4.68 ± 1.42</td>
<td>4.94 ± 1.38</td>
<td>4.95 ± 0.60</td>
<td>7.59 ± 1.27</td>
</tr>
<tr>
<td>700 ppm</td>
<td>4.70 ± 0.00</td>
<td>4.17 ± 1.46</td>
<td>4.61 ± 2.10</td>
<td>4.97 ± 1.83</td>
<td>4.63 ± 0.44</td>
<td>7.18 ± 1.47</td>
</tr>
</tbody>
</table>

Table 4.4. *p*-Anisidine value of the seed butter oil stored for 31 days at 50°C.
4.4.3. Totox value

Figures 4.3 and 4.4 show the TVs of seed butter oils stored at 30°C and 50°C for 31 days. The initial TV of the seed butter oil was 76.79. At 30°C, the TVs for the control and 100, 400, 700 ppm fortified seed butter oils increased to 128.09, 139.33, 141.78 and 147.81 on day 31 of storage, respectively. At 50°C, the TVs for the control and 100, 400, 700 ppm fortified seed butter oils rose up to 286.89, 297.75, 311.92 and 315.04 on day 31 of storage. The results showed that temperature significantly influenced the TV of the seed butter oils. In Figure 23, the seed butter oil fortified with 700 ppm α-tocopherol had significant (p<0.05) higher TV than the control after 31 days of storage at 30°C. At 50°C (Fig. 4.4), the TVs of seed butter oils fortified with 400 and 700 ppm α-tocopherol were significantly (p<0.05) higher than the control on day 31 of storage.
Figure 4.3. Totox value of the seed butter oil stored for 31 days at 30°C.

Figure 4.4. Totox value of the seed butter oil stored for 31 days at 50°C.
4.5. Discussion

In this study, the antioxidant ability of $\alpha$-tocopherol was investigated by testing its activity in the seed butter oil stored at 30°C and 50°C for 31 days. The results showed that temperature was a factor that influenced a higher level of oxidation in the samples. This is supported in previous studies reported by Mureșan et al. (2015). The fact that the 400 and 700 ppm $\alpha$-tocopherol significantly (p<0.05) increased the TV of the seed butter oil when compared with the control, means that $\alpha$-tocopherol did not inhibited but increased the rate of lipid oxidation in the samples. Shishkov, Shunanov, & Andreev (1979) found that the antioxidant ability of tocopherols decreased when the concentration was higher or lower than the optimum. This seed butter was made with sesame, sunflower and pumpkin seeds. Sunflower seeds are rich in $\alpha$-tocopherol (196.70 – 262.74 ppm) as reported by Zilic et al. (2010). Sesame and pumpkin seeds contain large quantity of $\gamma$-tocopherol, 408.3 – 437.9 and 293 – 569 ppm, respectively (Hajimahmoodi et al., 2010; Tuberoso, Kowalczyk, Sarritzu, & Cabras, 2007). This shows that the seed butter oil used in this study may have already contained optimal amount of $\alpha$-tocopherol. Therefore, the addition of $\alpha$-tocopherol did not inhibit or delay the lipid oxidation in seed butter samples.

4.6. Conclusion

The results of this study showed that temperature is a significant (p<0.05) factor influencing the PV, AV and TV of the seed butter oils. The addition of $\alpha$-tocopherol did not delay the lipid oxidation in seed butter oils at 30°C and 50°C after 31 days of storage
in the dark. However, α-tocopherol did not have an antioxidant effect in the seed butter oil at 30°C and 50°C when the addition of α-tocopherol was equal or higher than 400 ppm.

4.7. Reference

CHAPTER 5

CONCLUSION

This study explored the potential of selected food additives to extend the shelf life of seed butter made with sesame, sunflower and pumpkin seeds. The results showed that grape seed extract is potent as an antimicrobial agent against Salmonella enterica and Listeria innocua in the seed butter. Cinnamaldehyde was also able to reduce S. enterica but L. innocua in the seed butter. To maintain the texture and to prevent oil separation in the seed butter, hydrogenated rapeseed oil (HRO) was found to prevent excessive hardness and effectively control the oil separation of the seed butter during the storage time in this study. On the other hand, palm kernel oil (PKO) was weak in preventing excessive hardness, and it did not prevent oil separation in the seed butter. This showed that HRO was a more efficient stabilizer than PKO in the seed butter. This study also reported the antioxidant effect of α-tocopherol in the seed butter. The results showed that by adding α-tocopherol to the seed butter oil above the level of its natural background concentration did not effectively inhibit or delay lipid oxidation.
LIST OF REFERENCES


concern as vehicles of foodborne pathogens. *Journal of Food Protection*, 76(1), 150-172. doi:10.4315/0362-028X.JFP-12-211


Mechanical properties, release kinetics and antibacterial and antibiofilm activities. 

*Applied Microbiology and Biotechnology*, 96(4), 1029–1038. 
doi:10.1007/s00253-012-4091-3


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APPENDIX A: RAW DATA
### Log reduction of *S. enterica* in GSE fortified seed butter.

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rep 1</td>
<td>7.37</td>
<td>7.31</td>
<td>7.21</td>
<td>6.85</td>
<td>6.79</td>
<td>6.53</td>
</tr>
<tr>
<td>rep 2</td>
<td>7.41</td>
<td>7.08</td>
<td>6.83</td>
<td>6.94</td>
<td>6.59</td>
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<td>rep 3</td>
<td>7.50</td>
<td>7.20</td>
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<td>6.92</td>
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<td></td>
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<td>6.32</td>
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<td>5.83</td>
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<td>6.63</td>
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<td>6.02</td>
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</table>

### Log reduction of *S. enterica* in cinnamaldehyde fortified seed butter.

<table>
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<th>3</th>
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<th>7</th>
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<td>rep 1</td>
<td>7.37</td>
<td>7.31</td>
<td>7.21</td>
<td>6.85</td>
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<td>rep 2</td>
<td>7.41</td>
<td>7.08</td>
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<td>6.94</td>
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PV of α-tocopherol fortified seed butter at 30°C.

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PV of α-tocopherol fortified seed butter at 50°C.
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pAV of $\alpha$-tocopherol fortified seed butter at 30°C.

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TV of α-tocopherol fortified seed butter at 30°C.

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TV of α-tocopherol fortified seed butter at 50°C.
ANOVA and Tukey-Kramer for *S. enterica* in GSE fortified seed butter.

**Oneway Analysis of 1 By Antimicrobial levels**

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**Comparisons for all pairs using Tukey-Kramer HSD**

Connecting Letters Report

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### Comparisons for all pairs using Tukey-Kramer HSD Connecting Letters Report

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Oneway Analysis of 7 By Antimicrobial levels

Analysis of Variance
Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F
--- | --- | --- | --- | --- | ---
Antimicrobial levels | 3 | 5.5435000 | 1.84783 | 74.8616 | <.0001*
Error | 8 | 0.1974667 | 0.02468 | 0.02468 |
C. Total | 11 | 5.7409667 |

Means for Oneway Anova
Level | Number | Mean | Std Error | Lower 95% | Upper 95%
--- | --- | --- | --- | --- | ---
10% | 3 | -2.3667 | 0.09071 | -2.576 | -2.157
15% | 3 | -2.4300 | 0.09071 | -2.639 | -2.221
5% | 3 | -1.4600 | 0.09071 | -1.669 | -1.251
Control | 3 | -0.7900 | 0.09071 | -0.999 | -0.581

Comparisons for all pairs using Tukey-Kramer HSD
Connecting Letters Report
Level | Mean
--- | ---
Control | A
5% | B
10% | C
15% | C

Oneway Analysis of 9 By Antimicrobial levels

Analysis of Variance
Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F
--- | --- | --- | --- | --- | ---
Antimicrobial levels | 3 | 3.2520667 | 1.08402 | 11.6530 | 0.0027*
Error | 8 | 0.7442000 | 0.09302 |
C. Total | 11 | 3.9962667 |
### Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>3</td>
<td>-2.2267</td>
<td>0.17609</td>
<td>-2.633</td>
<td>-1.821</td>
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<td>0.17609</td>
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<td>-1.237</td>
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<td>-0.717</td>
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### Comparisons for all pairs using Tukey-Kramer HSD Connecting Letters Report

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<tbody>
<tr>
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<tr>
<td>5% A B</td>
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<tr>
<td>10% B C</td>
<td>-2.226667</td>
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<tr>
<td>15% C</td>
<td>-2.460000</td>
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ANOVA and Tukey-Kramer of *S. enterica* in cinnamaldehyde fortified seed butter.

### Oneway Analysis of 1 By Antimicrobial levels

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<th>Prob &gt; F</th>
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<tbody>
<tr>
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<td>1.1026917</td>
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<td>6.7879</td>
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<td>0.4332000</td>
<td>0.054150</td>
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<td>1.5358917</td>
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### Means for Oneway Anova

<table>
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<tr>
<th>Level</th>
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<th>Mean</th>
<th>Std Error</th>
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<th>Upper 95%</th>
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<td>-0.0235</td>
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<td>-0.2266667</td>
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<td>-0.536</td>
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### Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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<tbody>
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<tr>
<td>1%</td>
<td>A</td>
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<tr>
<td>1.5%</td>
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### Oneway Analysis of 3 By Antimicrobial levels

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Means for Oneway Anova

<table>
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<th>Upper 95%</th>
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</thead>
<tbody>
<tr>
<td>0.1%</td>
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<td>-0.7433</td>
<td>0.23860</td>
<td>-1.294</td>
<td>-0.1931</td>
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<td>-1.1500</td>
<td>0.23860</td>
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<td>-0.5998</td>
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<tr>
<td>1.5%</td>
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<td>-1.2200</td>
<td>0.23860</td>
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<td>-0.6698</td>
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<td>0.23860</td>
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<td>0.1735</td>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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<tbody>
<tr>
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<tr>
<td>0.1%</td>
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<tr>
<td>1%</td>
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Oneway Analysis of 5 By Antimicrobial levels

Analysis of Variance

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<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
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Means for Oneway Anova

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<th>Std Error</th>
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<th>Upper 95%</th>
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<tbody>
<tr>
<td>0.1%</td>
<td>3</td>
<td>-0.6933</td>
<td>0.22285</td>
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<td>1.5%</td>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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<tbody>
<tr>
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<tr>
<td>0.1%</td>
<td>A -0.693333</td>
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<tr>
<td>1%</td>
<td>A B -1.336667</td>
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<tr>
<td>1.5%</td>
<td>B -2.263333</td>
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Oneway Analysis of 7 By Antimicrobial levels

<table>
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<th>Prob &gt; F</th>
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<tr>
<td>Antimicrobial levels</td>
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<td>6.1183000</td>
<td>2.03943</td>
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Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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<tr>
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<tbody>
<tr>
<td>Control</td>
<td>A</td>
</tr>
<tr>
<td>0.1%</td>
<td>A B</td>
</tr>
<tr>
<td>1%</td>
<td>B C</td>
</tr>
<tr>
<td>1.5%</td>
<td>C</td>
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Oneway Analysis of 9 By Antimicrobial levels

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<th>Mean Square</th>
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<th>Prob &gt; F</th>
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<tbody>
<tr>
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<td>7.4627000</td>
<td>2.48757</td>
<td>13.3268</td>
<td>0.0018*</td>
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<td>C. Total</td>
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Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<tbody>
<tr>
<td>0.1%</td>
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<td>-1.2333</td>
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Comparisons for all pairs using Tukey-Kramer HSD
Connecting Letters Report

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<tr>
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<td>-1.123333</td>
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</tr>
<tr>
<td>0.1%</td>
<td>A</td>
</tr>
<tr>
<td>1%</td>
<td>B</td>
</tr>
<tr>
<td>1%</td>
<td>B</td>
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<tr>
<td>1.5%</td>
<td>C</td>
</tr>
<tr>
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ANOVA and Tukey-Kramer of *L. innocua* in GSE fortified seed butter.

### Oneway Analysis of d1 By Antimicrobial levels

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<th>Prob &gt; F</th>
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<tbody>
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<td>&lt;.0001</td>
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### Means for Oneway Anova

<table>
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<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<tbody>
<tr>
<td>10%</td>
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<td>-2.5667</td>
<td>0.16197</td>
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### Comparisons for all pairs using Tukey-Kramer HSD

**Connecting Letters Report**

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<tr>
<td>5%</td>
<td>A -0.706667</td>
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<tr>
<td>10%</td>
<td>B -2.566667</td>
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<tr>
<td>15%</td>
<td>B -3.153333</td>
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### Oneway Analysis of d3 2 By Antimicrobial levels

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<th>Prob &gt; F</th>
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### Analysis of Variance

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<th>Prob &gt; F</th>
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<tbody>
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### Analysis of Variance

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<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
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### Means for Oneway Anova

<table>
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<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<tbody>
<tr>
<td>10%</td>
<td>3</td>
<td>-2.6700</td>
<td>0.17985</td>
<td>-3.085</td>
<td>-2.255</td>
</tr>
<tr>
<td>15%</td>
<td>3</td>
<td>-3.6700</td>
<td>0.17985</td>
<td>-4.085</td>
<td>-3.255</td>
</tr>
<tr>
<td>5%</td>
<td>3</td>
<td>-0.8300</td>
<td>0.17985</td>
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### Comparisons for all pairs using Tukey-Kramer HSD

#### Connecting Letters Report

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<tr>
<td>5%</td>
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<tr>
<td>10%</td>
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Oneway Analysis of d7 By Antimicrobial levels

Analysis of Variance

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Means for Oneway Anova

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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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Oneway Analysis of d9 By Antimicrobial levels

Analysis of Variance

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**Means for Oneway Anova**

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**Comparisons for all pairs using Tukey-Kramer HSD**

**Connecting Letters Report**

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ANOVA and Tukey-Kramer of hardness of HRO and PKO stabilized seed butter.

**Oneway Analysis of 0 By Stabilizer Level**

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**Means for Oneway Anova**

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**Comparisons for all pairs using Tukey-Kramer HSD**

**Connecting Letters Report**

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### Means for Oneway Anova

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### Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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Oneway Analysis of 5 By Stabilizer Level

Analysis of Variance

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Means for Oneway Anova

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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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#### Analysis of Variance

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#### Means for Oneway Anova

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#### Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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### Oneway Analysis of 7 By Stabilizer Level

![Graph showing data distribution for different stabilizer levels.](image)

#### Analysis of Variance

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#### Means for Oneway Anova

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#### Comparisons for all pairs using Tukey-Kramer HSD Connecting Letters Report

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ANOVA and Tukey-Kramer of adhesiveness of HRO and PKO stabilized seed butter.

**Oneway Analysis of 0 By Stabilizer level**

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**Means for Oneway Anova**

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<th>Upper 95%</th>
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**Comparisons for all pairs using Tukey-Kramer HSD**

**Connecting Letters Report**

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Oneway Analysis of 1 By Stabilizer level

Analysis of Variance
Source      DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Stabilizer level  6  36.553986  6.09233  4.3420  0.0378*
Error        7  9.821850  1.40312
C. Total     13  46.375836

Means for Oneway Anova
Level  Number  Mean  Std Error  Lower 95%  Upper 95%
NSB      2   6.73500  0.83759  4.7544  8.7156
PAL1     2   5.48500  0.83759  3.5044  7.4656
PAL2     2   3.63500  0.83759  1.6544  5.6156
PAL3     2   2.11000  0.83759  0.1294  4.0906
PO1      2   5.08000  0.83759  3.0994  7.0606
PO2      2   3.24500  0.83759  1.2644  5.2256
PO3      2   2.21500  0.83759  0.2344  4.1956

Oneway Analysis of 3 By Stabilizer level

Analysis of Variance
Source      DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Stabilizer level  6  53.640390  8.94007  4.3787  0.0107*
Error        14  28.584133  2.04172
C. Total     20  82.224524

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Means for Oneway Anova

<table>
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<th>Level</th>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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<td>NSB</td>
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</tr>
<tr>
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<td>A, B</td>
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<td>PAL2</td>
<td>A, B</td>
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<td>A, B</td>
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Oneway Analysis of 5 By Stabilizer level

Analysis of Variance

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Means for Oneway Anova

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Comparisons for all pairs using Tukey-Kramer HSD
Connecting Letters Report

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Oneway Analysis of 7 By Stabilizer level

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Means for Oneway Anova

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Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
Connecting Letters Report

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Oneway Analysis of 14 By Stabilizer level

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Analysis of Variance

Means for Oneway Anova

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<th>Upper 95%</th>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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<td>A</td>
</tr>
<tr>
<td>PO2</td>
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</tr>
<tr>
<td>PAL2</td>
<td>A</td>
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<td>PAL3</td>
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Mean: 11.440000
Mean: 11.263333
Mean: 11.136667
Mean: 10.313333
Mean: 9.146667
Mean: 8.820000
Mean: 5.410000
Oneway Analysis of 21 By Stabilizer level

Analysis of Variance

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Means for Oneway Anova

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<th>Std Error</th>
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<th>Upper 95%</th>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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Oneway Analysis of 28 By Stabilizer level

Analysis of Variance

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<th>F Ratio</th>
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Means for Oneway Anova

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<th>Number</th>
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<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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Oneway Analysis of 35 By Stabilizer level

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Means for Oneway Anova

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<th>Upper 95%</th>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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</tr>
<tr>
<td>PO2</td>
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</tr>
<tr>
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<tr>
<td>PAL1</td>
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134
Oneway Analysis of 42 By Stabilizer level

Analysis of Variance

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Means for Oneway Anova

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<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<tbody>
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<td>0.63601</td>
<td>9.723</td>
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Comparisons for all pairs using Tukey-Kramer HSD

Connecting Letters Report

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<td>A B</td>
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ANOVA and Tukey-Kramer of $\alpha$-tocopherol fortified seed butter at 30°C.

### Oneway Analysis of 30 By Concentration

![Oneway Analysis of 30 By Concentration](image)

#### Analysis of Variance

<table>
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<tr>
<th>Source</th>
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<th>F Ratio</th>
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#### Means for One way Anova

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<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<tbody>
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#### Comparisons for all pairs using Tukey-Kramer HSD Connecting Letters Report

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ANOVA and Tukey-Kramer of $\alpha$-tocopherol fortified seed butter at 50°C.

### Oneway Analysis of 30 By Concentration

<table>
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<th>Mean</th>
<th>Std Error</th>
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<th>Upper 95%</th>
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<tbody>
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### Comparisons for all pairs using Tukey-Kramer HSD

#### Connecting Letters Report

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<td>A</td>
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