Light Effects on Soy Oil and Soymilk Influence Oxidation, Product
Quality, and Packaging Decisions

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Quality, and Packaging Decisions

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Abstract

The primary goal of this research was to evaluate the effects of
light on soymilk, including the oil component. Soybean oil with added
chlorophyll a (0, 1, and 2μg/ml), a photosensitizer, was exposed to light
(no light [control], broad-spectrum light) and narrow-band wavelengths
(430nm, 450nm, and 660nm] for 4h. Chlorophyll completely degraded
under broad-spectrum light and 430nm treatments; 64% degradation
occurred at 660nm. Oil with chlorophyll addition resulted in
significantly higher peroxide values and malondialdehyde
concentrations with light exposure to broad spectrum and 430nm
wavelengths. Light at 430 and 660 nm degraded chlorophyll and
increased risk of oxidation in soybean oil.

Soymilk contains low concentrations of chlorophyll, the
photosensitizer riboflavin, as well as highly susceptible oxidizable
substrates from the soy oil. Soymilk (1% fat from soybean oil) was
packaged under a positive flow hood into 5 high density polyethylene
(HDPE) packages and stored for 36 days at 4°C under fluorescent
lighting (1122 lux $\pm$ 439 lux). Control packaging had no light protective
additive (LPA; positive (foil-wrapped) and negative control) and the
experimental packaging treatments had three levels of LPA (low,
medium, high). Chemical and sensory analyses to measure oxidation
changes were completed on the product at days, 1, 4, 8, 15, 22, 29, and
36. HDPE packages with high LPA protected the sensory quality of the
product as well as the positive control (foil-wrapped) packages for a
minimum of 15 days. High-LPA HDPE protected soymilk for 29 days
from degradation of riboflavin and limited development of aldehyde
end-products associated with photooxidation.

Soymilk was treated with food grade TiO_2 at levels of 0, 0.5, and
1.0% by weight. TiO_2 significantly whitened the product as
demonstrated by L* values. TiO_2-treated soymilks resulted in
significantly improved hedonic scores for appearance, smell, taste,
mouthfeel, and aftertaste compared to control soymilk. However, in a
second experiment, overall acceptability of TiO_2-treated soymilk, at

additions of 0.1%, 0.3%, and 0.5% TiO_2, was not higher than control soy milk.

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Dedicated to Jack E. Henney, father and friend extraordinaire.

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Chapter 1 Introduction

Functional foods are those foods considered to have health benefits above and beyond providing nutrients to meet basic nutritional requirements (IFT Expert Report, 2005). There is no legal definition of a functional food, but both the Academy of Nutrition and Dietetics and the Institute of Food Technologists recognize that foods contain bioactive components that provide for a decreased risk of certain diseases (ADA, 2009; IFT Expert Report, 2005). Consumers have a growing interest in such foods (Mintel, 2012) and, because of this interest, the food industry has either marketed foods already available or developed foods with functional ingredients for providing health benefit. For example, consider the marketing of soy products containing soy protein and isoflavones. Soy protein itself can meet basic protein needs, but it has also been demonstrated to decrease risk of heart disease under certain conditions (Sacks et al., 2006). The phytochemicals, isoflavones, in soy products are not needed to meet basic nutrient requirements but they too have been shown to have antioxidant activity and estrogen-like activity; thus, they have been shown to provide decreased risk of bone disease (Reinwald and

Weaver, 2006) and some cancers (Korde et al., 2009). However, isoflavones have also been implicated as contributing to increased risk of some cancers (Ju et al., 2001). Labels on soy protein-containing foods are by law allowed to state the FDA authorized health claim, "25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. A serving of [name of food] supplies _ grams of soy protein." (Code of Federal Regulations, 2013).

Soymilk is one functional food that offers health benefits beyond basic nutrient requirements. It is a good source of polyunsaturated fatty acids and isoflavones, and also provides 6 grams of protein in a 240 ml (8 ounce) serving (Hammond, 1991; Gebhardt and Thomas, 2002; Xu and Change, 2009; Tucker et al., 2010). Soymilk popularity is increasing significantly in the U.S. Sales of soymilk have increased from over $250 million in 1996 to approximately $1.25 billion in 2011. (Soyfoods, 2014).

Food packaging is vital to the success of a product. Packaging has four purposes: 1) to protect food from contaminants and light; 2) to contain it in a way that it can be transported and stored; 3) to provide convenience such that the product is accessible and easily managed; and

4) to communicate information about the product to the consumer (Roberts, 2006).

Our work addresses how packaging can function to protect foods from light. Light can have damaging effects on foods that contain components that are photosensitive, resulting in photooxidation. Photooxidation is responsible for deleterious sensory and chemical aspects of many foods, particularly those that contain polyunsaturated fatty acids. Soymilk contains 1-4 g lipid per 240 ml (8 ounces) and approximately 66% of this lipid is polyunsaturated. Photooxidation of the lipids in soybean oil is known to result in 2-pentyl furan and 2-pentenyl furan, two compounds that are responsible for reversion flavor and odor in soybean oil.

Soymilk contains riboflavin, a light sensitive vitamin. Riboflavin and chlorophyll are both photosensitive molecules that can initiate photooxidation. Riboflavin has been extensively studied in this reaction, mostly in dairy products, but limited information relates chlorophyll as a contributor to light-induced oxidation reactions in foods, particularly in terms of packaging needs. While riboflavin has been studied extensively in this context, it has become apparent that

chlorophyll may also have an important role in photooxidation (Wold et al., 2005; Webster et al., 2011).

Chlorophyll is present in plants, and therefore is present in foods of plant origin. Chlorophyll and its degradative products are in soybean oil and are likely in soymilk as it is a plant-based product.

It is useful to understand how chlorophyll behaves when exposed to light and more importantly, what type of responses are seen in chlorophyll-containing foods when they are exposed to light. Understanding how chlorophyll responds at excitation wavelengths in different food matrices and how it subsequently affects food quality is a valuable contribution for characterizing food packaging requirements.

The increase in soymilk sales in the U.S. may be attributed to processing modifications for enhancing soymilk flavor and mouthfeel (Achouri et al., 2008; Lv et al., 2011; Zhang et al., 2012). In western cultures, soymilk has been described as having a beany flavor and chalky texture but substantial improvements have been made. The flavor attributes of soymilk have been described in several research studies (Torres-Penaranda and Reitmeier, 2001; Wansink, 2003a; Wansink, 2003b; N'Kouka et al., 2004; Chambers et al., 2006). However, the color of soymilk, which ranges from a brown/tan to creamy white, is

highly variable and potentially influences the perception of quality. The color of soymilk is different from the white-yellow of bovine milk; how this affects consumer perception of soymilk has not been studied. Whitening of soymilk to a standard color, similar to bovine milk, may have a valuable impact on quality perception of soymilk.

In this study, both soybean oil and soymilk were used for exploring the effects of light on the photoresponsive molecules riboflavin and chlorophyll. The outcomes of this study can provide benefit in understanding the implications of light on foods containing photoresponsive molecules such as riboflavin, chlorophyll or other pigments that respond to light. In addition to dairy milk, cheeses, and yogurts, and foods containing polyunsaturated fatty acids, many functional food beverages are being developed from spinach and spirulina, which contain chlorophyll (Esquivel, 2013). Chlorophyll is also used as a natural food coloring agent and there is an increased demand for natural colorings from consumers (FMI, 2011).

The primary goal of this research was to evaluate the effects of light on soymilk, including soy oil. Specific interest was focused on the implications to nutrient degradation and flavor quality, as measured by analytical and/or sensory methods. To accomplish this goal, three

studies were completed with the following objectives: 1) determine how chlorophyll responds under specific wavelengths of light and how that affects the lipid portion (soy oil) of soymilk; 2) determine how packaging can protect soymilk from the damaging effects of light on chemical and sensory characteristics; and 3) determine if modifying the color of soymilk can improve how much consumers like this product.

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Chapter 2 Literature Review

Functional Foods

Functional foods are of interest to consumers for the proposed benefit of adding nutritional value to a food for health benefits. No single accepted definition of functional foods that is used by all agencies in the United States. The American Dietetic Association defines functional foods as those that " ...move beyond necessity to provide additional health benefits that may reduce disease risk and/or promote optimal health" (Hasler and Brown, 2009). The Institute of Food Technologists defines functional foods as "...foods and food components that provide a health benefit beyond basic nutrition (for the intended population)" (IFT Expert Report, 2005).   In the United States and European countries there is no legal term of functional food; rather any reference to a functional food is strictly for marketing purposes. Currently Japan is the only country that has a definition of foods for specified health use (FOSHU) in which foods can be marketed as such if they have functional ingredients that affect specific aspects of health (Henry, 2010).   The U.S. Food and Drug Administration enforces U.S. law regarding functional foods only with jurisdiction over food label

claims. Structure/function claims are allowed to describe the amount

and the role of a nutrient or dietary supplement ingredient in relation to

how it affects normal structure or function in humans. Authorized

claims are allowed to describe a food or food component in relation to

reducing the risk of a specific disease or condition. Qualified health

claims allows the suggestion that a food or food component may reduce

the risk, but with the acknowledgment that evidence is not conclusive

(Hoadley and Rowlands, 2008).

Consumers are interested in functional foods and beverages that

have specific added nutrients and other compounds proposed to have

health benefits. Sloan (2012) summarizes marketing research on how

consumers are targeting functional foods. Consumers of the 18-24 year

old age group are the largest and fastest growing age sector of

functional food purchases. Up to 30% of consumers look for foods that

have labeling describing benefits of nutrients for disease. More than

50% of consumers choose foods to control diabetes, and to reduce

obesity, and control high cholesterol. Approximately one fourth of

consumers seek products rich in antioxidants (Sloan, 2012).

Soymilk as a Functional Food

Soymilk is a beverage of functional food interest as it is a plant source of calcium and other nutrients and thus, an alternative to cow's milk. Therefore, it is consumed by strict vegans, individuals who suffer from lactose intolerance, those who have cow milk protein allergies, and those who choose it for the health benefits associated with the myriad of functional components in the product (Tucker et al., 2010). Much research has been performed on isoflavones as components of soymilk that affect bone health, menopause symptoms, cardiovascular health, and risk of breast cancer (Ye et al., 2006; Reinwald and Weaver, 2006; Sacks et al., 2006; Tucker et al., 2010; Korde et al., 2009). Soy protein has been demonstrated to have beneficial effects on cardiovascular health and as such the FDA has allowed food claims related to this aspect of soy. The FDA allows the health claim, "25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. A serving of [name of food] supplies __ grams of soy protein." (Code of Federal Regulations, 2013). This increase in soy product consumption benefits Americans in the overall dietary goals of increasing fiber, decreasing saturated fat, cholesterol (Tucker et al.,

2010; Soyfoods Association of North America, 2012).

Soymilk is a water extract of soybeans, which contains proteins, essential fatty acids, antioxidants and some complex carbohydrates considered beneficial to health (Xu and Chang, 2009). While soy does contain the eight essential amino acids required by adults, soy is limited in the amounts of methionine. Typically, dried soybeans are cleaned and then soaked in water. The bean increases in weight, 2.2-2.3 times its initial weight. Some manufactures will dehull soybeans in an effort to improve flavor and extend shelf life. They are then ground; the fluid portion is extracted for soymilk (Chang, 2010; Sivanandan et al., 2008). A great deal of research has focused on processing methodology to improve sensory characteristics of soymilk, particularly targeted to meet expectations for consumers in western cultures. This soymilk is subject to ultra-high temperature for up to one minute to destroy pathogenic microorganisms; destroy anti-nutritional factors such as trypsin inhibitors and lectins; inhibit undesired enzymes; and improve flavor (Chang, 2007; and Xu and Chang, 2009; Kwok et al., 2002; Yuan et al., 2008).

Nutritional Composition of Soymilk

Nutritionally, soymilk closely resembles dairy milk (Table 1). They are comparable in protein in concentration; the primary soy proteins are glycinin and beta conglycinin versus casein and whey proteins, the primary milk proteins. Both soy and cow milk protein provide all nine essential amino acids (including histidine for infants) (Singh et al., 2008; Swaisgood, 1995). Soymilk and cow milk are both sources of beta-carotene and tocopherols. Soy contains many phytochemicals including isoflavones, including genistein and daidzein, phenolic acids, phytosterols, and saponins (Xu and Chang, 2009). Soymilk contains sucrose, stachyose, raffinose, and other oligosaccharides whereas the primary carbohydrate in cow milk is lactose. Furthermore, soymilk has a small amount of fiber.

Table 1. Nutrient composition of soymilk compared to 2% cow's milk per 240 mL (8 fluid ounces) (Pennington, 1994; Gebhardt and Thomas, 2010)

	Kcal	Prot g/8oz	Fat g/8oz	CHO g/8oz	Fiber g/8oz	Vit A IU/8oz	Ca mg/8oz	Ribo mg/8oz
Soymilk	79	6.6	4.6	4.3	1	208*	10	0.17
2% fat bovine milk	120	8.1	4.7	11.7	0	500*	297	0.4

* bovine and soymilk are fortified with vitamin A

While heat processing can destroy anti-nutritional factors, such as

trypsin inhibitors and lectins, in soymilk, others may not be inhibited. Therefore, soymilk nutrients may not be fully bioavailable due to interaction of goiterogens, saponins, phytates and oligosaccharides with nutrients (Anderson and Garner, 2000).

The lipid profile of soymilk has a higher proportion of unsaturated fatty acids compared to cow's milk. The primary fatty acids in soymilk are linoleic acid (LA, C18:2n-6), which makes up approximately 58% of the fatty acid content, and linolenic acid (ALA C18:3,n-3), which makes up 7.8% of the fatty acid content (Table 2) (Hammond, 1991). Soymilk lipids are low in saturated fatty acids and very few trans fats are produced during processing (Penalvo et al., 2004). Soymilk does not contain the nutritionally relevant omega-3 fatty acids, eicospentanoic, and docosahexanoic acids, but it does contain linoleic (an omega-6 fatty acid) and linolenic (an omega-3 fatty acid) acids; the latter is a precursor for omega-3 fatty acids that have nutritional relevance. Additionally, because soymilk is plant based, it contains only trace amounts of cholesterol. Thus, in terms of its lipid profile, soymilk also offers nutritional benefits to consumers and meets dietary recommendations for Americans, particularly since only 15% of the fat is saturated as compared to cow's milk, with approximately 62% (2%

fat milk) saturated fatty acids (Gebhardt and Thomas, 2010).

Table 2. Fatty acid composition of soy oil and 2% bovine milk
(Hammond, 1991; Jensen, 1995)

Percentage of total fat	Palmitic 16:0	Stearic 18:0	Oleic 18:1ω9	Linoleic 18:2:ω6	Linolenic 18:3ω3
Soymilk	11.4	3.7	22.9	53.6	8.4
Bovine milk	23.9	7.0	24	2.5	trace

Health Relevance of the Fatty Acid Profile of Soymilk

As mentioned above, soymilk is a good source of linoleic acid (LA, C18:2 ω-6) an omega-6-fatty acid and linolenic acid (ALA, C18:3, ω-3), an omega-3 fatty acid. Both linoleic acid and linolenic acids are polyunsaturated fatty acids (PUFAs) in which there are greater than one carbon-carbon double bond. Omega-6 fatty acids have one of the double bonds six carbons from the terminal methyl end of the fatty acid and omega-3 fatty acids have one of the double bonds three carbon atoms from the terminal methyl end of the fatty acid. The double bonds are in the cis configuration and are separated by a methenyl group and are referred to as methylene interrupted double bonds.

The carbon-carbon double bonds of PUFAs have been shown to be responsible for their dietary benefit because this type of structure

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results in a fatty acid that has a bend in the molecule. This keeps the fatty acids from "layering", hence it is more difficult for the fatty acids to form compact plaques in arteries (Spector, 2006). The ω-3 and ω-6 fatty acids that have relevance to human health are typically 18-22 carbons in length. Humans are unable to synthesize ω3 or ω6 fatty acids *de novo*; therefore, the ω6 and ω3 families are considered to be essential nutrients for health. If LA is consumed in the diet, arachidonic acid (ARA, 20:4, ω-6) can be synthesized by a 2-carbon elongation and desaturation. If ALA is consumed in the diet, eicosapentanoic acid (EPA, 20:5 ω-3) and docosahexanoic acid (DHA, 22:6 ω-3), which are two ω3s of health relevance, can be formed by 2-carbon elongations and desaturation. LA and ALA compete for the enzyme delta-6 desaturase, the first enzyme in the desaturation and elongation reactions, to create ARA and EPA/DHA respectively. Therefore when the ratio of LA (an ω6) is greater than ALA (an ω3), there is greater synthesis of arachidonic acid, a precursor of eicosanoids that promote inflammation. DHA is essential for brain development and EPA has been shown to be necessary for cardiac health and prevention of some cancers. Therefore, EPA/DHA synthesis is desired as well as consumption of these ω3s in the diet. ALA is a preferred substrate over LA yet LA tends to be higher

in most individuals' diets resulting in greater production of arachidonic acid (Lau, 2007).

Intake of PUFAs has health relevance in the human diet. When a greater percentage of the fat intake is from PUFAs, there is a lower risk for cardiovascular disease (Calder, 2004; Ferrucci et al., 2006). It is recommended that less than 30% of total calories consumed come from fat, with 5-10% of daily calories from polyunsaturated fatty acids and 0.6-1.2% of daily calories from linolenic acid (USDA, 2010). However, chemical aspects of these PUFAs render them challenging in foods.

Oxidation

While it is preferred to have a greater percentage of calories from PUFAs rather than saturated fatty acids, PUFAs are susceptible to two different forms of oxidation, autoxidation and photooxidation, in food systems and in the body. Oxidation of polyunsaturated fatty acids refers to the reaction of oxygen with a double bond in a fatty acid resulting in formation of intermediate compounds, such as hydroperoxides, and subsequently leading to aldehydes, ketones, and other degradative products. Both autoxidation and photooxidation require oxygen. Other components of foods, such as amino acids, sugars, carotenoids, and

polyphenols, can also be susceptible to oxidation.

Autoxidation

Autoxidation occurs when an initiator such as heat or metal ions generate reactive species that react with a PUFA to remove a hydrogen atom and renders the fatty acid in a state in which it has an unpaired electron or free radical state. This free radical form of the fatty acid then reacts with O_2 and produces a peroxyl radical, which in turn reacts with another fatty acid to produce another free radical fatty acid and peroxides (Frankel, 1984, Shahidi and Zhong, 2010) (Figure 1). Upon production of these compounds, the lipid peroxides decompose into a number of other components including aldehydes, ketones, alcohols, furans and other degradative products (Frankel 1984). The degree of oxidation that occurs can be determined by monitoring peroxide levels of the system.

Initiation:

(1) $RH \longrightarrow R\bullet + H\bullet$

(2) $R\bullet + O_2 \longrightarrow ROO\bullet$

Propagation:

(3) $ROO\bullet + RH \longrightarrow ROOH + R\bullet$

(4) $R\bullet + O_2 \longrightarrow ROO\bullet$

Termination:

(5) $R\bullet + R\bullet \longrightarrow R_2$

(6) $R\bullet + ROO\bullet \longrightarrow ROOR$

(7) $nROO\bullet \longrightarrow (ROO)_n$
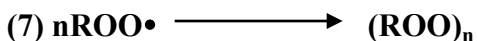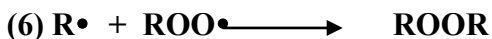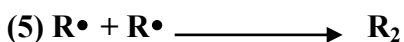
Figure 1. Diagrammatic representation of autoxidation of lipids. (reprinted with permission from Frankel, 1984).

Photooxidation

Photooxidation occurs when a photosensitizer molecule becomes excited upon absorbing energy from a photon; an electron on the photosensitizer is elevated from the ground state orbital to a higher energy orbital. The photosensitizer molecule can behave in three ways upon being excited; the one of importance in food quality is to transfer this energy to another molecule such as triplet oxygen, $^3O_2$.

Molecular Oxygen

To describe the role of photosensitizers in photooxidation, it is first necessary to understand the different states of molecular oxygen.

Triplet oxygen is the most stable form of this molecule. Oxygen upon

accepting energy is then transformed into singlet oxygen, $^1O_2$, which is a

higher energy state for molecular oxygen.

(a) Triplet oxygen ($^3O_2$)   (b) Singlet oxygen ($^1O_2$)
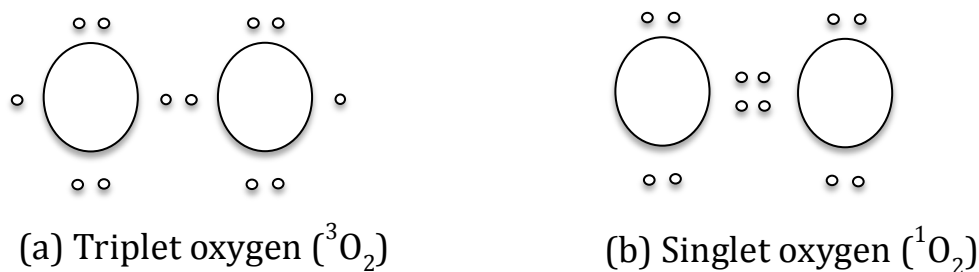
Figure 2. Lewis structure of oxygen atoms with electron orbitals.
Triplet oxygen (a) with two unpaired electrons, and singlet oxygen (b)
with empty orbital

This open orbital is not radical form of molecular O_2 and is electrophilic
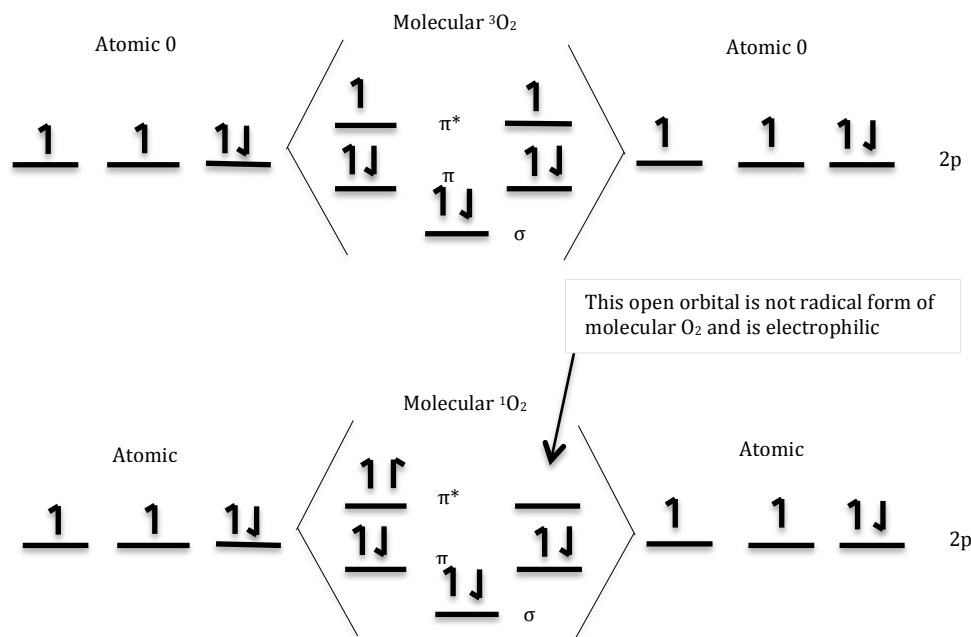
Figure 3. Illustration of molecular orbital of triplet oxygen ($^3O_2$)
and singlet oxygen ($^1O_2$) (reprinted with permission from Min and Boff,
2002).

The empty orbital shown in Figure 2 and Figure 3 illustrates that the $^1O_2$ is not a radical form of molecular oxygen but because the electrons in the outer shell lie in the one single orbital and have opposite spins, it is a molecule that is highly electrophilic. The electron rich double bonds of a polyunsaturated fatty acid are a powerful attractant to singlet oxygen. The resulting hydrogen atom transfer can be illustrated:

$$R\text{-}H \xrightarrow{\ ^1O_2\ } ROOH$$
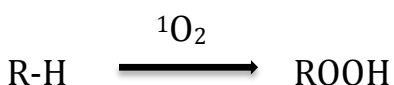
This hydroperoxide is susceptible to degradative pathways illustrating that not all attractions are beneficial.

Photosensitizers

Photosensitizers play an important role in the photooxidation process. Photosensitizers are compounds that initiate oxidation of fatty acids upon excitation due to light. Photosensitizers include different classes of compounds including metal ions and porphyrin ring structures. They absorb electrons from light energy due to their chemical structure. In the case of porphyrin ring structures, there are a number of conjugated double bonds that demonstrate resonance. This allows electrons to become highly excited upon exposure to light energy

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because the electrons are not tightly held within the structure. Thus the electrons are readily donated to triplet oxygen producing singlet oxygen. Riboflavin, chlorophyll, and hemoglobin are compounds that exhibit this conjugated double bond structure and all behave as photosensitizers.

Role of the Photosensitizer in Photooxidation - Type I and Type II Oxidation Pathways

There are two types of pathways that are described to illustrate how photosensitizers initiate photooxidation by reacting either with molecular oxygen or directly with the PUFA (Figure 4). When the ground state of the photosensitizer, Sen, absorbs energy in the form of photons, it is exited to the singlet state, ^{1}Sen*. ^{1}Sen* can then undergo intersystem crossing to its excited triplet state, ^{3}Sen*. At this point ^{3}Sen* may react with $^3O_2$ to form the excited $^1O_2$ described above. The $^1O_2$ formed may then react directly with a PUFA; the ^{3}Sen* returns to ^{1}Sen*. This is referred to as Type II pathway. It is a highly efficient pathway and is believed to be the primary pathway that occurs when chlorophyll is the photosensitizer (Min and Boff 2002).
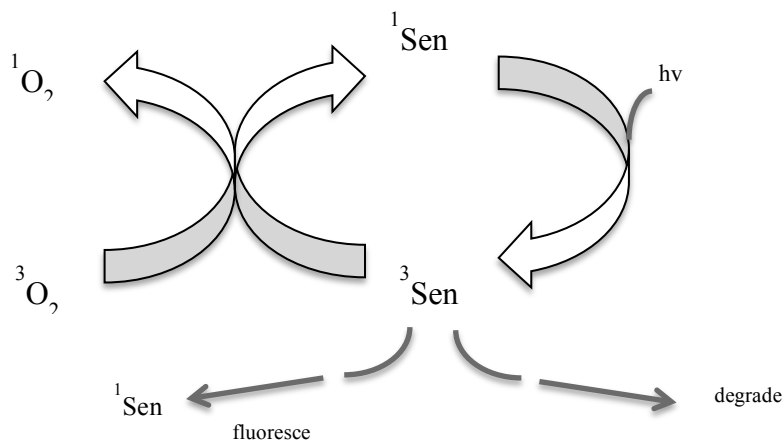
(a) Type II Pathway
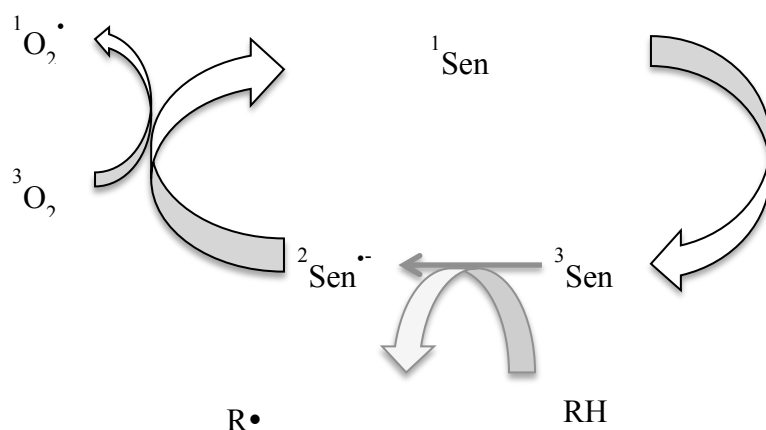
(b) Type I Pathway

Figure 4. Illustration of Type II and Type I pathways of photosensitization of sensitizer to create excited singlet oxygen (a or b) or react directly with a substrate such as a PUFA in which a radical is formed (b) (reprinted (adapted) with permission from Kristensen, D; Kroger-Ohlsen, M.V.; Skibsted, L.H. Radical formation in dairy products: predictions of oxidative stability based on electron spin resonance spectroscopy. In Free Radicals in Foods: Chemistry, Nutrition, and Health Effects. Eds M.J. Morello, F. Shahidi, C. Ho. Copyright 2002. American Chemical Society.)

Alternatively, ^{3}Sen* may react directly with a PUFA. In this case,

the [3]Sen* abstracts a hydrogen atom to create R•. This R• can go on to

react with other PUFAs with the oxidation process continuing as

described. This is referred to as a Type I pathway. It is favored more

under conditions with little oxygen in the food system and is more

typical of how riboflavin participates as a photosensitizer in the

photooxidation process (Min and Boff, 2002).

In Type II pathways, the $^1O_2$ will react with PUFAs in a number of

different reactions referred to as 1,4 – cycloaddition, 1,2 – cyclo

addition, and "ene' reactions. The 1,4-cycloaddition reaction will only

occur with conjugated double bonds (Figure 5, Figure 6, Figure 7).
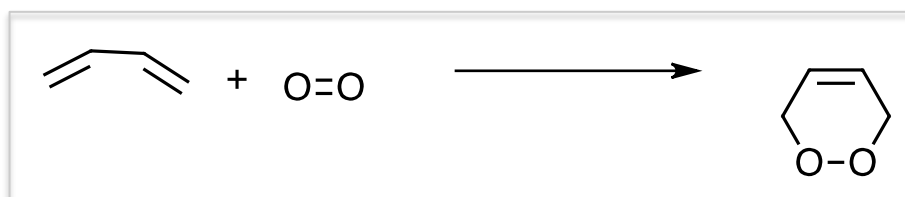
Figure 5. 1,4-cycloaddition of singlet oxygen to conjugated diene. Only occurs with a conjugated diene (reprinted with permission from Min and Boff, 2002).

The 1,2-cylcoaddition can only occur on a single double bond or a

methylene-interrupted double bond. However, the activation energy

necessary for this reaction to occur on a monounsaturated fatty acid is

quite high, so this reaction is most favored for polyunsaturated fatty

acids.

Figure 6. 1,2 cycloaddition of singlet oxygen to a methylene-interrupted double bond or single double bond (reprinted with permission from Min and Boff, 2002).
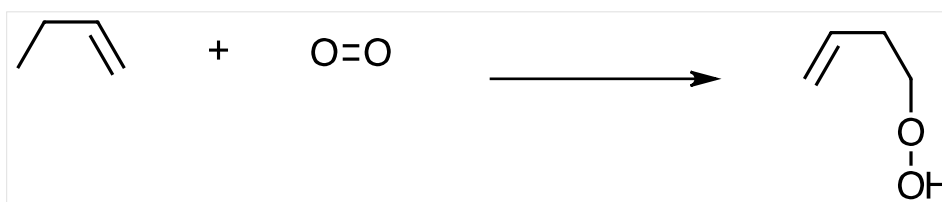
Figure 7. Ene reaction creating a hydroperoxide (reprinted with permission from Min and Boff, 2002).

The ene reactions result in formation of hydroperoxides (Figure 7).

Once the ene reactions have been initiated, a cascade of pathways

occurs that results in numerous hydroperoxides, including 2-pentyl

furan and 2-pentenyl furan, which are believed to contribute to the

"reversion flavor" in oxidized soy oil.

     Depending on which pathway occurs, the degradative products of

PUFA oxidation will differ.   The contribution of chlorophyll to this

oxidative process has been studied in isolated oils. Headspace volatiles

increase when the concentration of chlorophyll added to soybean oil is

increased (Min and Lee, 1988). This will be described in further detail,

but first it is necessary to understand the structure, function, and food concentration of common photosensitizers in foods.

Riboflavin as a Photosensitizer

Riboflavin has been studied extensively as a photosensitizer (Allen and Park, 1979; Webster et al., 2009; Bekbolet, 1990). Riboflavin, also called vitamin B_2, is a flavonoid that is highly susceptible to photo-degradation. It is a water-soluble vitamin found in large amounts in milk and meats. Riboflavin, so named for the ribityl side chain attached to a substituted isoalloxazine, is part of the coenzymes FAD and FMN required for redox reactions. The isoalloxazine ring structure has conjugated double bonds that readily give up an electron to oxygen as described above upon absorbance of light. Riboflavin absorbs light maximally at wavelengths 400, 446, and 570nm (Bekbolet, 1990). Degradation of this molecule in food systems is undesirable from a nutritional standpoint because it decreases the nutritional value of the food and also, the photooxidation that results from its degradation results in off flavors and odors. There have many numerous studies on protecting riboflavin photooxidation in cow and soymilk products as riboflavin naturally occurs in milk and plays a large role in the

deterioration of milk flavor and odor (Lee and Min, 2009; Huang et al., 2004a; Huang et al., 2004b; Webster et al., 2009). Concentrations of riboflavin in cow milk and soymilk are 1.88 µg/ml and 0.71µg/ml respectively.

Chlorophyll

Physical Characteristics and Chemical Structure

Chlorophyll is also a photosensitizer that has been studied extensively. Chlorophyll is a chromophore that exists in two primary forms, chlorophyll a and b. There are other forms but they exist in small amounts. The molecular weights of chlorophyll a and b are 893.4 and 907.4 respectively; the ratio of chlorophyll a to b in the foods is 3:1. Chlorophyll is similar in structure to riboflavin in that it contains ring structures with conjugated double bonds. However, chlorophyll is more hydrophobic and is soluble in organic solvents such as acetone, methanol, or ethanol (Jackson, 1976; Schwartz and Lorenzo, 1990; Schwartz et al., 2008; Lichtenthaler, 1949). It is well known that this compound is the plant pigment that participates in photosynthesis, functioning as the molecule that absorbs energy from the sun in the form of photons and transferring that energy to ultimately

biosynthesize carbohydrates (Jackson, 1976).

Chlorophyll a absorbs light at wavelengths, 433nm and 663nm thus projecting a bluish green color. Chlorophyll b absorbs light at 452nm and 640nm, within the red and blue spectra, thus appearing green (Lichtenthaler, 1949; Jackson, 1976; Tymoczko et al., 2010). However, in foods that are high in chlorophyll, we typically perceive a green color likely due to the interactions of other chromophores or pigments that are present in the plant.
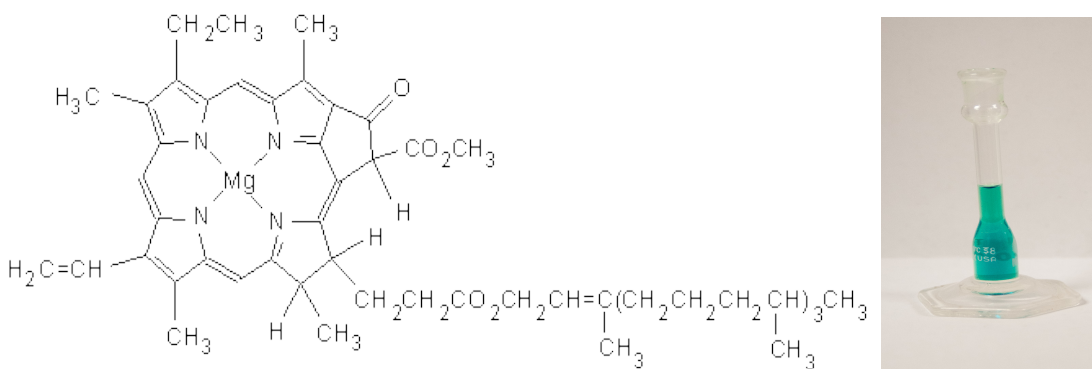
Figure 8. Chemical structure of chlorophyll a (copied with permission, Schreiner, R.)
http://scifun.chem.wisc.edu/chemweek/chlrphyl/chlrphyl.html)
Photograph courtesy of Jian Wu.

Chlorophyll is a tetrapyrrole with magnesium bound at its center similar to the manner of iron binding to hemoglobin (Figure 8). It consists of an esterified isoprenoid alcohol tail, phytol, and a carbomethoxy group at the C-10 position of the molecule (Jackson, 1976).

Degradation of Chlorophyll

The loss of the magnesium atom at the center results in pheophytin, which occurs when chlorophyll is heated under acidic conditions. However, under basic conditions, when heated, chlorophyll is stable. Upon further heating, pheophytin further degrades into pyropheophytin in which not only is the Mg atom lost, but the carbomethoxy group at the C-10 position is replaced with a hydrogen atom. This is actually the degradation product that is responsible for the olive green color of heated green vegetables. Another degradative product occurs upon enzymatic cleavage of the phytol ester by chlorophyllase with the resultant product of chlorophyllide. When this compound loses its Mg atom upon heating, it degrades into pheoborbide. Subsequent loss of the carbomethoxy group results in pyropheoborbide (Jackson, 1976; Schwartz et al., 2008). Thus when processing foods that are high in chlorophyll, it is desirable to do so under basic conditions to

Regreening may occur in which salts of Cu^{2+} and Zn^{2+} complex with magnesium depleted chlorophyll to form a more stable product to food processing. These complexes can be added to foods to maintain maintain color (Schwartz and Lorenzo, 1990).

Table 3. Causes of degradative products of chlorophyll, the aspect of the molecule that is lost on the molecule upon that degradation, and the name of the degradative product.

Cause of degradation	Loss of:	Results in:	+ the loss of:	Results in:	+ the loss of:	Results in:
Heat under acidic conditions	-Mg++	**Pheophytin**	-CO2CH3	**Pyropheophytin**		
Chlorophyllase	Phytol ester	**Chlorophyllide**	-Mg++	**Pheoborbide**	-CO2CH3	**Pyropheoborbide**

color. Another alternative that provides green coloring for foods is

sodium copper chlorophyllin (SCC), in which chlorophyll is treated with

methanolic sodium hydroxide; the magnesium atom within the

porphyrin rings is replaced with copper. There are a number of

resultant products, but importantly, these products are water-soluble.

Furthermore, they have been shown to be bioavailable (Schwartz and

Lorenzo, 1990; Schwartz et al., 2008).

Because chlorophyll is susceptible to degradation under acidic

conditions, it is widely believed that natural chlorophyll as found in

fruits and vegetable is not bioavailable. However, because it is also

found in fruit and vegetable tissues at a high concentration, as much as

five times as that of carotenoids, it is hypothesized that there may be

some absorption *in vivo* and that it may contribute to some health

benefit (Ferrruzzi and Blakesee, 2007). After chlorophyll is subject to

the low pH of the stomach, its degradative products may be

micellularized during digestion. Thus, chlorophyll derivatives have been studied as phytochemicals that may have health benefit, specifically in the field of cancer prevention. An epidemiological study (Balder et al., 2006) did show a correlation with decreased risk of colorectal cancer. However, one must consider that when eating a diet that is rich in chlorophyll-containing foods, those foods tend to be rich in other nutrients and functional food components and may be displacing foods that are high in carcinogens. Likely those who have diets high in nutritient-dense foods participate in other lifestyles that are conducive to healthy living, e.g., high physical activity, non-smoking, etc.

Role of Chlorophyll as a Photosensitizer

There are limited studies regarding how chlorophyll affects photooxidation of PUFAs specifically on the photooxidation of PUFAS in soy oil (Endo et al., 1984; Usuki, et al., 1984). Lee and Min (1988) studied the relationship between chlorophyll and soybean oil in an effort to determine if β-carotene would quench singlet oxygen during photooxidation.   Increased molar concentrations of soybean oil and increased concentrations of chlorophyll were added to methylene

chloride and exposed to fluorescent light over time at 10°C. As expected, as oxidation proceeded, headspace oxygen (HSO) decreased indicating that there was a reduced amount of oxygen as it was incorporated into hydroperoxides. Also as the concentration of soybean oil, and hence PUFAs, and the concentration of chlorophyll increased, so did oxidation. The inference was that chlorophyll was responsible as the photosensitizer and could increase the degree of photooxidation with more substrate (PUFAs) available. When β-carotene was added, HSO depletion decreased indicating diminished photooxidation. This only occurred in the light indicating that photooxidation was responsible. In the dark, β-carotene had no effect. This study supports chlorophyll behaving as a photosensitizer that can be influenced by the presence of an antioxidant, β-carotene.

Lee and Min (1990) then added chlorophyll to purified soybean oil and exposed the mixture to light again to determine the kinetics of β-carotene and other carotenoids quenching singlet oxygen. Peroxide value and headspace oxygen were measured endpoints of oxidation. β-carotene, lutein, zeaxanthin, and lycopene all resulted in quenching of singlet oxygen and therefore inhibition of photooxidation due to chlorophyll. This supported their earlier study that photooxidation

occurred as a result of chlorophyll and that these carotenoids behave as an antioxidants.

The above studies have been done on soybean oil, which is a valuable food matrix for studying the effects of chlorophyll on PUFAs. However, little has been done on other food matrices that contain PUFAs and chlorophyll. Understanding the effects of chlorophyll on soybean oil, soymilk and dairy products is important in protection from photooxidation, particularly as functional food beverages become more popular and in demand. Studies on the photooxidation of PUFAs in soymilk have shown that riboflavin acts as a photosensitizer with hexanal as a primary product (Huang et al., 2004; Huang et al., 2006).

Photooxidation may occur at a different rate in soybean oil vs. soymilk vs. cow milk as the proteins, sugars, and fatty acids are different within the three products. Soymilk naturally contains antioxidants that cow milk does not contain and concentrations of chlorophyll in the products are likely different. Riboflavin concentrations are different also. While cow's milk has PUFAS, the concentration of these fatty acids is greater in soymilk thus this soymilk has a higher susceptibility to photooxidation. Additionally, soymilk may be higher in chlorophyll since it is a plant product. However, soymilk also has antioxidants

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naturally present that cow's milk does not have, such as isoflavones.

Wold et al. (2005) studied the chlorophyll and porphyrin content of cheeses in relation to photooxidation of lipids and off flavors and aromas. They determined that chlorophyll and other porphyrins as well as riboflavin are responsible for photooxidation of cheese. They also suggest that different off flavors and aromas may be due to specific photosensitizers.

A number of beverages are being developed from foods such as spinach, spirulina, and algae. Chlorophyll is high concentrations in such beverages. If stored in clear packages and exposed to light, the chlorophyll may degrade rapidly. Perhaps the food industry will employ the use of SCC as a natural food coloring agent or "regreen" the chlorophyll with copper or zinc as pasteurization will affect the color of these foods. Nonetheless, the goal is to maintain as much of the product as possible; currently there is a demand from consumers for more natural products. Mintel research shows that the use of natural coloring for beverages is growing such that there is a 77% increase in natural coloring use in developing new products. Whether or not chlorophyll-containing foods are being used for this purpose is unknown; however, it is evident that products containing spinach, algae, chlorella, spirulina,

etc. are available in supermarkets (Mintel Esquivel Food Product Design, 2013; Inanc, 2011; Ferruzzi and Blakesee, 2007; Bomgardner, 2014). Thus protecting the color and nutrients within chlorophyll-containing beverages does have relevance. Drinks in clear packages allow the consumer to make a sensory judgment on the appearance of the product, but it should not be at the expense of depleting the nutritional composition. Complete protection may be more desirable from a nutritional and compositional perspective, and a protected product will be more beneficial to the consumer than one that is visible.

Protecting Foods From Photooxidation

There are a number of ways that foods can be protected from photooxidation. The most obvious method would be to protect the food from light. This will be discussed in detail below. Antioxidants are often employed not only in the processing of foods, but the food product may often contain naturally occurring antioxidants. For example, although soybean oil is highly susceptible to photooxidation, it is known to contain tocopherols, a naturally present antioxidant. Ascorbic acid and some carotenoids such as beta carotene and lutein are also known to behave as antioxidants. There are also antioxidants that are

synthesized and added to foods because they work so effectively as antioxidants, such as BHT, etc.

Another method for minimizing the degree of oxidation is to remove oxygen as much as possible from the food product. Vacuum packaging can be done with meats, however this is not a reasonable method with liquid products such as dairy or soymilks.

Temperature can increase the rate of autoxidation and so keeping foods such as vegetable oils from heat is useful for minimizing oxidation. However, as stated before, the most efficient means of reducing photooxidation in foods is to protect the food from light. Thus packaging has been studied extensively for this purpose.

Protecting Food from Photooxidation

Milk is exposed to light while in retail stores and even to some extent in the home. The extent of photooxidation in milk will depend on distance to the light sources, the intensity of the light source, wavelengths, and the surface area of milk that is exposed to the light source (Robertson, 2006; Duncan and Webster, 2010). The food industry does make attempts to decrease the photooxidation that occurs in food products. Specific food processing methodologies

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(Robertson, 2006) have been studied to determine if emulsification, heating treatments, and antioxidants can improve oxidative stability of PUFAs in products. Another way in which photooxidation can be inhibited is the type of packaging for the food products.

Role of Food Packaging

The role of packaging has proven to be important in reducing the degree of photooxidation of polyunsaturated fatty acids.   Because riboflavin is a photosensitizer, known to absorb light at wavelengths 400, 446, and 570 nm, packaging has been designed to reduce transmission of these wavelengths in order to protect milk quality and protect riboflavin from degrading (van Aardt et al., 2001; Webster et al., 2009; Duncan and Change 2012). Typical types of packaging for fluid milk products include HDPE, paperboard, glass, high density polyethylene (HDPE), and poly(ethylene) terephthalate (PET). HDPE and PET can be pigmented to reduce wavelengths of light that transmit to the milk (Duncan and Chang, 2012).   Paperboard is commonly, though not exclusively, used to package soymilk. Titanium dioxide (TiO$_2$) has also been added to HDPE and PET to reduce transmission of light and to absorb damaging UV wavelengths (Robertson, 2006;

Duncan and Hannah, 2012). It is one possible packaging innovation that has shown to be useful in protecting against photooxidation due to its ability to scatter light. It scatters light via two properties of refracting and diffracting light. It can be added at different concentrations to a material. Furthermore, the particle size of TiO_2 can be adjusted to affect color of light transmitted (DuPont, 2007).

Inhibition of photooxidation in dairy and fruit beverage products has been studied extensively, including evaluation of packaging materials, lighting, specific wavelengths, and added antioxidants (van Aardt et al., 2005a; van Aardt et al., 2005b; Kline et al., 2011). Soymilk can be protected from photooxidation by blocking all light. The paperboard primarily used in packaging soymilk blocks out light. There may be alternative ways to package it such that consumers can see the product while still protecting from those wavelengths that result in the greatest amount of photooxidation (Duncan and Chang, 2012).

Packaging effectiveness in protecting bovine milk from photooxidation has been determined by studying changes in oxidative products, as measured by sensory analysis of off flavors and aromas and monitoring volatile chemistry (van Aardt et al., 2005; Webster et al., 2011; Duncan and Webster, 2009; Duncan and Webster, 2010; Duncan

and Hannah, 2012). Sensory analysis has shown that off flavors/aromas can be deterred with specific types of packaging that eliminate certain wavelengths of light to penetrate through to the product. Many studies consistently demonstrate that specific volatiles produced from photooxidation are responsible for off flavors and aromas in bovine milk; however, they do not demonstrate consistently the wavelengths that are responsible for the photooxidation.

van Aardt et al. (2001) demonstrated that amber PET was most effective in reducing off flavors/aromas of cow's milk compared to clear PET and HDPE. Amber PETE reduces light wavelengths in the 450 nm region and partially in the 450-700 nm region.   This is consistent with the wavelengths at which chlorophyll and riboflavin absorb light, suggesting that these photosensitizers are at least partly responsible for the off flavors and aromas seen. Intawiwat et al. (2010) demonstrated that sensory characteristics of milk were affected by presence of and types of light-blocking filters applied to the package surface. However, they found that filters that allowed different transmissions of visible light with red (transmission of 570-800 nm) and green (transmission of 500-800 nm) filters showed lower rancid (oxidized) odors. Admittedly, it is difficult to compare studies that have overlap of wavelength

transmission.

Webster et al. (2009) studied the feasibility of reducing light oxidation in milk by reducing specific wavelengths that transmit through packaging. Because the photosensitizer riboflavin becomes absorbs energy primarily at 400, 446, and 570 nm wavelengths, iridescent films that reduced light transmittance at those wavelengths were studied. Using sensory analysis, it was determined that despite the fact that riboflavin wavelengths were reduced, there were still off flavors due to light oxidation. They suggest that other compounds in the milk are acting as photosensitizers at different wavelengths or that perhaps there are other mechanisms that result in the formation of off flavors and aromas as previously reported by Wold et al. (2005).

Light exposure in milk products results in formation of hexanal, pentanal, dimethyl disulfide, 1-octene-3-one, acetaldehyde, and 1-hexen-3-one (Cadwallader and Howard, 1998; Cladman et al., 1998). Headspace oxygen is also a factor in the level of formation of these off-flavor products as oxygen is a component of the photooxidation process (Schröder 1982). In addition to studying sensory analysis of milk exposed to light, van Aardt et al. (2001) also demonstrated the amber PET was useful in reducing the formation of hexanal and dimethyl

disulfide, but not acetaldehyde. Other materials that have been shown

to reduce hexanal concentrations include PET/PEN which allows a

transmittance level of 40% of wavelengths at the 365 nm range

(Lennersen and Lingert, 2000).   They also found that blue light bulbs

emitted light at 365, 405, and 435, and caused the greatest lipid

oxidation as measured by hexanal concentration.   Intawiwat et al.

(2010) identified that aldehydes were the higher concentration of

predominant compounds formed during light exposure of milk. Milk

stored with an orange filter (blocking transmittance of 520-800)

showed higher concentrations of oxidative products than red, amber, or

green filters. Webster et al. (2011) exposed milk to specific and narrow

(50 nm) wavelength bands of light to determine which volatile

compounds were produced in milk. They found that both hexanal and

pentanal were produced in high amounts at a wavelength range of 200-

400 (UV).

There are many factors that may contribute to the wide variety of

results seen in these studies, but what appears to be a common theme is

that different wavelengths affect photooxidation of foods in different

manners, most likely due to the wide variety of chemical compositions

and physical structures that exist in foods (Duncan and Chang, 2012).

There is no definitive answer to which packaging material will best

eliminate only specific wavelengths that are responsible for specific

photosensitizers. Soymilk has not been studied and possibilities for

different methods of packaging are a large unexplored area for research.

This can have particular relevance if the consumer can see the soymilk

product in a relatively inexpensive package that can protect the

nutrients and flavor.

Consumer Acceptability of Soymilk

Soymilk sales are on the rise. It is becoming a more popular and

acceptable product in western cultures. The functional food benefit of

this product and the fact that it is a healthy alternative to bovine milk

for lactose intolerance, vegetarianism etc. has contributed to the

increase in sales. However it cannot be overlooked that there has been

advances made in the area of processing of this beverage to make it a

more palatable product to consumers also.

Numerous studies have studied the use of different types of beans,

and processing methods to improve the consumer acceptability of this

product and to determine how to best maintain the nutritional

composition of it. Kwok et al. (2002) found that an ultra high

temperature processing method of 143°C for 60 sec allows for

inactivation of trypsin inhibitors, and a low rate of Maillard browning

reactions but allows for sensory quality to be maintained at "slightly

like" (6) and higher on a hedonic scale of 1-9. Other methods to

quantify sensory aspects of processing methods on the quality of

soymilk are to measure compounds known to adversely affect sensory

quality. Zhang et al. (2012) used different grinding temperatures

combined with different heating methods and measured production of

volatiles known to be associated with off odors such as hexanal, 2-

pentylfuran, 1-octen-3-ol. They found that hot grinding and a two-

phase UHT processing method resulted in the formation of less off-

flavor volatiles. Type of heat provided to soymilk has also been shown

to play a role in odor compounds produced during processing. Yuan

and Chang (2007) evaluated a direct steam injection compared to

traditional indirect cooking and demonstrated that the former resulted

in lower odor components produced as measured by gas

chromatography. Storage time and temperature, variety of the soybean

used have also been studied (Achouri et al., 2008). One common theme

of these studies is that lipoxygenase activity is important as well as the

degree to which the Maillard browning reaction occurs when producing off odors.

While the work in this area focuses on flavor and odor compounds produced during storage and processing, very few studies assess how the appearance of soymilk is affected. Appearance is the first sensory attribute that a consumer will make a judgment on regarding a product. and color is an important part of appearance (Hutchings, 1999). Degradation of chlorophyll can alter the appearance, which might influence the perception of quality. It is recognized that visual appearance can indirectly affect perception of flavor and aroma (Hutchings, 1999). It is possible to alter the color of soymilk as shown by Xiang et al. (2011). They used CIE L*, a*, b* colorimetry which provides numerical quantification for light-dark, red-green, and blue – yellow ranges respectively. This study demonstrated that higher voltages of pulsed electric fields caused a decrease in the CIE L*, a* and B* values of soymilk. This higher voltage actually results in a darker product (lower L* value) than the lower voltages. Thus processing methods affectively alter the color of the soymilk. This is still a largely unexplored area to improve consumer acceptability of soymilk.

Conclusions

Protecting soymilk from photooxidation, determining the amount of chlorophyll in this product and to what degree it contributes to photooxidation are areas that are unexplored. Determining how important color of this product is to consumers is also unknown. The intent of the projects that are described within this dissertation is to address most of these questions in an effort to improve consumer acceptability and nutritional quality of this beverage.

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Chapter 3 Contribution of Chlorophyll to Photooxidation of Soybean Oil At Specific Visible Wavelengths of Light

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Abstract

Photosensitizers in foods and beverages are important considerations during selection of packaging materials. Chlorophyll is found at low concentrations in many functional food products; however, chlorophyll's contribution as a photosensitizer to oxidation of foods is not well known. The objective of this study was to determine the photosensitizing effect of chlorophyll on soybean oil (SO) using broad-spectrum light and 2 visible wavelength regions of light.

SO with added chlorophyll (0, 1, or 2 µg chlorophyll added/ml SO) was exposed to four light conditions, using a photochemical reactor (10°C; 4h). Light treatments included broad-spectrum (BS; no filter; 157.6±4.7mW intensity), 430nm (10 nm bandpass; 1.8±0.7mW), and 660nm (10 nm bandpass; 0.332±0.05 mW) wavelengths compared to a no-light control. Chlorophyll a absorbs light in the selected visible wavelength regions. Chlorophyll degradation was evaluated by HPLC. Oxidative changes in SO were assessed by peroxide value (PV) and thiobarbituric acid reactive substances (TBARS), which measures malondialdehyde, assays.

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Chlorophyll was completely degraded at BS and 430nm conditions and degraded to 36% of original concentration at 660nm wavelength. PV and malondialdehyde concentration significantly increased due to chlorophyll addition (1 μg/ml) at BS (3.9 mEq/kg oil and 1.26 mg MDA/kg oil, respectively) and 430nm wavelengths (2.4 mEq/kg oil and 0.28 mg MDA/kg oil, respectively) compared to no-light control (0.28 mEq/kg oil and 0.08 mg MDA/kg oil, respectively). Even lower light intensity at 660 nm initiated oxidation reactions as measured by PV. There were differences in PV (BS, 430nm) and TBARS (BS) between the no-light and light-exposed SO without chlorophyll added.

This study suggests that broad-spectrum light, and at least light wavelengths at or near 430nm and 660nm, can excite chlorophyll, resulting in initiation of oxidation reactions. Packaging material selection for foods and beverages should consider blocking excitation wavelengths of photosensitizing molecules, including chlorophyll, to protect product quality.

Key Words: chlorophyll, HPLC, peroxide value, TBARS, photooxidation, photosensitizer, packaging, light wavelengths

Introduction

Chlorophyll is a chromophore that exists in several forms in plants, with the primary forms being chlorophyll a and b. Chlorophyll, which is a tetrapyrole, contains magnesium at its center. It also consists of phytol, which is an esterified isoprenoid alcohol tail, and a carbomethoxy group at the C-10 position of the molecule (Figure 9). Typically in the foods that we eat, chlorophyll exists in a 3:1 ratio of a:b. Chlorophyll a absorbs light at wavelengths 433nm and 663nm, thus projecting a bluish green color (Figure 10) (Jackson, 1976; Schwartz and Lorenzo, 1990; Schwartz et al., 2008). However, in plant foods that are high in chlorophyll, such as spinach, broccoli, or green beans, we typically perceive a green color likely due to the interactions of other chromophores or pigments that are present in the plant. Chlorophyll is important as a chromophore in plants as it is the photosynthesis pigment responsible for absorbing energy in the form of photons and through a process of redox reactions, biosynthesizing that energy into carbohydrate with a release of oxygen (Jackson, 1976).
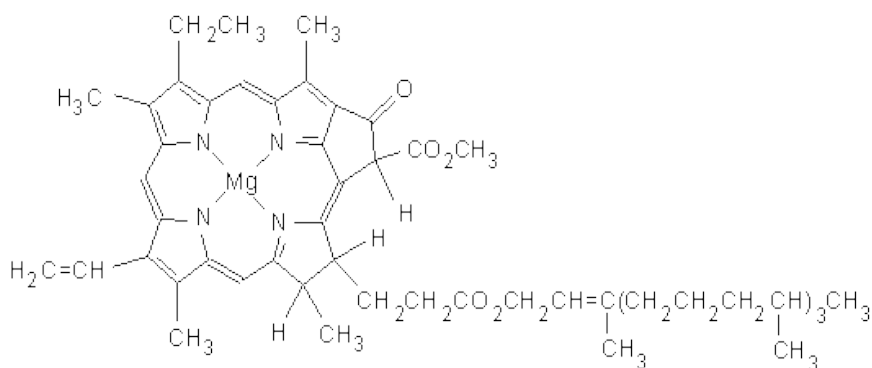
Figure 9. Chemical structure of chlorophyll a (copied with permission, Schreiner, R. http://scifun.chem.wisc.edu/chemweek/chlrphyl/chlrphyl.html)
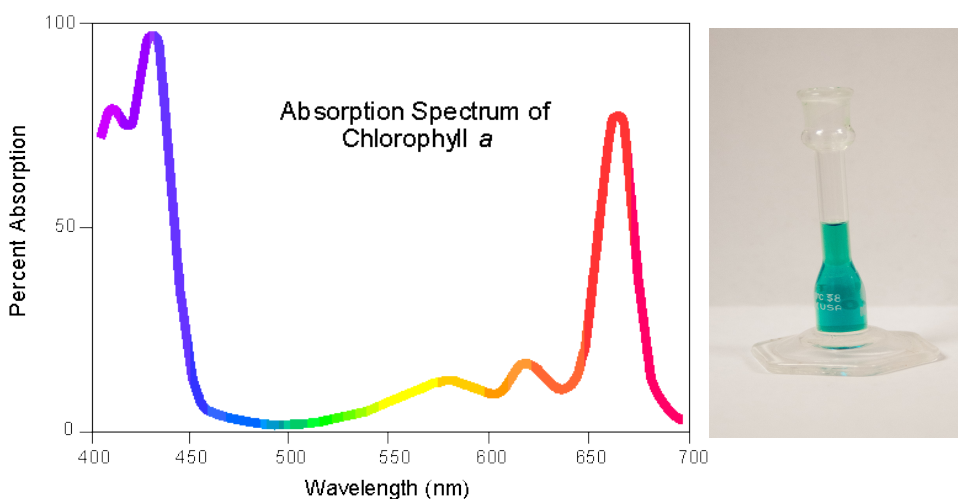
Figure 10. Absorption spectrum of chlorophyll a (copied with permission, Schreiner, R., http://scifun.chem.wisc.edu/chemweek/chlrphyl/chlrphyl.html) and photograph of chlorophyll a in acetone (courtesy of Jian Wu).

As a chromophore, chlorophyll also behaves as a photosensitizer in photo-oxidation (Endo et al., 1985). Photosensitizers are molecules that absorb photons. An electron is then elevated in the photosensitizer from ground state to a higher energy orbital. Compounds that

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structurally contain many double bonds demonstrate resonance structure that allows for this excitation from photons. Chlorophyll contributes to photooxidation using Type II photo-oxidation pathway as it has been shown to use singlet oxygen in the production of photooxidation products (Ho et al., 1978). The oxidation products resulting from the Type II photooxidative pathway contribute to reversion odors and flavors (Smouse and Change, 1967). These products, including 2-pentyl furan, 2-pentenyl furan, peroxides, and aldehydes, are undesirable from a nutrition and quality standpoint. It is well known that SO is not oxidatively stable in light conditions.

SO is a highly unsaturated oil, containing approximately 53% linoleic acid and 7% linolenic acid. Linoleic acid has one bis-allylic C-H bond and linolenic acid has two; only 50 Kcal/mole is needed for formation of an alkyl radical (Frankel, 1985; Chapman et al., 2009). The amount of and the degree to which tocopherols behave as antioxidants and prooxidants in SO have also been studied. However, the degree to which chlorophyll contributes to photooxidation of retail foods under different lighting conditions has not been well studied. We suggest that chlorophyll's role as a photosensitizer in different food matrices should

be a consideration in packaging selection for protecting nutrients and product quality.

Packaging is an important aspect of protecting foods from nutrient and quality deterioration. While packaging technology often focuses on protecting riboflavin from degradation and the subsequent quality deterioration, other photosensitizers, such as chlorophyll, will be present in foods and contribute to deterioration also.

The purpose of this study was to determine the extent to which peroxides and aldehydes were produced in SO with added chlorophyll at wavelength regions at which chlorophyll absorbs light. Our goal is to provide the packaging and food industries with information for optimizing food packaging to protect foods from loss of quality due to light and its effects on chromophores, specifically chlorophyll.

Materials and Methods

Materials

Vegetable oil (100% SO) was purchased from a retail supermarket and stored at 4°C in the dark; the package was flushed with nitrogen after each use to minimize oxidation. Chlorophyll a (extracted from

spinach) was purchased from Sigma (Lot SLBF340V, $\geq$85% chlorophyll a, $\leq$0.4% chlorophyll b) and stored in the freezer (-10°C). Prior to use, it was dissolved in acetone, allocated into smaller vials, and placed back in the freezer (-10°C) to avoid losses from repeated access. All work was performed under incandescent lights with green coating (Mood Lites®) that emitted low intensity light (49.25 ±3.2 lux). Room lighting in experimental lab was shut off during all experiments.

Chlorophyll was added to the SO at two different treatment levels, 1µg/ml and 2µg/ml. A control had no added chlorophyll. To avoid experimental error due to additional acetone added to the oil, acetone was added to the control and to the 1ug/ml treatment such that all treatment groups had equivalent amounts of acetone. Chlorophyll was mixed in the oil using a Vortex Genie (Scientific Industries Inc., Bohemia, NY). SO (30ml) was placed in foil-wrapped test tubes, flushed with nitrogen and immediately placed in refrigeration until use. Mixtures were made immediately before experimental procedure.

Exposure of SO with Added Chlorophyll to Light in Photo-reactors

SO mixtures (13.7 ± 0.1gm) were weighed into a 15 ml quartz crystal sample cell (Fisherbrand, Fisher Scientific, Pittsburgh, PA) for

exposure to light. Sample cells were exposed to light using a photo-reactor (Model 66902 Universal Arc Lamp Housing, Model 66910 Power Supply, Thermo Oriel Instruments, Stratford CT) (Figure 11). A mercury bulb was used to provide high intensity, broad-spectrum light or narrow bandwidths of light using bandpass filters. Infrared wavelength was filtered out for all light exposures. Bandpass filters with peak transmittance at 430nm, 450nm, and 660nm (Thermo Oriel) and 10nm bandwidth at 50% transmittance were placed between the light source and the sample cell (Figure 4). A broad-spectrum light control with no filter and a no light control were also employed. Two photo-reactors were used. They provided equal light intensities and a preliminary oxidation of oil under full light was completed to validate equal peroxide value data between each photo-reactor.

Samples were placed in an aluminum block through which antifreeze coolant was pumped (Masterline Forma Scientific, Marietta OH), allowing samples to be kept at consistent temperature of 11.9°C ± 1.9. Samples were stirred via magnetic stir bars in the cell throughout the entire experimental conditions. Light meter readings were taken for each light group at the back of the sample cell with a Nova Laser Power Energy Monitor (Ophir Optronics, Newport Corporation, Stratford, CT).
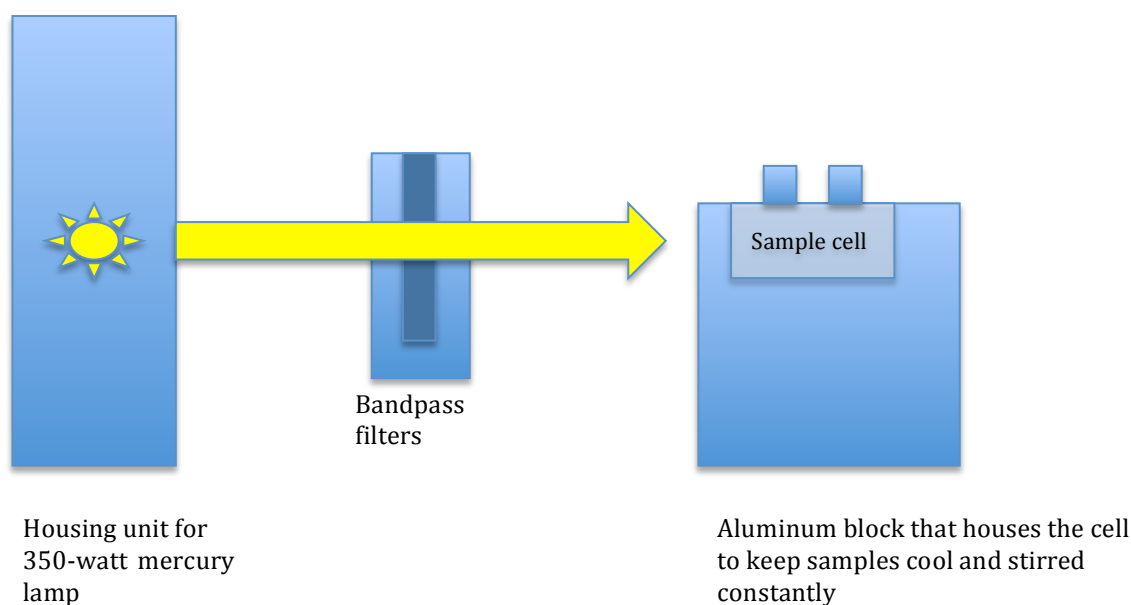
Figure 11. Illustration of photo-reactor unit used to provide light to samples.

Samples of SO were exposed to light in the photo-reactors for four hours. At the end of the four hours, temperature of the SO was recorded, and the SO was prepared for HPLC analysis of chlorophyll concentration, peroxide values (PV) and thiobarbituric reactive substances (TBARS) analysis. Analysis of the source oil was completed on each sample before each replication.

HPLC Analysis.

Analysis of chlorophyll concentration in the SO was performed following the method of Gauthier-Jaques et al. (2001) by reverse phase HPLC using a Waters 1525 separations module equipped with a column

heater and Waters 717 autosampler. Separations were performed using

a Phenomenex Kinetex 5u C18 100 Å column (250x 4.6mm). A Waters

2487 Dual Absorbance Detector (Waters Corporation, Milford, MA) was

set to detect chlorophyll at wavelengths 430m and 660nm. Solvent A

consisted of 1:4 ratio of 1M aqueous ammonium acetate to methanol,

and Solvent B consisted of 1:4 ratio of acetone to methanol. Conditions

were a 1.5ml flow rate with a linear gradient from 100% solvent A to

100% solvent B over a 10-minute period. Solvent B was held for 20

minutes, returned to solvent A over 5 minutes and equilibrated with

solvent A for 5 minutes. SO samples (0.25ml) were pipetted from the

photo-reactor cell and immediately mixed 1:3 with acetone and placed

in the autosampler; sample temperature was kept at 4°C. Injection

volumes were 10 μL. The source oil with the known amount of

chlorophyll added was run on the HPLC and compared to external

standards before it was exposed to light. The external standards

consisted of stock solutions of chlorophyll added to acetone to create

concentrations of 0μg/mL, 0.1μg/mL, 0.4μg/mL, 1.0μg/mL, and

2.0μg/mL. Duplicates of concentrations were analyzed on the HPLC per

the methods described above. Peaks from these were plotted against

concentration, to create a standard curve (regression analysis resulted

in an R^2 value of 0.9968); using y=mx+b, peaks of samples were calculated to obtain concentration amounts; the curve was not forced through zero. The concentration of chlorophyll at the four hour end point was calculated based on the same external standard curves and compared to pre-light exposure concentrations. Thus the comparisons were made from the same batch of chlorophyll in SO mixtures. External standards and additions of chlorophyll to soybean oil were obtained from the same stock solutions.

Limit of blank (LOB) was determined by the method of Armbruster and Pry (2008). HPLC analysis of twenty SO samples with no added chlorophyll (blanks) was completed as described above. The peaks at the retention time at which chlorophyll show were manually integrated. Concentrations were calculated based on standard curves. Serial dilutions of chlorophyll added to SO were also analyzed on the HPLC until a peak could not be detected. This concentration is the low concentration sample. The LOB was calculated based on mean$_{blank}$ ± 1.645 x standard deviation$_{blank}$. The limit of detection was calculated based on LOB + 1.645 x standard deviation$_{low\ concentration\ sample}$.

Percent recovery was completed by adding 0.4µg chlorophyll/mL to acetone and SO, each in triplicate. Each of these was diluted 1:3 with

acetone and analyzed via HPLC methods described above.

Concentrations of these were calculated according to the standards as described above. Percent recovery was calculated as $0.4\mu g/mL$ in SO ÷ $0.4\mu g/mL$ standard x 100.

Oxidative Endpoints.

PV was completed on each sample by the AOAC method Cd 8b-90 (1997) using isooctane (Appendix A-1). PV was completed on source oil immediately after chlorophyll was added to SO and immediately after completion of the four-hour exposure to light on treated and control samples.

TBARS was completed in duplicate on each experimental sample and source oil with stirring based on modifications of Spanier and Taylor (1976) (Appendix A-2). One ml of each light-exposed and control SO sample was immediately placed in polypropylene centrifuge tubes in a freezer (-72°C) (VWR Brand Model 5479, Forma Scientific Inc. Marietta, OH) until the assay could be completed. Spectrophotometric measurements of the concentration of malondialdehyde (MDA) were completed on a Milton Roy Spectronic 21D spectrophotometer with measurements taken at 532nm.

Analysis of tocopherols in SO.

δ-tocopherol was measured in the SO used in the light exposure experiment using the HPLC experimental methods of Carpenter (1979). SO (5g) was dissolved in 100 ml hexane and placed on a Macherey-Nagel normal phase column (25cm x 0.46cm i.d.). An Agilent Technologies 1260 Infinity HPLC using a fluorescence detector was used in which a mobile phase of 99 parts hexane to 1 part isopropanol was run at 1.0 ml/min. The fluorescence detector was set at 295nm. Amounts of δ-tocopherol were quantified by comparing to a standard curve in which dilutions of δ-tocopherol (Sigma) was analyzed on the same HPLC conditions. Amounts of α- and ϒ-tocopherols were estimated from the δ-tocopherol curves as all tocopherols have equal fluorescence intensities (Sikorska et al., 2012).

Chlorophyll Degradation.

Chlorophyll was added to SO at 1µg/ml, stirred, flushed with nitrogen, placed in the photo-reactor cell, and exposed to broad-spectrum light as described above. Samples (0.25ml) were taken every 30 minutes for 4 hours. This was completed in duplicate at three different light wavelength treatments (broad-spectrum light, 430nm, and 660nm).   Samples were diluted 1:3 with acetone and analyzed in

the HPLC as previously described. Temperature was monitored throughout the four hours and light intensity was recorded as described above.

Effect of Acetone on SO Photooxidation.

Acetone can be photooxidized and contribute to peroxide formation (Marcotte and Noyes, 1957; Caldwell and Hoare, 1962). The effect of acetone on photooxidation of SO was determined at broad-spectrum light. Kroger brand vegetable oil (100% SO) was used. Acetone (Fisher Scientific) was added at 0.01ml/ml and vortexed in a foil-wrapped test tube and kept refrigerated until placement in the photo-reactor. This mixture was placed in the crystal sample cells in the photo-reactor as described above. The samples were exposed to broad-spectrum light for 4 hours. Light readings and temperature were monitored as described above. PV was completed as described above prior to exposure to light and at the four-hour endpoint.

Color Analysis.

Color of the SO was measured on samples without chlorophyll added as well as SO with 1μg/ml chlorophyll added before and after

four-hour exposure to broad-spectrum light. A handheld Minolta

Chroma MeterCR 200 (Osaka, Japan) was used to assess the L*a*b*

values for the oil. Hue and chroma were calculated from a Graphic CIE

L*a*b* calculator (carmil.tripod.com/colorpro/convert.html). Samples

were placed in the photo-reactor as described above. Light meter

readings and temperature were measured as described above.

Statistical Analysis.

Statistical analysis was completed on three replications of the

experiment on JMP Pro 10.0.0 software (SAS Institute Inc., Cary, NC).

Analysis of variance was completed to determine statistical significance

using $\alpha = 0.05$. Because light intensity varies depending on the

bandpass filters, one-way ANOVA analyses were only made within each

wavelength treatment group. Where appropriate, comparisons of

significant differences were done using Tukeys-Kramer HSD test.

<div align="center">

Results and Discussion

</div>

Effect of Light on Chlorophyll in SO

Experimental Conditions.

Light meter readings were taken at the back of the sample cell

holder prior to placement of sample cell with oil for broad-spectrum,

430nm, 450nm, and 660nm wavelengths; readings were

157.6±4.7mW/4.15cm^2, 1.8±0.7mW/4.15cm^2, 0.33±0.05 mW/4.15cm^2,

and 0.3±0.06 mW/4.15cm^2 respectively (Appendix A-3). As expected,

broad-spectrum light provided much higher light intensity than those

wavelengths that were filtered. The light intensities of 660nm and

450nm compared to 430nm was lower but not significantly different.

This was consistent with other work in our lab (Webster et al, 2011).

Because the light intensity is different for each wavelength, statistical

comparisons were not made between wavelengths; rather, comparisons

were made between differing levels of chlorophyll additions to SO

within each wavelength region. It should also be noted that broad-

spectrum light from this photo-reactor is significantly more intense

than what would be found under retail storage conditions.

The source oil was evaluated for oxidative stability over the time

period of the testing period. Peroxide value of the source SO used

throughout the experiment did not increase significantly over time

($p > 0.05$), ranging from 0.23±0.06 on day one to 0.42±0.14 on day 13 of

the experiment. Thus oil used throughout the study period was not

oxidized due to storage conditions over the study period. Acetone did

not contribute significantly to the oxidation of SO.   After four hours of

exposure to broad-spectrum light, peroxide value of oil with and without acetone was 2.7±1.1 and 3.4±0.2 respectively (p= 0.587).

Because δ-tocopherol is an antioxidant that could potentially limit photooxidation and affect experimental results, we analyzed the concentration of this vitamin in the SO used for the replications. α-, δ-, and γ-Tocopherol were present at a concentration of 73.5±0.7, 339±3.8, 679.2±5.7 µg/ml respectively (Appendix A-4).

Limits of detection of chlorophyll were determined to be 0.1 µg/ml. Percent recovery of chlorophyll was determined to be 99.44%.

Changes in Chlorophyll Concentrations At Different Wavelengths of Light.

Placing SO spiked with chlorophyll in the dark, under the same temperature and stirring conditions as the other light treatments, resulted in no significant decreases in chlorophyll concentrations (p>0.05) (Figure 12). Chlorophyll in SO exposed to broad-spectrum light for 4h was degraded to the degree in which measurable amounts could not be detected for both levels of chlorophyll addition.   This degree of degradation also occurred with chlorophyll in oil exposed to light at 430nm; when added at 2µg/ml chlorophyll to oil, concentrations were still significantly lower than the source oil, but were detectable (p= 0.0004 and 0.0011 for 1µg/ml and 2µg/ml respectively). The light

intensity at 430nm is significantly lower than that of broad-spectrum light. This is a specific wavelength region at which chlorophyll absorbs energy, apparently resulting in significant degradation of the compound at this light intensity. When exposed to light at 660nm, chlorophyll also degraded, even at this lower level of light intensity. Although it did not show complete degradation, it was significantly lower than baseline (p=0.001 and 0.0121 for 1μg/ml and 2μg/ml respectively). Chlorophyll did not degrade at 450nm, with recovery of chlorophyll equivalent to the "no light" control treatment.
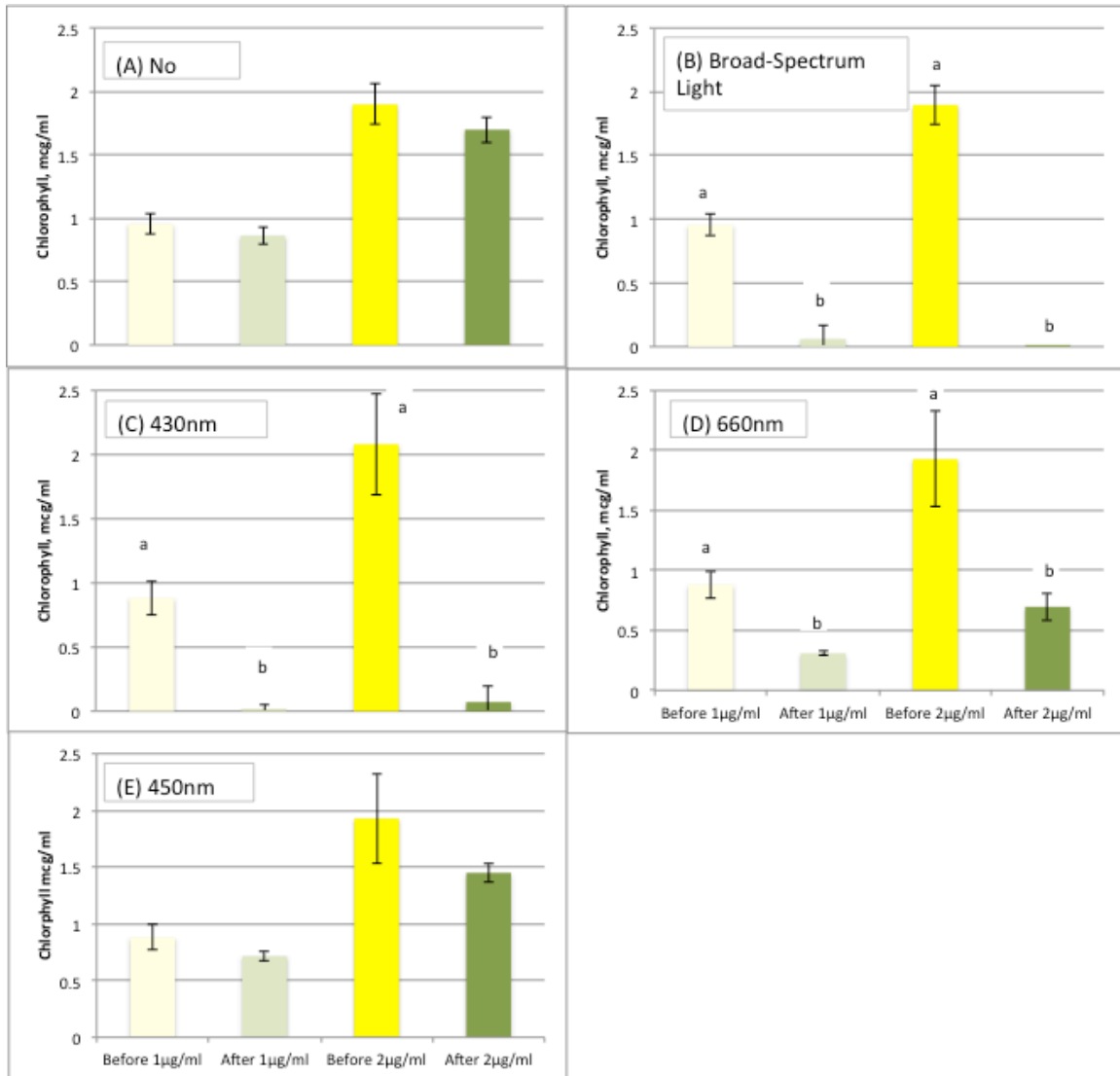
Figure 12. Chlorophyll concentration in SO with 2 different chlorophyll additions, 1µg/ml and 2µg/ml, before and after exposure to 5 different light treatments, no light, broad-spectrum light (157.6±4.7mW/4.15cm^2), 430nm (1.8±0.7mW/4.15cm^2), 450nm (0.33±0.05mW/4.15cm^2), and 660nm (0.3±0.06mW/4.15cm^2). Letters that differ between treatments and their respective baselines indicate statistical significant differences, α=0.05.

Chromatograms of chlorophyll added at 2µg/ml before and after

four-hour light exposure (Figure 13) show degradation per reduced

peak size.
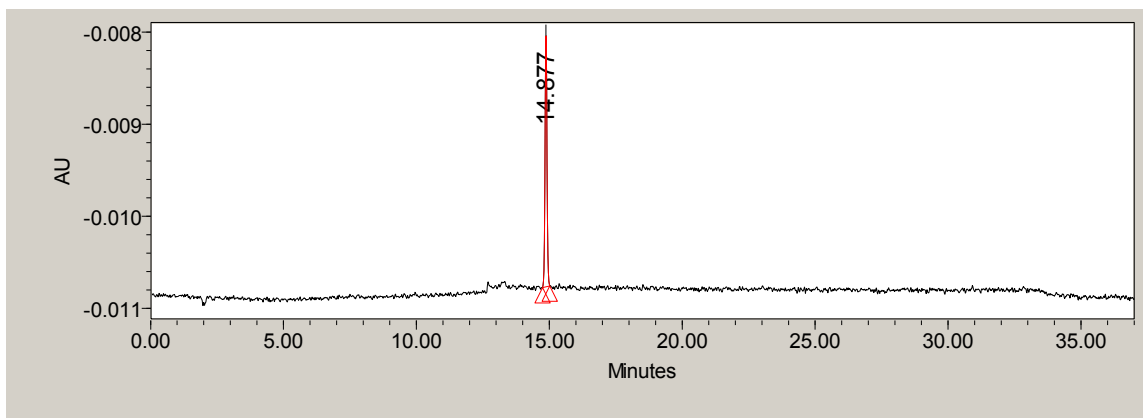
(a) before
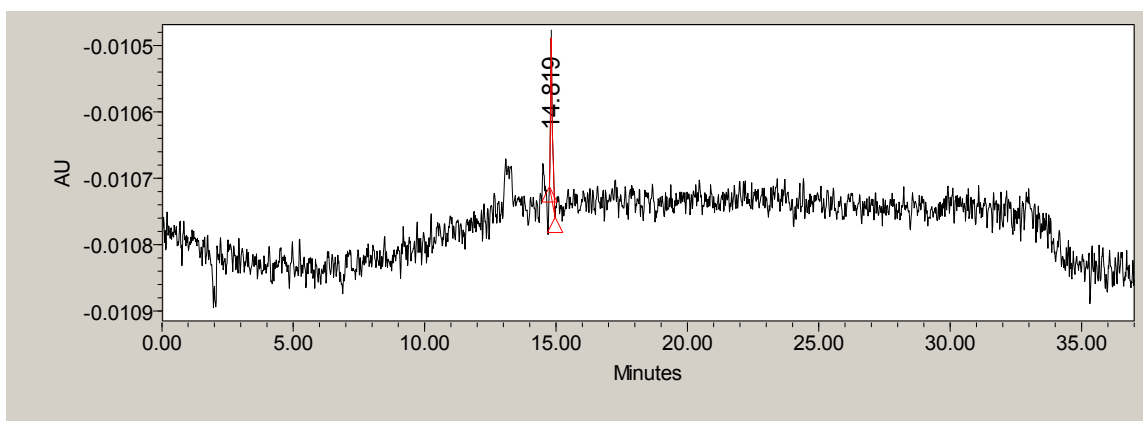
(b) after

Figure 13. Profile of chlorophyll, 2µg/ml, in SO using reversed phase
HPLC before (A) and after (B) 4 hrs of exposure at 430nm
(1.8±0.7mW/16.6cm^2) at 11.9°C.

Chlorophyll Degradation.

Chlorophyll degradation at 12°C was below the detection limits within

30 minutes in broad-spectrum light (Figure 14). At 430nm wavelength

of light, it degraded 90.5%, but not until after 4 hours of exposure. This was expected, as chlorophyll was not detected in the oil after four hours in the experiments evaluating oxidative products; however we did not expect chlorophyll to degrade so rapidly under broad-spectrum light conditions. At 660nm, chlorophyll degraded 55.5% over the four hour time period.

Changes in Peroxide Values and Malondialdehyde Concentrations.

PV and TBARS were significantly higher in the oil exposed to broad-spectrum light regardless of how much chlorophyll was added. In fact, even in the oil that had no added chlorophyll, PV and aldehydes were higher when exposed to broad-spectrum light compared to oil that was kept in dark conditions ($p < 0.0001$ for PV; $p < 0.0001$ for TBARS). There were no statistically significant differences in PV or MDA among chlorophyll concentrations added to the different oils exposed to broad-spectrum light ($p = 0.4569$ for PV; 0.6434 for MDA) (Figure 15). We hypothesize that 1) there is either chlorophyll in the oil at concentrations below the limits of detection that contributed to the photooxidation of the oil, or 2) other compounds in the oil act as prooxidants under such intense light conditions, such as the tocopherols present in the oil.
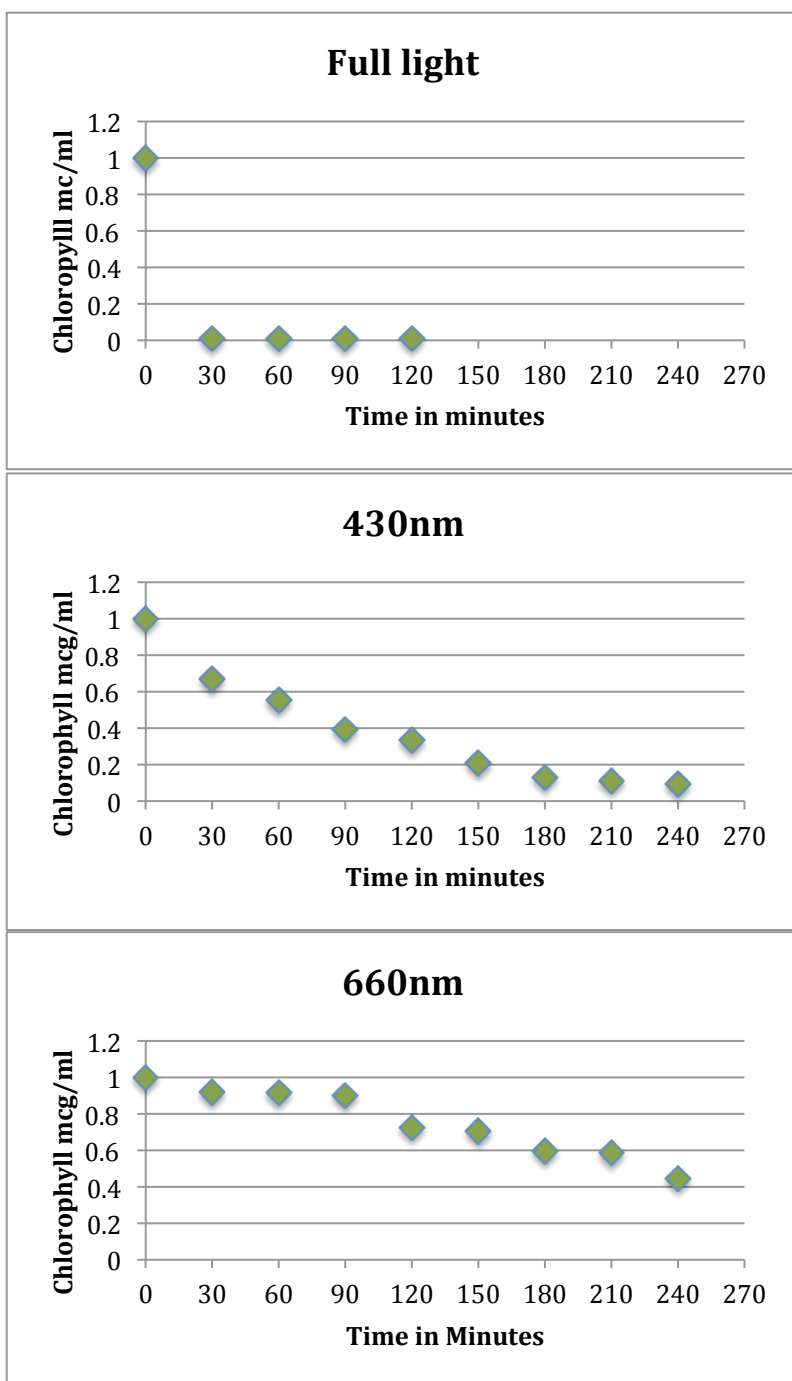
Figure 14. Chlorophyll degradation in SO over four-hour time period (11.9°C) at broad-spectrum light (157.6±4.7mW/4.15cm2), 430nm (1.8±0.7mW/4.15cm2) and 660nm (0.3±0.06mW/4.15cm2) wavelengths of light. Chlorophyll was added to the SO at 1µg/ml. Broad-spectrum light tested was stopped at 120 minutes because there was no measurable concentration of chlorophyll remaining.

Previous studies have shown α-tocopherol behaves as a prooxidant (Jung and Min, 1990; Warner, 2005; Kim et al., 2007; Chapman, et al., 2009). We quantified α-, δ-, and ϒ-tocopherol in the SO. The intensity of light that was provided in these experiments may have played an important role in initiating tocopherols to function as prooxidants in the SO.

At broad-spectrum light, peroxide and aldehyde formation decreased, albeit not significantly, when 2μg/mL chlorophyll was added compared to the 1μg/mL. We suspect that the peroxide concentration decreased as it underwent further oxidation pathways and subsequently peroxides were converted into secondary byproducts of oxidation; aldehydes formed were likely undergoing further degradative pathways also.

At the 430nm wavelength region, peroxide value was increased ($p<0.05$) at both concentrations of chlorophyll addition and even in the oil that had no added chlorophyll ($p <0.0001$). Tocopherols do not absorb light at this wavelength, supporting the conclusion that there is chlorophyll naturally present in the SO below our detection limits. MDA concentrations were only significantly increased in oil with 1ppm chlorophyll added ($p= 0.002$) (Figure 16).
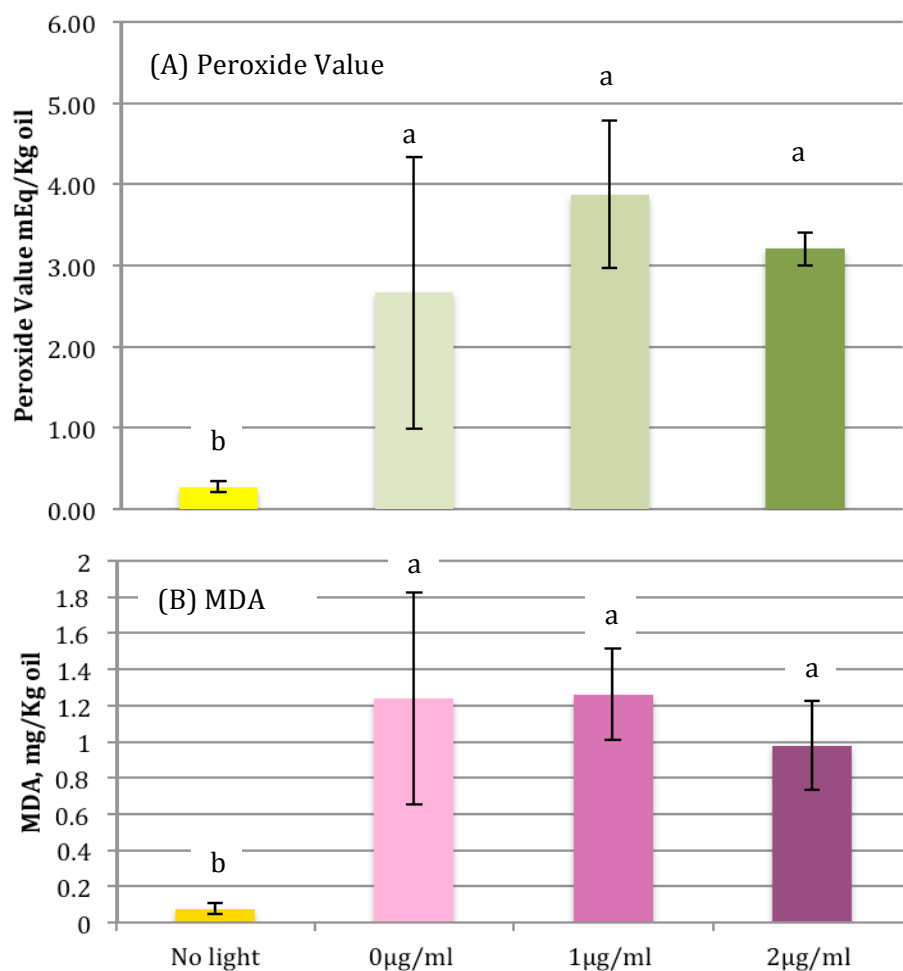
Figure 15. Peroxide value (mEq/Kg oil) (A) and malondialdehyde (mg/Kg oil) (B) formation in SO with 0, 1, and 2μg/ml chlorophyll added, exposed to broad-spectrum light (157.6±47mW /4.15cm^2) for 4 hours (11.9°C) compared to no light control. Lowercase letters that differ indicate significance difference (α=0.05).
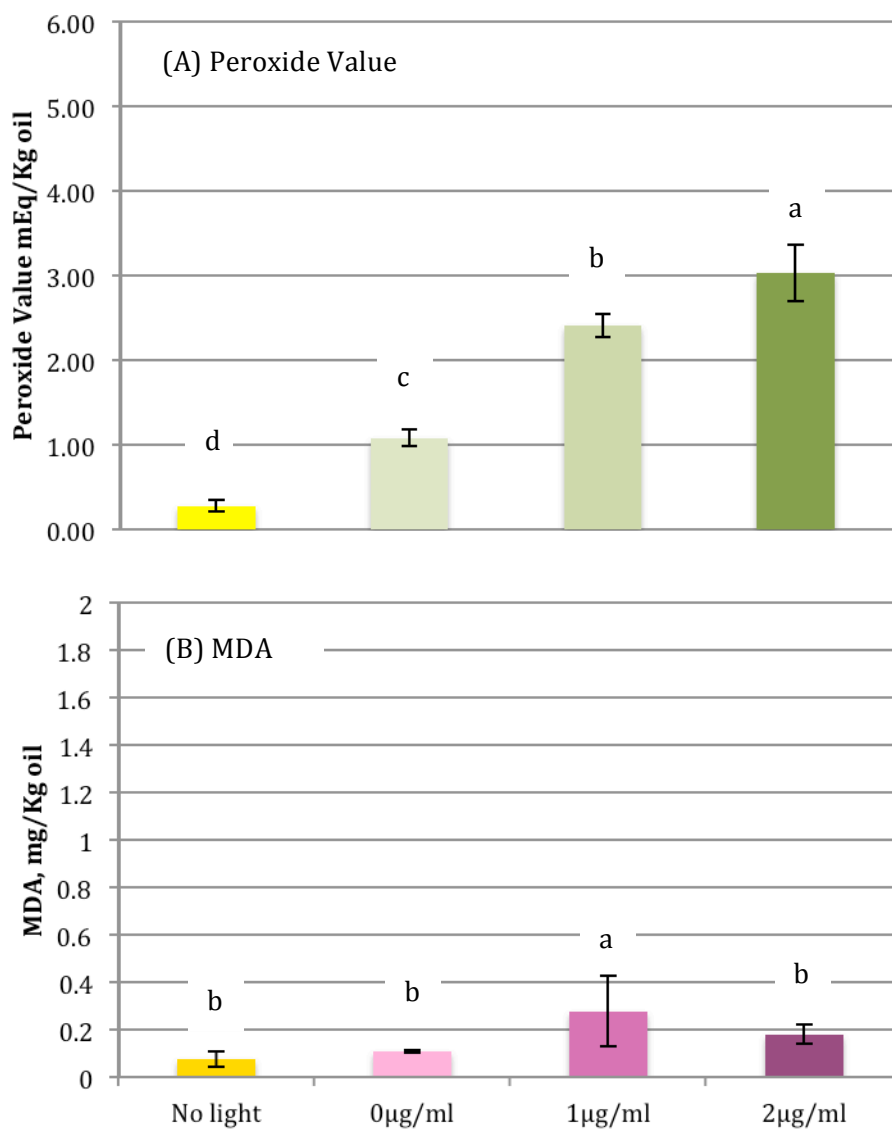
Figure 16. Peroxide value (mEq/Kg oil) (A) and malondialdehyde (mg/Kg oil) formation (B) in SO with 0, 1, and 2µg/ml chlorophyll added, exposed to 430nm (1.8±0.7mW/4.15cm2) wavelength of light for 4 hours (11.9°C) compared to no light control. Lowercase letters indicate statistical significant differences (α=0.05).

At the 660nm wavelength, there were no significant differences between oil with no chlorophyll added compared to oil receiving no light exposure. Chlorophyll added at 1µg/ml and 2µg/ml had

significantly higher PV than oil without chlorophyll ($p<0.0001$) and also between each other ($p<0.0001$). The MDA concentration was only significantly higher in the 1μg/ml chlorophyll addition compared to no light ($p=0.0344$). Among the 0, 1, and 2μg/ml chlorophyll, there were no significant differences at 660nm ($p=0.2209$) (Figure 17).

While there are statistically significant differences at different wavelengths, these levels of peroxides and malondialdehyde are likely to cause no to mild olfactory sensory changes in the product. Sensory studies have shown that panelists may rate oil with PV of 3-5 as mildly oxidized (Warner and Nelson, 1996; Choppin and Pike, 2001). Under the conditions of this study, some treatments did increase PV greater than 3 but oils did not reach a PV above 5. MDA concentrations that are correlated with sensory detection of off flavors and odors are approximately 1.5mg/Kg oil (unpublished data) and the oil in this study only reached approximately 1.2 mg/Kg oil. This suggests that only those individuals who are highly sensitive to such oxidation by-products may detect a change in aroma or flavor. However visual effects, which will be discussed below, may create a bias that influences perceptions of flavor and aroma differences.
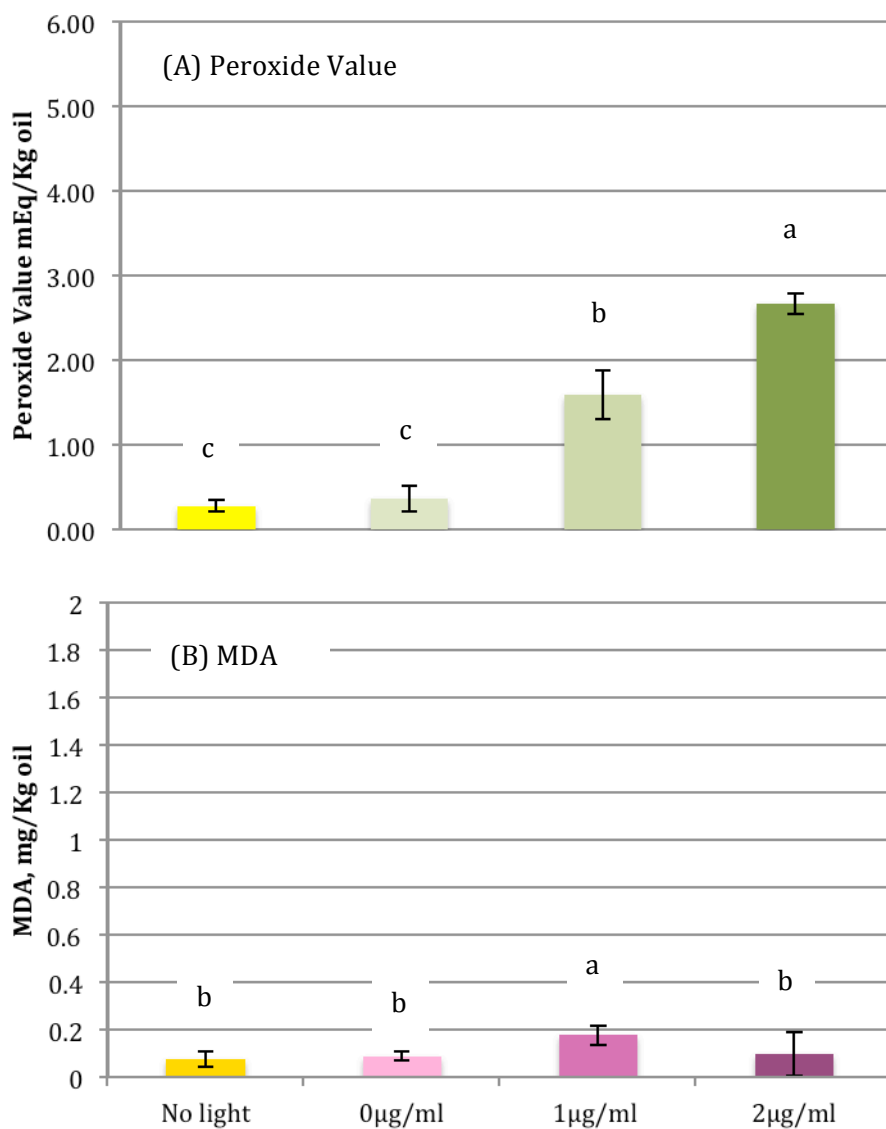
Figure 17. Peroxide value (mEq/Kg oil) (A) and malondialdehyde (mg/Kg oil) formation (B) in SO with 0, 1, and 2μg/ml chlorophyll added, exposed to 660nm (0.3±0.06mW/4.15cm2) wavelength of light for 4 hours. Letters indicate statistical significant differences (α=0.05).

At 450nm there were no differences in peroxide and aldehyde formation from the no light control (data not shown). Thus it appears that region of light has no effect on chlorophyll photosensitization on

the oil. This wavelength was studied because riboflavin is a highly responsive photosensitizer at 450nm. Both riboflavin and chlorophyll are found in soymilk although riboflavin is not in soybean oil. We wanted to demonstrate that chlorophyll responds and has an effect at wavelengths separate from riboflavin.

While we did not deliberately compare results from specific wavelength of light to each other, it is notable that despite the much lower intensity of light at 660nm vs. 430nm, both wavelengths provided measurable differences in peroxide and MDA formation. We can only make inferences that because the intensity of light is lower with the 430nm and 660nm bandpass filters, they would have more of a damaging impact if the intensity of light were higher.

The degree of lipid oxidation can be measured by PV in oils or aldehyde levels (TBARS or GC-MS) in liquid foods such as milk or solid foods such as meats. PV has been associated with sensory deterioration of oil due to rancidity. While PVs of SO oil of 3-5 are considered only mildly oxidized, PV values of 10-12 are perceived as moderately oxidized (Warner and Nelson, 1996). Added chlorophyll under broad-spectrum light conditions oxidized the SO to conditions that would be considered mildly oxidized. Thus, chlorophyll effects on oxidation in

and of itself may not necessarily contribute to significant sensory deterioration of a food product with oil. There is concern however, that initiation of autoxidation due to photooxidation of chlorophyll could contribute to a lower quality and shorter shelf-life of the product. In addition, other food products, such as milk or soymilk contain other photosensitizers such as riboflavin that also provide sensory deterioration if not protected from light. While the trend of packaging may focus on protecting wavelengths at which riboflavin is excited, we believe that the wavelengths of 430nm in particular may also be a source of photooxidative products that contribute to off flavor and odor production associated with cumulative effects of chlorophyll, riboflavin and other photosensitizers.

We have adapted the use of the TBARS assay to measure degree of oxidation in milk and soymilk. Our lab (data not published) has shown that an MDA concentration of 1.5mEq/kg oil is indicative of sensory detection of oxidized dairy milk. Once again, the MDA concentration of chlorophyll-sensitized oil is not in the range of expected sensory deterioration, but it must be noted, this is from chlorophyll alone and combined with oxidation due to other photosensitizers, the cumulative effect could be above sensory detection and thus applicably relevant.

Numerous studies have elucidated the role of chlorophyll in photooxidation of SO including the specific compounds that result in off flavors and odors. Previous studies have demonstrated that singlet oxygen is involved in the production of 2-pentyl furan and 2-pentenyl furan both of which contribute to reversion flavor of SO (Smouse and Chang, 1967, Ho et al., 1978, Chang et al., 1983).

We wanted to determine to what degree the wavelengths of light at which chlorophyll absorbs contributes to formation of photooxidative products. While we did not characterize the specific oxidative products, we were able to determine that the wavelength of 430nm is primarily responsible for the degradation of chlorophyll and its contribution to photooxidation as expected. As packaging technology develops such that it is capable of protecting from specific wavelengths, products that contain chlorophyll should be provided complete protection in the 430nm region. Webster et al. (2011) showed the importance of protecting milk, and specifically riboflavin, using different wavelengths and different types of films that only allow certain wavelengths of light through to the food product. They found that wavelengths in the UV range of 200-400nm produced significantly higher levels of hexanal in dairy milk, despite the lower intensity of light, implicating riboflavin.

However, pentanal was produced in higher amounts at wavelength in the 610 region implicating something other than riboflavin as this is not a wavelength region at which riboflavin has an absorption maximum. This may provide support for the hypothesis that chlorophyll or its derivatives are indeed in milk.

Our study is in agreement with Thron et al. (2001) who demonstrated that light in the 400nm region had a greater impact on oxidation outcomes of sunflower oil including oxygen consumption and pentane formation. They found that oxygen consumption was higher at 665nm than 408nm, but the absorption maximum for chlorophyll was significantly higher at 408nm. Chlorophyll had a higher extinction coefficient at 408nm and thus while the intensity of light was lower, the impact at 408nm was more profound. Wold et al. (2005) showed that sensory off flavors and odors were produced in cheese that was exposed to wavelengths of light within the 661-672nm region and suggests that chlorins may be present in this dairy product. It is reasonable to hypothesize that chlorins are in bovine milk since chlorophyll is in high amounts in the feed of cows. How it is digested and absorbed has been minimally studied; however, Ferruzzi and Blakeslee (2007) have

reviewed possible mechanisms of bioavailability of chlorophyll in the diet.

We evaluated how chlorophyll responded to specific wavelengths of light in an oil matrix. It would also be useful to evaluate how chlorophyll responds to light within an emulsion such as soymilk or dairy milk. Because chlorophyll is lipid soluble and within a fat globule that is in an aqueous matrix, it may be protected from light due to the phospholipids or other components that emulsify the lipid phase. Riboflavin is within the aqueous phase of milk and responds at a number of wavelengths to different degrees (Webster et al., 2011). How light is refracted and absorbed within an emulsion that contains both riboflavin and chlorins would be a potential area of research for photo-protection of milks.

One limitation of our study is associated with the sensitivity of the HPLC dual absorbance detector. We were only able to emit 2 specific wavelengths of light on the analyte (430nm and 660nm). Furthermore, we did not employ fluorescence detection.   Thus, we did not see degradative products on the chromatogram.   Even at the lower intensity of 660nm, in which chlorophyll degradation did not occur broad-spectrum, degradative peaks of pheophytin or pheoborbide were

not seen. Those studies that demonstrated good separation of chlorophyll and pheophytin/pheoborbide compounds employed photodiode array detectors and fluorescent detectors. Canjura and Schwartz (1991) reported absorbance maxima for pheophytin a, pyropheophytin a, pheoborbide a and pyropheoborbide a of 409/668, 411/666, 409/667, and 411/666 respectively. It should be noted that the detection wavelength of 430nm provided the majority of peak production.

Another possibility of our results may be that at such intense light, these compounds are decomposed into fragments that are not detectable. Indeed the light intensity of the broad-spectrum light is significantly higher than that of normal lighting. Struck et al. (1990) demonstrated that chlorinated chlorophylls degrade into linear tetrapyroles.

Color Changes in SO with Added Chlorophyll

Changes in color in SO containing added chlorophyll were affected by light exposure (

Table 4). The a* value is a measurement of green to red; the more

negative the number on this scale, the more green is being reflected.

Since chlorophyll absorbs light at wavelengths 430 and 660nm,

wavelengths in the blue-green spectrum are reflected; thus chlorophyll

a is observed as a greenish-blue color. This value decreased when

chlorophyll was added to SO. Upon chlorophyll degradation, the a*

value behaved as expected; it increased indicating that green reflectance

decreased. After four hours exposure, the a* value was similar to SO

values without any added chlorophyll.

The b* value is an indicator of color on the yellow-blue spectrum.

Some yellow color is reflected as expected due to tocopherols in the SO.

Interestingly, this value of color did increase albeit not significantly,

likely due to the chlorophyll not masking the yellow reflectance upon

degradation.

The CIE L* value, a measurement of darkness to lightness showed

that added chlorophyll to the oil does "darken" the oil. After four hours

of exposure to light, SO containing 1µg/ml chlorophyll returned to the

same lightness as oil with no chlorophyll added. This is easily explained

by the fact that as the chlorophyll degraded, neither it nor its

degradative products contributed to darkening of the oil.

Table 4. CIE-L*a*b* and chroma (C*) and hue (H°) mean and standard deviation values of SO without added chlorophyll and SO with added chlorophyll, 1µg/ml, before and after four hour broad-spectrum light treatment (157.6±4.7mW/16.6cm^2). Statistical significant differences for L*, a*, b*, C*, and H° are shown with differing letters.

	L*	a*	b*	C*	H°
No chlorophyll add	34.87±0.05a	-1.74±0.02a	5.58±1.16b	6.01±0.6b	111.9±6ab
1ppm chlorophyll added, before light treatment	33.44±0.29b	-3.90±0.51b	6.29±0.19ab	7.04±0.3ab	121.2±4a
1ppm chlorophyll added, after light treatment	34.18±0.70ab	-1.32±0.31a	7.04±0.70a	7.47±0.5a	100.38±4.8 b

Conclusions

Chlorophyll has been studied extensively as a photosensitizing agent. We have demonstrated that packaging intended to protect foods containing chlorophyll from organoleptic deterioration should protect wavelengths in the 430nm region. This region of light wavelength appears to contribute to chlorophyll degradation and contribute to peroxide and aldehyde formation.   Future studies should determine how chlorophyll might contribute to photooxidation of protein at this

wavelength and hence any organoleptic deterioration of products containing this chromophore, particularly since plants do contain small amounts of protein. Numerous plant derived food products may have a change in color, flavor, and odor that could potentially be perceived as negative characteristics by consumers if light is allowed to photooxidize the product due to chlorophyll.   Future studies should also be carried out to determine if bovine milk contains chlorophyll. In addition, beverage products containing high chlorophyll containing plants should be studied for light induced sensory changes over their shelf life periods.

Acknowledgements

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Chapter 4 Protecting Soymilk Flavor and Nutrients from Photodegradation

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Abstract

Five different packaging treatments were studied over a 36-day period to determine if they protected soymilk from photooxidation. Soymilk was packaged in high density polyethylene (HDPE) bottles with and without light protective additives (LPA). Two controls [(1) no LPA (translucent appearance); (2) a light-protected control (foil overwrap over no LPA control)] and three LPA-containing treatments (low, med, high) were studied. Bottles were stored in a lighted refrigerated display case (average light intensity: 2200 lux; 4°C) for 36 days and evaluated weekly. Soymilk packaged in high LPA bottles protected soymilk from developing light-oxidized off flavors and odors for a minimum of 15 days. High LPA bottles provided optimal protection for riboflavin and photooxidative products for approximately 29 days.

Keywords

Introduction

Soymilk (SM) sales have increased significantly in recent decades from $250 million per year to $1.25 billion per year within 15 years (1996- 2011) (Soyfoods Association of North America, 2014). The increase in sales may be attributed to improved sensory attributes of soymilk, increased consumer interest in functional foods for health value, and improved processing methods. Soymilk contains heart-healthy polyunsaturated fatty acids (PUFA; 63% of total fat), including the omega-3 fatty acid linolenic acid (18:3n3), which are susceptible to autoxidation and photooxidation (Frankel, 1984; Min and Boff, 2002).

Photooxidation of soymilk occurs when photosensitizing molecules (e.g., riboflavin (Rb), are activated by light energy and contribute to degradation of PUFA. This leads to decreased health value of the product as well as development of off-flavors. Bovine milk, which is composed of approximately 31% unsaturated fatty acids (Mansonn, 2008), is also highly susceptible to photooxidation. Riboflavin is found naturally in bovine milk at a concentration of 1.87μg/ml and occurs in soymilk naturally at 0.7μg/ml (USDA Food Composition Tables, 2002). However, riboflavin is listed as an added ingredient on soymilk food

labels. Kwok et al. (2006) reported Rb in soymilk at concentrations of 2.44mg/kg. Porphyrin structures, such as chlorophyll, also may function as photosensitizers; their presence in milk is suggested but not confirmed (Wold et al., Webster et al., 2011) nor is there any reported concentration of chlorophyll in soymilk.

Because soymilk is higher in PUFA and Rb than bovine milk, the potential for photooxidation is higher, suggesting that photooxidation could rapidly decrease shelf-life of soymilk. Other components of soymilk complicate this theory however.   Soymilk contains polyphenols that are also known to be antioxidants but under certain conditions can act as prooxidants (Sisa et al., 2010).   Additionally, the carotenoid vitamin A, which is enriched in bovine milk (2.08 IU/ml) and in enriched in soymilk (O.87 IU/ml) may behave as antioxidants or prooxidants. Therefore there are many factors in these two beverages that may result in different responses to light.

Soymilk and bovine milk are commonly packaged in paperboard or high-density polyethylene (HDPE) packaging. Packaging for light sensitive products often has light protective additives (LPA), such as titanium dioxide (TiO_2), added. LPA molecules interfere with light transmission, creating a visually opaque or colored packaging material

and prevent light penetrating the product. Novel approaches for modifying polymer material opacity and light interference for protecting food quality can improve product quality (Webster et al., 2009).

The purpose of this study was to determine the effect of light on the oxidative stability of soymilk quality, measured with sensory testing and chemical analyses. Efficacy of light protective additives (LPA) in HDPE packaging in protecting soymilk quality was assessed.

Materials and Methods

Packaging

HDPE bottles were manufactured with three LPA levels (experimental TiO_2 characteristics) as well as controls with no LPA. Bottle dimensions were 7.16"ht x 3.29"w x 2.1" deep and a volume of 528 ml to overflow. Four HDPE bottle treatments were supplied (DuPont™, Wilmington, DE); treatments were differentiated by LPA (three titanium dioxide modifications) contained within the bottle resin, yielding bottles with different levels of light protection as well as one treatment that did not contain LPA (control). Compositional differences of the LPA were confidential to the supplier and were not revealed to

the researchers. LPA-containing treatments were identified as Low, Medium (Med), and High to indicate the targeted level of light protection. Control packaging treatments included foil-wrapped light-protected control bottles (F= foil-wrapped), which provided no light penetration to the product, and control bottles (no LPA, no foil overwrap), identified as clear (C) because the product could be readily seen through the bottle although the package was translucent. Bottles and caps were sanitized with 100ppm chlorine solution, rinsed with deionized distilled water, and drained prior to filling with soymilk product.

Product

Soymilk (PS Lite, one half gallon paperboard containers, Kroger Co., Cincinnati, OH) was purchased from a local supermarket on the delivery date and transported immediately (5 minute drive) to the VT Food Science and Technology Department in chilled portable coolers. The product label stated the following ingredients: filtered water, whole organic soybeans, organic evaporated cane syrup, calcium carbonate, natural flavors, sea salt, carrageenan, vitamin A palmitate, vitamin D_2, Rb, and vitamin $B_{12.}$

Packaging Filling and Product Storage

Package Filling.

Cartons of soymilk were commingled into a clean, sanitized
bucket (5 gallon GeFcontainer, General Films, Inc. Covington, OH).
Product was kept cold on ice throughout the filling process. Each bottle
was filled with soymilk (450 ml), using a peristaltic pump (Wheaton
Unispense II, Millville, NJ) and sanitized tubing, under a positive air flow
hood (Atmos Tech Industries, Ocean NJ) to minimize incidental
microbial contamination from the air. Packages were immediately
capped and stored in a darkened portable cooler on ice, then
transferred to a refrigerated cooling system. Product was stored at
refrigerated conditions, with product evaluation occurring at seven
intervals over 36 days.

Shelf-life Storage Conditions.

Filled bottles were placed into a refrigerated Friedrich Floating
Air beverage case (Friedrich 60-10-1056; San Antonio, TX) to simulate
retail conditions. The cooler was glass fronted with 5 doors and
equipped with cool white, 32-watt light bulbs (Alto II, Panasonic, Maple
Grove, MN), simulating retail conditions. Above each of three shelves, 2
fluorescent light bulbs ran the horizontal length of the dairy case over

each shelf at 1/5th and 4/5th the distance from the front of the shelf. Bottles were 11.43 cm below horizontal lights. Six light bulbs were positioned vertically at the front of the dairy case at the ends of the case and in between each door junction.

Bottle placement was randomized so that all treatments were distributed randomly within the dairy case to reduce the effects of different lighting intensity within the case. Temperature and light intensity were measured routinely. The average temperature of the dairy case was 2.7°C $\pm$ 0.8. Over the 5-week storage study, light intensity measurements were taken in three general locations on each treatment for each day of analysis. Light intensity averaged 1122 lux $\pm$ 439 (range: 355-1942 lux), depending on sampling location. Because of this broad range of light intensity, 10 of each treatment bottles and 20 of each control bottles were randomly selected for sampling on each day of analyses. Product from at least two bottles per treatment was used for each analytical assay.

Sensory Testing

Sensory Testing.

IRB approval for use of human subjects in research was obtained

from the Virginia Tech Institutional Review Board (IRB #11-477,

approved Oct 14, 2011) (Appendix B-1). Panelists were recruited from

student, faculty and staff at VT and local community members

(Appendix B-2). Ninety-eight panelists were targeted to complete the

sensory testing for each day of evaluation. Panelists completed

informed consent (Appendix B-3) prior to completing the sensory test.

Panelists evaluated the samples in partitioned sensory booths, equipped

with a touchscreen monitor, under white lighting. Data from a touch

screen monitor was collected using Sensory Information Management

Software (SIMS 2000; Sensory Computer Management, Morristown, NJ).

A triangle test for difference and for similarity was completed on

each day of evaluation. Testing for similarity in soymilk flavor was

completed for the three LPA-HDPE experimental treatments compared

to the foil-wrapped light-protected (F) control (F:Low, F:Med, F:High);

testing for difference was completed for all experimental treatments

compared to the no LPA light-exposed (C) bottles (C:Low, C:Med,

C:High). A comparison of the two controls (F:C) was also completed.

Testing for similarity provided determination that the opacity of the

package protected as well as light-protected control bottles. Testing for

difference allowed determination that the packaging opacity protected better than light-exposed controls.

Sensory Statistical Design and Parameters.

An incomplete randomized block design was used, with each panelist receiving three 3-sample sets (9 total samples). This design reduced biases due to sensory fatigue. The order of samples within a comparison set was organized in a balanced order so that all possible combinations were presented an equal number of times. The order of the three sample sets was randomized so that there would be no bias based on the order of presentation, to reduce the influence of fatigue. Each set of three samples had two identical samples from one packaging treatment and one sample from a different treatment. The panelists were instructed to smell and taste the samples from left to right and choose the sample that was different, using the touch screen monitor. Panelists were required to choose a sample even if they could not detect a difference. Water and unsalted oyster crackers were given to each panelist for consumption to cleanse the palette between each triangle test.

Statistical parameters for the triangle test for similarity, comparing treatment packages to foil-wrapped controls, targeted an α

of 0.20, β of 0.05, and a proportion of discriminators (p_d) of 0.30. For

triangle test for difference, comparing treatment packages to clear

controls, an α of 0.05, a β of 0.20, and a p_d of 0.30 was targeted.

However because the number of panelists participating on any given

day of evaluation varied, the actual parameters varied.

Chemical Analysis

Packaging and Storage.

Chemical analyses were completed in two experimental blocks (A,

B) and separate from the sensory study. Block A was completed in April

and block B was completed in August. A failure in the refrigerated dairy

case to maintain temperatures under the conditions of the summer heat

required a change in sample storage for the second block.

Soymilk was obtained and filled into bottles as described

previously. Each bottle was labeled on the bottom, filled and randomly

placed into the dairy case used for the sensory tests (block A) or a walk-

in cooler (block B) (Tonka, Hopkins, MN). Randomization was

completed to ensure that bottles from each treatment group were

pulled out from random areas of the cooler on each day of analysis. In

the first study (Block A), light intensity was measured over each bottle

and averaged 2186 lux ±, 867 with a range from 396-3970 lux,

depending on sampling location. Temperature averaged 3.0°C ± 0.87.

For the second study (Block B), temperature was taken in two different

areas of the walk-in cooler. For Block B, temperatures ranged from 5-

8°C with an average of 5.68°C ± 0.91. Light intensity was measured in 5

random locations above the bottles on day 1, with an average light

intensity of 794.2 lux ± 43.1. Philips 34-W fluorescent light bulbs

(Panasonic, Maple Grove, MN) were placed 12.7cm over the bottles on

two length-sides of the area of the treatment bottles. Treatment bottles

were pulled out of the dairy case/cooler on days 1, 4(block A)/3(block

B), 8, 15, 22, 29, and 36. Averages of all data for both blocks are

presented. There was a statistically significance difference between the

light intensities of the two blocks using a t-test (p<0.0008).

Analytical Assessment.

Chemical analyses included Rb degradation, thiobarbituric acid

reactive substances (TBARS), and GC-MS analysis of headspace volatiles.

Analysis of bacterial contamination was completed on 3M aerobic petri

films on each day of chemical analysis. Bottles were pulled out each

morning and soymilk portioned into appropriate vials for analysis.

Rb analysis was done according to a modified assay of the AOAC number 960.65, in which the fluorescence of Rb was measured on a spectrofluorometer (Appendix B-4) (Shimadzo Scientific Instrument, Inc., Columbia, MD) (AOAC, 1998; Bradley 2000; Webster et al, 2009).

Thiobarbituric reactive substances (TBARS), reported as mg malondialdehyde/kg product, were assayed in each sample. The TBARS assay is a measurement of formation of malondialdehyde, a secondary product of oxidation and an indicator of the production of other secondary volatile oxidation products. The procedure was modified from Spanier and Taylor (1991) for milk analysis (Appendix A-2).

Volatile analysis was completed on control and treatment samples. Volatile compounds from the soymilk were extracted and concentrated using gas chromatography (GC). An HP 5890 GC with 5972 series mass selective detector HP5MS (Hewlett Packard, Palo Alto, CA) was used to separate and identify volatile headspace compounds. Samples were heated to 45°C on an RCT basic heater with an ETS-D4 Fuzzy Controller (IKA Werke, Wilmington, NC) while being stirred. An 85um carboxen-polydimethyl siloxane (PDMS) solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA) was used to adsorb volatile compounds. The fiber was exposed to the headspace

while 8 ml of milk was heated for 20 minutes. Volatiles were desorbed

from the fiber onto an HP-5MS capillary column (30 m x 0.25mm id x

0.25um film thickness) to separate and analyze volatiles. The following

procedure was used: Helium gas flow: 28-36 cm/sec; injector

temperature: 280^0C; and detector temperature: 280^0C. The initial

temperature of 35^0C was held for 0.5 min then ramped by 15^0C/min to

180^0C and held for 0.5 min. The temperature then was ramped by

20^0C/min to 260^0C with the final temperature held for 0.5 min. The

total run time was 15.17 min and the program was run in splitless

mode. The chromatograms were integrated using HP ChemStation

software (Hewlett Packard, Palo Alto, CA).

Statistical Analyses.

Results of the chemical analyses of the soymilk were evaluated

using ANOVA with a two factorial design and blocking for each

experimental block. Main effects of package and time were the two

factors. Interaction was tested as was each individual factor using

α=0.05 (JMP 9.0 software, SAS Institute Inc, Cary, NC). Contrasts were

performed for all bottle treatments to determine failures over time

compared against themselves, foil-wrapped and clear and against Foil,

day 1 for each response of Rb, TBARS, and hexanal. These are described

in detail in results and discussion.

Results And Discussion

Effectiveness of Packaging in Protecting Sensory Quality

Packaging treatments were tested for similarity of soymilk

sensory quality (β=0.05, α=0.30, p_d = 0.30) compared to soymilk in foil-

wrapped (F) packages to test the hypothesis that LPA packaging

treatments protected the milk as well as an optimum light-protected

condition. The statistical parameters for similarity testing were set to

reduce type II error. Difference testing was performed against the no

LPA bottles (C) to test the hypothesis that LPA-packaging treatments

provided better protection to oxidation. The statistical parameters

(α=0.05, β=0.30, and p_d=0.30) for difference testing were set to reduce

Type I error. Proportion of discriminators is a reasonable estimate that

30% of the population would be able to discriminate oxidized flavor and

odor in soymilk; this estimate was based on previous experience in our

laboratory. It also must be noted that some of the panelists consistently

participated in sensory tests over the testing period where as others

participated only a limited number of times.

Sensory difference testing illustrated that experimental packaging

treatments protected soymilk from photooxidation related to the LPA

design and the duration of light exposure (Table 5).

Table 5. Summary table of statistical significance (p<.05) of sensory testing for soymilk by triangle tests, for each packaging treatment (low, med, high)[1] compared to controls (Clear: C, Foil-wrapped: F) for each day of evaluation.

Package Treatment Comparison	Day 1	Day 4	Day 8	Day 15	Day 22	Day 36
Difference test: $\alpha=0.05$, $\beta=,0.30$, $p_d=0.30$						
C : low	0.1548	0.4225	0.8384	0.5578	0.0008*	0.0000*
C : med	0.7788	0.8384	0.3364	0.0084*	0.0214*	0.1733
C : high	0.0085*	0.5624	0.0507	0.0384*	0.0039*	0.0187*
F : C	0.0442*	0.0754	0.0096*	0.0001*	0.0000*	0.0034*
Similarity test: $\alpha=0.30$, $\beta=0.05$, $p_d=0.30$						
F: low	0.07788	0.7692	0.0135*	0.0290*	0.0000*	0.0034*
F : med	0.05157	0.6496	0.0135*	0.5578	0.0000*	0.0084*
F : high	0.2521	0.1354	0.7605	0.5578	0.0187*	0.0013*
Panelist/Test	35	36-38	37-41	41-42	40-42	41-42

*P<0.05
[1]Study Codes for Treatments: for Controls: F=Foil (HDPE with foil overwrap); C=Clear (HDPE bottles with no TiO_2 or foil)

[2]Power analysis: triangle test for difference against foil-wrapped bottles, $\alpha=0.05$, $\beta=,0.30$, $p_d=0.30$; triangle test for similarity against clear bottles, $\alpha=0.30$, $\beta=0.05$, $p_d=0.30$
[4]Exposure to light (1172 lux ± 439) in hours: day 1=20, day 4=92, day 8=188, day 15=356, day 22=524, day 36= 860

As expected, there were significant differences between the control (F:C) packaging treatments within 20 hr of light exposure (day 1; p=0.044) and consistently (exception on day 4 (92 hr); p=0.075) from day 8 (188 hr), indicating that soymilk is photo-responsive to low to moderate retail lighting conditions within relatively short storage times with resulting impact on sensory quality. Similarity testing showed the high LPA treatment was as effective as the light-protected control (F) in protecting soymilk flavor for at least 15 days but was differentiated on day 22; however, this treatment package still afforded some protection as the soymilk was still differentiated from the soymilk packaged in the clear control during the latter part of the storage study. With the exception of day 4, the other two treatments (low and med LPA-treatments) were statistically different (p<.05) by day 8 from light-protected control (F), indicating that they did not protect from off-flavors or aromas as well as full light protection. In addition, they were not statistically different from clear packaging for at least the first two

weeks also supporting that they did not provide protection any better than the clear bottles during the early exposure. However, soymilk packaged in low and med LPA treatments did not change in flavor as extensively as product packaged in the clear control, as there were product differences at days 15 (med) and 22 (low). Soymilk within the clear (no LPA) packaging had significant photooxidation occurring within these bottles that exceeded that of the product in the low and med LPA packaging.

The lack of sensory difference between the two controls (F:C) on Day 4 appears to be an anomaly, which may be partially explained by the participants on that day of evaluation. When the portion of discriminators were assessed for each sensory day, the number of discriminators was lower on day 4 of sensory testing indicating that not as many panelists were able to discriminate oxidized aroma/flavor of the soy milk. The challenge is to determine if that is because there truly was not a difference among the products or was the portion of the population that completed the test on that day less sensitive (than the population on day 1, for example). Although many participants routinely participated throughout the study, there was no way to get

100% compliance in participation so there were some changes in the participant pool on each date.

While changes in product quality were obvious over time, we also acknowledge that participants also learned from experience. We are 95% confident that the true population that can distinguish differences is lower at the beginning of the study, when sensory differences are relatively small, and increases with time as product quality changes with light exposure. We also acknowledge, however, that inadvertent "training" of the panelists may also occur over time. As many of the panelists returned throughout the study, they may have become more adept at detecting differences. There also appears to be a pattern in which the true populations that can distinguish between the foil-wrapped and clear control treatments is higher than the other comparisons, as there would be the greater difference in sensory quality between products in these two controls.

Effectiveness of Packaging at Protecting Riboflavin and Limiting Oxidative Degradation

The chemical analyses of the evaluation were treated as blocks because there were differences in experimental conditions and to reduce effects associated with greater light intensity and variation in the

first study (block A) than the second study (block B). Nonetheless, each

block demonstrated similar trends. Furthermore, statistical analysis of

block effects indicated that each block did not differ from one another

significantly (Table 6,Table 7). Power was determined for each block

and each had a power estimate of 1, demonstrating that the blocks can

stand independently.   This indicates that the treatment packages are

effective for the lower, less variable light intensity (block B) as well as

under conditions of higher light intensity and greater variability in

intensity (block A). Both block A and B showed that the high LPA

treatment provided better protection for Rb degradation and

photooxidation products (TBARS, hexanal). Results shown are the

average of all data from both blocks. However, because the lighting

conditions of block B were dissimilar from the lighting conditions of the

sensory experiment, comparisons between sensory analysis and

chemical analysis were only discussed regarding block A

Table 6. ANOVA table for effects test for response of Rb concentration testing for package, time (days), and block and interactions. $\alpha = 0.05$

Source	DF	Sum of Squares	F Ratio	Prob > F
Package	4	3.4601683	36.6817	<.0001*
Block	1	0.0572344	2.4270	0.1238
Day	6	4.0805960	28.8393	<.0001*
Package*Block	4	0.2958369	3.1362	0.0198*
Package*Day	24	2.0330449	3.5921	<.0001*
Block*Day	6	0.5527603	3.9066	0.0020*
Package*Block*Day	24	0.9395797	1.6601	0.0532

Table 7. ANOVA table for effects test for response of malondialdehyde concentration testing for package, time (days), and block and interactions. α = 0.05

Source	DF	Sum of Squares	F Ratio	Prob > F
Package	4	4.9064436	124.7502	<.0001*
Block	1	0.0235323	2.3933	0.1264
Day	6	8.3244725	141.1042	<.0001*
Package*Block	4	0.5042532	12.8210	<.0001*
Package*Day	24	2.7434866	11.6259	<.0001*
Block*Day	6	2.0762859	35.1941	<.0001*
Package*Block*Day	24	0.9852940	4.1753	<.0001*

*pvalue <0.05 indicates statistical significant difference.

Rb concentration in fresh soymilk was 2.33µg/ml. This is in agreement with Kwok et al. (1998) who found riboflavin to be present in soymilk at a concentration of 2.44mg/kg. In soymilk packaged in foil-wrapped control bottles, Rb remained relatively unchanged over time, with only 5.4% loss of Rb over the 5-week period (Figure 18).   The effect of packaging and storage time ("day") were statistically significant (p<0.05) as were the two-way interactions (package*block, package*day, and block*day). In the clear (no LPA), low, and med LPA packaging treatments, the degradation of Rb was greater than in foil-wrapped bottles, with losses of 28.3%, 20.9% and 23.7%, respectively,

over the 5-week storage. High LPA packaging treatment provided

optimal protection up to day 29 as the Rb concentration was not

significantly different from the light-protected control (F) up to that

time, with only 0.8% loss of Rb. However degradation in soymilk in the

high LPA packaging did occur from day 29 to day 36, with a 5.4% loss of

Rb in the light-protected soymilk (F) and an 18.1% decrease in the high

LPA-packaged soymilk.
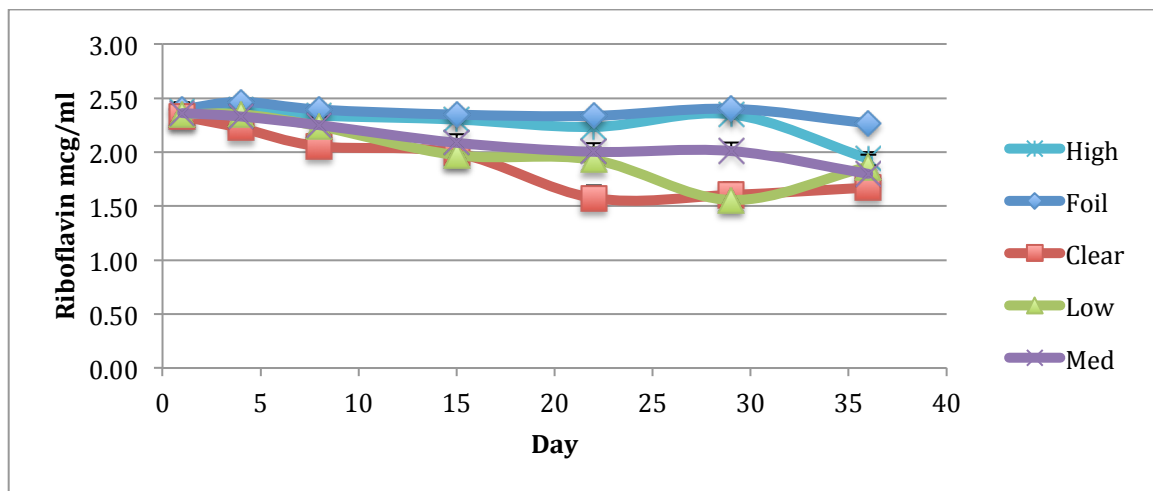
Figure 18. Rb degradation in packaging treatments, low, med, and high
compared to foil-wrapped and clear packaging over a 36 day period.
Average of data in both Block A and Block B.
[1]Exposure to light in hours: day 1=23.5, day 4=84, day 8=191, day
15=359, day 22=527, day 29=696, day 36=864.

Statistical contrasts comparing the Rb concentration of the light-

protected soymilk (F) from day 1 to the concentration on each

subsequent day of analysis and all of the other bottles on all days were

completed (Table 8). As expected a contrast completed between foil-

wrapped control, day 1 (F1) vs. other TiO$_2$-packages demonstrated significant differences.   However, comparison of the Rb concentration in F1 indicated no differences compared to high LPA bottles.   There were no differences in Rb concentration among any of the packaging treatments at the beginning of the study.   Differences in Rb concentration in high TiO$_2$-HDPE packaged soymilk were not evident compared to F1 control until day 36.

Table 8. p values for specific contrasts for responses Rb concentration, MDA, and hexanal. $\alpha = 0.05$

Contrast	Rb	MDA	Hexanal
F1 : F4-36	0.7324	0.0791	0.0951
F1 : C1-36	<0.0001*	<0.0001*	<0.0001*
F1 : Low 1-36	<0.0001*	<0.0001*	<0.0001*
F1 : Med 1-36	0.0012*	<0.0001*	0.0025*
F1 : High 1-36	0.14	0.0679	0.1042
F1 : C1,L1,M1,H1	0.6206	0.2779	0.9253
F1 : H1	0.8198		
F1 : H4	0.9418		
F1 : H8	0.5862		0.0023*
F1 : H15	0.3855		0.3398
F1 : H22	0.1322	0.0142*	0.3593
F1 : H29	0.6709	<0.0001*	0.2008
F1 : H36	<0.0001*	<0.0001*	0.0350*
F4 : H4	0.4866	0.2759	0.9418
F8 : H8	0.6083	0.5441	0.7681
F15 : H15	0.6793	0.7627	0.7715
F22 : H22	0.3381	0.9688	0.9454

F29 : H29	0.6414	0.0976	0.6067
F36 : H36	0.0034*	<0.0001*	0.1978

F1 : C1	0.5465	0.3097	0.9146
F1 : L1	0.6738	0.4272	0.9477
F1 : M1	0.7539	0.2420	0.9810

H1 : H29	0.8436	<0.0001*	0.2008
H1 : H36	0.0002*	<0.0001*	0.0350*

F1 : C8	0.0023*	0.0063*	<0.0001*
F1 : L8		0.0002*	0.0004*
F1 : L15	0.0002*		
F1 : M8			0.0015*
F1 : M15	0.0056*	0.0029*	

*pvalue <0.05 indicates statistical significant difference.
F=foil-wrapped control, C=clear control, L=low TiO_2, M=med TiO_2,
H=high TiO_2
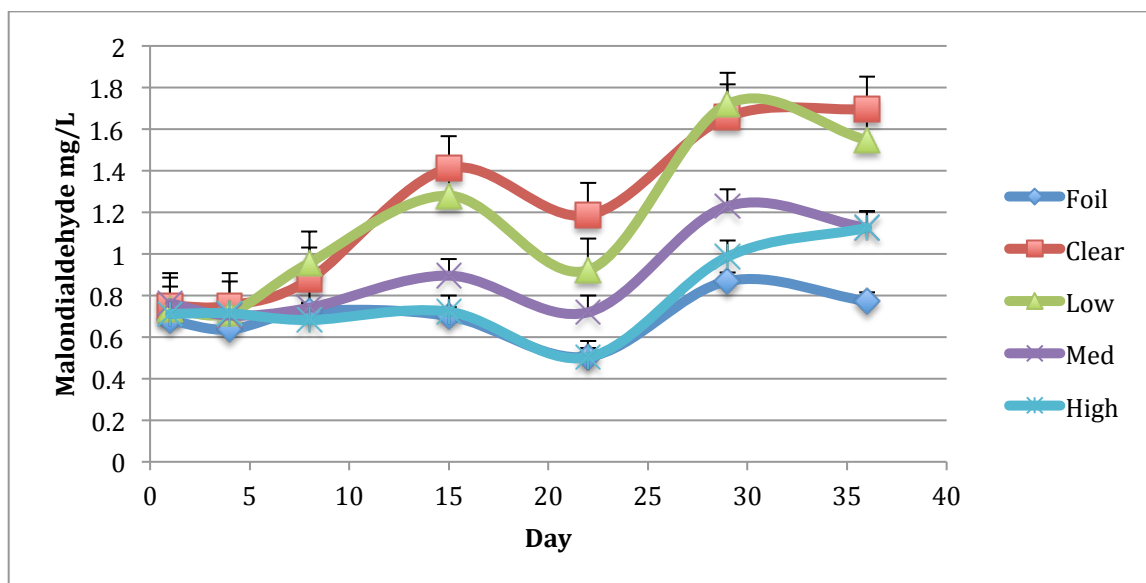
Figure 19. Malondialdehyde concentration in packaging treatments, low, med, and high TiO_2 compared to foil-wrapped and clear packaging over a 36 day period. Average of data in both block A and B
[1]Exposure to light in hours: day 1=23.5, day 4=84, day 8=191, day 15=359, day 22=527, day 29=696, day 36=864

Malondialdehyde (MDA) is an end product of photooxidation. It has been studied extensively in meat and milk oxidation and been shown to increase in concentration as photooxidation occurs. It has also been shown to correlate with changes in sensory characteristics of rancidity (Wang et al., 2001; van Aardt et al., 2005). MDA concentration for both block A and B of study increased over time, as shown in Figure 2. As expected, foil-wrapped packages protected the soymilk optimally as MDA production was lowest in soymilk from this packaging treatment over time; significant differences did not occur until day 36. Soymilk in high LPA-HDPE packages had low MDA, mimicking MDA of soymilk in foil-wrapped bottles up to day 29. However between day 29 and 36, MDA production increased in soymilk in high LPA-HDPE packages, following the trend observed for Rb, and still significantly lower than clear packages. Differences were noted in MDA at day 29 (Table 9 (ANOVA); Table 8(contrasts). In a previous study in our lab on fluid milk (Johnson, 2012), sensory detection between control and packaging treatments were frequently associated with MDA levels of 1.3mg/L in the product. MDA in the soymilk was 1.5mg/L on day 15 in soymilk in Low and Med LPA bottles and this is the point at which

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sensory differences were first detected. Although Rb degradation was not significantly different at day 29, the secondary products of photooxidation increased compared to day 1 concentrations. Other components of soymilk, such as protein, may be undergoing oxidation, or chlorophyll, another known photosensitizer, may be contributing to additional photooxidation pathways of components in the soymilk. Mendiola et al. (2008) attempted to determine chlorophyll concentration in a mixture of orange juice and soymilk; however they could not detect it under their specific HPLC conditions.   Wold et al. (2005) suggests that chlorophyll in dairy-based cheese contributed to photooxidized off-flavors. Determining if or how chlorophyll contributes to off-flavors associated with photooxidized soymilk is important to optimizing packaging for protecting soymilk flavor and nutrient quality. Additionally, soymilk contains a complex mixture of antioxidants including isoflavones (Chiarello et al., 2006; USDA, 2007). These antioxidants may also play a role in the inhibition of photooxidation, yet, they may also be sensitive to photooxidation after some degree of light exposure. In soymilk, how all these photosensitizers and antioxidants should best be protected from light using packaging is a relevant question.

Table 9. ANOVA table for effects tests for response of malondialdehyde concentration testing for package, time (days), and block and interactions. α = 0.05

Source	DF	Sum of Squares	F Ratio	Prob > F
Package	4	4.9064436	124.7502	<.0001*
Block	1	0.0235323	2.3933	0.1264
Day	6	8.3244725	141.1042	<.0001*
Package*Block	4	0.5042532	12.8210	<.0001*
Package*Day	24	2.7434866	11.6259	<.0001*
Block*Day	6	2.0762859	35.1941	<.0001*
Package*Block*Day	24	0.9852940	4.1753	<.0001*

*pvalue <0.05 indicates statistical significant difference.

It is also evident that there is a dip in MDA concentration at day 22. This trend has been seen before (Wang et al., 2000).   These authors studied characteristics of flavored soymilk over time and demonstrated a decrease in MDA concentrations in refrigerated soymilk by day 14 and also at day 28. They hypothesize that the decrease is due to secondary photooxidation reaction products binding to proteins in the soymilk. They further postulate that this binding may be the formation of Schiff bases due to Strecker degradation in which carbonyls formed from oxidation are reacting with amine groups in the protein (Damodaran 1996). We saw a subsequent increase in MDA after the decrease though. Wang et al. (2000) corrected for possible sugar oxidation that may occur from the sucrose in the soymilk whereas we did not. Perhaps

the subsequent increase in MDA is due to oxidation of proteins. Alternatively, perhaps the binding of carbonyls to amine groups had reached a saturation point and oxidative products continued to accumulate.

The values of MDA that coincide with the sensory changes in the milk are lower than what has been seen in similar studies with cow milk. Our hypothesis is that there are other changes in the soymilk that are occurring that are contributing to sensory changes which will be discussed in more detail below.

Hexanal is also a known secondary oxidative product of photooxidation. Hexanal concentration of soymilk packaged in the different bottles is shown in Figure 20. As expected, the high LPA treatment protected similarly to foil-wrapped bottles up to day 29 and even at day 36 protected significantly better than clear bottles. There is a wider variation of hexanal concentration in the packaging treatments over the study period, but this is not surprising as hexanal is a secondary oxidative product and it may be produced in a more variable manner because the subsequent scission reactions of photooxidation are numerous.
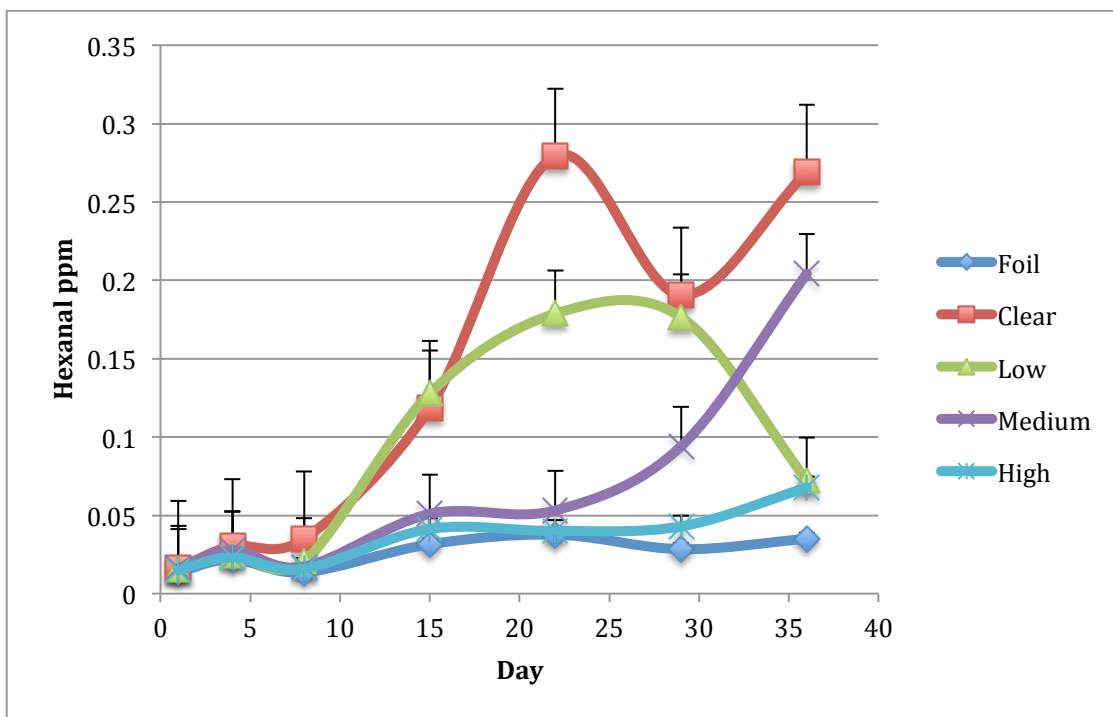
Figure 20. Hexanal concentration in packaging treatments, low, med, and high TiO_2 compared to foil-wrapped and clear packaging over a 36 day period. Average of data in both block A and B.
[1]Exposure to light in hours: day 1=23.5, day 4=84, day 8=191, day 15=359, day 22=527, day 29=696, day 36=864.

The ANOVA table for interactions and effects are shown in Table 10. These statistics are not blocked. However once again, the results are an indication that packaging treatment and time do have an effect on hexanal concentration and that there is an interaction of packaging and time.

Table 10. ANOVA table for effects tests for response of hexanal concentration testing for package, time (days), and block and interactions. α = 0.05

Source	DF	Sum of Squares	F Ratio	Prob > F
Package	4	0.18003449	40.5672	<.0001*
Day	6	0.24596801	36.9493	<.0001*
Package*Day	24	0.17917666	6.7290	<.0001*

*pvalue <0.05 indicates statistical significant difference.

Contrasts for hexanal are identical to the results of the Rb analysis. Shown in Table 8, hexanal concentration in soymilk in different packaging treatments as early as day 1 is not significantly different from one another, and over time, foil-wrapped and high LPA bottles protect well as there are no significant differences in these bottles over a 4 week period. However by day 36, soymilk in high LPA bottles has increased hexanal production compared to foil-wrapped bottles. Furthermore, clear, low and med TiO_2 bottles are unable to prohibit hexanal production.

While the chemical analyses are all in agreement regarding how long the high LPA package can maintain protection similar to the foil-wrapped packaging, the sensory testing indicates that high LPA treatment was not able to maintain protection from off flavors and aromas by panelists after 15 days. Possible explanations for this include the fact that human sensory is much more sensitive than chemical

analysis and perhaps human sensory testing is detecting compounds

that we have not yet evaluated or are simply are not yet in high enough

concentrations to be detected analytically. Certainly there are other end

products of photooxidation that we have not analyzed including 2-

pentyl furan and 2- pentanyl furan, both of which are known oxidative

products that contribute to soy oil reversion flavor (Ho, et al., 1983;

Frankel, 1984). Furthermore, single compounds in and of themselves do

not necessarily cause sensory responses, so it may be that a

combination of the compounds that we do have analyses for and/or

other compounds not analyzed caused detection of rancidity earlier

than the chemical assays.

Another explanation is that the soymilk package indicates that

natural flavorings are added. It may be that a natural flavoring such as

vanillin is oxidized or undergoing time degradation. Perhaps the

degradation of the natural flavorings added are what panelists were

able to detect as a difference rather than an increase in off flavors and

odors.

Rb decreased and MDA and hexanal increased in the foil-wrapped

bottles over time, but never to the point of significant differences from

F1 control. In the high LPA bottles, the Rb decreased and MDA and

hexanal increased over time, such that it was significantly different from initial concentrations of foil and day 1 high LPA bottles by day 36. However, if one accounts for the "natural" amount of Rb degradation, oxidation per MDA and hexanal that occurs, only differences that were significant were Rb and MDA at day 36 indicating that high LPA bottles protect as well as foil-wrapped for at least 29 days.

Conclusions

High LPA packages protected soymilk from photooxidation as effectively as foil-wrapped bottles for at least 15 days in terms of human detection of off flavors and aromas. This does not necessarily mean that the high LPA package is only capable of protecting for 15 days. Normally consumers are not handling soymilk in their homes and comparing to an optimal product for each use. The sensory test conducted did not assess acceptability of the products over time, so significant sensory differences do not necessarily translate to poor quality. From a practical standpoint, the question is how long do most consumers keep their soymilk before it is used and can the high LPA package allow for consumer acceptability of the product for this length of time.

Abbreviations

PUFA: Polyunsaturated Fatty Acid

HDPE: High Density Polyethylene

TiO_2: Titanium Dioxide

TBARS: Thiobarbituric Acid Reactive Substances

MDA: Malondialdehyde

GC-MS: Gas Chromatography-Mass Spectrometry

SPME: Solid Phase Microextraction

PDMS: Polydimethyl Siloxane

Acknowledgments

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Chapter 5 Color Modification Improves Soymilk Attribute Acceptability

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Abstract

Visual appearance (e.g. color) can influence perception of
unrelated characteristics. Titanium dioxide (TiO_2) is an inert substance
approved for food use for whitening purposes that may be used to
improve color of food products. The aim of this study was to determine
if whitening soymilk, using TiO_2, could improve sensory attributes
(appearance, flavor, and mouthfeel) and consumer acceptability of this
beverage.

Titanium dioxide was added at two treatment levels (0.05% and
1.0% by weight) and compared to a soymilk control with no TiO_2. Color
was measured using CIE-L* a* b* scale. Affective sensory testing for
attributes (appearance, smell, taste, mouth feel, aftertaste) was
completed using a 9-point hedonic scale (1 = "dislike extremely"; 9 =
"like extremely"; n = 83 panelists). In a separate study, TiO_2 was added
at 0.01%, 0.3%, and 0.5% (by weight) and compared for overall
acceptability (9-point hedonic scale; n = 66 panelists) to a soymilk
control with no TiO_2.

As expected, soymilk increased in whiteness (L-value) with
increasing TiO_2 addition. Color (green to red:a*; blue to yellow:b*) also
differed among all treatments. The change in visual appearance from

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the addition of TiO_2 increased acceptability of all attributes. Appearance acceptability increased significantly from 4.2 (control; dislike slightly) to 6.0 to 6.3 (like slightly) for 0.5% and 1.0% TiO_2, respectively. Taste scores increased significantly from 5.8 (control) to 6.3 and 6.5 (0.5% and 1.0% TiO_2 respectively), corresponding to "like slightly". Mouth feel scores increased significantly from 5.5 (control) to about 6.1. Although consumer acceptability of individual attributes increased with addition of TiO_2, overall acceptability was not significantly increased

Adding TiO_2 as a whitener to enhance the visual appearance provides a halo effect for other attributes. However, the improved affective perception in attributes was not sufficient to alter the overall acceptability of the product.

Key words: soymilk, titanium dioxide, sensory attributes, consumer acceptability, color, halo effect

Introduction

Sales of soymilk, a popular beverage, have increased from approximately $250 million in 1996 to over $1 billion in 2011 (Soyfoods, 2014). The increase in sales may be partially attributed to consumer motivation for an alternative to bovine milk in their diet. Soymilk is often selected by consumers who suffer from lactose intolerance, have cow milk protein allergies, as well as by vegetarians. Soymilk offers many nutritional and functional food benefits.

Soymilk contains 6g protein per 240ml (8oz) serving, including all eight essential amino acids required by adults, as well as histidine, which is an essential amino acid for infants. The main proteins are glycinin and beta conglycinin, which are easily digested (Singh et al., 2008; Swaisgood 1995). Scientific evidence of the relationship between soy protein and decreased risk of heart disease has led to an FDA authorized health claim. This health claim stipulates that 25 grams soy protein are needed each day to reduce the risk of heart disease (Code of Federal Regulations, 2013). Therefore consuming soymilk as part of the daily diet may contribute to long-term health benefits. Soymilk has a fatty acid profile that approximates the nutritional guidelines of the

USDA, with a high percentage of polyunsaturated fatty acids (61%); the major fatty acids, linoleic acid (53%) and α-linolenic acid (8%) are essential fatty acids (Hammond 1991). α-Linolenic acid is a precursor for eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), which are valuable omega-3 fatty acids for health (Masson et al., 2013; Luchtman et al., 2013). Soymilk contains phytochemicals including isoflavones and phytosterols (Xu and Chang 2009), which are considered to be important for health, as well as small amounts of fiber.

Sensory studies of soymilk have shown that those in Western cultures view soymilk as having an undesirable beany flavor and chalky, dry mouthfeel characteristics, leaving the consumer with a negative perception of soymilk (Torres-Penaranda and Reitmeier, 2001; Wansink, 2003a; Wansink, 2003b, N'Kouka et al., 2004; Chambers et al., 2006). There is a discrepancy between perceived negative sensory characteristics of soymilk and observed increase in sales over the past decade; the latter is attributed to consumer interest in health benefits of soy, as well as cultural and ethical factors (William Reed Business Media SAS, 2011). Market research into consumer attitudes regarding the motivation for consuming soy products indicates that forty-seven

percent of consumers seek foods with soy for health reasons (United Soybean Board, 2013).

Realizing that sensory characteristics of soymilk are a limiting factor for market share, methods of changing the flavor of soymilk have been studied. For example, processing methodologies that have improved soy flavor include blanching and grinding Lv et al., 2011; Endo et al., 2004), grinding methods, and choice of variety of bean (Zhang et al., 2012). Soybean varieties combined with storage methods have also been shown to affect flavor (Achouri et al., 2008). While flavor of soymilk has been studied, the color of this beverage has primarily been studied in relation to dairy milk. Villegas et al. (2008) studied the color of vanilla-flavored bovine milk and soymilk beverages. Vanilla bovine milk was more yellow in color than the vanilla soymilk. Increasing yellow color intensity positively influenced rankings and acceptability of bovine milk over soymilk.

Packaging artwork illustrates a dazzling white soymilk product, which is not typical of the actual product. Coloring bovine nonfat milk with titanium dioxide resulted in improved consumer acceptability scores with increased whiteness of dairy milk (Phillips and Barbano, 1997). Thus it seems plausible that increasing the whiteness of soymilk

might alter product appearance in a positive manner and influence overall product acceptability. Visual appearance on packaging can lead to product expectations (Koutsimanis et al., 2012; Kim et al., 2013). In addition, appearance of a product can influence overall product acceptability (Johnston et al., 2011; Wadhani and McMahon, 2012; Bloomberg et al., 2011). An interesting phenomenon is the role of visual appearance on perception of other sensory characteristics and overall acceptability.

Titanium dioxide (TiO_2) is a widely used whitener. TiO_2 is chemically inert; it demonstrates light scattering properties and has a high refractive index, thus only a small amount is necessary to achieve whitening (Diebold, 2003; DuPont, 2007). It is also water soluble (Skocaj et al., 2011). There is no current evidence on how TiO_2 affects mouthfeel characteristics in beverages. It is used to whiten food products, cosmetics, paints, paper, and food packaging. Food grade TiO_2 is found in many consumer food products including, but not limited to chewing gums, donuts, flavored drink products, candies, frostings, puddings, coffee creamers, and marshmallows (Weir et al., 2012). Food grade TiO_2 can be added at up to 1% by weight of product (FDA 2012).

The objective of this study was to determine if the addition of titanium dioxide to soymilk, for the purpose of enhancing white color, improved consumer perception of specific sensory attributes and overall acceptability of this beverage.

Materials and Methods

Study 1. Affective Analysis of Sensory Attributes

Materials.

Soymilk (Kroger brand PS Lite Soymilk, Cincinnati, OH) was purchased from a local supermarket (Kroger, Blacksburg, VA). Powdered food grade titanium dioxide (Coloreze™ Natural Color Powder Product number TIO$_2$, Lot #20732) was donated by International Foodcraft Corporation (Linden NJ).

Product Preparation. For color analysis TiO$_2$ was dispersed in 1000g of soymilk at two treatment levels, 0.5% and 1% by weight of soymilk, and blended in an Osterizer® blender (Jarden Corporation, Boca Raton, FL) for 2 minutes. The mixture was prepared by mixing one half of the weight of the soy milk with the full weight of TiO$_2$ in the blender. Once the TiO$_2$ was solubilized with the soymilk the entire volume of soymilk

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was mixed together in one-gallon plastic containers by shaking vigorously.

Color Analysis.

Color was analyzed using a handheld Minolta Chroma MeterCR 200 (Osaka, Japan).   The colorimeter was first calibrated using a white calibration tile. Each treatment group was measured in triplicate in a 2.54cm diameter crystal tube and color measured by colorimeter for L*, a*, and b* values based on the CIE-L* a* b* scales. Values were compared using one-way ANOVA among treatments for each.

Affective Evaluation.

Soymilk was prepared the night before, as described above, 18 hours prior to evaluation and stored at 2°C for 18 hours. Treatment were portioned (28 ml) in two-ounce portion cups, capped, and stored in the dark at 4°C until the sensory study began. Three digit codes were assigned to each treatment group using Sensory Information Management System Software 2000 (SIMS Sensory Software, Berkeley Heights, NJ).

Affective Testing of Attributes.

Panelists were recruited by email from a list of individuals who had participated in a previous soymilk sensory study and other sensory studies (Appendix C-1:). A goal of 100 panelists was established.

IRB approval (#12-181) was obtained through the VT Office of Research Compliance (Appendix C-2). Informed consent was obtained from each panelist prior to beginning the sensory test (Appendix C-3).

Consumer perception of attributes was assessed using an affective hedonic test (Meilgaard et al., 2007). Panelists were seated in separated sensory booths, each equipped with a touch screen monitor. Data was captured using Sensory Information Management Software 2000 (SIMS Sensory Software, Berkely Heights, NJ). Samples (n=3) were presented sequentially in a balanced order of presentation using a complete block design (Appendix C-4). Panelists were served one sample at a time. Panelists were asked to look at, smell, and then taste and rate each soymilk sample on appearance, smell, taste, mouth feel, and aftertaste using a scale of 1 to 9 (1 = "dislike extremely"; and 9 = "like extremely") (Appendix C-5).

Upon completion of the final sample, panelists completed a survey (Appendix C-6) regarding demographics, as well as consumption and frequency of purchase patterns, and preference for soymilk products. In

addition, they were asked the following question, "Would the addition of titanium dioxide (a food colorant used for whitening foods) to soymilk affect your acceptability of soymilk?"

Statistical Evaluation.

The average (± s.d.) for each attribute for each treatment was calculated from the hedonic scores. Scores were assessed for normality using Shapiro-Wilk Goodness of Fit test. They were then blocked by panelist and analyzed by the Wilcoxon/Kruskal-Wallis Rank Sum Test with $\alpha=0.05$ to determine statistical differences among treatments for each attribute (Gacula et al.,2009). Where statistical differences were found, Wilcoxon Each Pair was used for nonparametric comparison of means (Gacula et al., 2009).

Survey data was summarized as percent of responses. In addition, survey questions regarding consumption of soymilk were scored and compared to those survey questions expressing opinions of soymilk and TiO_2.

Study 2. Consumer Acceptability

Materials, Product Preparation, and Color Analysis.

Kroger brand soymilk and food grade TiO_2 were used as previously described. TiO_2 was added at 0.5%, 0.3%, 0.1% by weight, and a control (no TiO_2). Color of the soymilk was measured as described above. Preparation of samples for sensory analysis was previously described.

Overall Acceptability Testing.

Individuals who had not participated in the previous sensory study were recruited by email, with a goal of attaining 60 participants (Appendix C-7). IRB approval (#13-278) was obtained through the VT Office of Research Compliance (Appendix C-8). Informed consent was obtained from each panelist prior to beginning the sensory test (Appendix C-9).

Overall product acceptability was determined using an affective hedonic test employing a nine point scale (1= "dislike extremely"; 9= "like extremely") (Meilgaard, et al 2007). Each of the four treatments was presented in a balanced order using a complete block design. Participants followed the same sample evaluation process (look, smell, taste) and rated their overall acceptability of the product (Appendix C-10).

Upon completion of the final sample, panelists were asked to complete the same survey used in the sensory attributes portion of the study (Appendix C-6).

For both studies it was desirable to target those individuals who typically consume soymilk; however due to the difficulty of recruiting panelists, particularly individuals who consume soy on a regular basis, non-soymilk drinkers were also allowed to participate.

Statistical Evaluation.

The average (± s.d.) was obtained for each treatment for acceptability. Scores were assessed for normality using the Shapiro-Wilk Goodness of Fit test. They were then analyzed by the Wilcoxon/Kruskal-Wallis Rank Sum Test with $\alpha=0.05$ to determine statistical differences among treatments (Gacula and others 2009). Where differences were found, Wilcoxon Each Pair was used for nonparametric comparison of means. Survey responses were assessed as previously described.

Results and Discussion

Although the same product brand was used in the two studies, the color characteristics were different suggesting that color varies greatly

by production date. The product used for the consumer acceptability sensory was much lighter in color. The addition of TiO_2 lightened the product, as expected. Soymilk from the sensory attributes study with 0.5% and 1.0% TiO_2 were significantly different (p <0.05) from the control and each other on all color planes. In the consumer acceptability study, the color of soymilk with 0.3% TiO_2 was not different from the 0.1% or 0.5% TiO_2 color measurements (Table 11)

Table 11. Mean (n=3) ± s.d CIE-L*a*b*[1] values of soymilk with and without TiO_2[2]

Treatment: % TiO_2 added	L* Mean $\pm$ s.d.	a* Mean $\pm$ s.d.	b* Mean $\pm$ s.d.
Sensory Attribute Study			
0.0%	38.17 $\pm$ 0.04c	-4.94 $\pm$ 0.04a	13.74 $\pm$ 0.08a
0.5%	44.46 $\pm$ 0.05b	-6.80 $\pm$ 0.01b	12.50 $\pm$ 0.04b
1.0%	46.42 $\pm$ 0.04a	-7.74 $\pm$ 0.01c	11.18 $\pm$ 0.02c
Consumer Acceptability Study			
0.0%	66.03$\pm$0.39c	-2.21$\pm$0.13c	12.42$\pm$0.24a
0.1%	73.08$\pm$1.04b	-1.31$\pm$0.08b	11.99$\pm$0.27ab
0.3%	75.06$\pm$0.76a	-1.08$\pm$0.10ab	11.01$\pm$0.53b
0.5%	76.20$\pm$0.37a	-0.84$\pm$0.07a	9.82$\pm$0.55c

Statistical differences are shown by different letters after each mean, n=3
[1]CIE L*: 0=black, 100=white; a*: green-red (+ numbers = more red); b*: blue-yellow (+ numbers = yellow)
[2]%TiO_2 added (wt/wt)

Data was not normally distributed for attributes (Appendix C-11) so non-parametric statistical analyses were used. Mean hedonic scores

for both treatments for all attributes were significantly higher (p<0.05) from the scores for the control indicating improved perceptions for attributes with the addition of TiO_2 (Table 12). The mean score for appearance increased by approximately 2 integers, from 4.19 (dislike slightly) for the control to over 6.0 (like slightly) for the 0.5% and 1% treatments respectively. The addition of TiO_2 at 0.5% to 1% significantly increased the mean hedonic score for smell, taste, mouthfeel and aftertaste by about a half integer over that of the control. While the mean scores do not appear to be different from one another between treatments, it is important to remember that each panelist served as his/her own block. Thus variation that might have existed due to how an individual perceives soymilk in general is removed, focusing instead on the direct comparison of different treatments. The overall hedonic scores for the two treatment groups did not differ from each other (p>0.05). Therefore, while the higher level of 1% TiO_2 addition created a lighter milk than the 0.5% TiO_2 based on analytical color measurements, it did not improve the perception of different sensory attributes to a degree in which cost would outweigh benefit.

Table 12. Hedonic scores (means ± s.d.)[1] of sensory attributes for soymilk with (0.5%, 1.0%) and without TiO2.

Treatment	Appearance	Smell	Taste	Mouthfeel	Aftertaste
Control	4.19 ± 1.87b	6.07 ± 1.53	5.76 ± 1.97b	5.51 + 1.99b	5.24 + 1.81b
0.5% TiO_2	6.33 ± 1.80a	6.53 ± 1.37	6.30 ± 1.84a	6.10 + 1.90a	5.71 +1.89a
1.0% TiO_2	6.01 ± 2.09a	6.46 ± 1.56	6.47 ± 1.73a	6.06 + 1.78a	5.80 + 1.73a

Significant differences are shown with different letters after each mean.
[1] 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither dislike or like, 6=like slightly, 7=like moderately, 8=like very much, 9=like extremely. n=83

Since consumers rated each sensory attribute of lightened soymilk with more positive hedonic ratings for appearance and taste, we hypothesized that consumers would also rate TiO_2-lightened soymilk as generally more acceptable. However, the actual ratings were not significantly different and fell between like slightly and like moderately (6.21-6.50) (Appendix C-12) These values are in the same range as the means for the attributes. The results may have been confounded by the differences in color of the control soymilk. TiO_2 addition between 0.1% and 0.5% did provide significant differences in lightness of the soymilk ($p<0.05$) (Table 11). However, the base product in the consumer acceptability study was already much lighter than the TiO_2 treated soymilk in the sensory attribute study (Table 11).

One hundred forty-nine people participated in the two studies and completed the survey (83 as part of the sensory attributes study and 66 in the overall acceptability study). The majority of panelists were female (69%), between18-24 years old (70%), and were students at the university. While 39% indicated that they consumed soymilk at least monthly, most panelists reported that they did not (rarely/never) drink soymilk (Appendix C-13).

It was important whether the addition of a synthetic ingredient (TiO_2) would affect consumer acceptability of soymilk. The question, "Would the addition of titanium dioxide (a food colorant used for whitening of foods) to soymilk affect your acceptability of soymilk?," was asked to gain that perspective. Twenty-seven percent responded "Yes" and provided a comment that suggested that they would be more likely to buy the soymilk if it was whiter and more closely resembled dairy milk. Twenty percent responded with "Yes" and provided a comment that suggested that they would be less willing to buy the soymilk if it had TiO_2 added to it. Fifty-one percent responded "No."

We segmented the soymilk drinkers from non-drinkers for the question, "Would the addition of titanium dioxide (a food colorant used for whitening foods) to soymilk affect your acceptability of soymilk?" Of

the panelists who regularly consumed soymilk, 18% (n= 4/20), of the

panelists answered that it would affect their acceptability in a positive

way such that the product would be more pleasing to them. Of the non-

drinkers of soymilk, 26% (n=33/128) answered that it would affect

their acceptability of the product in a positive way.

Those panelists who routinely drink soymilk were asked to

identify sensory attributes of the product they found appealing. They

were allowed to choose more than one attribute.   Taste was chosen by

85% of the panelists, smell and mouth feel were each chosen by 44%,

and color was chosen by 11% of the panelists. We also asked a

question, "Why do you drink soymilk?" Again panelist could choose as

many answers that applied. Taste was chosen by 61% of the panelists,

functional food benefit was chosen by 56% of the panelists, lactose

intolerance and other was chosen by 22%, vegetarianism and cultural

(e.g., grew up drinking soymilk) were each chosen by 9%, and cow milk

protein allergies was chosen by 2% of panelists.   These results are in

agreement with other recent reports (Teratanavat and Hooker, 2011;

United Soybean Board, 2013).

Those panelists who did not routinely drink soymilk were asked

to identify what sensory aspects of soymilk they found displeasing.

They could choose as many sensory characteristics that applied. Taste was chosen by 61% of panelists, mouth feel by 36%, color by 18%, and smell by 13% of panelists. We also asked if there were any other reasons besides sensory characteristics that kept them from preferring soymilk. Cost was chosen by 35% of the panelists, "other" was chosen by 37%, and packaging was chosen by 1%. For the "other" category, contributed reasons (written in) included habit, nutrition content of the milk, don't drink any milk at all, and simply never purchased or considered soymilk before.

A confounding factor in the interpretation of these results is that the control soymilk in the consumer acceptability study was already lighter in color than the whitened product used in the Sensory Attribute portion of the study. There is apparently a wide range of color variation in soymilk that may be dependent on agricultural conditions or even processing or storage conditions from one soybean lot to another (Achouri et al., 2008, Zilic et al., 2013, Xiang et al., 2011). This is not unusual as many crops may yield a variety of different attributes of a product based on soil, weather conditions, farming location, etc. Thus it may be that once a threshold of lightness is reached, consumers would

be satisfied with this product. However, there are other explanations for our results that cannot be ruled out that will be discussed below.

The first sensory judgment that an individual makes about a product is appearance (Lawless and Heyman, 2010; Dematte et al., 2009). This includes an inventory of color, texture, consistency, preparedness, safety, etc. If a food does not appear the way that the individual anticipates, the food may not even be tasted. These appearance judgments are made on a daily basis.

Color is a very valuable aspect of the visual judgment one makes about the appearance of a food. Color masking has been shown to dramatically reduce one's ability to correctly identify flavors in beverages; in addition, color can affect product acceptability. DuBose and others (1980) showed that when panelists were unable to distinguish color of beverages they were unable to correctly identify the flavor of those beverages. This has also been shown with wine (Morrot 2001). When panelists were unable to see the color the wine, they were unable to describe the wine with language typical to white vs. red. Color has also been shown to have an effect on how panelists rated odor intensity (Zellner and Kautz 1990; Christensen, 1983).

Soymilk is not truly milk but rather a beverage that has been so named because its nutrient profile is similar to that of bovine milk. The association of nutrition and the use of the term "milk" may influence the perception that it should also look like milk. However, there is a distinct color difference between soymilk and bovine milk. Villegas and others (2008) have shown that these two beverages are different in both L* and b* values values of CIELAB.

Furthermore, Villegas et al (2008) demonstrated that the differences in color values had a strong correlation with hedonic scores of bovine milk and soymilk, both plain and flavored with vanilla. They found that higher a* color values (more red) were correlated with lower acceptance scores. This is in agreement only with our sensory attribute study as there were lower hedonic scores for the control soymilk which had higher a* values. Results from the overall acceptability study did not agree with this, possibly because the soymilk was already lighter.

A previous study blended TiO_2 with a sodium caseinate and added that mixture to nonfat bovine milk. They demonstrated that whitening nonfat milk did yield improved scores for appearance, creamy aroma, and texture (Phillips and Barbano, 1997). While this is in agreement with the sensory attribute part of our study, it conflicts with our

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colorimeter data. Nonfat milk with added TiO_2 had lower values for a*

and b* color values on the CIE L* a* b* scheme compared to nonfat milk.

These lower a* and b* values of the nonfat milk were associated with

lower scores of that nonfat milk. Our data indicates that the TiO_2-

treated soymilk had lower a* and b* values and these TiO_2-treated

soymilks had higher scores for appearance. However, it must be

recognized that the significant difference in L* value for our TiO_2-

treated milk may have obscured any effects of the a* and b* values on

consumer preference for appearance.

It is noteworthy that in asking panelists to evaluate specific

attributes, an apparent halo effect was observed with the whitened

milk. The halo effect is a type of response correlation in which the

positive perception of one attribute favorably influences the perception

of another unrelated attribute. TiO_2 should not change the taste or

smell of the soymilk, yet panelists rated these attributes higher in the

lightened product. The halo test would need further investigation and it

would need to be determined that indeed TiO_2 does not have an effect

on taste, smell, mouthfeel, and aftertaste. Other studies have shown

halo effects in which adding vanilla extract to milk results in increased

ratings in sweetness, thickness, and creaminess (Clark and Lawless,

1994). While the halo effect is important for specific attributes, our study demonstrates that it may not necessarily be important for overall acceptability of a product.

There is the possibility that when consumers are asked specific questions about a product, they are more likely to focus on those attributes and provide more deliberate critiques of those attributes. It has been suggested that when consumers are asked to focus on a specific attribute, they will carefully consider their response and may even take longer to consider how they conscientiously like or dislike it. Whereas, to be asked in general how they like a product, even if prompted to look, smell, and rate it in other way, they may provide more of a universal product assessment response rather than a more analytical evaluation (Lawless and Heyman, 2010).   When making a general evaluation of a product, Anderson (1971) proposed a theory in which many different types of information are used to make an evaluation of a product, including advertising exposure, attitude, and packaging. Asking panelists in general how they like a product involves different cognitive skills/tasks than asking panelists to try a sample and evaluate it for specific sensory attributes. In the former, the panelist may rely on historical experiences, knowledge of the product, attitude,

and then overall experience with the product at that moment. In the latter, while the panelist may bring previous experiences and knowledge of the product to the table, they are asked to focus on just one item at a time and perhaps the historical experience and knowledge is not weighed as heavily in their decision making process (Anderson, 1971).

Based on the dichotomy of results between the two studies, one must also consider that perhaps use of just one type of sensory analysis of a product should not be used to definitively make a decision regarding a product. As sensory scientists, we may also want to revisit the use of the nine point hedonic scale. This was originally designed by Quartermaster and the University of Chicago to determine food preferences of soldiers for ready-to-eat meals (Jones et al., 1955). At this time, it was desired to simply determine if MRE's would be acceptable. Furthermore, while color plays an important role in how an individual will assimilate information about flavor and smell, there are other important factors that determine how that individual will *accept* the product. Deliza and MacFie (1996) outline many models in which expectations of a product play an integral role in our acceptance of the product and whether or not we will purchase that product again.

Perhaps in a more complex food market in which consumers are basing so many of their choices on nutritional value, organoleptic properties, aesthetics, packaging, cost, cultural ideals, etc., a more reliable and complex method to ascertain how consumers truly respond to a product should be developed for use in the sensory laboratory. Aaron et al (1994) demonstrated that information about a product shifts hedonic scores toward consistency with subject attitude. The fact that there was large percentage of panelists who do not drink soymilk or had no previous experience with it (74%) may have also led to ambivalence regarding how they felt about the product.

The survey indicated that the sample of population who participated was, largely, non-soymilk drinkers and skewed towards young educated adults. Typically for consumer acceptability studies, it is desirable to sample that portion of the population who regularly consume the product being tested. Mintel (2009) documented that the young adult age group is the top consumer of functional foods. Therefore, these results are useful for targeting that segment of the population who is interested in functional foods. We also demonstrated that a large number of those individuals who consume soymilk do so for the functional food benefit. These results agree with Teratanavat and

Hooker (2006) who demonstrated that consumers value products for health benefit.

Food Marketing Trends showed that 47% of consumers purposefully do not buy products that contain added chemicals (FMI 2011). In our study, only a small percentage of individuals indicated they would not consume soymilk with TiO_2. However, many of the participants (97%) were affiliated with the Food Science and Technology Department at Virginia Tech and this segment of the population may be more open to added ingredients in foods. Therefore, our results need to be considered in the context of the tested population. Future research that pertains to improving consumer acceptability of soymilk should focus on a more diverse demographic and should also include greater representation of the population who regularly consumes soymilk.

There were no significant results in the consumer acceptability study in which the soymilk was already lighter to the soymilk in the sensory attribute study which did provide perceived preferences for lighter soymilk. The variation in the color of the milk and the different results may provide the industry with motivation to provide a more consistent product. While the actual use of TiO_2 may not be the

preferred way to do this, the industry may want to consider processing methods or choice of soybeans that allow for a more consistent and/or lighter colored product. Xiang et al. (2011) applied pulsed electric field treatment to soymilk and showed that the product can be lightened. However, given that taste was shown to be a primary driver of whether or not panelists choose soymilk over dairy milk, focus of research in this area should be supported.

Conclusions

Many studies have shown that color has a large influence on further sensory judgments of a food and that color can influence one's perceptions and preferences of a food. Our study provides support to this knowledge but only when consumers are asked to purposefully evaluate specific sensory attributes of the product. However, when evaluating all aspects of consumers' motivation to consume this product, in view of the response to TiO_2 as an additive and the response to general acceptability, it may not necessarily enhance sales of soymilk. Further studies are needed to determine if the addition of TiO_2 and at

what level, can be used to provide a more consistent product if it is to be

used as a whitening agent. However, in view of the fact that many

consumers are adverse to additional chemicals in their food, the soymilk

industry may want to explore other processing methodologies to

improve the consistency of this beverage. Certainly, our data provides

the sensory science with validation of the halo effect. We also

demonstrate that using only one method of sensory analysis may not

provide a clear picture of how consumers respond to a new product.

Acknowledgements

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Appendices

Appendix A-1

Peroxide Value Assay: Acetic Acid – Isooctane Method.

Materials
Burret
2 - 50 ml volumetric flask
3 Beakers
Hot plate
Spatula
Pipettes and tips
250 Erlenmeyer Flasks
Glacial acetic acid
Potassium Iodide (PI), saturated in Dionized (DI) water
Isooctane
DI water
Sodium Lauryl sulfate, >98% (SDS)
Soluble Potato Starch solution for Iodometry
0.1N Sodium Thiosulfate (Na ThioS)

Procedure
1. Place 5gm oil in Erlenmeyer flask.
2. Add 50 ml 3:2 Acetic acid to isooctane. Mix well.
3. Add 0.5 ml PI
4. Time for 1 minute while stirring.
5. Add 30 ml water
6. Titrate with Na ThioS until yellow almost gone
7. Add 0.5 ml SDS
8. Add 0.5 ml starch
9. Titrate with Na ThioS until blue just disappears
10. Note the amount of Na ThioS used.

Calculations
PV mEq/1000g = $\dfrac{(B) \times N \times 1000}{\text{Wt sample, g}}$

Where   B= tritration of sample, ml
        N= Normality of Na ThioS

Thiobarbituric Acid Reactive Substances Assay

Materials
TBA
SDS
80% acetic acid
NaOH
n-Butanol
pyridine
propyl gallate
EDTA
TMP
D.D. water
2x 500 ml volumetric flasks
Bottles in which pH probe can fit
cuvettes
pH meter
Pipetter
1000 ml dilution flask
50 ml volumetric flask
5x 100 ml volumetric flask
Water Bath
15 ml glass test tube
Vortex mixer
15 ml disposable centrifuge tubes
Ice water
Cenrifuge

Solution I (500 ml volumetric flask):
Use the same batch for each replication.
Store in fridge for up to a month.

Dissolve in distilled water:
1.875 g TBA
2.53 g SDS

Add 59.5 ml 80% acetic acid.
Bring up to 500 ml using distilled water.

Adjust pH to 3.4 using NaOH

Solution III (500 ml volumetric flask):
Store in fridge for up to 2 months.

Dissolve in distilled water:
.05 g propyl gallate
.10 g EDTA

Bring up to 500 ml with distilled water.

Solution II (size varies):
Make fresh daily under a hood.

Mix n-butanol and pyridine at a ratio of 15:1
5.0 mM TMP stock solution (1000 ml dilution flask) Store in fridge for up to 2 months.

Add .829 ml TMP into the 1000 ml dilution flask.
Bring up to 1000 ml using distilled water.

Invert several times to mix.

.1 mM TMP solution (50 ml volumetric flask) Store in fridge for up to 2 months.

Add one mL of the 5.0 mM TMP stock solution.
Bring up to 50 mL using distilled water.

Standard TMP solutions (5x 100 ml volumetric flasks) Store in fridge for up to 2 months.

Pipette 0.0, 2.5, 5.0, 7.5, 10.0 ml of .1 mM TMP solution into their respective, labeled flasks.
Add 10 ml of Solution III to each flask.
Bring up to 100 ml with D.D. water.

The TMP concentrations for these standards will be 0.0, 2.5, 5.0, 7.5, and 10.0

Procedure

Turn on the water bath and set the temperature to 95° C. Turn the safety switch clockwise to the end. When the temperature reaches 95° C, set up the temperature control using the safety switch. Allow all standards to come to room temperature, Solution I must be stirred. Sample preparation and absorbance measurement:
1. Shake each sample in order to homogenize
2. Transfer 1.0 ml of each sample into a 15 ml glass test tube using a pipetter.
3. Dilute each sample to 5.0 ml by adding 4.0 ml distilled water.
4. Mix each sample using a Vortex mixer.
5. Transfer 1.0 ml of the diluted samples into their own 15 ml disposable centrifuge tubes.
6. Transfer 1.0 ml of each of the standard solutions into their own 15 ml disposable centrifuge tubes.
7. Add 4.0 ml of Solution I into each tube.
8. Add .1 ml of Solution III into each tube.
9. Cap the tubes and mix using the Vortex.
10. Incubate tubes in the hot water bath for 60 minutes.
11. During the incubation period, make Solution II.
The following steps should be done within 1 hour**.**
12. Cool the tubes in tap water and ice water.
13. Under the hood, add 5.0 ml of Solution II to each tube.
14. Mix each tube for 10 seconds using the vortex.
15. Centrifuge the solution at room temperature at 3000 rpm for 20 minutes.
16. Transfer 2.5-3.0 ml of the top, organic layer of the solution into a cuvette.
17. Read the absorbance of the organic solutions at 532 nm under the hood.

Appendix A-3

Light Meter Readings for Broad Spectrum, 430nm, 450nm, and 660nm Wavelengths of Light
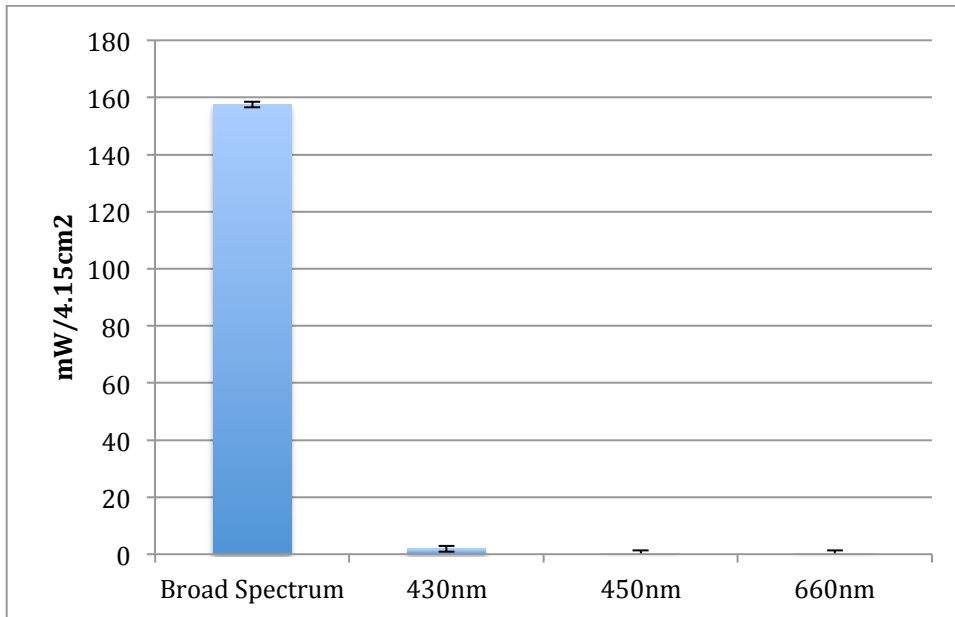
Appendix A-4

Tocopherol Chromatogram

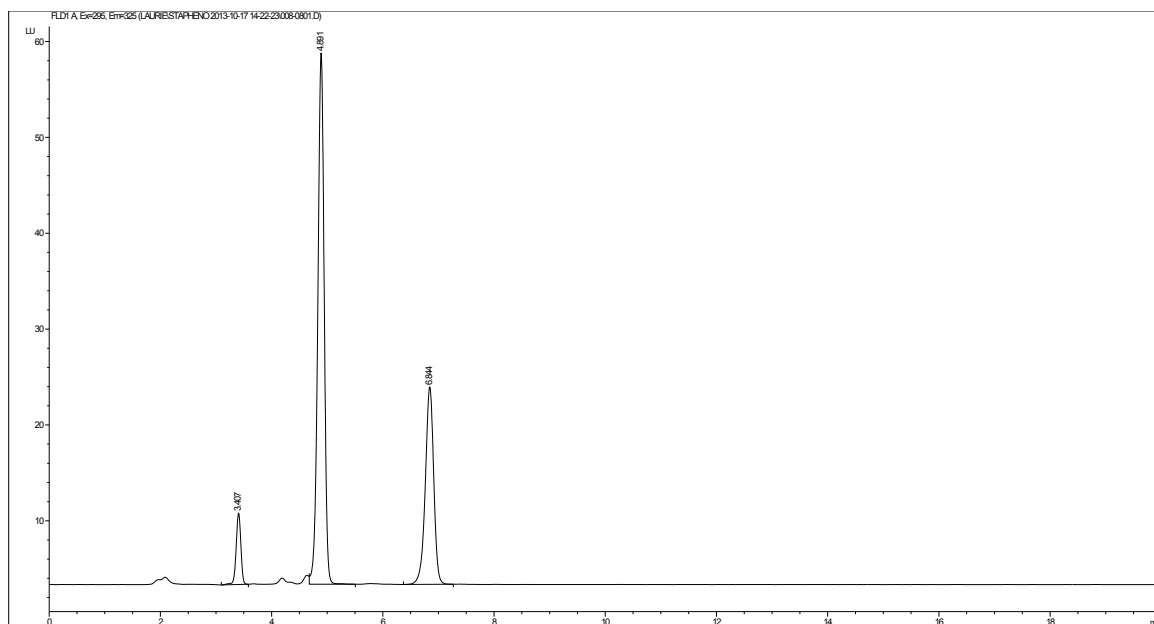HPLC chromatogram of fluorescence detection of α-, δ-, ϒ- tocopherols in soybean oil.

Appendix B-1

Virginia Tech IRB Approval Letter, IRB#11-477

VirginiaTech

Office of Research Compliance
Institutional Review Board
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24060
540/231-4606 Fax 540/231-0959
e-mail irb@vt.edu
Website: www.irb.vt.edu

MEMORANDUM

DATE: July 7, 2011

TO: Susan E. Duncan, Virginia Fernandez-Plotka, Laurie Bianchi

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires May 31, 2014)

PROTOCOL TITLE: Effect of TiO2 on the Stability of Riboflavin in Food Systems. Phase II: Protecting Sensory Quality and Riboflavin Integrity in Value-Added Milk and Soy Products Based on TiO2 Opacity of Polyethylene Films

IRB NUMBER: 11-477

Effective July 7, 2011, the Virginia Tech IRB Chair, Dr. David M. Moore, approved the new protocol for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at http://www.irb.vt.edu/pages/responsibilities.htm (please review before the commencement of your research).

PROTOCOL INFORMATION:
Approved as: **Exempt, under 45 CFR 46.101(b) category(ies) 2, 6**
Protocol Approval Date: **7/7/2011**
Protocol Expiration Date: **NA**
Continuing Review Due Date*: **NA**
*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:
Per federally regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals / work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

Invent the Future

Date*	OSP Number	Sponsor	Grant Comparison Conducted?
7/7/2011	11161102	Dupont	Not Required (not federally funded)

*Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

cc: File

Email sent to recruit sensory panelists.

Hello,

My name is Laurie Bianchi and I am a doctoral student in the Food Science and Technology department. Our lab is conducting two sensory studies on dairy milk and soymilk to determine if different types of packaging can protect the flavor quality of these milks. We are looking for panelists to smell and taste dairy and soymilk (1 oz each, nine samples) that have been packaged in different packaging treatments. The sensory studies will occur on several days this spring.

If you are interested, please complete the VT survey below. **Please be sure to leave your email address and your time availability on the survey.** Each time you participate in a sensory panel, you will receive a small edible snack. In addition, if you participate in all seven panels, your name will be entered into a drawing for a $100 Kroger gift card. If you have soy or milk allergies, we discourage you from participating.

The sensory panels are scheduled on Tuesdays, Wednesdays, and two Fridays, over a period of 5 weeks, and will occur from 9am to 2pm or until enough panelists have participated. Do to the number of panelists needed, we will schedule a convenient time for you to participate and will contact you with that time. Below are the dates for the studies:

Dairy milk: Wednesdays: February 1, 8, 15, 22, 29, and March 7 and Friday: February 3

Soymilk: Tuesdays: February 7, 14, 21, 28, March 6, and 13 and Friday: February 10

If necessary, please copy and paste in your browser.

https://survey.vt.edu/survey/entry.jsp?id=1327627869185

Don't hesitate to contact me if you have any questions. Thank you,

Laurie Bianchi
Graduate Student
Food Science and Technology
Virginia Tech
lbianchi@vt.edu

Appendix B-3

Informed Consent

Virginia Polytechnic Institute and State University

**Informed Consent for Participants in Research Projects Involving
Human Subjects (Sensory Evaluation)**

Title Project: Protective Effect of Packaging Materials on Sensory
Quality of Soy Milk

Investigators: Susan E. Duncan, PhD, RD, Laurie Bianchi, Virginia
Fernandez-Plotka,

I. Purpose of this Research/Project
You are invited to participate in a study to determine whether
consumers can detect a difference in the aroma and flavor of soy milk
protected with different packaging.

II. Procedures
You will be evaluating three sample sets of soy milk for sensory quality.
Each set will have three samples. You will identify the sample that is
different, based on aroma and flavor, within each set and respond using
a touchscreen computer monitor. There are seven sensory sessions over
five weeks. You are encouraged to participate in all sessions, if possible.

III. Risks
There are only minimal risks associated with this study. Individuals
with allergies to certain food components, particularly soy beans, may
be at risk. Soy milk is manufactured from soybeans.

IV. Benefits
The goal of this research is to determine if an alternative packaging
product may provide protection for soy milk flavor and nutrient quality.
Your participation in this study will provide valuable information to the
packaging industry about the best way to protect flavor and
nutritionally quality of soy milk.

V. Extent of Anonymity and Confidentiality

The results of your performance as a panelist will be kept strictly confidential except to the investigator. Individual panelists will be referred to by a code number for data analyses and for any publication of the results.

VI. Compensation

You will be compensated with a small edible treat at the end of every session in which you choose to participate. Your name will be entered into a drawing for a $100 Kroger gift card each time you participate. Chances of winning are based on the number of times you participate (maximum number is 7). The anticipated number of entries is estimated at 756. The drawing will occur at the end of the study.

VII. Freedom to Withdraw

If you agree to participate in this study, you are free to withdraw from the study at any time without penalty.

VIII. Subject's Responsibilities

I voluntarily agree to participate in this study. I have the following responsibilities:

- Evaluate 3 sets (3 samples each) of soy milk, as presented, and provide answers using a touch screen computer.

IX. Subject's Permission

I have read the consent form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

Date: _____

Subject Signature: _____

Subject Printed Name: _____

- - - - - - - - - - For Human Subject to Keep - - - - - - - - - - - -

Should I have any pertinent questions about this research or its conduct, and research subjects' rights, and whom to contact in the event of a research-related injury to the subject. I may contact:

Susan Duncan, Faculty/Investigator   (540) 231-8675;
                                                      duncans@vt.edu

Laurie Bianchi, Graduate Student/Investigator   (540) 231-8675;
                                                      lbianchi@vt.edu

Virginia Fernandez-Plotka, Research Associate,   (540) 231-9843;
Investigator   tplotka@vt.edu

Daryan Johnson, Graduate Student/Investigator (540) 231-8675;
                                                      daryan21@vt.edu

David Moore
Chair, Virginia Tech Institutional Review   (540) 231-4991;
Board for the Protection of Human Subjects   moored@vt.edu
Office of Research Compliance
1880 Pratt Drive, Suite 2006 (0497)
Blacksburg, VA 24061

Appendix B-4

Riboflavin Assay. Modified AOAC 960.65.

Materials
0.02N Glacial acetic acid
Riboflavin
5 100ml volumetric flasks
1 500ml volumetric flasks
pipette and tips
0.1N HCl
10N HCl
NaOH
Centrifuge bottle in which pH probe can fit and be capped
Foil wrapped test tubes
Cuvettes
100µl pipette and tips
10 ml glass pipettes
pH meter
0.45micron syringe filters
syringe filters
Autoclave
Fluorometer

Standards
0.02N Acetic acid

Stock: 100µg riboflavin/ml acetic acid. 50mg rbfn bvt 500ml 0.02N acetic acid
Intermediate Solution: 10µg rbfn/ml acetic acid. 10 ml stock bvt 100ml 0.02N acetic acid. Must be kept in dark and in refrigerator. Good for one week.
Work Standards:

Procedure
1. Measure 10ml of milk in bottle that pH probe can fit into and can be capped.
2. Adjust milk to pH 5.0-6.0 with dilute HCl
3. Add 0.1 ml 10N HCl/10ml milk

4. If material is not readily soluble comminute so that it may be evenly dispersed in liquid. Then agitate vigorously and wash down sides of flask with 0.1N HCl.
5. Heat mixture in autoclave 30 minutes at 121-123°C and cool.
6. Adjust pH to 6.0-6.6 with NaOH.
7. Adjust pH to 0.01N HCl to 4.5
8. Centrifuge for 10 minutes
9. Filter through the syringe filters.
10. Should get a clear supernatant, this needs to be kept in foil wrapped tubes. Place in cuvettes and measure on fluormeter at excitation 450 and emission 520. Compare readings to standard curve and account for dilutions with acids/bases.

Appendix C-1:

Email used for recruitment of panelists

Subject line: Invitation to participate in soymilk sensory study, Tuesday,

March ...., 9 am – 2 pm

Hello,

Our laboratory would like to determine if the addition of food ingredients can improve the sensory quality of soymilk, making it more acceptable and pleasing to consumers. Because you participated in sensory testing before, we are requesting your participation in this study. This particular study consists of approximately 15-minute sensory test in which you will taste three samples of soymilk and rate them on how well you like the appearance, color, mouth feel, smell, and taste. If you are interested, please hit reply and in the email subject line indicate "Yes for soymilk study". I will contact you with more details.

The sensory study will occur on Tuesday, March 20th. If you would like the same booth time you have had for other sensory studies, please let me know. You will need to report to room 127 of the Food Science Building at the corner of Washington Street and Duck Pond Drive. It will take approximately 15 minutes of your time, which will include tasting the 3 samples and completing a short survey.

You will be provided with a small snack for your participation.

Thank you,

Laurie Bianchi
Graduate Student
Food Science and Technology
lbianchi@vt.edu

Appendix C-2

Virginia Tech IRB Approval Letter #12-181

VirginiaTech

Office of Research Compliance
Institutional Review Board
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, VA 24060
540/231-4606 Fax 540/231-0959
email irb@vt.edu
website http://www.irb.vt.edu

MEMORANDUM

DATE: March 29, 2013

TO: Susan E Duncan, Laurie M Bianchi, Allie Sivak

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires May 31, 2014)

PROTOCOL TITLE: Effect of Titanium Dioxide on Consumer Acceptability of Soy Milk

IRB NUMBER: 13-278

Effective March 29, 2013, the Virginia Tech Institution Review Board (IRB) Chair, David M Moore, approved the Amendment request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

http://www.irb.vt.edu/pages/responsibilities.htm

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As:	**Exempt, under 45 CFR 46.110 category(ies) 2,6**
Protocol Approval Date:	**March 22, 2013**
Protocol Expiration Date:	**N/A**
Continuing Review Due Date*:	**N/A**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY
An equal opportunity, affirmative action institution

Date*	OSP Number	Sponsor	Grant Comparison Conducted?

* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

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Appendix C-3

Informed Consent

IRB Approval Date:
IRB Approval Number:
Participant ID Number:

Virginia Polytechnic Institute and State University

Informed Consent for Participants in Research Projects Involving Human Subjects (Sensory Evaluation)

Title Project: Consumer Acceptability of Sensory Characteristics of Soymilk

Investigators: Susan E. Duncan, PhD, RD and Laurie Bianchi

I. Purpose of this Research/Project
You are invited to participate in a study to determine if the addition of a food grade ingredient might improve sensory characteristics of soymilk.

II. Procedures
You will evaluate three samples of soymilk for sensory quality. For each sample, you will be asked to rate it for the following sensory characteristics: appearance, smell, taste, and mouth feel. You will rate each of those sensory characteristics on a scale of Like Extremely to Dislike Extremely. You will score each of the characteristics in each sample on a touch screen monitor. You will also complete a questionnaire after tasting the three samples. The questionnaire asks demographic and soymilk consumption questions. There is one sensory session that will take approximately 15 minutes.

III. Risks
There are only minimal risks associated with this study. Individuals with allergies to certain food components, particularly soybeans, may be at risk. Soymilk is manufactured from soybeans.

IV. Benefits
The goal of this research is to determine if a food grade ingredient added to soymilk can improve consumer acceptability of soymilk characteristics. Your participation in this study will provide valuable information to the soymilk industry about the use of ingredients to improve consumer acceptability of soymilk.

V. Extent of Anonymity and Confidentiality
The results of your performance as a panelist will be kept strictly confidential except to the investigator. Individual panelists will be referred to by a code number for data analyses and for any publication of the results.

VI. Compensation

You will be compensated with a small edible treat at the end of the session.

VII. Freedom to Withdraw

IRB Approval Date:
IRB Approval Number:
Participant ID Number:
If you agree to participate in this study, you are free to withdraw from the study at any time without penalty.

VIII. Subject's Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

- Evaluate 3 samples of soymilk, as presented, and provide answers using a touch screen computer.
- Complete a short questionnaire regarding demographics and soymilk consumption.

IX. Subject's Permission

I have read the consent form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

Date: _____

Subject Signature: _____

Subject Printed Name: _____

IRB Approval Date:
IRB Approval Number:
Participant ID Number:
- - - - - - - - - - - For Human Subject to Keep - - - - - - - - - - - -

Should I have any pertinent questions about this research or its conduct, and research subjects' rights, and whom to contact in the event of a research-related injury to the subject. I may contact:

Susan Duncan, Faculty/Investigator   (540) 231-8675;
                                                    duncans@vt.edu

Laurie Bianchi, Graduate Student/Investigator   (540) 231-8675;
                                                    lbianchi@vt.edu

David Moore
Chair, Virginia Tech Institutional Review   (540) 231-4991;
Board for the Protection of Human Subjects   moored@vt.edu
Office of Research Compliance
Blacksburg, VA 24061

Appendix C-4

Test Administration Worksheet

Date: March 20, 2012___	**Worksheet** 5T1R3D	**Test Code** 1M

Post this sheet in the area where trays are prepared. Code scoresheets in advance. Label serving containers in advance.

Type of Samples: Soymilk
Type of Test: Affective, hedonic scale, 1-9 for appearance, smell, taste, mouth feel, and aftertaste

Sample Identification	Code	
Soymilk with 0.5% TiO2 added	A	289
Soymilk with 1.0% TiO2 added	B	839
Soymilk with 0 TiO2 added	C	579

Code serving containers as follows:

Panelist Number	Order of Presentation
1,7,13,19.25,31,37,43,49,55,61,67,73,79	A-B-C
2,8,14,20,26,32,38,44,50,56,62,68,74,80	B-C-A
3,9,15,21,27,33,39,45,51,57,63,69,75,81	C-A-B
4,10,16,22,28,34,40,46,52,58,64,70,76,82	A-C-B
5,11,17,23,29,35,41,47,53,59,65,71,77,83	B-A-C
6,12,18,24,30,36,42,48,54,60,66,72,78	C-B-A

1. Place pencil and Informed Consent with panelist's number on tray.
2. Select cups of A, B, or C from those previously coded and place on tray and serve one at a time with corresponding score sheet and instruction sheet. Panelist will score samples on SIMS.
3. Provide panelist with survey to complete.

Appendix C-5

Scorecard placed on SIMS touchscreen monitor

Please make sure that the sample code matches the sample code on the cup.

Please look first at the following sample and then smell and taste it. If needed, you may expectorate it in the cup provided. Please drink a small amount of water and eat a couple of oyster crackers in between each sample.

Please rate the sample on the following:

Appearance:
- o Like extremely
- o
- o
- o
- o Neither like nor dislike
- o
- o
- o
- o Dislike extremely

Smell
- o Like Extremely
- o
- o
- o
- o Neither like nor dislike
- o
- o
- o
- o Dislike Extremely

Taste
- o Like Extremely
- o
- o
- o
- o Neither like nor dislike
- o
- o
- o
- o Dislike Extremely

Mouth feel
- o Like Extremely
- o
- o

- o
- o Neither like nor dislike
- o
- o
- o
- o Dislike Extremely

Aftertaste
- o Like Extremely
- o
- o
- o
- o Neither like nor dislike
- o
- o
- o
- o Dislike Extremely

Appendix C-6

Survey of Participants

Questionnaire for Sensory Studies

The goal of our research is to determine if a food additive can improve consumer acceptability of soymilk. Your participation in this study will provide valuable information to the soymilk industry regarding consumer acceptability of soymilk beverages.

Please answer all of the following questions.

1. Gender
   o   Male
   o   Female

2. Age
   o   18-24
   o   25-34
   o   35-45
   o   45 -55
   o   55 -65
   o   65 and older

3. Please check the category that best describes you:
   o   Student
   o   Faculty
   o   Staff
   o   Community member

4. How often do you **purchase** soymilk, including regular, vanilla, or chocolate flavored milks?
   o   Daily (at least once/day)
   o   Weekly (once per week up to 5-6 times/week)
   o   Monthly (1 to 3 times/month)
   o   Rarely/Never

5. How often do you **drink** soymilk?
   o   Daily (at least once/day)
   o   Weekly (once per week up to 5-6 times/week)
   o   Monthly (1 to 3 times/month)
   o   Rarely/Never

6. Would the addition of titanium dioxide (a food colorant used for whitening foods) to soymilk affect your acceptability of soymilk?
   o   Yes
   o   No

     o   If Yes, please describe how this would affect your perception of the product

If you **do** drink soymilk please answer questions 7 through 9 and then stop.

If you **do not** drink soymilk, skip questions 7 through 9 and go directly to questions 10 through 11.

7. What types of soymilk do you drink? Check ALL that apply.
   o Regular
   o Lite
   o Vanilla
   o Chocolate
   o Omega-3 Fatty Acid enriched
   o Other (please do not include almond milk here)

8. Why do you drink soymilk? Check ALL that apply.
   o Lactose intolerant
   o Cow milk protein allergies
   o Vegetarian
   o Functional food benefits
   o Taste
   o Cultural (e.g., grew up drinking soymilk)
   o Other, please list here

9. What sensory aspects of soymilk do you like?
   o Taste
   o Smell
   o Mouthfeel
   o Color
   o Please describe what it is about these characteristics that you especially like.

If you do not drink soymilk, please answer the following questions:

10. What sensory aspects of soymilk do you find displeasing?
    o Taste
    o Smell
    o Mouthfeel
    o Color
    o Please describe what it is about these characteristics that you especially DO NOT like.

    ┌───┐
    │   │
    │   │
    │   │
    │   │
    └───┘

11. Are there other reasons besides sensory characteristics why you do not prefer soymilk?
    o Cost
    o Packaging
    o Soy protein allergies
    o Other, please list

    ┌───┐
    │   │
    │   │
    │   │
    │   │
    └───┘

Appendix C-7

Email used for recruitment of panelists

Subject line: Invitation to participate in soymilk sensory study

Hello,

Our laboratory would like to determine if the addition of food ingredients can affect consumer acceptability of soymilk. This particular study consists of approximately 15-minute sensory test in which you will taste four samples of soymilk and rate them on how well you like the overall product. If you are interested, please hit reply and in the email subject line indicate "Yes for soymilk study". I will contact you with more details.

The sensory study will occur on September 10th between 9am and 4pm. Please report to room 127 of the Food Science Building at the corner of Washington Street and Duck Pond Drive between 9am-4pm. The test will take approximately 15 minutes of your time, which will include tasting the four samples and completing a short survey.

The FST Sensory Lab has a marketing campaign of "Serving Science and Society", which provides incentives that help participants feel good about participating. Each time you participate in an FST Sensory Lab study, panelists are rewarded for their participation with a $2 gift card (Kroger, Panera, or other local store), snacks, as well as canned foods (total value about $5). Panelists can keep the gift card and snacks and may keep or choose to donate the canned food, through the FST Sensory Lab, to the Montgomery County Emergency Assistance Program (MCEAP). MCEAP provides assistance to families and individuals in immediate, temporary, and emergency situations. By participating in each FST Sensory Lab sensory study, you have the opportunity to help scientific research and assist yourself and others in the local community in filling your cupboard and theirs. [NOTE: some studies are not conducted through the FST Sensory Lab but are conducted for other projects and may not be part of the "Serving Science and Society" incentive program].

Thank you,

Laurie Bianchi, RDN
Doctoral Candidate
Food Science and Technology
Virginia Tech
lbianchi@vt.edu

Appendix C-8

Virginia Tech IRB Approval Letter #13-278

WVirginiaTech

Office of Research Compliance
Institutional Review Board
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24060
540/231-4606 Fax 540/231-0959
e-mail irb@vt.edu
Website: www.irb.vt.edu

MEMORANDUM

DATE: March 21, 2012

TO: Susan E. Duncan, Laurie Bianchi

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires May 31, 2014)

PROTOCOL TITLE: Effects of the Addition of Food Grade Titanium Dioxide on Sensory Characteristics of Soy Milk

IRB NUMBER: 12-181

Effective March 21, 2012, the Virginia Tech IRB Chair, Dr. David M. Moore, approved the new protocol for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at http://www.irb.vt.edu/pages/responsibilities.htm (please review before the commencement of your research).

PROTOCOL INFORMATION:
Approved as: **Expedited, under 45 CFR 46.110 category(ies) 7**
Protocol Approval Date: **3/21/2012**
Protocol Expiration Date: **3/20/2013**
Continuing Review Due Date*: **3/6/2013**
*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:
Per federally regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals / work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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Date*	OSP Number	Sponsor	Grant Comparison Conducted?

*Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

cc: File

Appendix C-9

Informed Consent

IRB Approval Date: March 29, 2013
IRB Approval Number: 13-278
Participant ID Number:

Virginia Polytechnic Institute and State University

Informed Consent for Participants in Research Projects Involving Human Subjects (Sensory Evaluation)

Title Project: Consumer Acceptability of Soymilk

Investigators: Susan E. Duncan, PhD, RD, Laurie Bianchi, and Allie Sivak

I. Purpose of this Research/Project
You are invited to participate in a study to determine if the addition of a food grade ingredient might affect consumer acceptability of soymilk.

II. Procedures
You will evaluate four samples of soymilk for sensory quality. For each sample, you will be asked to rate it for how you will like the product on a scale of Like Extremely to Dislike Extremely. You will score each sample on a touch screen monitor. You will also complete a questionnaire after tasting the four samples. The questionnaire asks demographic and soymilk consumption questions. There is one sensory session that will take approximately 15 minutes.

III. Risks
There are only minimal risks associated with this study. Individuals with allergies to certain food components, particularly soybeans, may be at risk. Soymilk is manufactured from soybeans.

IV. Benefits
The goal of this research is to determine if a food grade ingredient added to soymilk can improve consumer acceptability of soymilk.

Your participation in this study will provide valuable information to the soymilk industry about the use of ingredients to improve consumer acceptability of soymilk.

V. Extent of Anonymity and Confidentiality
The results of your performance as a panelist will be kept strictly confidential except to the investigator. Individual panelists will be referred to by a code number for data analyses and for any publication of the results.

VI. Compensation

The FST Sensory Lab has a marketing campaign of "Serving Science and Society", which provides incentives that help participants feel good about participating. Each time you participate in an FST Sensory Lab study, panelists are rewarded for their participation with a $2 gift card (Kroger, Panera, or other local store), snacks, as well as canned foods (total value about $5). Panelists can keep the gift card and snacks and may keep or choose to donate the canned food, through the FST Sensory Lab, to the Montgomery County Emergency Assistance Program (MCEAP). MCEAP provides assistance to families and individuals in immediate, temporary, and emergency situations. By participating in each FST Sensory Lab sensory study, you have the opportunity to help scientific research and assist yourself and others in the local community in filling your cupboard and theirs. [NOTE: some studies are not conducted through the FST Sensory Lab but are conducted for other projects and may not be part of the "Serving Science and Society" incentive program].

VII. Freedom to Withdraw
If you agree to participate in this study, you are free to withdraw from the study at any time without penalty.

VIII. Subject's Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

- Evaluate 4 samples of soymilk, as presented, and provide answers using a touch screen computer.
- Complete a short questionnaire regarding demographics and soymilk consumption.

IX. Subject's Permission

I have read the consent form and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

Date: _____

Subject Signature:

_____

Subject Printed Name:

_____

- - - - - - - - - - - For Human Subject to Keep - - - - - - - - - - - -

Should I have any pertinent questions about this research or its conduct, and research subjects' rights, and whom to contact in the event of a research-related injury to the subject. I may contact:

Susan Duncan, Faculty/Investigator   (540) 231-8675;
        duncans@vt.edu

Laurie Bianchi, Graduate Student/Investigator   (540) 231-8675;
                                                            lbianchi@vt.edu

Allie Sivak, Undergraduate Student/Investigator   (240) 328-8052

                                                            allie22@vt.edu

David Moore
Chair, Virginia Tech Institutional Review   (540) 231-4991;
Board for the Protection of Human Subjects   moored@vt.edu
Office of Research Compliance
Blacksburg, VA 24061

Appendix C-10

Scorecard placed on SIMS touchscreen monitor

Please make sure that the sample code matches the sample code on the cup.

Please look first at the following sample and then smell and taste it. If needed, you may expectorate it in the cup provided. Please drink a small amount of water and eat a couple of oyster crackers in between each sample.

Please rate the sample on the following:

- Like extremely
-
-
-
- Neither like nor dislike
-
-
-
- Dislike extremely

Appendix C-11

Histograms of Frequency of Hedonic Scores

Distribution of frequency of hedonic scores (1=dislike extremely; 9=like extremely) chosen by panelists (n=88) for each attribute, appearance, smell, taste, mouthfeel, aftertaste for all treatments (0%, 0.05% and 1% TiO_2) combined.

Appearance

Smell

Taste

Frequency of hedonic scores chosen by panelists for scoring of taste

Mouthfeel

Frequency of hedonic scores chosen for mouthfeel

Aftertaste

Frequency of hedonic scores chosen for aftertaste

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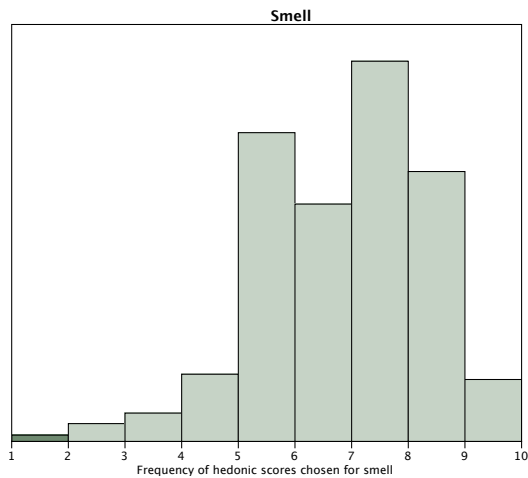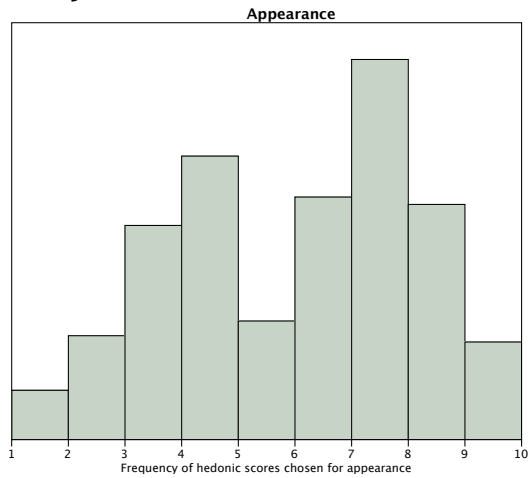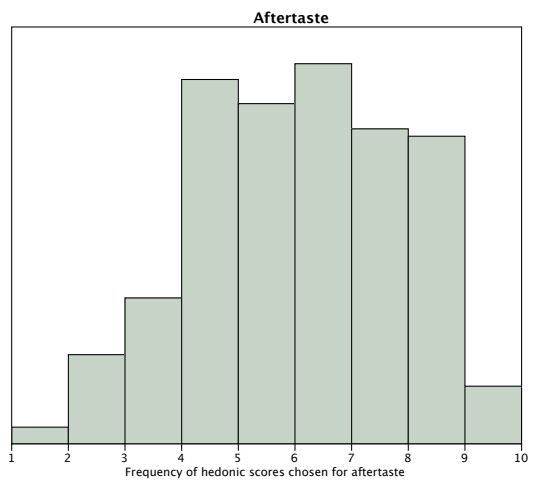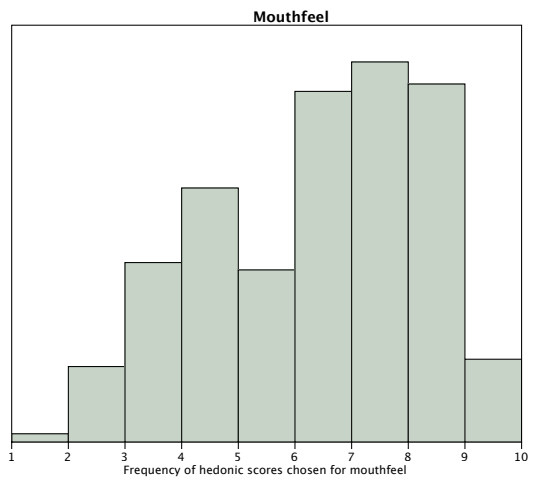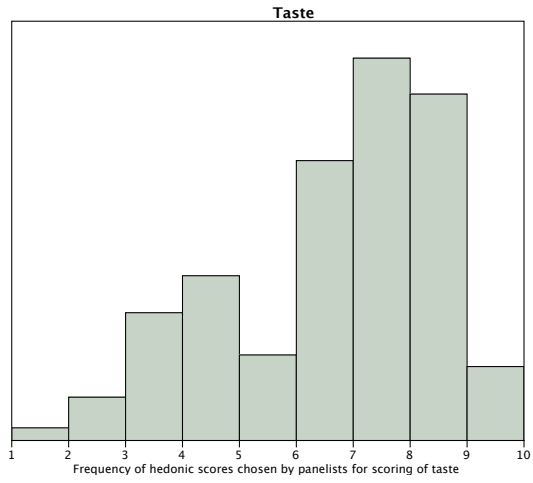
Appendix C-12

Hedonic Scores for Consumer Acceptability Study

Hedonic scores means ± sd for overall acceptability of control, 0.1% TiO_2, 0.3% TiO_2, and 0.5%TiO_2

Treatment: %TiO_2 added	0 (Control)	0.1%	0.3%	0.5%
Hedonic Score: mean±std dev	6.36±1.16	6.21±1.15	6.50±0.92	6.30±1.07

Hedonic scores correlations: 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither dislike or like, 6=like slightly, 7=like moderately, 8=like very much, 9=like extremely. n=66

Appendix C-13

Demographic Data of Panelists

Demographic information and soymilk consumption information for panelists who participated in the sensory attribute study and consumer acceptability study.

	Percentage of Respondents
Female	69
Male	31
18-24 yrs age	70
25-34	13
35-44	5
45-54	7
55+	5
Consume Soymilk:	
Rarely/Never	66
Monthly	20
Weekly	11
Daily	3

Appendix D-1

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