THE ANTIOXIDANT FUNCTION OF LUTEIN IN CONTROLLING PHOTO-OXIDATION OF A COLLOIDAL BEVERAGE SYSTEM

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(ABSTRACT)

.The effect of light on a model colloidal beverage system under two different test conditions, refrigerated storage (14 d, 4°C) and accelerated storage at room temperature (12 hr, 25°C), was investigated. The addition of lutein to provide protection against photo-oxidation of susceptible compounds also was investigated.

Fluorescent light-exposure (14 d, 4°C) of the control beverage system led to a decline in sensory quality based on triangle test results. Sensory quality also declined in the lutein-fortified beverage due to light-exposure. Sensory quality of light-exposed, lutein-fortified beverage compared to the light-protected control did not yield significant sensory differences for two out of three replications giving positive implications towards the use of lutein for photo-protection. Overall, panelists preferred beverages that were protected from light exposure with no specific preference towards control or lutein-fortified beverage, indicating lutein did not inhibit photo-chemical reactions leading to a decline in sensory quality. Chemical analysis showed limonene concentration was significantly higher in the lutein-fortified beverage compared to the control beverage after light exposure (14 d, 4°C). Hexanal concentration, however, was not closely correlated with sensory differences.

Results of the accelerated storage (12 hr, 25°C) study showed that the most damaging wavelengths to lutein stability were UV (200-400 nm) and 463 nm wavelengths. Degradation of lutein at 463 nm was expected and can be attributed to lutein's absorption of blue light at 450

nm. Hexanal formation was highest in the control beverage when exposed to full spectrum light and specifically UV (200-400 nm) wavelengths. Hexanal was also formed in the lutein-fortified beverage under full spectrum light and UV (200-400 nm) wavelength but to a significantly lesser degree. Limonene degraded significantly under all treatment conditions, with the most occurring during full spectrum light exposure. Lutein-fortification did not effectively protect limonene from degradation under these conditions.

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CHAPTER I

INTRODUCTION

Consumers are always looking for products that fulfill their need for convenience and concerns for health. In response, the food industry has developed innovative products aimed at matching these needs (Hasler 2002). Through advances in ingredient technology and product formulation, functional foods are rapidly emerging in the market place (Fass and Jones 2005). The 2005 IFT Expert Report defines functional foods as foods and food components that provide essential nutrients that impart health benefits beyond basic nutrition (Anonymous 2005).

Popularity of functional foods' has grown over the past few years and includes energy/sports beverages, probiotic products, heart-healthy yogurts and beverages, and many others. Industry projections estimate that the functional beverage market will be close to $6.4 billion by 2009 (Brewster 2006) with a growth rate of five times the standard food and beverage growth rate of 2% per year (Weststrate and others 2002).

Functional sports beverages incorporating vitamins, minerals, proteins and phospholipids have been developed to focus not only on athletic performance, but overall health and well-being (Maria 2006). Many energy and sports beverages that contain proteins are colloidal systems in which the protein is stabilized through the use of phospholipid emulsifiers.

Colloidal systems are very common in the beverage market and include dairy-based beverages and also many soft drinks, which are diluted colloidal systems (Anonymous 2003). Value-added colloidal systems with proteins emulsified by lipids have the potential to be oxidized from visible and ultraviolet light exposure.

Light oxidation results in the formation of reactive species that significantly damage other components (Horspool and Armesto 1992). Secondary lipid oxidation products can have strong off-flavors that can lead to a decrease in consumer appeal (Kamal-Eldin 2003). Oxidation also can result in the loss of vitamins and other nutrients that provide functional health value to the beverage. Molecules such as riboflavin can act as photosensitizers by absorbing specific light wavelengths and then initiating free radical reactions. Pigments, such as carotenoids and anthocyanins, can have alteration in color as an effect of light oxidation (Chen and others 1995; Iverson 1999). Overall, oxidation can lead to a decrease in shelf-life for functional beverages.

Due to the effects of light exposure, many vitamin-enriched beverages have been placed in opaque or tinted bottles to reduce the formation of off-flavors and protect nutrients from oxidation. Light-barrier packaging has been shown to significantly reduce the effects of photo-oxidation on milk degradation during extended storage (Mestdagh and others 2005; Webster 2006; van Aardt and others 2001). Although light-barrier packaging proves beneficial in reducing photo-oxidation incorporation of antioxidants within the beverage matrices may reduce oxidation due to light exposure and allow transparent packaging.

In the eye, the antioxidants lutein and zeaxanthin exhibit a protective effect against photo-oxidation of susceptible polyunsaturated fatty acids in photoreceptors responsible for central vision (Sujak and others 1999). These antioxidants are fortified in foods for their health implications, but it is unknown whether they may also provide protection from photo-oxidative degradation in vitro.

The overall objective of this project is to determine the effects of different light wavelengths on chemical and sensory quality of a colloidal beverage system and evaluate

antioxidant function of a lutein and zeaxanthin mixture in controlling photo-degradation of molecular constituents.

The first research objective of this project was to determine if sensory quality of a colloidal beverage system formulated with protein and phospholipids degraded after exposure to broad spectrum light. The hypothesis was that a colloidal beverage system without antioxidants would demonstrate sensory quality degradation. The colloidal system that included the antioxidants lutein and zeaxanthin would retain sensory quality under conditions of light exposure.

The second objective of this project was to determine if lutein and zeaxanthin can reduce photo-oxidation of whey proteins and phospholipids when exposed to various wavelengths in a colloidal beverage system. The hypothesis was that there would be a reduction in the level of oxidative by-products within the colloidal beverage system when lutein and zeaxanthin were added. A related hypothesis was that the antioxidant compounds lutein and zeaxanthin would be less stable and degraded with increased exposure to photo-oxidative conditions over the product shelf-life.

CHAPTER II

LITERATURE REVIEW

2.1 CAROTENOIDS AS ANTIOXIDANTS

In nature, there are over 600 carotenoids. Carotenoids can be defined as the yellow, orange, and red pigments produced by many plants and also some animals. In plant and animal systems, carotenoids reside on the interior of cell membranes and associate with lipoproteins due to their lipophilic nature. Carotenoids are lipid soluble due to a conjugated double bond system and long hydrocarbon chain (Groff and Gropper 2000).

Two carotenoid structures, lutein and zeaxanthin, are present in the macular pigment of the macular region of the human retina (Krinsky and others 2003, Bone and others 1993). Presence of these xanthophylls in the macular region was first reported by George Wald in 1945. Wald based his characterization of these structures on the spectrum and solubility similarities in the yellow spot of dissected foveas to plant xanthophylls (Krinsky and others 2003). Within the eye these xanthophylls act to protect the eye in two ways. They protect against damage of lipid membranes from photo-oxidation and they also act as a shield from short-wavelength radiation (Sujak and others 1999). If short-wavelength radiation is not shielded it could damage the outer segments of photoreceptors which are primarily composed of lipid membranes (Curran-Celentano and others 2001). Damage to this area could result in loss of visual acuity and central vision (Gottlieb 2002). Epidemiological evidence suggests that the amount of macular pigment is inversely associated with incidence of age-related macular degeneration, the major cause of blindness in the elderly (Krinsky and others 2003).

Carotenoids function in plants as photo-protectors by capturing and transferring light energy during photochemical reactions (Frank and others 1999). Many carotenoids act as antioxidants due to their conjugated double bond system. This system allows carotenoids to react with and quench singlet oxygen and also quench free radical reactions that occur in lipid membranes and potentially in solution. Free radical reactions that carotenoids can quench include lipid peroxidation caused by peroxyl radicals (Groff and Gropper 2000).

2.2 LUTEIN AND ZEAXANTHIN

Lutein and zeaxanthin are oxygenated carotenoids (xanthophylls) commonly found in the pigment of red, orange, yellow and green fruits and vegetables (Subagio and Morita 2000). Chlorophyll in green fruits and vegetables masks the red, yellow, or orange pigment from visual perception (Groff and Gropper 2000). Lutein and zeaxanthin concentration is highest in spinach (156.9 μg/g), but also can be found in lesser amount in summer squash, broccoli, green peppers, raspberries and many other fruits and vegetables at varying concentration levels (USDA 2005). Lutein also has been extracted from marigold flowers and used as a food colorant in commercial products (Subagio and Morita 2000). It is difficult to separate lutein and zeaxanthin in the extraction process so many lutein sources contain small amounts of zeaxanthin.

2.2.1 STRUCTURE AND CHEMISTRY

Lutein and zeaxanthin have the same empirical formula of $C_{40}H_{56}O_2$ with a molecular weight of 568.85 (Figure 1). The melting point of both compounds is 190°C and they are insoluble in water and soluble in fats and fat solvents.

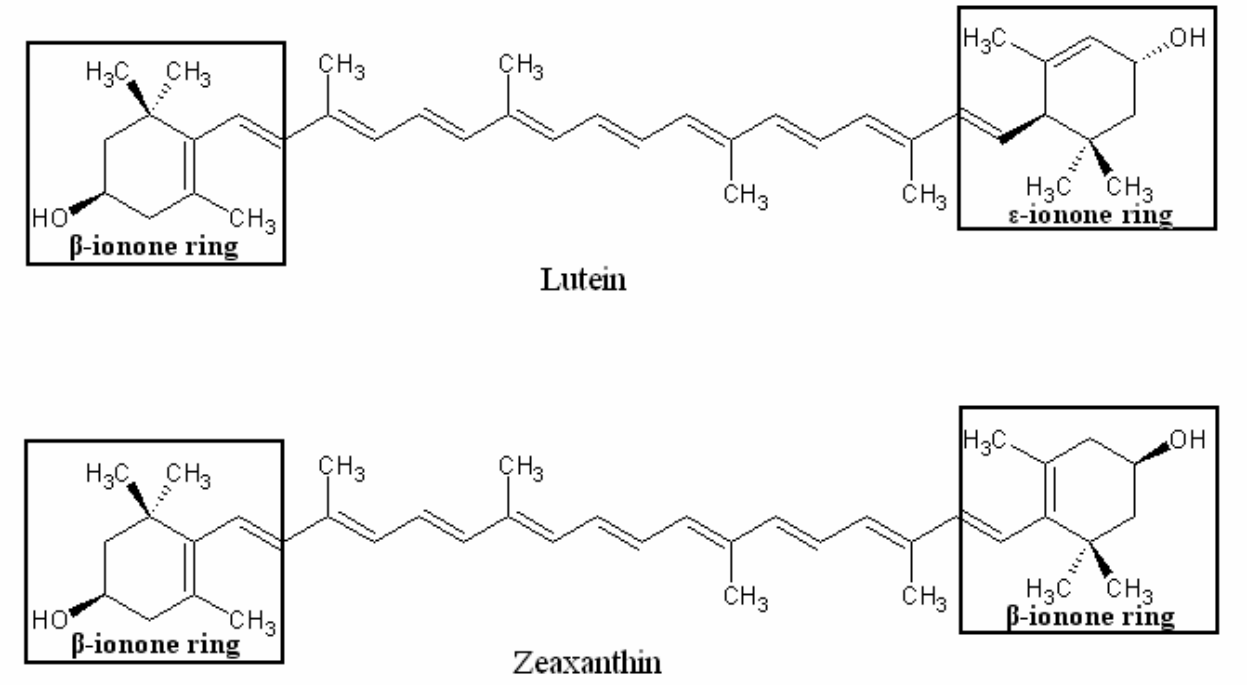

Figure 1. Chemical structure of lutein and zeaxanthin. Adapted from Krinsky and others 2003.

Lutein and zeaxanthin are isomeric dihydroxy-carotenoids containing ionone ring systems substituted at the 3 and 3' carbons (Fig 1). Lutein contains a β-ionone ring and a ε-ionone ring which have similar stereochemical configurations with the exception of the hydroxyl group in the ε-ring faces the opposite direction in respect to the hydroxyl group in the β-ring. The β-ionone ring is oriented in an axial direction while the ε-ionone ring is oriented in an equatorial direction which may affect how it orients in a membrane matrix. Oxidation of the

secondary alcohols in the lutein structure causes formation of ketones. It also appears as if the alcohol of the ε-ionone ring is more prone to being oxidized than the β-ionone ring.

Zeaxanthin contains two β-ionone rings, in which the hydroxyl group of each ring faces the same direction as contrasted to the hydroxyl groups in the lutein ring structures. This orientation difference between the two xanthophylls may affect molecular recognition by proteins and also affect preferential site selection when incorporated into membrane systems. Unlike lutein, zeaxanthin structure contains two axially directed rings which may explain orientation differences in membrane matrices between the two xanthophylls. When placed in a membrane, zeaxanthin spans the lipid bilayer with the hydroxyl groups residing in proximity to the phospholipid head on each side (Krinsky and others 2003). Due to this orientation, zeaxanthin stabilizes lipid membranes thus reducing the occurrence of lipid peroxyl radical chain propagation (Young and Lowe 2001). Zeaxanthin contains one more conjugated double bond than lutein which may explain why zeaxanthin is a more potent singlet oxygen quencher (Bone and others 1993).

Heat and ultraviolet light stability of lutein and its myristate esters were studied by Subagio and others (1999). Degradation of free lutein occurred much more readily than lutein monomyristate and lutein dimyristate when incubated at 60°C for 4 days in the dark. Similar results were found when compounds were exposed to ultraviolet light at 10°C for 3 days. Overall, free lutein appeared to have a significantly higher rate of degradation compared to the myristate esters under heat and ultraviolet light conditions.

2.2.2 LIGHT ABSORPTION BY ANTIOXIDANT AND PROOXIDANT FUNCTIONS

Lutein and zeaxanthin function as antioxidants by reducing free radical formation or through singlet oxygen quenching. Lutein and zeaxanthin were found to protect against oxidative damage of egg yolk lecithin liposomal membranes exposed to UV radiation with similar efficacy. Under long term UV exposure, zeaxanthin worked better than lutein possibly due to the photooxidation of lutein itself (Sujak and others 1999).

The level at which antioxidant function of lutein and zeaxanthin occurs is based on the concentration of oxygen, the chemical structure of the carotenoid, and the presence of other oxidants. An alteration to the chemical structure of the carotenoid has been shown to enhance carotenoid function. It has been reported that esterification of hydroxyl groups with fatty acids in lutein acts to stabilize the carotenoid against heat and UV light (Subagio and Morita 2000).

The antioxidant mechanism of lutein and zeaxanthin can occur through the electron transfer, hydrogen abstraction and radical addition pathways when the carotenoid is exposed to a reactive species. Electron transfer occurs when the reactive species ($ROO^{\bullet}$) takes an electron from the polyene chain of the carotenoid (CAR) forming a cation radical (Young and Lowe 2001).

Electron Transfer:

1) $ROO^{\bullet} + CAR \rightarrow ROO^{-} + CAR^{\bullet+}$

It is hypothesized that the radical cation formed could be regenerated to the unaltered carotenoid through a reaction with ascorbate (Krinsky and others 2003). Hydrogen abstraction occurs when a peroxyl radical reacts with the carotenoid and results in the formation of a neutral radical (Young and Lowe 2001).

Hydrogen Abstraction:

2) $ROO^{\bullet} + CAR \rightarrow ROOH + CAR^{\bullet}$

The radical addition pathway can occur when the polyene chain of the carotenoid is oxidized through the attachment of a peroxyl radical. This creates a carotenoid peroxyl radical which has a prooxidant effect at high partial pressures (Krinsky and others 2003; Young and Lowe 2001).

Radical Addition Pathway:

3) $ROO^{\bullet} + CAR \rightarrow (ROO - CAR)^{\bullet}$

Lutein and zeaxanthin also are characterized by an unusual passive antioxidant light filtering characteristic. These structures absorb blue-light which indirectly reduces photosensitization and the oxidative chain reactions induced by peroxyl radicals (Krinsky and others 2003). The blue light filtering mechanism of lutein and zeaxanthin was investigated in liposomes at various concentrations by Junghans and others (2001). Lutein and zeaxanthin function as broad-band filters thus reducing the intensity of blue light. Zeaxanthin appears to have a slightly higher absorption maximum (451 nm) than lutein (445 nm) in ethanol (Krinsky and others 2003). Junghans and others (2001) proposed that the specific orientation of the carotenoids in the membrane of the liposomes was related to a reduction in blue light emission. They found that zeaxanthin reached an optimal blue light filtration at 2-3.5 nmol/mg phospholipids and lutein at 4-7 nmol/mg phospholipids (Junghans and others 2001).

Under certain conditions, carotenoid structure may act as pro-oxidants. Subagio and Morita (2003) found that free lutein exhibited prooxidant effects in corn-triacylglyeride. They hypothesized that oxidative products of lutein itself played a role in the observed prooxidant effect.

Prooxidation is defined by the generation of more radicals than those being consumed. This reaction occurs through the radical addition pathway in which a peroxyl radical adds to the carotenoid (Krinsky and others 2003). The end product of the radical addition pathway is a carbon-centered carotenoid radical that can react directly with O_2 (Young and Lowe 2001).

4) $CAR^{\bullet} + O_2 \rightarrow CAR - OO^{\bullet}$

Formation of the carotenoid radical is dependant on the partial pressure of oxygen (ppO_2) since, at high partial pressures, additional radicals are generated through the cleaving of peroxy bonds (Krinsky and others 2003). Thus at low ppO_2 of less than 150 torr the carotenoid acts as an antioxidant and at high ppO_2 it can act as a prooxidant (Krinsky and others 2003; El-Agamey and others 2004). At ground level, the partial pressure of oxygen is close to 150 torr.

2.3 OXIDATION

Oxidation of molecular structures, such as proteins and lipids, within food systems frequently leads to reduced nutritional and sensory quality. Understanding oxidative processes and control mechanisms is essential in protecting product quality. Oxidation can be defined as the loss of electron density by carbon caused by carbon bond formation to a more electronegative atom such as oxygen, nitrogen or a halogen (McMurry 2004). Ability of oxygen to oxidize organic molecules depends on its oxidation state (Hudlický 1990). Triplet oxygen is characterized by two electrons with parallel spins whereas the two electrons of singlet oxygen have antiparallel spin (Hudlický 1990). Figure 2 and Figure 3 show the molecular orbital for triplet oxygen and singlet oxygen. Triplet oxygen is also known as ground-state oxygen and is more stable than its counterpart, the excited-state singlet oxygen (Hudlický 1990).

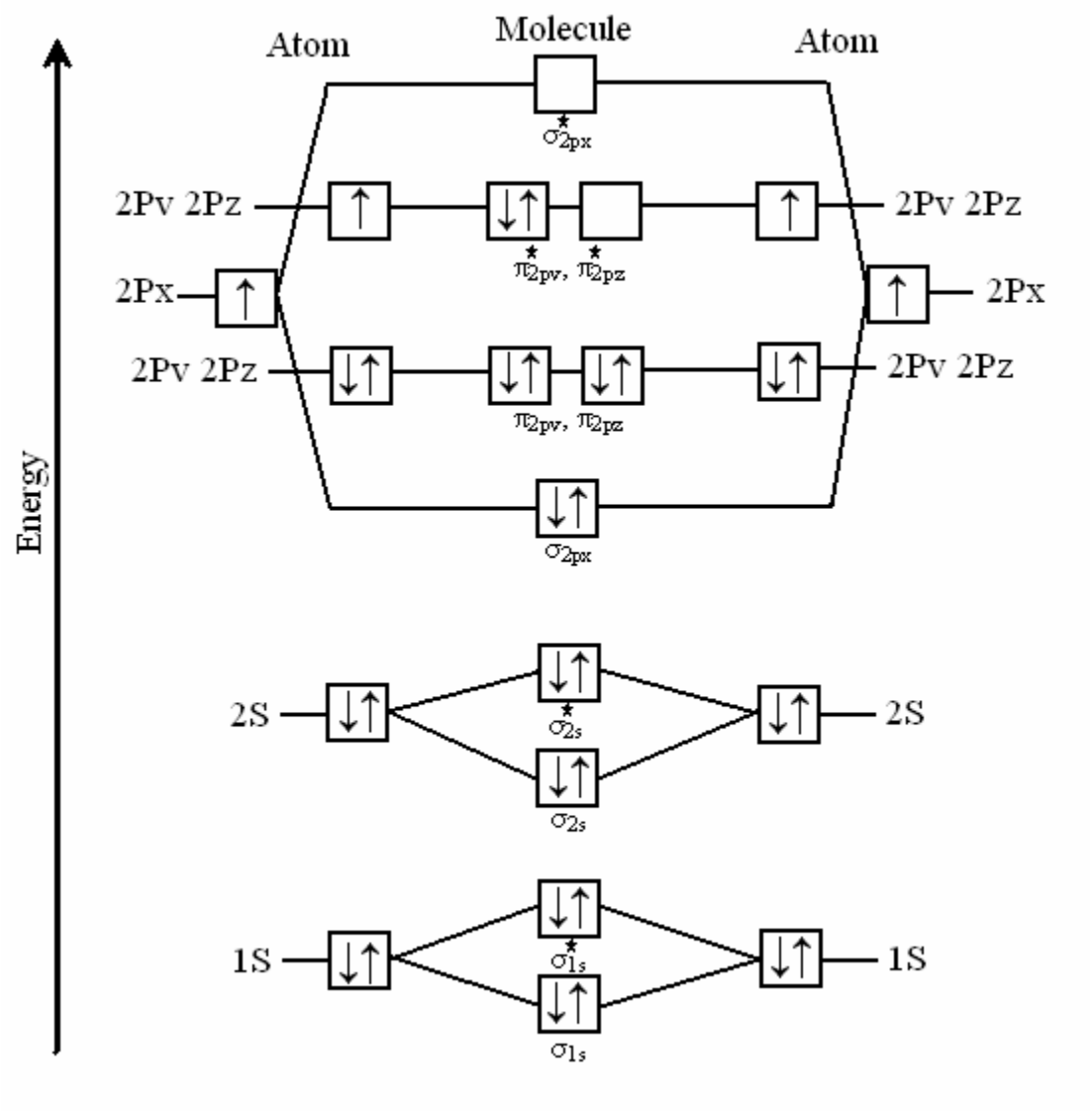

Figure 2. Singlet oxygen molecular orbital diagram. Adapted from Min and Boff 2002.

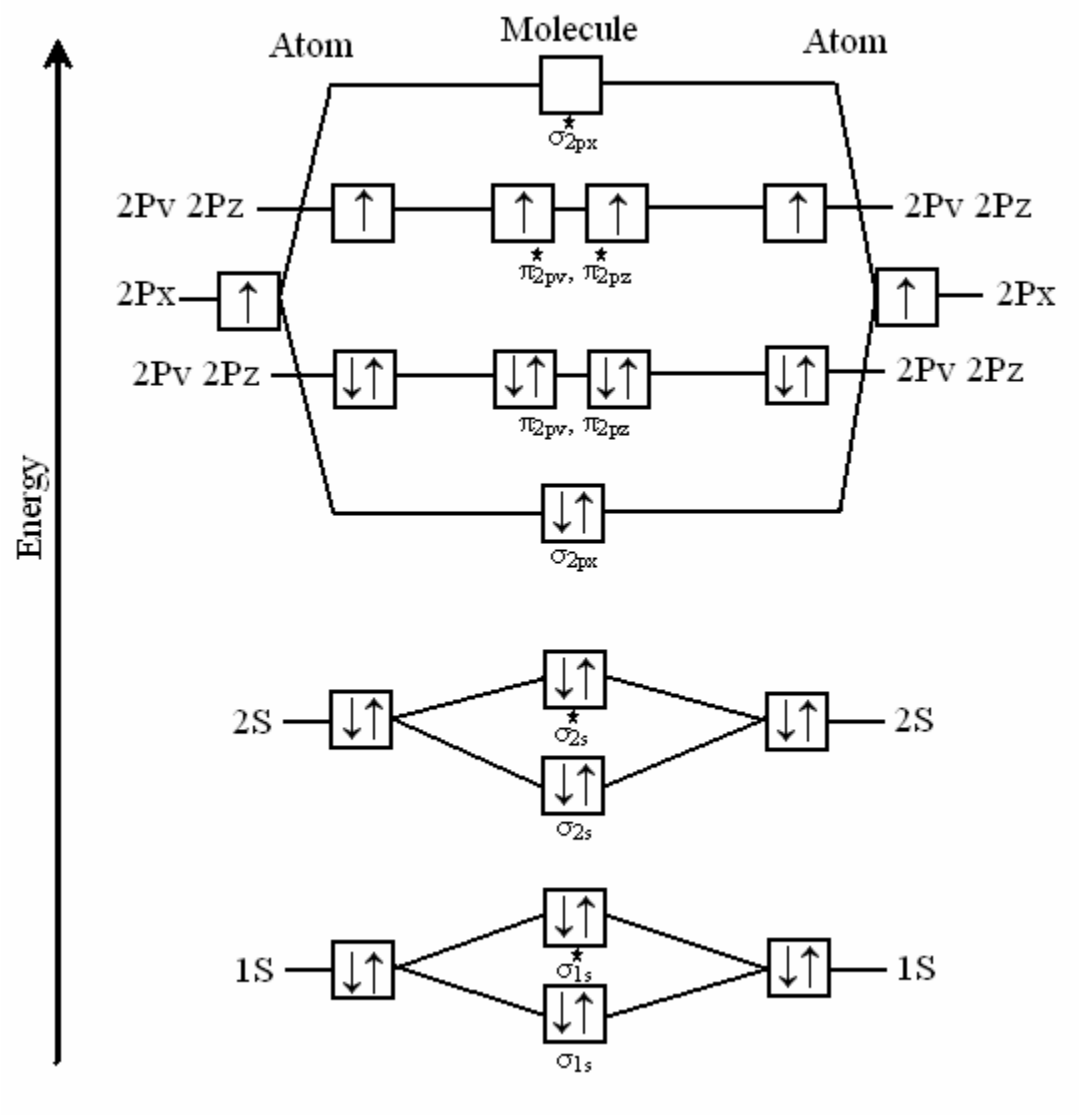

Figure 3: Triplet oxygen molecular orbital diagram. Adapted from Min and Boff 2002.

The process of conversion from triplet oxygen to singlet oxygen through the absorption of light is a very rapid process and the resultant singlet oxygen has only a short lifetime (10^{-3} to 10^{-12} second) at room temperature in a fluid solution. To regenerate the ground state oxygen the singlet oxygen must move from a highest energy to lowest energy excited state, also known as intersystem crossing. This process is relatively fast; however, to move from the lowest energy

excited state to the ground state is slow due to the larger energy gap between these two states (Horspool and Armesto 1992).

2.3.1 LIPID OXIDATION

Lipids are an essential element in food systems and most often occur in the form of triacylglycerides (Kamal-Eldin 2003). Triacylglycerides are composed of saturated, polyunsaturated or monounsaturated fatty acids linked to a glycerol backbone. The major component of lipid oxidation occurs when alkenes, such as unsaturated fatty acids, interact with excited singlet oxygen (Horspool and Armesto 1992) through free radical intermediates (Kamal-Eldin 2003) and can lead to metabolic byproducts (Horspool and Armesto 1992). For example, polyunsaturated fatty acids are attacked at the allylic position by oxygen which causes the formation of unsaturated hydroperoxides. Hydroperoxides are the primary products of lipid oxidation and are initiators of oxidative chain reactions when they are broken down through further oxidation or cleavage to form free radicals. The result of hydroperoxide decomposition leads to secondary oxidation products which include low molecular weight products such as aldehydes, ketones, alcohols, acids and lactones, esters and furans. Off-flavors and rancidity in oxidized beverage systems are due to these secondary oxidation products (Kamal-Eldin 2003).

Three different mechanisms can induce lipid peroxidation: autoxidation by free radical reaction, photo-oxidation and enzyme action. The first, autoxidation, can be divided into three stages: Initiation, propagation and termination. Initiation is characterized by the abstraction of hydrogen from a carbon atom (RH) next to the double bond in an unsaturated fatty acid to form a free radical ($R^{\bullet}$) (deMan 1999).

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

Hydrogen abstraction can be caused by initiators such as heat, light, or possibly a metal. After the formation of an initial free radical, oxygen will attach to form a peroxy-free radical ($RO_2^{\bullet}$).

6) $R^{\bullet} + O_2 \rightarrow RO_2^{\bullet}$

The propagation step in autoxidation involves the peroxy-free radical undergoing further hydrogen abstraction with other unsaturated fatty acids to form a peroxide (ROOH) and another free radical (deMan 1999).

7) $RO_2^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

Propagation can occur repeatedly, creating a chain reaction that forms many more free radicals. UV light and temperature were found to increase free radical reactions. Also, the rate of initiation dramatically increases once initial hydroperoxides are formed (Kamal-Edin 2003).

The third step in autoxidation, termination, follows propagation when a nonactive product is formed from the reaction of one free radical with another (deMan 1999).

8) $R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$

9) $R^{\bullet} + RO_2^{\bullet} \rightarrow RO_2R$

10) $nRO_2^{\bullet} \rightarrow (RO_2)n$

The hydroperoxides formed during propagation reactions are the primary products of oxidation. These compounds are relatively unstable and upon decomposition yield secondary oxidation products such as esters, ketones, lactones, furans, hydrocarbons and free fatty acids (deMan 1990). Odor and flavor characteristics associated with oxidation arise from these secondary oxidation products.

Lipids in colloidal food systems can be protected from oxidation through inclusion of antioxidants ($A^{\bullet}$). Antioxidants slow the free radical chain process of autoxidation through

chain-breaking inhibitors and preventive inhibitors but the overall efficiency is dependant on structure of the antioxidant molecule, the structure of the molecule being oxidized and the conditions under which oxidation is occurring (Kamal-Edin 2003). An efficient antioxidant traps peroxyl radicals, resulting in the halt of free radical chain oxidation.

12) $A^{\bullet} + LOOH \rightarrow AH + LOO^{\bullet}$

13) $A^{\bullet} + LOO^{\bullet} \rightarrow A\text{-}OOL$

Extent of oxidation can be plotted vs. time (Figure 4). Antioxidants produce a lag in oxidation during the "induction period" (Kamal-Edin 2003) (Figure 5). The induction period is defined as the initial stages of oxidation when hydroperoxide formation is increasing slowly (deMan 1990).

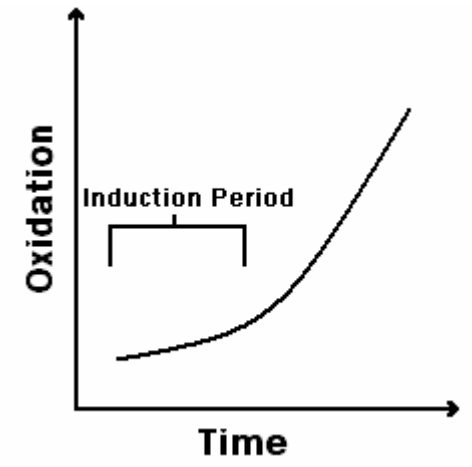

Figure 4. Extent of oxidation vs time

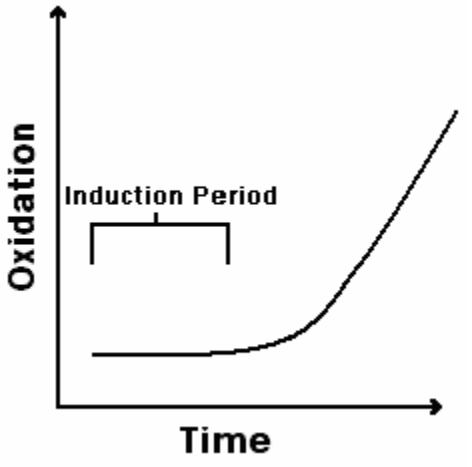

Figure 5. Extent of oxidation vs time with antioxidant lag

The duration of the lag is dependent on antioxidant concentration. Once the antioxidant has been consumed, the induction period ends and oxidation continues at the rate of an unprotected lipid. Antioxidant effectiveness can be characterized by the length of the induction period which shows the extent in time that oxidation was slowed. This also represents the

antioxidants interactions with peroxyl radicals which slow chain radical process from occurring (Kamal-Edin 2003).

2.3.2 PROTEIN OXIDATION

Early photo-oxidative studies of proteins and amino acids documented photo-induced oxygen interactions for several proteins. More recent studies have shown that radical formation may be a key factor in protein oxidation. Protein-damaging radical formation can occur by two processes. The first process occurs by direct cleavage of a molecular bond, resulting in two molecules containing unpaired electrons. The following equation shows that two reactive species can be created when UV light interacts with hydrogen peroxide (Davies and Dean 1997).

14) $H_2O_2 + \text{UV light} \rightarrow 2HO^{\bullet}$

This process also can occur in the presence of visible light however this most often occurs in the presence of a sensitizer molecule such as riboflavin.

The second process for protein-damaging radical formation involves the process of electron transfer. The transfer of an electron from one molecule to another generates a species which rapidly fragments resulting in a component with an unpaired electron. The following equations (15-17) show the transfer of electrons with hydrogen peroxide and water to form an intermediate that rapidly fragments, resulting in a component with an unpaired electron (Davies and Dean 1997).

15) $H_2O_2 + e^- \rightarrow (H_2O_2)^{\bullet -} \rightarrow HO^- + HO^{\bullet}$

16) $H_2O_2 - e^- \rightarrow (H_2O_2)^{\bullet +} \rightarrow H^+ + HOO^{\bullet}$

17) $H_2O - e^- \rightarrow (H_2O)^{\bullet +} \rightarrow H^+ + HO^{\bullet}$

The exact chemistry for these protein interactions is not well understood; however addition reactions with unsaturated systems, such as double bonds and aromatic rings, appear to be more rapid than those reactions with C-H bonds. The relative target selectivity of the reactive species also is based on the level of reactivity. Highly reactive species may not be more damaging than less reactive species since they are not as selective for targets and diffuse less before undergoing reactions (Davies and Dean 1997).

Besides radical induced oxidation of proteins, singlet oxygen can readily react with amino acid side chains. Lipid peroxidation also can cause oxidative damage to proteins especially in membrane systems where protein oxidation occurs near the interface between the lipid and protein regions of the membrane (Davies and Dean 1997). Lipid peroxides can react with methionine to form methionine sulfoxide. Reactive carbonyl compounds, produced from unsaturated fatty acid decomposition, can react with proteins in similar ways to nonenzymatic browning (deMan 1999).

Protein oxidation has been shown to form odor and flavor compounds such as a burnt feather or burnt protein flavor in milk exposed to light (Aurand and others 1966). Methionine can be converted to methional (β-methylmercaptopropionaldehyde) during sunlight exposure (deMan 1999). Further exposure of methional to sunlight yielded a burnt feather flavor (Allen and Parks 1975).

Antioxidants have been shown to slow protein degradation resulting from radical formation. Vitamin E acts as protein antioxidant in membrane systems; however it also transfers the damage from the protein to lipid phase which can cause further oxidation of lipids. Vitamin C also has been shown to have a protective antioxidant effect against protein derived radicals.

The protein concentration, nature and relative concentration of the array of proteins and the nature, extent and location of radical flux are all key factors that influence protein-radical interactions (Davies and Dean 1997).

2.3.3 PHOTOOXIDATION

Sunlight is often the source of photochemical reactions in living organisms however it is hard to apply natural sunlight into a laboratory setting. Changes in spectral distribution, intensity and discontinuous availability based on geographic location, time of day and year all play factors into the limitations of natural sunlight use (Kagan 1993). Artificial fluorescent lighting, used in food storage areas, emits a broad spectra from both ultraviolet to visible and into infrared however the glass tube absorbs wavelengths below 320 nm while allowing visible wavelengths to pass through (Rosenthal 1992). Wavelengths perceptible to the human eye are within the range of 380 to 780 nm, also known as the visible portion of the spectrum. Ultraviolet radiation which is imperceptible by the human eye ranges from 200 to 380 nm in length (Rosenthal 1992).

Photooxidation is defined as oxidative reactions induced by light through "the loss of one or more electrons from a chemical species as a result of photoexcitation of that species" or through "the reaction of a substance with oxygen under the influence of light (IUPAC 2006)." Photodeactivation can occur through four processes: photochemical reaction, luminescence, nonradiative deactivation and quenching processes. Nonradiative deactivation is defined as the conversion of excess energy into heat, which is a characteristic feature of carotenoids (Halyanasundaram and Gratzel 1993).

Photo-oxidation in food systems results when photo-sensitive molecules absorb light energy and subsequently transfer that energy to initiate oxidation of other molecular structures. Auto-oxidation reactions often occur as a result of a photo-initiation. Singlet oxygen and other excited molecules act as sensitizers which can raise the ground state of other molecules to the excited state (Horspool and Armesto 1992). Examples of photosensitizers include riboflavin, porphyrins and chlorines (Wold and others 2005). The whey portion of milk contains a high level of riboflavin, increasing milks susceptibility to photo-oxidative degradation (Sattar and others 1976, Bekbolet 1990). Photosensitized reactions have detrimental affects on food products stored in well lit areas such as supermarkets. Food products containing high concentrations of food colorants and unsaturated lipids are particularly susceptible to photo-oxidation (Rosenthal 1992).

Luminescence occurs in the general light induced oxidation manner, when a ground state molecule absorbs light energy (photons) and is promoted to the excited state. Photon energy, which causes molecular excitation when absorbed, is related to wavelength. Photon energy at a given wavelength is calculated by dividing the speed of light (c) by the wavelength in nanometers (λ), $E = c/\lambda$. Planck's constant shows the energy difference from the first excited state to ground state for a molecule, $\Delta E = E_1 - E_0 = hc/\lambda$ (Kagan 1993).

Molecules that absorb photons of energy are termed chromophores. Lutein and zeaxanthin are both recognized as chromophores due to their conjugated double bonds in the polyene chain and also situated in the β-rings (Niizu and Rodriguez-Amaya 2005). Absorption of energy promotes a chromophore to an excited state. For a chromophore to return back to ground state, energy must be released. Energy can be released in the form of heat, energy given to surrounding molecules and also through the emission of photons or luminescence (Albani

2004). The spontaneous emission of photons, fluorescence, allows the molecule, fluorophore, to return to a ground state (Wolfbeis and others 2005). The energy level of emission is always lower than that of absorption (Albani 2004).

The intensity of light energy used to cause excitation also plays a role in the amount of excited fluorophores. Excitation of fluorophores in low energy light is very dependant on the interaction of those molecules with the surrounding environment. Molecules embedded deep within a protein's structure, with very large energy gaps between ground and excited state, will not fluoresce (Lakowicz 1992). High intensity light, on the other hand, causes photoquenching. Molecules are excited to the point where they are "photobleached" and unable to fluoresce, resulting in a decrease in overall fluorescent intensity (Albani 2004).

The term quenching refers to the inactivation of molecules such as singlet molecular oxygen which is electronically excited through a transfer of energy. The excitation of molecules such as oxygen can be due to lipid peroxidation of membranes, photochemical reactions, or enzymatic reactions. Quenching these excited molecules is important because they can damage susceptible components such as proteins and lipids. The quenching process leads to the carotenoid moving into an excited state; however instead of damaging susceptible components, the carotenoid releases the energy in the form of heat (Groff and Gropper 2000).

2.3.4 MOLECULAR EXCITATION

Molecular excitation of compounds by photochemical reactions involves chemical and physical effects, some of which arise from radiation by ultraviolet light and others through the visible light range. Molecules are excited through the absorption of photons that cause their

electronic energy to move from a lower to higher state (Rosenthal 1992). This can be characterized by the equation $A + hv \rightarrow {}^{*}A$ (Halyanasundaram and Gratzel 1993).

Most photochemically active molecules contain π-systems (Kagan 1993). Specifically, electrons are excited from either π or n orbitals to π^* for most photochemical reactions involving alkene structures ($\pi \rightarrow \pi^*$) or heteroatoms ($n \rightarrow \pi^*$) (Rosenthal 1992). π is a bonding orbital with its complimentary π^* antibonding orbital. The nonbonding orbital (n), however, is composed of lone pairs of electrons (Arnold and others 1974), that can be occupied by oxygen, nitrogen or other heteroatoms and does not have a complimentary antibonding orbital.

Molecules can be excited into a singlet state which is an important initiation mediator in photochemical reactions, specifically oxygen in photooxidation. Molecules in the singlet state contain two unpaired electrons and can readily take hydrogen atoms or add to double bonds during through interactions with other molecules in the medium (Rosenthal 1992). Transfer of energy from electronically excited molecules has the potential to damage other components of a biological system which can be expressed using the following equation ${}^{*}A + B \rightarrow A + {}^{*}B$ (Halyanasundaram and Gratzel 1993). Type II photooxidation is characterized by photosensitized oxidation through a singlet oxygen mechanism (Rosenthal 1992).

2.3.5 INTERFACE EXCITATION WITHIN COLLOIDAL SYSTEMS

A colloidal beverage system can be defined as a suspension of small particles dispersed in an aqueous solution. Colloidal systems are very common in the beverage market and include dairy-based beverages and also many soft drinks, which are diluted colloidal systems (Anonymous 2003). Milk is a natural colloidal beverage composed of protein, lipid,

carbohydrate, minerals and vitamins. In milk, both the milk fat and the proteins are dispersed through the aqueous phase. The proteins exist in micelle form with a water soluble carbohydrate tail. Fat droplets in homogenized milk range from 0.2 to 2 μm in size and are stabilized by native milk fat globule membrane fragments containing phospholipids molecules, casein molecules and whey proteins on the lipid surface. These molecules function as emulsifiers, reducing the interfacial tension between the lipid and aqueous phase. The inclusion of a lipid soluble carotenoid, such as lutein, into a nonpolar matrix would necessitate the use of an molecules to reduce the interfacial tension. Interface excitation reactions studied in colloidal systems containing micelle structures have shown electric charge on surface-active components such as proteins and phospholipids can affect rate of lipid oxidation reactions (McCelments and Decker 2000).

In some formulated beverage systems micelles are aggregates of phospholipids in which the hydrophobic region is located towards the inside of the structure and the hydrophilic region is located towards the outside interacting with the solvent. Micelles can be spherical or disc-like in shape and of many sizes. Micelles are typically larger in size when they are in higher NaCl solution or when surfactant concentration is increased (Thomas 1984).

The use of micelles allows for the distribution of proteins and fat-soluble lutein into an aqueous medium. Distribution of proteins into micelles is not exactly uniform (Thomas 1984). This is due to factors such as whether solubilization of the protein disturbs the micelle, whether another solute already in the micelle will change the solubilization of the protein, and whether the solubilization of the protein is completely random. Oxidation reactions develop in micelle systems (Decker and others 2002). Surfactants can promote free-radial initiators to rapidly polymerize small emulsion particles (Thomas 1984).

2.3.6 ANALYTICAL MEASUREMENT BY CHROMATOGRAPHY

Chromatography is a useful way to physically separate compounds based on a solute's interactions with two phases, a mobile phase which moves through or along a stationary phase. Three types of mobile phases exist including liquid and gas (Nollet 2000). High performance liquid chromatography (HPLC) and gas chromatography (GC) have been used to determine the identity of compounds in food matrices and their relative concentrations.

HPLC is advantageous over other methods of analyses since it is completely automated and has high rate of reproducibility. One downside to HPLC is that complex extraction or isolation sample preparations can lead to error in quantitation. HPLC can be used to separate nonvolatile lipid-soluble components such as antioxidants. Lutein quantitation has been studied using high performance liquid chromatography (HPLC) by Katchek and others (1986). Since carotenoids, such as lutein, are fat soluble, an organic solvent must be used for extraction. During extraction, isomerization of carotenoids may occur. To alleviate this, Tetrahydrofuran (THF) can be used to solubilize carotenoids without causing isomerization and also denature proteins that may be associated with the carotenoids. Magnesium carbonate is also used in the extraction process to inhibit organic acids from degrading the carotenoids (Nollet 2000).

Carotenoid extracts can be separated based on their hydrophobicity using reverse phase HPLC. Reverse phase HPLC uses a non-polar stationary phase and a polar mobile phase. More polar carotenoids are retained longer than less polar carotenoids, so they elute at later retention times. Often in carotenoid analysis, the polar mobile phase is composed or partially composed of acetonitrile and methanol (Weston and Brown 1997).

Gas chromatography with solid phase microextraction (SPME) allows for the analysis of volatile compounds, such as the end products of oxidation, without destroying the sample matrix. SPME involves the concentration of volatile components onto a coated fiber that is then desorbed into a gas chromatogram. Several coated fibers exist, however poly(dimethylsiloxane) PDMS is one of the most useful since it can isolate both polar and non-polar compounds fairly well (Rouseff and Cadwallader 2001).

SPME fibers may be exposed to a sample in several ways including direct extraction and headspace extraction. In analyzing a beverage system, direct extraction would involve exposing the fiber directly to the liquid. Problems may arise with this approach since non-volatile compounds such as proteins could negatively affect the fiber. Headspace analysis is a better alternative since only volatile components should concentrate on the fiber (Rouseff and Cadwallader 2001). Headspace analysis using SPME in light oxidized milk has been studied by Marsili (1999) and other researchers (van Aardt and others 2001, Webster 2006).

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CHAPTER III

EFFECT OF LUTEIN FORTIFICATION ON THE SENSORY QUALITY OF A COLLOIDAL BEVERAGE SYSTEM EXPOSED TO LIGHT

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3.1 ABSTRACT

The addition of lutein into a model colloidal beverage system was studied to determine if lutein provides protection against photo-oxidation of susceptible compounds. Light-protected and light-exposed treatments of the control and lutein-fortified beverage systems were exposed to fluorescent light for 14 d. Triangle tests for difference and rank preference tests were used to evaluate differences between treatments. Chemical analysis consisted of volatile compound evaluation using gas chromatography with solid-phase microextraction.

Triangle tests showed light-exposure caused significant changes to the control beverage system. Light exposure, also caused sensory differences in the lutein-fortified beverage suggesting that lutein may not fully inhibit photochemical reactions that have sensory impact. Overall, panelists significantly preferred treatments that were light-protected over treatments exposed to light with no significant preference for the lutein-fortified beverage over the control. Limonene concentration was higher in the lutein-fortified beverage compared to the control after 14 days of light exposure. Hexanal concentration was not closely correlated with sensory differences.

Addition of lutein for photo-protection may not maintain sensory quality to a degree comparable to light-protection. The addition of lutein to reduce photo-oxidative degradation alone is not recommended however protection may be an added benefit when fortifying beverages with lutein for health benefits.

Key words: Photo-oxidation, Sensory, Hexanal, Limonene, Lutein

3.2 INTRODUCTION

To meet consumer needs for convenience and concern for health, beverage manufacturers have been increasingly adding vitamins, minerals, proteins and phospholipids to functional beverages (Hasler 2002; Maria 2006). Many protein-added beverages are colloidal systems in which phospholipids emulsify and thus stabilize the protein.

Light exposure during storage and retail display is detrimental to flavor, nutritional quality, and shelf-life of dairy-based beverages containing lipids and proteins (Bekbolet 1990, Wold and others 2005). Functional beverages containing susceptible compounds such as whey protein, phospholipids and even flavoring components may undergo oxidative degradation. Oxidation involves the loss of electron density of carbon caused by carbon bond formation to a more electronegative atom such as oxygen (McMurry 2004). Oxidation initiated by light exposure, known as photo-oxidation, occurs most commonly when a sensitizer, activated by light, reacts directly with a substrate or causes ground-state oxygen (triplet oxygen $^3O_2$) to convert to an excited state (singlet oxygen $^1O_2$) (deMan 1999). In this excited state, oxygen has the ability to oxidize organic molecules, such as lipids and proteins through free radical mechanisms (Hudlický 1990).

Photo-oxidation of lipids and proteins results in off-flavor formation and can lead to degradation in sensory quality (McClements and Decker 2000). A correlation between photo-oxidation initiated processes and off-flavor formation has been noted in milk, a natural colloidal system with lipids and proteins. Formation of aldehydes and carbonyl compounds, primarily hexanal, pentanal and 1-octen-3-ol are responsible for off-flavor/off-odor development in milk due to light oxidation (van Aardt and others 2005; Frankel 1998). Sensory descriptive analysis

has shown that a cardboard-like off-flavor is commonly associated with light oxidized milk and is attributed to lipid oxidation (Hedegaard and others 2006). Protein oxidation has been shown to form odor and flavor compounds such as a burnt feather or burnt protein flavor in milk exposed to light (Aurand and others 1966).

Flavoring components of a beverage system undergoing oxidation conditions also can result in off-flavor formation. A common flavoring agent in orange flavored beverages is citrus oil. Citrus oil contains unsaturated hydrocarbon terpenes, such as limonene, linalool and myrcene that are susceptible to oxidation (Taylor 2002). Limonene, the major constituent in orange oil, readily oxidizes to form carveol, carvone and p-cymene. Limonene and its oxidation products have been detected in essential oils, juices and juice beverages by Marine and Clemons (2003) using gas chromatography with solid-phase microextraction. Out of several fiber compositions studied, they found that a 7-μm-thick bonded polydimethylsiloxane (PDMS) fiber showed the best results for detecting these compounds.

In autoxidation, the rate at which lipid oxidation occurs increases as the degree of unsaturation increases in fatty acids (deMan 1999). Unsaturated lipids are also susceptible to photo-oxidation since singlet oxygen can react directly with the double bonds forming hydroperoxides. Breakdown of hydroperoxides form secondary oxidation products, which leads to off-flavor formation (deMan 1999).

Antioxidants protect against photo-oxidation by quenching free radicals before they damage components such as lipids and proteins. Efficiency of numerous antioxidants on photo-degradation has been reported in various food matrices. β-Carotene has been reported by Rahmani and Csallany (1998) to function as a quencher of singlet oxygen in photo-oxidation

studies of virgin olive oil. Addition of the antioxidants, BHA and BHT, were shown to control or limit milk off-flavor formation due to light oxidation (van Aardt and others 2005).

Lutein and zeaxanthin function as antioxidants in the human eye and may provide antioxidant function in food systems. In the macular region of the human retina, lutein and zeaxanthin act as antioxidants by shielding damaging short-wavelength radiation and passively filtering blue light that could damage the unsaturated fatty acid components of photoreceptors (Bone and others 1999). In egg yolk lecithin liposomal membranes exposed to UV radiation, lutein and zeaxanthin protected against oxidation damage (Sujak and others 1999). Fortifying a beverage system with lutein and zeaxanthin has the potential to reduce off-flavor formation due to oxidation of lipids, proteins and flavoring components.

The objectives of this study were to determine if lutein fortification would protect sensory quality of a colloidal beverage system, containing milk proteins and phospholipids, when exposed to light for 14 d; and to determine the effects of 14 d light exposure on the concentration of aroma-active flavor compounds as an indicator of oxidation reactions occurring within the system.

3.3 MATERIALS AND METHODS

3.3.1 Beverage System Processing:

A model colloidal beverage system (MCBS), with and without lutein, was formulated from whey protein, lecithin, orange-flavored instant beverage mix and water. Whey protein (Provon 292 Instantized, Glanbia, Monroe, WI) (146.52 g) was hydrated in filtered water (10 L) for 15 min under agitation. Lecithin granules (Annie Kay's Whole Foods, Blacksburg, VA) (7.32 g) were heated in 400 mL filtered water until dissolved and allowed to cool. Lecithin granules contained phosphatidylcholine (260 mg/g), phosphatidylinositol (140 mg/g), and phosphatidylethanolamine (200 mg/g). Lutein extract 20% powder (Pharmline, Florida, NY) (0.44 g) was mixed into dissolved lecithin mixture to formulate the MCBS with lutein but was not added to the control system. Lutein extract contained 21.9% lutein and 2.25% zeaxanthin, of which 99.15% were free xanthophylls (Pharmline, Florida, NY). Lutein extract contained tricalcium phosphate as a carrier. Lecithin mixture was stirred into hydrated whey protein. Orange-flavored instant beverage mix (Gatorade, Chicago, IL) (615.4 g) was incorporated into the whey and lecithin solution, slowly, using a handheld mixer. Gatorade instant mix was composed of sucrose, dextrose, citric acid, salt, sodium citrate, natural orange flavor with other natural flavors, monopotassium phosphate calcium silicate (a flow agent), partially hydrogenated soybean and cottonseed oils, and yellow 6.

Both (lutein, no lutein) beverage systems were homogenized in a two stage homogenizer (10,339 kpa (1500 psi) – 1st stage; 3,446 kpa (500 psi) – 2nd stage) (Type DX, Cherry Burrel Corp, Delavan, WI), and Pasteurized at 90.6-92.2ºC for 2 sec using a tubular heat exchanger

(UHT/HTST Lab 25 HV, Microthermics Inc., Raleigh, NC). After heat treatment, beverage was cooled to less than 25ºC. Beverage (800 mL) was stored in sterile 1 L glass bottles. Seven bottles of each beverage system were filled.

3.3.2 Beverage Storage and Handling

Bottles of MCBS treatments were either wrapped in aluminum foil, as a light-protective barrier (light-protected), or were not wrapped (light-exposed); and glass bottles were stored under 900-1100 lux fluorescent lighting for 14 d of refrigerated storage (4°C) in a Tonka refrigeration unit (Hopkins, MN). Lighting consisted of two 30 W cool white fluorescent bulbs. Light intensity was measured using a light meter (Extech Instrument Corp., Walthaun, MA). On Day 14, 20-mL aliquots of light-exposed and light-protected beverage treatments were poured into 25-mL plastic cups with plastic lids. Light-exposed beverage samples and light-protected beverage samples were identified with three-digit codes (Appendix A, Table A3). Coded samples were stored at 4ºC for no more than 3 hrs until sensory testing.

To ensure formulation of the beverage systems was consistent, protein and soluble solids were measured on day 0. Protein concentration was determined using a Bio-Rad DC dye-binding protein assay (Bio-Rad Laboratories, Hercules, CA). One milliliter of beverage system was diluted to 25 mL with distilled water and assay completed following the assay kit instructions.

Protein standards were prepared using a 1.36 mg/mL stock bovine serium albumin (BSA) solution (Bio-Rad Laboratories, Hercules, CA) at the following concentrations: 0, 0.2, 0.3, 0.6, 0.9, 1.2 mg/mL. The standards were prepared in distilled water.

Absorbance of each sample was read at 750 nm using a spectrophotometer (Spectronic 1001, Milton Roy Company, Rochester, NY). A standard curve was prepared using the absorbance measurements from the protein standards. The beverage system protein absorbance readings were compared to the standard curve to determine a concentration value. Similar protein concentration of 0.49 ± 0.02 mg/mL was measured from control and lutein-fortified beverage systems. Theoretical concentration was calculated to be 0.56 mg/mL.

Soluble solids were measured using a ABBE MARK II digital refractometer (Reichert Analytical Instruments, Depew, NY). The formulation had a soluble solids content of 6.8. There was not a significant difference in soluble solids content between the lutein-fortified and unfortified beverage systems. Control and beverage system pH was measured using a digital AR14 pH meter (Fisher Scientific, Pittsburgh, PA). A pH of 3.50 was measured with no significant difference detected between the two beverage systems.

Color space values were determined to ensure sensory testing would not be influenced by lutein pigmentation. L*a*b* values were measured for all beverage treatments on day 14 using a Minolta CR-200 Chroma Meter (Ramsey, NJ). Lightness (L*), a* and b* chromaticity were not significantly different at p=0.05 level for all beverage treatments (Table A3). Research investigators also did not visually detect differences based on informal testing. Sensory testing proceeded under white lighting.

3.3.3 Sensory Analysis

Triangle tests are useful in situations when differences in product quality result from changes in formulation, processing, packaging or storage (Meilgaard and others 1999). A

triangle test for difference was used to determine the effect of light oxidation on beverage flavor (Appendix A, Figure A2). Untrained panelists (n = 30) from students, staff, and faculty in the Department of Food Science and Technology at Virginia Tech (Blacksburg, VA) were recruited based on availability and willingness to participate in the study. Testing was conducted in the sensory laboratory of the Food Science and Technology Department at Virginia Tech (Blacksburg, VA). Panelists were seated in individual sensory booths under white lighting and asked to complete a human subject's consent form. The project was approved by the University Institutional Review Board at Virginia Tech.

After completion of the consent form, a tray with a three-sample set, spring water, spit cup, napkin and pencil was presented to each panelist. Each panelist was asked to determine the "different" sample. Samples were identified with 3-digit randomly selected codes. The 3-sample set included two light-protected samples (F – Foil covered bottle) and one light-exposed sample (C – Clear bottle) or two light-exposed samples (C) and one light-protected sample (F). The position of the "odd" sample was randomized to remove positional bias with six possible orders presented (CCF, FFC, CFC, CFF, FCC, FCF). Samples were presented at approximately 7°C.

In a separate test, a rank preference test was conducted. This test compared all four treatments: lutein light-protected, lutein light-exposed, no lutein light-protected, no lutein light-exposed. The panelists were asked to rank the samples from 1 to 4 with "1" being the most preferred and "4" being the least preferred. The position of the samples and letter codes were randomized to remove bias. Samples were presented at approximately 7°C. Fifty-seven panelists completed the test.

3.3.4 Sensory Statistical Analysis

Triangle test data was analyzed by summing the number of correctly identified "different" sample responses. This value was compared with the critical values found in Table 8 in Meilgaard and others (1999) to determine if significant differences existed. Statistical parameters of $\alpha = 0.5$, $\beta = 0.30$ and a $p_d = 30\%$ were used. Thirty panelists were recruited to achieve the minimum number of assessments needed in a triangle test for the selected parameters. The critical number of correct responses in a triangle test for 30 panelists with an $\alpha = 0.05$ is 15 (Meilgaard and others 1999). An $\alpha = 0.05$ was used so that the risk of a Type I error is low to minimize the risk of finding a difference when no difference exists. A Type I error means that the null hypothesis is rejected when it is actually true, or in other words, a difference exists when it does not. A relatively high β (0.30) was used to reduce the number of panelists needed to conduct the triangle test but this did increase the risk that a Type II error could occur. Type II errors occur when the null hypothesis is accepted when it is false, which means that the results fail to detect a difference that actually exists. The p_d value refers to the maximum allowable proportion of distinguishers in the population (Meilgaard and others 1999). A p_d value of 0.30 was chosen for this study since it was assumed that moderate sensory differences would exist.

Rank preference test was analyzed by determining the rank sums for each treatment and then conducting Friedman analysis. A critical significance of 0.05 was used with $n = 57$. Fisher's protected LSD for rank sums was 27.016. For two treatments to be considered significant at the 0.05 level, the rank sum had to differ by at least 27.016.

3.3.5 Gas Chromatography with Solid Phase Microextraction

Gas chromatography (GC) analysis was conducted with solid phase microextraction (SPME) absorption of volatiles from the beverage system pool for both light-exposed and protected dark treatments. Aliquots (3 mL) of beverage were pipetted into 8 ml clear glass bottles wrapped in aluminum foil to protect against light-exposure and fitted with Teflon septa. Sodium chloride (0.75 g) was added to each glass bottle to increase volatility of flavor compounds. A 75-µm carboxen poly(dimethyl siloxane)-coated SPME fiber was exposed to the sample headspace for 22 min at 45ºC with magnetic stirring of the sample.

Volatile compounds from the SPME fiber were desorbed into the injector port of a HP 5890 series II plus chromatograph equipped with a flame ionization detector (FID). The injector temperature was 280°C and detector temperature was 300°C. Separation was completed on a 30 m x 0.32 mm capillary column with helium carrier gas flow rates of 1.5 ml/min and linear velocity of 25.5 cm/sec and a temperature ramp of 15ºC/min for 0.50 minutes and then 20ºC/min for 5.50 min.

Hexanal and limonene concentrations for the light-exposed and light-protected treatments were quantified by comparing peak area counts to standard curves based on addition standards of hexanal and limonene. Stock solutions of hexanal (Sigma-Aldrich, St. Louis, MO) and (R)-(+)-limonene (Sigma-Aldrich, St. Louis, MO) in the orange beverage were prepared at 0, 0.5, 1, 5, and 10 µg/mL concentrations. Duplicate samples (3-mL each) were analyzed using GC-SPME and area counts were used to create a standard curve (Appendix B, Figure B2). Amount of hexanal and limonene in the model beverage with no added hexanal and limonene were also determined and factored into the calibration curve.

3.3.6 Analytical Statistical Analysis

Hexanal and (R)-(+)-limonene concentrations (n = 2) for Day 0 and Day 14, light-exposed, and light-protected treatments were analyzed using SAS (Statistical Analysis Software) Proc GLM Factorial ANOVA. The main effects were light levels (Day 0, Day 14), lutein levels (lutein, no lutein) and replication (1, 2, 3). Two and three-way interactions were tested as well. Mean separation was determined using LS means with a $p = 0.05$.

3.3.7 Microbiological Analysis

Model colloidal beverage system was evaluated using Petrifilm (3M, St Paul, MN) standard aerobic plate count and yeast and molds plate count according to Petrifilm standard methods to ensure treatments and bottles were of similar microbiological quality. Beverage system was evaluated on Day 0 and Day 14 of light exposure (Table A1 and A2).

3.4 RESULTS AND DISCUSSION

Lutein fortification of a model colloidal beverage system that undergoes photo-oxidation could potentially reduce off-flavor formation resulting in increased shelf-life and overall quality. In this study, 14 d light-exposed and light-protected treatments of the model colloidal beverage system with and without lutein were compared through sensory triangle and rank preference tests. Volatile components were compared for 14 d light-exposed and light-protected treatments.

To truly determine if lutein fortification was actually inhibiting light oxidation in the model beverage system, it was important to ensure that changes occurred during light exposure. Light-exposure caused a difference in sensory quality of the control colloidal beverage system (Table 1). Overall, 39 of 60 panelists were able to correctly identify the different samples in the triangle test comparing the light-protected against the light-exposed control beverage. Beverage system changes from light-exposure can be attributed to degradation of susceptible compounds including whey protein, phospholipids and flavorings. A sensory change caused by light degradation of proteins and lipids has been noted in milk, a natural colloidal system and other dairy products (Jung and others 1998; Webster 2006; Shiota and others 2002).

It was also necessary to ensure that the addition of lutein into the colloidal beverage system did not cause a significantly perceivable sensory difference. A triangle test comparing the light-protected beverage system with and without lutein found no difference between samples, validating that lutein addition did not contribute to the sensory profile. These results are consistent with Aryana and others (2006) who found that lutein fortification did not affect flavor in strawberry yogurt.

Table 1. Number of correct responses for each replication of compared beverage system treatments for sensory triangle difference test.

Beverage treatments compared	# of Correct Responses Replication 1	2	3
Control Beverage, Light-protected Control Beverage, Light-exposed	17[1]	22[1]	N/A
Beverage with Lutein, Light-protected Control Beverage, Light-protected	5	13	N/A
Beverage with Lutein, Light-exposed Control Beverage, Light-protected	8	14	15[1]
Beverage with Lutein, Light-protected Beverage with Lutein, Light-exposed	16[1]	16[1]	17[1]
Beverage with Lutein, Light-exposed Control Beverage, Light exposed	16[1]	10	N/A

Total observations = 30 per replication; critical response # = 15; $\alpha = 0.5$, $\beta = 0.30$; $p_d = 30\%$
[1] Significant difference
Overall Significant (p value) based on sum of correct responses compared to critical response.

Viljanen and others (2002) reported that lutein, lycopene, and β-carotene acted as antioxidants during photo-oxidation by inhibiting formation of methyl linoleate hydroperoxides from methyl linoleate in methanol solution. Ideally, lutein fortification would inhibit light-induced sensory degradation to a level comparable to light-protection. To determine if this was true, a triangle test comparing light-exposed beverage with lutein against light-protected control beverage was conducted. No significant difference was detected for 2 of the 3 replications giving positive implications towards the use of lutein to decrease off-flavor formation due to photo-oxidation (Table 1). It is important to note that to reduce the number of panelists needed for sensory analysis; a relatively high β-error was used. This increases the chances of having a Type II error where the results would fail to detect a difference that actually exists.

With one replication showing a significant difference it was important to also determine if the beverage with lutein changed under light-exposure. Light-exposure did cause significant

differences in the lutein-fortified beverage. This suggests that photo-oxidation is still occurring in the lutein-fortified light-exposed sample, indicating that lutein may not fully inhibit photochemical reactions that have sensory impact. As carotenoids absorb light they are consumed and can degrade possibly leading to loss in antioxidant function (Viljanen and others 2002). Exposure to cold white fluorescent light (4600 lux) at 25°C was reported by Shi and Chen (2006) to degrade lutein in solution at 0.8-10.7% per day.

Although light-exposure affected both the lutein-fortified and control beverage, lutein fortification may have lowered the amount of sensory change occurring. A triangle test comparing the light-exposed control against the lutein-fortified beverage yielded mixed results. Replication 1 yielded a significant difference in which 16 of 30 panelists (critical # = 15) were able to correctly identify the different samples in the triangle test comparing the light-protected against the light-exposed lutein-fortified beverage (Table 1). Replication 2 however resulted in no significant difference between samples.

A rank preference test was used to determine panelists' preference of each treatment after 14 d light-exposure (Table 2). Panelists significantly preferred the light-protected treatments over the light-exposed treatments. Overall this suggests that 900-1100 lux light-exposure of the model colloidal beverage system negatively impacts sensory attributes. Panelists did not significantly prefer one light-exposed treatment over the other or light-protected treatment over the other. This may mean that the extent to which photo-oxidation is reduced by lutein fortification is not sufficient to cause panelists to prefer a fortified model colloidal beverage system (Table 2).

Table 2. Average overall scores from preference rank test comparing control beverage system and beverage system with lutein, Light-exposed and Light-protected.

Treatment	Beverage w/ Lutein, Light-protected	Control Beverage, Light-protected	Control Beverage, Light-exposed	Beverage w/ Lutein, Light-exposed
Mean	1.77A	1.89A	3.14B	3.19B

Note: Means not followed by the same letter are significantly different at the 5% level

Value of 1 represents "Most Preferred" with value of 4 representing "Least Preferred."

It is valuable to protect natural flavor systems in formulated beverages from photo-oxidation. Limonene, a natural citrus flavoring component in the beverage system, is susceptible to oxidation due to its unsaturated hydrocarbon structure (Taylor 2002). It was hypothesized that limonene concentration would degrade due to full light and specifically UV (200-400 nm) wavelengths. At d 0, limonene concentration was the same for both beverage systems. Comparison of control treatments light-exposed vs light-protected at 14 d indicated no difference in limonene concentrations. However, the light-protected beverage with lutein had a higher limonene concentration (26.76 ± 5.2 μg/mL) than the light-protected control beverage (22.86 ± 4.3 μg/mL) after 14 d. This may mean that degradation of limonene was still occurring under light-protected conditions. Breakdown of limonene can result in off-flavors due to the formation of α-terpineol, carveol, carvone and p-cymene (Haleva-Toledo and others 1999). The limonene concentration in the light-exposed control beverage and beverage with lutein were not significantly different at 14 d. It can be concluded that under light-protection, lutein may prevent or slow down the degradation of limonene; however under light-exposure conditions lutein was not effective.

Hexanal is the product of linolenic acid oxidation and is characterized by a "green/grassy" or "wet cardboard-like" off-flavor in systems such as milk. Hexanal

concentration was significantly higher in the d 0 control beverage system (2.49 ± 0.24 μg/mL) when compared to the lutein-fortified beverage (2.14 ± 0.28 μg/mL). Overall, hexanal concentration did not significantly change due to the effects of light-exposure on the beverage systems. Hexanal concentration was not closely correlated with the differences seen in the sensory work. This may mean that there are other compounds involved which were not detected using gas chromatography with solid-phase microextraction.

The estimated cost of lutein used in this study was $520/kg (20% lutein) (Pharmline, Florida, NY). Fortified-beverage system was formulated to have 2 mg lutein per 8 oz serving which is a suggested level for energy, sport and isotonic drinks (Kemin Foods, Des Moines, IA). Overall the addition of lutein would cost roughly $0.01 per 16 oz beverage bottle. The costs associated with lutein fortification must be considered when determining the efficacy of using lutein for photo-protection of susceptible compounds. In this study lutein degraded during temperature and light wavelength exposure. Beverage manufacturers may find it necessary to over-fortify (overage) with lutein to achieve the desired delivery level.

3.5 CONCLUSION

Colloidal beverage systems with whey protein, phospholipids and natural citrus flavor are negatively affected by light exposure. Susceptibility can be attributed to photo-oxidation of lipid, protein and flavor components. Consideration should be given to using photo-protective barrier packaging for colloidal beverages. Addition of lutein for photo-protection may not maintain sensory quality to a degree comparable to light-protection. However, it may offer some value in protecting degradation of citrus flavoring in a light-protected system.

The cost of lutein fortification is prohibitive for the function of protection of sensory quality. The addition of lutein to reduce photo-oxidative degradation alone is not recommended. A photo-protective effect may be an added benefit when fortifying beverages with lutein for health benefits.

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CHAPTER IV

LIGHT WAVELENGTH EFFECTS ON A LUTEIN-FORTIFIED MODEL COLLOIDAL BEVERAGE

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4.1 ABSTRACT

The effect of specific light wavelength exposure on quality and stability of control and lutein-fortified beverage systems was evaluated. Lutein degradation was the highest in the full spectrum light, UV (200-400 nm) and 463 nm wavelengths. Degradation during 463 nm wavelength exposure is attributed to riboflavin sensitization and lutein's blue light absorption capability at 450 nm. Lutein degradation occurred in all other wavelength treatments with no light having the least degradation.

At Hr 0, hexanal was present in the control and fortified beverage systems at low concentrations. Hexanal concentration significantly increased in the control beverage, and to a lesser degree in the lutein-fortified beverage, when exposed to full spectrum light and specifically UV (200-400 nm) wavelength. Limonene degraded under all light treatments. The most significant degradation occurred during full spectrum light exposure for both the control and lutein-fortified beverages. Lutein-fortification did not demonstrate a protective effect against degradation of limonene.

The extent of lutein photo-protection may not be great enough to negate lutein degradation when fortifying beverage systems for health benefits. Consideration should be given to using photo-protective barrier packaging to preserve lutein for health benefits.

Key words: Photo-oxidation, Light Wavelengths, Hexanal, Limonene, Lutein

4.2 INTRODUCTION

Colloidal systems such as dairy-based beverages and soft drinks are very common in the beverage market (Anonymous 2003). Functional beverages that impart health benefits beyond basic nutrition are increasingly emerging in the market place (Fass and Jones 2005, Anonymous 2005). Protecting light-susceptible ingredients such as vitamins, proteins and phospholipids in beverages is a challenge to the food industry.

Fluorescent light, commonly used in beverage cases in food service and retail establishments, emits a broad spectrum of ultraviolet and visible light (Rosenthal 1992). Ultraviolet and visible light can cause photochemical reactions in food systems leading to molecular excitation of susceptible compounds (Rosenthal 1992). Molecular excitation is caused by photon energy which is directly related to light wavelengths (Kagan 1993).

When a ground state molecule is promoted to an excited state through light absorption, energy release can occur in several ways. Excited state molecules can release energy through heat, transfer of energy to other molecules, or emission of photons, also known as fluorescence (Albani 2004). Transfer of energy to other molecules can cause damage and also lead to off-flavor formation and other negative sensory characteristics. Riboflavin, a sensitizer found in the whey portion of milk, can become excited by absorbing light at specific wavelengths. Excited state riboflavin can initiate free radical reactions (Dimick 1982). Free radical reactions can lead to lipid and protein oxidation. Hydroperoxides are formed during the initial stages of lipid oxidation, and can be further decomposed to form aldehydes, ketones, lactones, esters and furans. These secondary products lead to off-flavors and rancidity in oxidized beverages (Kamal-Eldin 2003).

Components susceptible to oxidation of a colloidal beverage system include unsaturated fatty acids such as ω-6, flavor compounds (limonene) and whey proteins. Linolenic acid oxidation can form products such as hexanal which can cause off-flavor formation. Limonene is the main odor constituent of citrus and is found in high concentration in orange oil (Taylor 2002). Although it is found in high concentration, aroma impact is low compared to other orange oil odor compounds. Orange oil is a main flavor component in orange flavored beverages. Limonene oxidation results in off-flavor and aroma formation and the production of degradation products such as carveol, carvone and p-cymene. Limonene and its oxidation products have been detected by Marine and Clemons (2003) using using gas chromatography with solid-phase microextraction (SPME).

Light-barrier packaging significantly reduces off-flavor formation by protecting nutrients from oxidation (Mestdagh and others 2005; Webster 2006; van Aardt and others 2001). Antioxidant fortification may lead to a reduction in oxidized components due to light exposure without need for light-protection packaging by delaying oxidation during its initial stages when hydroperoxide formation is increasing slowly (Davies 1997). Efficiency of an antioxidant is based on the structure of the molecule, the structure of the molecule being oxidized and the conditions under which oxidation is occurring (Kamal-Eldin 2003). Carotenoids act as quenchers of oxidation by acting as an acceptor of energy from other excited state molecules and then releasing the energy in the form of heat (Groff and Gropper 2000).

Lutein and zeaxanthin function as antioxidants in the human eye. These compounds have recently been studied in food systems. In the macular region of the human retina, lutein and zeaxanthin act as a shield from short-wavelength radiation that could damage the polyunsaturated fatty acid components of photoreceptors (Sujak and others 1999). Lutein and

zeaxanthin have also been shown to have passive light filtering characteristics in that they absorb blue-light at an absorbance maximum of 450 nm (Sujak and others 1999). In egg yolk lecithin liposomal membranes exposed to UV radiation, lutein and zeaxanthin protected against oxidation damage. Lutein and zeaxanthin may have optimal wavelengths at which they function. Degradation may occur more rapidly at some specific wavelengths than others. However the relationship of wavelength to functionality of lutein and zeaxanthin is not well known.

Lutein for food fortification purposes is typically extracted from marigolds (Subagio and Morita 2000). Marigold pigment is primarily composed of lutein and to a lesser extent zeaxanthin (3-6%) (Hadden and others 1999). It is difficult to separate lutein and zeaxanthin in the extraction process so many lutein sources contain small amounts of zeaxanthin. With this said, it is important to note that when discussing lutein in this paper, some zeaxanthin is present. Lutein is lipid soluble and has been shown to degrade under full light illumination and high storage temperatures (35°C) (Groff and Gropper 2000, Lin and Chen 2005). Stability of lutein in tomato juice under various processing conditions showed a high-temperature-short-time method generated the highest yields of all-*trans* and *cis* forms of lutein (Lin and Chen 2005).

The objective of this study was to determine lutein's stability and effect on aroma-active flavor compounds in a colloidal beverage system when exposed to narrow bandwidths of light.

4.3 MATERIALS AND METHODS

4.3.1 Beverage System Processing:

A model colloidal beverage system (MCBS), with and without lutein, was formulated from whey protein, lecithin, orange-flavored instant beverage mix and water. Whey protein, (Provon 292 Instantized, Glanbia, Monroe, WI) (146.52 g) was hydrated in filtered water (10 L) for 15 min under agitation. Lecithin granules (Annie Kay's Whole Foods, Blacksburg, VA) (7.32 g) were heated in 400 mL filtered water until dissolved and allowed to cool. Lecithin granules contained phosphatidylcholine (260 mg/g), phosphatidylinositol (140 mg/g), and phosphatidylethanolamine (200 mg/g). Lutein extract 20% powder (Pharmline, Florida, NY) (0.44 g) was mixed into dissolved lecithin mixture to formulate the MCBS with lutein but was not added to the control system. Lutein extract contained 21.9% lutein and 2.25% zeaxanthin, of which 99.15% were free xanthophylls. Extract contained tricalcium phosphate as a carrier. Lecithin mixture was stirred into hydrated whey protein. Orange-flavored instant beverage mix (Gatorade, Chicago, IL) (615.4 g) was incorporated into the whey and lecithin solution, slowly, using a handheld mixer. Gatorade instant mix was composed of sucrose, dextrose, citric acid, salt, sodium citrate, natural orange flavor with other natural flavors, monopotassium phosphate calcium silicate (a flow agent), partially hydrogenated soybean and cottonseed oils, and yellow 6.

Both (lutein, no lutein) beverage systems were homogenized in a two stage homogenizer (10,339 kpa (1500 psi) – 1^{st} stage; 3,446 kpa (500 psi) – 2^{nd} stage) (Type DX, Cherry Burrel Corp, Delavan, WI) and Pasteurized at 90.6-92.2 ºC for 2 sec using a tubular heat exchanger (UHT/HTST Lab 25 HV, Microthermics Inc., Raleigh, NC). After heat treatment, the beverage

was cooled to less than 25ºC. The beverage (800 mL) was collected in 7 sterile 1 L glass bottles per treatment. This process was replicated three times.

4.3.2 Preparation of Samples for Specific Light Wavelengths

Beverage systems with and without lutein were exposed to specific (50 nm) light wavelength bands using a Thermo Oriel Photo-Reactor with a 350 W mercury lamp (Model 66902 Universal Arc Lamp Housing, Model 66910 Power Supply, Thermo Oriel Instruments, Stratford, CT) with specific light filters. The specific light wavelength filters included ultraviolet (UV) 200-400 nm and very narrow bandwidth (50 nm) light with maximum absorbance peaks at 463, 516, 567, 610 nm. A full spectrum light treatment was also studied. Beverage system (13 mL) and a stirring bar were placed in a 15 mL quartz crystal vial (Fisher-brand, 50 mm cylindrical cell) and capped with rubber septa. The vial was inserted into a cooling block set to maintain sample temperature at 25° C ± 1°C, and stirred continuously for 12 hours. A constant temperature for the sample was created by continuously pumping 50% solution of antifreeze through the cooling block. A stainless steel thermocouple (Omega Instruments) was inserted into the rubber septa to monitor sample temperature. Light was transmitted from the 350 W mercury lamp through the wavelength filter and onto the crystal vial face. Transmitted light energy was measured using an Oriel energy meter (Model 70260, Oriel Instruments, Stratford, CT). A light-protected control exposed to the same temperature and stirring conditions was also prepared for comparative analysis.

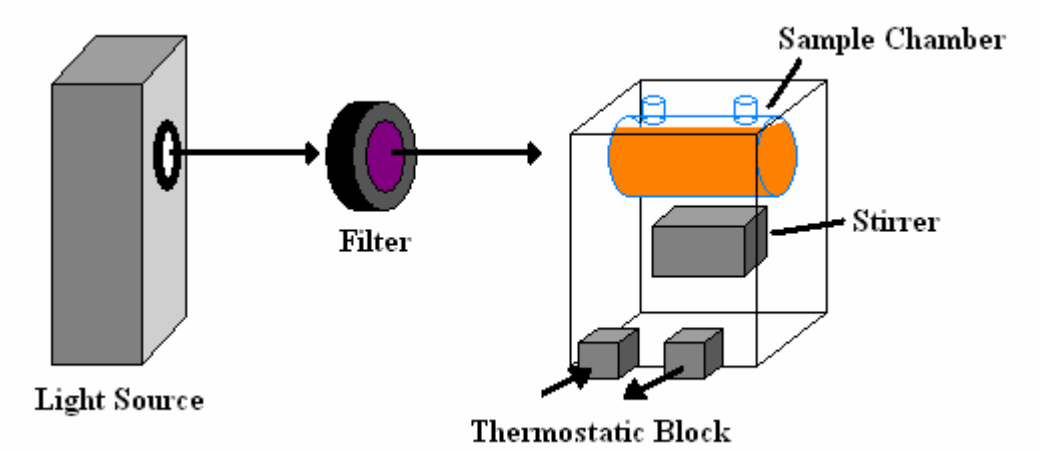

Figure 1. Thermo Oriel Photo-Reactor set up. A 350 W mercury lamp transmitted light through specific wavelength filters onto sample chamber. Thermostated block maintained sample temperature at 25°C.

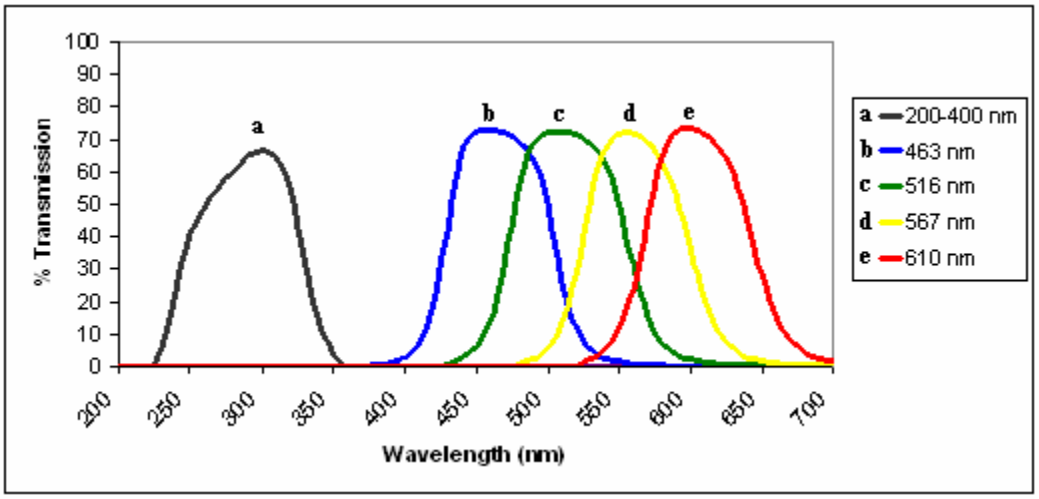

Figure 2. Percent transmission and wavelength bandwidth curves for filters used in the Thermo Oriel Photo-Reactor (adapted from Webster 2006).

4.3.3 Volatile Headspace Analysis Using Gas Chromatography

After light treatment, triplicate samples (3 mL) were transferred, using a hypodermic syringe, to borosilicate glass vials (8 mL) containing 0.75 g NaCl and fitted with Teflon septa. Samples were stirred and heated at 45° C on an RCT basic (IKA Werke, Wilmington, NC) heater

with an ETS-D4 Fuzzy Controller (IKA Werke, Wilmington, NC) for 22 minutes while a 75 μm carboxen polydimethyl siloxane (PDMS) coated solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA) was exposed to the headspace to adsorb volatile compounds. The fiber was positioned approximately 1 cm above the model beverage system surface. Several studies have shown that polydimethylsiloxane fibers are effective at separating limonene and aroma-active products of oxidation (Marine and Clemons 2003, Webster 2006)

Volatile compounds on the SPME fiber were then desorbed into the injector port of an HP 5980 Series II gas chromatograph (GC) with Flame Ionization Detector (FID detector, 30 m RTX-5 column) (Hewlett-Packard Co., Palo Alto, CA). Injector temperature was set to 280° C, detector temperature 300 °C, and the program was run in splitless mode. Volatiles were separated using an RTX-5 (crossbond 5% diphenyl-95% dimethyl polysiloxane, Restek Corp., USA) 30 m x 0.32 mm id x 1.00 μm film thickness capillary column with helium carrier gas at a flow rate of 1.50 mL/min and linear velocity of 25.5 cm/sec. A temperature ramp was used starting with 15° C/min for 0.50 min, then 20° C/min for 5.50 min. HP ChemStation software (Rev. A.05.02[273], Hewlett Packard, Palo Alto, CA) was used to plot and integrate the chromatograms.

Identification of hexanal and limonene compounds was confirmed by standard addition, prepared from stock solutions of hexanal (Sigma-Aldrich, St. Louis, MO) and limonene (Sigma-Aldrich, St. Louis, MO) following the same methodology as above. Compounds were quantified by comparing peak area counts to standard curves prepared using 0, 0.5, 1, 5, and 10 μg/mL concentrations of hexanal and limonene in the orange beverage. Duplicate samples (3 mL each) were analyzed using GC-SPME and area counts were used to create the standard curve.

4.3.4 High Performance Liquid Chromatography (HPLC) Analysis of Lutein

After exposure to wavelength treatment for 12 hours, HPLC extractions were carried out using a similar procedure for extraction of carotenoids from vegetable juice and tomato products (Khachik and others 1992, Tonucci and others 1995). Model beverage (2.5 mL), magnesium carbonate (0.3 g), tetrahydrofuran (4.0 mL), and a stir bar were placed into a 50 mL Erlenmeyer flask wrapped in aluminum foil. Beta-carotene (MP Biomedicals, Inc, Solon, OH) (4,000 µg/mL) internal standard was added to the mixture at 10 µl. Sample was blended for 20 minutes in an ice bath under minimal light, and then filtered through a Whatman No. 1 filter paper on a 250 mL Buchner funnel wrapped in aluminum foil. Components were partitioned into dichloromethane (~ 13 mL) and salt water (25 mg/mL) (~ 8 mL) in a separatory funnel covered in aluminum foil.

After allowing the components to remain undisturbed for 10 minutes, the lower organic layer was removed. The organic layer was washed with salt water (24 mL). Organic layer containing carotenoids was dried over anhydrous sodium sulfate (powder) and filtered through a Whatman No. 42 filter paper on a Buchner funnel wrapped in aluminum foil. Organic layer was brought to volume in a 25 mL volumetric flask with dichloromethane. Solution was then filtered through a 0.45 µm filter into a 12x32 mm Amber crimp top vial. Vial was flushed with nitrogen gas and sealed with an 11 mm aluminum seal with PTFE/Butyl rubber septum. Vials were stored at 0 °C until HPLC analysis.

The dichloromethane extracts were placed in an auto sampler and analyzed on a Waters 2695 HPLC system with Waters 2487 Dual Wavelength using a Luna 5u C18 Reversed Phase

Column (250 x 4.6 mm) (Phenomenex, USA). The mobile phase consisted of 75% acetonitrile: 15% methanol: 5% hexane: 5% dichloromethane at a flow rate of 1 mL/min.

Lutein and internal standard (β-carotene) (MP Biomedicals, Inc, Solon, OH) were quantified (mg/mL of beverage system) by comparing peak area counts to standard curves created from 0, 0.2, 0.4, 0.8, 1.0 mg/mL standards of lutein (Pharmline, Florida, NY) and β-carotene (in dichloromethane).

4.3.5 Analytical Statistical Analysis

GC and HPLC results were analyzed using SAS (Statistical Analysis Software) Proc GLM Factorial ANOVA. The main effects were wavelength (Hr 0, UV (200-400 nm), 463 nm, 516 nm, 567 nm, 610 nm, full light, no light), lutein levels (lutein, no lutein) and replication (1, 2, 3). Two and three-way interactions were tested as well. Mean separation was determined using LS means with a $p = 0.05$.

4.4 RESULTS AND DISCUSSION

Caution must be used when making comparisons of wavelength effect on aroma-active volatile components. In our laboratory variation in light energy due to wavelength filters was determined by Webster (2006). Similar results were determined by Wold and others (2005). In our lab, Webster (2006) found significant energy output (mW) differences between wavelengths studied (Table 1). The highest energy output (8.1 mW) was noted with full light exposure. The lowest energy output (0.301 mW) was found with the UV (200-400 nm) filter. Direct comparison between wavelengths could only be made between 463, 516 and 610 nm due to similar energy measurements at ~ 0.5 mW ($p < 0.05$) while inferences could be made about other wavelengths. Overall energy emission measures were < 1.0 mW for all filters used (Webster 2006). In this study, conditions for the photochemical reactor were identical to those of Webster (2006).

Table 1. Light intensities (mW) for each wavelength filter treatment: UV (200-400 nm), 463, 516, 567, 610 nm, Full Light.

Peak Transmission Wavelength of Filter (nm)	mW (x±sd)
UV (200-400)	$0.301^{d} \pm 0.006$
463	$0.561^{c} \pm 0.002$
516	$0.573^{c} \pm 0.002$
567	$0.951^{b} \pm 0.001$
610	$0.571^{c} \pm 0.005$
Full Light	$8.113^{a} \pm 0.014$

Means followed by the same letter are not significantly different at the $p<0.05$ level. Statistical signitifance was determined using Tukey's HSD.

The effect of specific light wavelength on the concentration of lutein in the fortified beverage system was studied using a modified extraction method and quantification using HPLC. It was hypothesized that lutein concentration would decrease when exposed to full

spectrum light and 463 nm specific wavelength. Lutein has been shown to degrade under full light illumination and high storage temperatures (Lin and Chen 2005). At 450 nm, the sensitizer riboflavin degrades rapidly, forming free radicals that could lead to degradation of lutein (Singleton and others 1963, Sattar and others 1977, Fanelli and others 1985, Webster 2006). Absorption of blue light energy at approximately 450 nm may also play a role in lutein degradation at 463 nm. Significant differences were found as a result of light wavelength. Experimental conditions of heating (25°C) and stirring (12 hrs) also affected lutein concentration.

Hr 0 lutein concentration, measured prior to heating and stirring, was significantly higher than all other treatments including the light protected (no light) treatment (Figure 3). The Hr 0 control was stored under refrigeration temperatures whereas the light protected treatment was stored at 25±1°C (12 hrs) under continuous stirring.

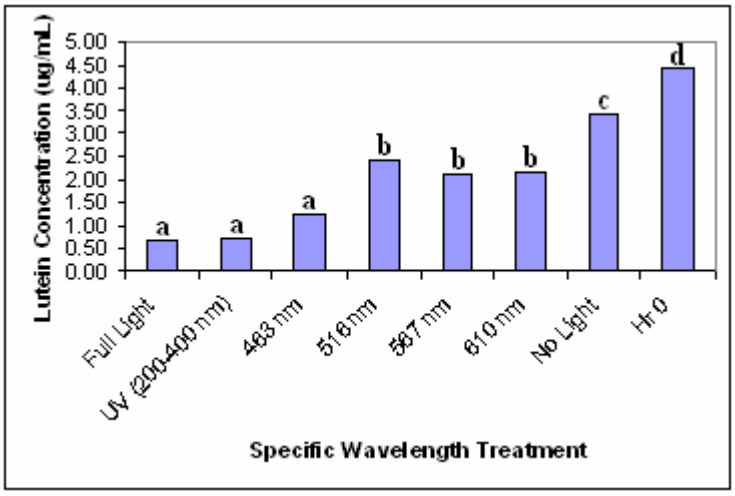

Figure 3. Effect of light wavelength exposure (12 hrs) on lutein concentration (µg/mL) in fortified beverage system quantified by HPLC. **Compounds with the same letter are not significantly different at the p=0.05 level. Statistical analysis conducted using one-way ANOVA with Fisher's LSD to determine differences among treatments.

A 23% loss in lutein occurred in the "no-light" control during 12-hour storage at 25°C. This may mean that some light exposure during preparation of samples occurred. Also the effects of stirring may have incorporated more oxygen into the system from the sample headspace. Temperature differences may also have affected lutein stability.

Exposure to full broad spectrum light, 463 nm, UV-specific wavelengths of light significantly decreased lutein concentration compared to all other treatments (Figure 3). Full broad spectrum light treatment had the highest energy output, so it was expected to cause significant damage to the lutein molecule. It can be inferred that UV light (200-400 nm) is very important in degradation of lutein since the 200-400 nm filter had the second lowest energy output of all treatments. Stability of lutein to light exposure and storage has been previously studied in tomato juice by Lin and Chen (2005). The researchers found that, although all-*trans*-lutein degraded in dark storage conditions, all-*trans*-lutein had a greater degradation under full light illumination. When the researchers compared the concentration of all-*trans*-lutein at storage temperatures of 4 °C, 25 °C and 35°C, degradation was found to be increased at the higher temperatures, further supporting the results observed in our study.

The decrease in lutein concentration when exposed to 463 nm light wavelengths can be attributed to lutein and zeaxanthin's absorption of blue light wavelengths at 450 nm. Since light energy levels were similar for 463, 516 and 610 nm filters, it can be concluded that 463 nm wavelengths cause significantly more damage to lutein than 516 and 610 nm wavelengths. Junghans and others (2001) reported that lutein and zeaxanthin had a blue light filter effect by diminishing fluorescence emission of lucifer yellow in the hydrophilic core of liposomes. Excitation by absorption of blue light can lead to degradation of the carotenoid molecules.

Overall, the results of HPLC quantification of lutein in a beverage system exposed to various wavelength treatments suggest that lutein does degrade during 12 hours of full light exposure at 25 °C. It can be inferred that UV (200-400 nm) and 463 nm wavelengths are the most important wavelengths attributed to lutein degradation. The main food industry implication of these findings is that functional beverages fortified with lutein that are not light protected may undergo lutein degradation resulting in a product with substantially less lutein than initially fortified.

Two peaks on the GC chromatogram, corresponding to hexanal and limonene, were affected by specific wavelength treatment and lutein fortification.

Table 2. GLM Factorial ANOVA output for hexanal concentration, displaying df (degrees of freedom), ms (mean square), F-value, and p-value for main effects: lutein level (lutein, no lutein), replication (1, 2, 3), wavelength (Hr 0, UV (200-400 nm), 463 nm, 516 nm, 567 nm, 610 nm, full light, no light). Two and three-way interactions also are displayed.

Source	DF	MS	F-value	Pr > F
Lutein	1	39.67636	7.99	0.0056
Rep	2	25.48647	5.13	0.0075
Wavelength	8	315.4611	63.53	<0.0001
Lutein*Rep	2	7.200936	1.45	0.2392
Lutein*Wavelength	8	11.94185	2.4	0.0201
Wavelength*Rep	16	31.5756	6.36	<0.0001
Lutein*Wavelength*Rep	16	3.541663	0.71	0.775

Mean separation using LS means, helped to provide further delineation of the light wavelengths that had effects. Factorial ANOVA output for hexanal concentration is shown in Table 2. All main effects (lutein, replication and wavelength) were significant at the $p = 0.05$ level, meaning that differences existed due to lutein fortification, replication and wavelength. We hypothesized that there would be differences associated with lutein fortification and specific wavelength exposure while no differences would exist between replication. The interaction between lutein

and replication was not significant, meaning that there is not a strong relationship between these two effects. A strong relationship, however, did exist between lutein fortification and wavelength treatment. Three-way interaction did not show a strong relationship between lutein, wavelength and replication ($p<0.05$).

Table 3 shows the factorial ANOVA output for limonene concentration. Results were similar to hexanal output. However; lutein fortification did not cause a significant effect. Three-way interaction was significant meaning that a strong relationship existed between the main effects lutein, replication and wavelength.

Table 3. GLM Factorial ANOVA output for limonene concentration, displaying df (degrees of freedom), ms (mean square), F-value, and p-value for main effects: lutein level (lutein, no lutein), replication (1, 2, 3), wavelength (Hr 0, UV (200-400 nm), 463 nm, 516 nm, 567 nm, 610 nm, full light, no light). Two and three-way interactions also are displayed

Source	DF	MS	F-value	Pr > F
Lutein	1	0.103943	0	0.9535
Rep	2	1072.188	35.21	<0.0001
Wavelength	8	527.2599	17.31	<0.0001
Lutein*Rep	2	15.81816	0.52	0.5964
Lutein*Wavelength	8	176.8473	5.81	<0.0001
Wavelength*Rep	16	144.2159	4.74	<0.0001
Lutein*Wavelength*Rep	16	121.369	3.99	<0.0001

Hexanal concentration was significantly higher in the full light and UV (200-400 nm) exposed lutein-fortified beverages (Figure 4). In food systems, breakdown of ω-6 peroxides has been shown to form hexanal, most frequently detected during lipid oxidation (Frankel 1998).

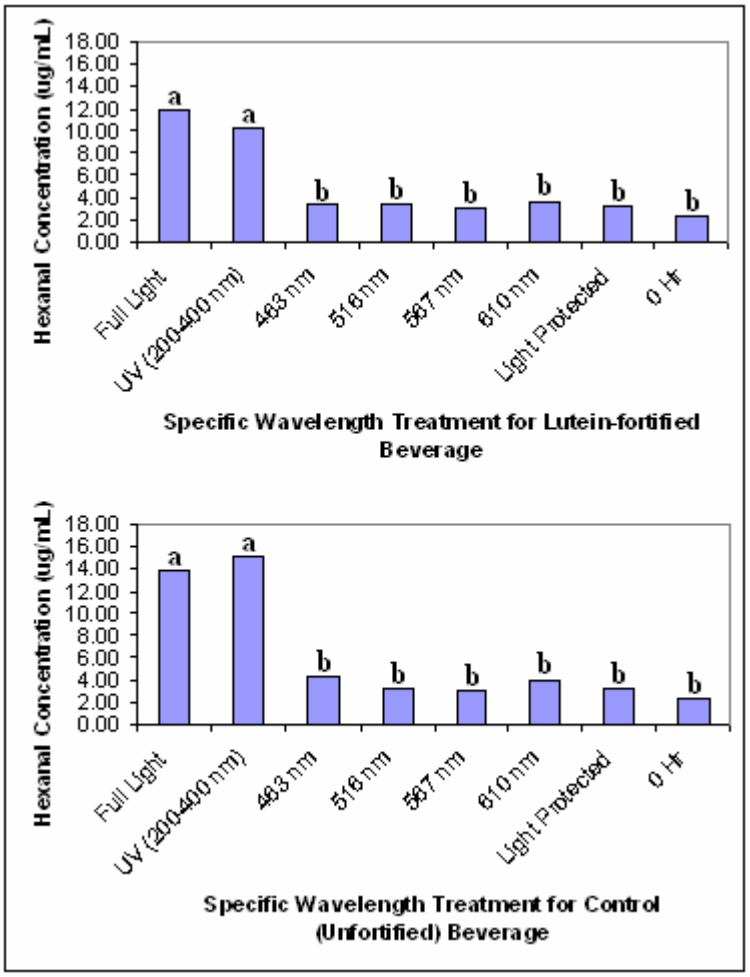

Figure 4. Effect of light wavelength exposure (12 hrs) on hexanal concentration (µg/ml) in lutein-fortified and unfortified beverage systems quantified by gas chromatography with flame ionization detector using solid phase microextraction. **Compounds with the same letter are not significantly different at the p=0.05 level. Statistical analysis conducted using using Proc GLM Factorial ANOVA with mean separation by LS means.

It can be inferred that UV light (200-400 nm) is very important in hexanal formation since the 200-400 nm filter had the second lowest energy output of all treatments. Hr 0 control, light protected, 463 nm, 516 nm, 567 nm and 610 nm wavelength exposures all resulted in relatively the same hexanal concentration (Figure 4). Hexanal is present in the beverage system at low concentrations and attributed to formulation and processing conditions, as evident in the control samples at hr 0 and light-protected (no light) samples. Hexanal concentration at 463, 516 and 610 nm can be directly compared since light energy levels were equivalent. No significant

differences ($p>0.05$) in hexanal concentration among the 463 nm, 516 n, and 610 nm narrow-band wavelengths suggest that these wavelengths do not impact hexanal concentration. This may mean these wavelengths do not cause photo-oxidative damage to the phospholipids (lecithin) in the beverage system.

A low concentration of hexanal in the lutein-fortified beverage at some specific wavelengths could mean that lutein is reducing the degree at which the degradative compound is formed. It was important to compare these findings to the unfortified control beverage system with the same specific wavelength treatments (Figure 4). Similar relationships in hexanal concentrations by wavelength region in the control beverage were found. Hexanal concentration was significantly higher after full light and UV (200-400nm) wavelength exposure compared to all other treatments.

Hexanal concentrations for the lutein fortified and control beverages at each wavelength treatment were compared to determine if lutein fortification affected hexanal production (Figure 6). Significant differences were only found when comparing beverages with and without lutein at the full light and UV (200-400 nm) wavelength treatments. Hexanal concentration was significantly lower for the lutein-fortified beverage compared to the control beverage when exposed to UV (200-400 nm) wavelengths (10.80 µg/mL and 15.93 µg/mL respectively) and full light (11.68 µg/mL and 13.95 µg/mL respectively). A reduction in hexanal concentration during full spectrum light and specifically UV (200-400 nm) could mean that lutein is inhibiting photochemical reactions that result in the formation of hexanal.

Limonene was the major peak observed in the GC chromatogram and was found in all beverage samples. Orange oil is a common flavoring for orange-flavored mixes such as the one used to make the beverage system. Orange oil is composed primarily of limonene which does

not have a major flavor impact however limonene degradation may be an indicator of other flavor component degradation. Limonene has the potential to degrade during light exposure, so it was of interest to determine the effects of specific light wavelength treatments and lutein fortification on relative limonene concentration in the beverage system.

Mean limonene concentration was compared between treatments for lutein-fortified and also for the unfortified control beverages respectively. It was hypothesized that the limonene concentration would decrease due to light-exposure. The hr 0 control was significantly higher in limonene concentration than all other wavelength treatments, including the light-protected sample (Figure 5).

Limonene concentration was not significantly different at 463, 516 and 610 nm and can be directly compared since light energy levels were equivalent. It can be inferred that under wavelength treatment conditions (12 hr, 25°C) limonene concentration declined in general. Limonene concentration under full light conditions was significantly lower than all other treatments.

Mean limonene concentrations were also examined in the unfortified beverage at the same wavelength treatments as above (Figure 5). The light protected and hr 0 control treatments limonene concentrations were significantly higher than the full light exposed treatment. Overall, limonene concentration declined as wavelength decreased.

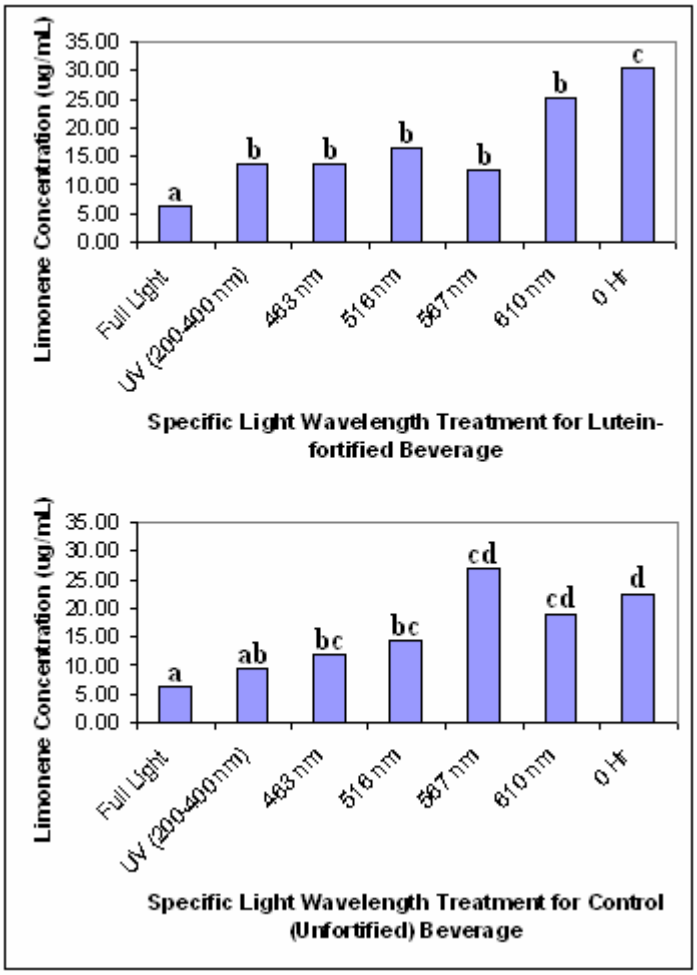

Figure 5. Effect of light wavelength exposure (12 hrs) on limonene concentration (µg/ml) in lutein-fortified and unfortified beverage systems quantified by gas chromatography with flame ionization detector using solid phase microextraction. **Compounds with the same letter are not significantly different at the p=0.05 level. Statistical analysis conducted using using Proc GLM Factorial ANOVA with mean separation by LS means.

A comparison based on wavelength treatment for the lutein-fortified and unfortified beverages was also examined. Overall, changes in limonene concentration did not suggest lutein has a protective effect for limonene against photo-oxidation.

In conclusion, hexanal is an important degradation molecule formed during full light and UV (200-400 nm) exposure in a model beverage system with added proteins and phospholipids. Lutein fortification of the beverage system resulted in a significantly lower hexanal

concentration when compared to the unfortified beverage for full light and UV (200-400 nm) wavelength treatments, however hexanal concentrations were still significantly higher than baseline values. Limonene was also affected by full light exposure. Limonene concentration was significantly lower for full light exposed samples compared to hr 0 control values for both beverage systems. Lutein fortification did not significantly reduce limonene degradation due to light exposure. This may imply that lutein fortification does not inhibit reactions that cause limonene degradation. Lutein's antioxidant function towards other molecules may be dependant on the structure and location within the complex matrices of the beverage system.

The costs associated with lutein fortification must be considered when determining the efficacy of using lutein for photo-protection of susceptible compounds. The fortified-beverage system, used in this study, was formulated to have 2 mg lutein per 8 oz serving which is a suggested level for energy, sport and isotonic drinks (Kemin Foods, Des Moines, IA). The estimated cost of lutein used in this study was $520/kg (20% lutein) (Pharmline, Florida, NY). Overall lutein addition would cost roughly $0.01 per 16 oz beverage bottle.

4.5 CONCLUSION

Exposure to full broad spectrum light and specific wavelengths negatively affects colloidal beverage systems with whey proteins and phospholipids. Susceptibility can be attributed to photo-oxidation of lipid, protein and flavor components. Lutein fortification exhibited a photo-protective effect by reducing hexanal formation under full spectrum light and UV (200-400 nm) wavelength exposure.

Lutein degradation was shown to be a problem in light-exposed functional beverages. The extent of photo-protection may not be great enough to negate lutein degradation when fortifying beverage systems for health benefits. Consideration should be given to using photo-protective barrier packaging to preserve lutein for health benefits.

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APPENDICES

APPENDIX A

Table A1. Standard aerobic bacterial counts (CFU/ml) for sensory shelf-life of control and lutein-fortified colloidal beverage system.

	Standard Aerobic Plate Count (log CFU/mL)	
Treatment	Day 0	Day 14
Replication 1		
Control Beverage, No Light	0	0
Control Beverage, Full Light		0
Beverage with Lutein, No Light	0	0
Beverage with Lutein, Full Light		0
Replication 2		
Control Beverage, No Light	0	0
Control Beverage, Full Light		0
Beverage with Lutein, No Light	0	0
Beverage with Lutein, Full Light		0
Replication 3		
Control Beverage, No Light	0	0
Control Beverage, Full Light		2
Beverage with Lutein, No Light	0	0
Beverage with Lutein, Full Light		0

Day 0 counts are only light-protected counts since light exposure has not occurred yet.

Table A2. Yeast and Mold Bacterial Counts (CFU/ml) for sensory shelf-life of Model Colloidal Beverage system with and without added Lutein

Treatment	Yeast and Mold Plate Count (log CFU/ml) Day 0	Day 14
Replication 1		
Control Beverage, No Light	0	0
Control Beverage, Full Light		0
Beverage with Lutein, No Light	0	0
Beverage with Lutein, Full Light		0
Replication 2		
Control Beverage, No Light	0	2
Control Beverage, Full Light		0
Beverage with Lutein, No Light	0	0
Beverage with Lutein, Full Light		0
Replication 3		
Control Beverage, No Light	2	0
Control Beverage, Full Light		0
Beverage with Lutein, No Light	0	0
Beverage with Lutein, Full Light		1

Day 0 counts are only light-protected counts since light exposure has not occurred yet.

Table A3. Colorimetry L*a*b* color values for 14 d sensory treatments exposed to fluorescent light at 4°C.

	Control Beverage, Light-exposed	Lutein-fortified Beverage, Light-exposed	Control Beverage, Light-protected	Lutein-fortified Beverage, Light-protected
L*	40.27	36.97	37.60	37.17
a*	5.50	4.64	4.98	4.75
b*	18.30	15.30	15.94	15.60

Virginia Tech

Human Subjects Forms for Sensory Evaluation

Protocol for Projects of Sensory Evaluation

If the project involves sensory evaluation, please complete the following questions about the project to assist you and the Institutional Review Board in determining the risk level of the project.

Definition: Sensory evaluation is the evaluation of food or other substances by the senses including taste, touch, smell, sight and hearing.

Check all that apply:

1. The procedure for sensory evaluation in this project involves:
   __x__ Tasting in the mouth (includes tests where the panelist is instructed to spit it out)
   _____ Substances applied to the skin
   __x__ Substances smelled for odor components
   _____ Substances evaluated by sound when chewed
   __x__ Substances evaluated by visual senses

2. The product/s to be evaluated are:
   __x__ Made entirely of ingredients approved by FDA for consumption or application under approved conditions of processing
   _____ Made of ingredients approved by FDA but not approved for the use in the project (e.g. heating of aspartame, fat substitutes approved only as an emulsifier).
   _____ Made partially or entirely of experimental ingredients pending FDA approval.
   _____ Made partially or entirely of experimental ingredients not approved for human consumption or topical use
   _____ Made from materials from or altered by biotechnology

3. The processing or preparation of the product is:
   __x__ By usual approved good manufacturing or preparation practices for that food or topical product.
   _____ By experimental procedures including non-good manufacturing practices. Briefly describe the procedures.

4. The packaging of the product includes:
   __x__ Processing or storage in FDA-approved packaging materials.
   _____ Processing or storage in packaging materials not approved by FDA.

5. Describe the storage protocols for the product that are necessary to maintain the product in safe condition.

Figure A1. Institutional Review Board (IRB) forms.

Product will be stored in sterile sealed containers at 2°C (refrigeration temperature) for less than 15 days

6. If microbiological cultures are a part of the food processing or preparation procedure, describe what cultures will be used, if they will be active on consumption, and give evidence that these cultures are known to be safe for human consumption.
   Microbiological cultures will not be used.

7. Allergies
   __x__ Are any ingredients to be used potentially allergenic as consumed or by topical application? If yes, describe. Have panelists been made aware of these ingredients?
   Whey Protein
   Lecithin
   Lutein
   Zeaxanthin

Panelists will be alerted to the ingredient list and asked if they have any known food allergies.

Virginia Polytechnic Institute and State University
Informed Consent for Participation in Sensory Evaluation

Title of Project: Sensory Evaluation of a Model Beverage System

Principal Investigator: Dr. Susan Duncan, Mark Kline

I. THE PURPOSE OF THIS PROJECT

You are invited to participate on a sensory evaluation panel for a model beverage system. The purpose of this evaluation is to determine if sensory degradation in a model beverage system can be detected when exposed to fluorescent lighting compared to a non-exposed sample. This project will also determine if fortification of the model beverage system with lutein and zeaxanthin changes the flavor profile significantly. The information collected from this sensory evaluation will be used for preliminary work for my thesis research to determine the effects of photo-oxidation on sensory quality and consumer appeal in a beverage system.

II. PROCEDURES

There will be six sensory sessions involving about 15 minutes of your time. In the first four sensory sessions, you will be presented with three triangle tests. In the last two sensory sessions you will be presented with two triangle tests and a rank preference test. As a panelist, it is critical to the project that you attend the session and give your full attention to evaluating the samples. Should you find a sample unpalatable or offensive, you may choose to spit it out and continue to other samples.

Certain individuals are sensitive to some foods such as milk, eggs, wheat gluten, strawberries, chocolate, artificial sweeteners, etc. The ingredient list for this product is:
Sucrose
Dextrose
Citric acid
Salt
Sodium citrate
Natural orange flavor with other natural flavors
Monopotassium phosphate
Calcium silicate (flow agent)
Partially hydrogenated soybean and cottonseed oils
Yellow 6
Whey protein isolate
Lecithin (with ethoxylated monodiglycerides and propylene glycol)
Water
Lutein and zeaxanthin

If you are aware of any food or drug allergies, list them in the following space.

_____

III. BENEFITS/RISKS OF THE PROJECT

Your participation in the project will provide the following information that may be helpful. Results of this session will be used to determine if sensory quality degrades in a model beverage system when it is exposed to fluorescent light compared to a dark control. Your participation will also help to determine if the fortification of the beverage system changes the flavor profile in an adverse way.

Some risks may be involved if you have an unknown food allergy.

IV. EXTENT OF ANONYMITY AND CONFIDENTIALITY

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

V. FREEDOM TO WITHDRAW

It is essential to sensory evaluation projects that you complete the session in so far as possible. However, there may be conditions preventing your completion of the session. If after reading and becoming familiar with the sensory project, you decide not to participate as a panelist, you may withdraw at any time without penalty.

VI. APPROVAL OF RESEARCH

This research project has been approved by the Institutional Review Board for projects involving human subjects at Virginia Polytechnic Institute and State University and by the human subjects review of the Department of Food Science and Technology.

VII.SUBJECT'S RESPONSIBILITIES

I know of no reason why I cannot participate in this study which will require one 10-minute session.

_____

Signature/Date

Please provide your address and phone number so the investigator may reach you in case of emergency or schedule changes.

Address _____

Phone _____

----------(tear off)----------

IX. SUBJECT'S PERMISSION (human subjects copy)

I have read the information about the conditions of this sensory evaluation project and give my voluntary consent for participation in this project.

I know of no reason why I cannot participate in this study which will require one 10-minute session.

_____

Signature Date

Should I have any questions about this research or its conduct, I should contact:

Mark Kline (540) 230-6017
Investigator/Phone

Dr. Susan Duncan (540) 231-8675
Faculty/Phone

Dr. David Moore (540) 231-6077
Chair, IRB/Phone for Research Division

Outline for Protocol to Accompany IRB Request

To assist the IRB with your request, please attach a protocol to the appropriate form with the following information. Typically, the protocol should be not more than two to three pages in length.

JUSTIFICATION OF PROJECT

The purpose of this evaluation is to determine if sensory degradation in a model beverage system can be detected when exposed to fluorescent lighting compared to a non-exposed sample. This evaluation will also determine if fortification of the beverage system with lutein and zeaxanthin changes the flavor profile of the beverage adversely. The information collected from this sensory evaluation will be used for preliminary work for my thesis research to determine the effects of photo-oxidation on sensory quality and consumer appeal in a beverage system.

PROCEDURES

An e-mail will be sent through the Food Science and Technology Department listserv, requesting human subjects in the sensory evaluation of a model beverage (see attached email) Human subjects will be selected based on willingness to participate in the study and will not be limited by age or gender. A total of thirty untrained panelists from the faculty, staff and students in the Food Science and Technology Department at Virginia Tech will be selected to evaluate the model beverage for each session.

There will only be one sensory session per day of sensory analysis, which will take approximately 15 minutes per session. A total of six sensory sessions will be conducted. Each human subject will be asked (but is under no obligation) to participate in one or more sensory sessions. Human subjects may attend all, one, two, three, four, five, or no sessions. No training prior to sessions will be expected from the human subjects.

Human subjects will evaluate the appearance, aroma, and flavor of three sets of three samples (difference test) of the model beverage system for the first four sessions. In the last two sessions, human subjects will evaluate the appearance, aroma, and flavor of two sets of three samples (difference test) of the model beverage system and also one set of four samples (rank preference test) of the beverage system. A difference test will be performed to determine if the subject can choose the sample different from the other two. A rank preference test will be performed to determine subjects' preference for each of the four samples.

The primary investigator for this project is Mark Kline. His training is based on a certificate in Culinary Arts and the background and skills acquired through years of working in the food service industry. He also has achieved a Bachelor of Science Degree in Nutrition: Dietetics Track.

RISKS AND BENEFITS

Some risks may be involved if panelists have an unknown food allergy. Panelists will be alerted to the list of food ingredients, potential food allergens within the ingredient list, and requested to identify any known food allergies.

Results of this test will be used to determine if model beverage system exposed to fluorescent lighting has a different flavor profile compared to one unexposed to the light. The results will also show whether fortification of the beverage system with lutein and zeaxanthin affects the flavor profile in a adverse way.

CONFIDENTIALITY/ANONYMITY

The results of individual panelist performance will be kept strictly confidential. Individual panelists will be referred to by code for analyses and in any publication of the results.

Table A4. Example worksheet for triangle test difference testing for light-exposed versus light-protected, beverage treatments.

Date 1/2/2006	WORKSHEET	Test code	Mark

Post this sheet in the area where trays are prepared.
Code score sheets ahead of time. Label serving containers ahead of time

Type of samples: Model beverage
Type of test: Triangle test

Sample identification:		Codes used for: : Sets with 2 F's		Sets with 2 C's	
C:	Clear container	914		277	821
F:	Dark container	113	122	772	

Code serving containers as follows:

Panelist #	Order of Presentation	Codes in order		
1, 7, 13, 19, 25	C - F - F	914	113	122
2, 8, 14, 20, 26	F - C - F	113	914	122
3, 9, 15, 21, 27	F - F - C	113	122	914
4, 10, 16, 22, 28	F - C - C	772	277	821
5, 11, 17, 23, 29	C - F - C	277	772	821
6, 12, 18, 24, 30	C - C - F	277	821	772

1. Place stickers with panelist's number on tray
2. Select cups "C" or "F" from those previously coded and place on tray from left to right.
3. Write codes selected on panelist's score sheet.
4. Serve samples.
5. Receive filled-in score sheet and note on it the order of presentation used, and whether reply was correct (c) or incorrect (I).

Panelist #: _____

Triangle Test for the Sensory Evaluation of a Model Beverage System

Name: _____ Date: _____

INSTRUCTIONS: Three sets of three samples each are presented in front of you. Two of the samples out of each set are identical and one out of each set is different. Sip the samples in the order they are presented for each set. (You may expectorate the sample if you wish.) Rinse your mouth with water before tasting the next sample. Select the odd/different sample out of each set and indicate by placing an X on the code of the odd sample.

Set 1: **Indicate odd sample**

☐	☐	☐
512	**257**	**442**

Remarks:

Set 2: **Indicate odd sample**

☐	☐	☐
310	**626**	**193**

Remarks:

Set 3: **Indicate odd sample**

☐	☐	☐
776	**272**	**934**

Remarks:

Thank you for your time!

Figure A2. Sample triangle test score sheet 14 d refrigerated storage study.

Table A5. Significance of null and alternative hypotheses for sensory triangle difference test.

Sensory Difference Test	Hypothesis	Result
	H_0: $^1P_{correct\ response} < 15/30^2$	(no significant difference)
	H_1: $^1P_{correct\ response} \geq 15/30^2$	(significant difference)

[1] $P_{correct\ response}$: Proportion of correct identification of the "different" sample in each triangle set of samples
[2] n= 30 independent observations with $\alpha = 0.5$, $\beta = 0.30$ and $p_d = 30\%$

Table A6. Summary sheet for replication 1 panelist responses to a preference rank test comparing control beverage with lutein-fortified beverage, Light-exposed and Light-protected.

	Ranks Assigned to Samples			
Judge	Beverage w/ Lutein, Light-exposed	Control Beverage, Light-exposed	Beverage w/ Lutein, Light-protected	Control Beverage, Light-protected
1	3	4	1	2
2	3	4	1	2
3	3	4	1	2
4	3	4	1	2
5	3	2	4	1
6	2	3	1	4
7	4	1	3	2
8	3	4	1	2
9	3	4	2	1
10	4	2	1	3
11	2	4	1	3
12	3	4	2	1
13	4	3	1	2
14	4	3	2	1
15	4	3	2	1
16	4	3	1	2
17	4	3	1	2
18	2	4	1	3
19	4	3	2	1
20	4	3	1	2
21	4	3	1	2
22	4	3	2	1
23	2	4	3	1
24	3	4	2	1
25	4	3	1	2
26	2	4	1	3
27	4	3	1	2
28	4	3	2	1
Sum	93	92	43	52
Sum^2	8649	8464	1849	2704

Value of 1 represents "Most Preferred" with value of 4 representing "Least Preferred."

Table A7. Summary sheet for replication 2 panelist responses to a preference rank test comparing control beverage with lutein-fortified beverage, Light-exposed and Light-protected.

	Ranks Assigned to Samples			
Judge	Beverage w/ Lutein, Light-exposed	Control Beverage, Light-exposed	Beverage w/ Lutein, Light-protected	Control Beverage, Light-protected
1	2	3	1	4
2	2	4	3	1
3	3	4	2	1
4	4	2	3	1
5	4	3	1	2
6	4	3	1	2
7	2	1	3	4
8	4	3	2	1
9	3	4	2	1
10	4	3	1	2
11	1	2	4	3
12	3	4	1	2
13	4	3	1	2
14	4	2	1	3
15	4	3	2	1
16	2	3	4	1
17	3	4	1	2
18	4	3	1	2
19	4	3	2	1
20	1	4	2	3
21	2	4	1	3
22	4	3	1	2
23	4	2	1	3
24	2	3	4	1
25	3	4	2	1
26	4	3	2	1
27	4	3	1	2
28	2	1	4	3
29	2	3	4	1
Sum	89	87	58	56
Sum^2	7921	7569	3364	3136

Value of 1 represents "Most Preferred" with value of 4 representing "Least Preferred."

APPENDIX B

Table B1: Mean lutein concentrations ± standard deviation (µg/ml) from three experimental replications for all specific light wavelength treatments by HPLC. Statistical analysis conducted using one-way ANOVA with Fisher's LSD to determine differences between treatments.

	Mean Lutein Concentration		
Hr 0	4.44^{a}	±	0.47
Light Protected	3.41^{b}	±	0.39
516 nm filter	2.43^{cd}	±	0.71
610 nm filter	2.18^{d}	±	0.21
567 nm filter	2.14^{d}	±	1.04
463 nm filter	1.24^{e}	±	0.12
UV (200-400 nm) filter	0.69^{e}	±	0.17
Full Light (no filter)	0.68^{e}	±	0.09

Means followed by the same letter are not significantly different at the p = 0.05 level.

Table B2: Mean hexanal concentrations ± standard deviation (µg/ml) from three experimental replications comparing each specific wavelength treatment for lutein-fortified and unfortified control beverage system by GC SPME. Statistical analysis conducted using Proc GLM Factorial ANOVA. Mean separation was determined using LS means with a p = 0.05.

	Mean Hexanal Concentration					
	Lutein-fortified			Unfortified Control		
Hr 0	2.38[b]	±	0.62	2.44[b]	±	0.65
Light Protected	3.40[b]	±	1.58	3.23[b]	±	0.49
516 nm filter	3.43[b]	±	0.57	3.37[b]	±	0.67
610 nm filter	3.57[b]	±	0.88	3.91[b]	±	1.1
567 nm filter	3.05[b]	±	0.33	3.06[b]	±	0.74
463 nm filter	3.53[b]	±	0.72	4.41[b]	±	1.87
UV (200-400 nm) filter	10.22[a]*	±	5.92	15.2[a]*	±	10.23
Full Light (no filter)	11.79[a]*	±	2.07	13.85[a]*	±	2.11

Means within a column followed by the same letter are not significantly different at the p = 0.05 level.
Means within a row followed by an asterisk are significantly different at the p = 0.05 level.

Table B3: Mean limonene concentrations ± standard deviation (µg/ml) from three experimental replications comparing each specific wavelength treatment for lutein-fortified and unfortified beverage system by GC SPME. Statistical analysis conducted using Proc GLM Factorial ANOVA. Mean separation was determined using LS means with a p = 0.05.

	Mean Limonene Concentration					
	Lutein-fortified			Unfortified		
Hr 0	30.48[b]	±	20.54	22.38[d]	±	10.24
Light Protected	12.84[b]	±	4.28	25.9	±	13.14
516 nm filter	16.56[b]	±	6.72	14.52[bc]	±	3.81
610 nm filter	25.42[b]	±	31.73	18.86[cd]	±	9.54
567 nm filter	12.58[b]	±	3.58	26.84[cd]	±	9.91
463 nm filter	13.53[b]	±	3.66	11.7[bc]	±	2.34
UV (200-400 nm) filter	13.65[a]	±	11.93	9.4[ab]	±	3.74
Full Light (no filter)	6.57[a]	±	2.34	6.43[a]	±	2.68

Means within a column followed by the same letter are not significantly different at the p = 0.05 level.
Means within a row followed by an asterisk are significantly different at the p = 0.05 level.

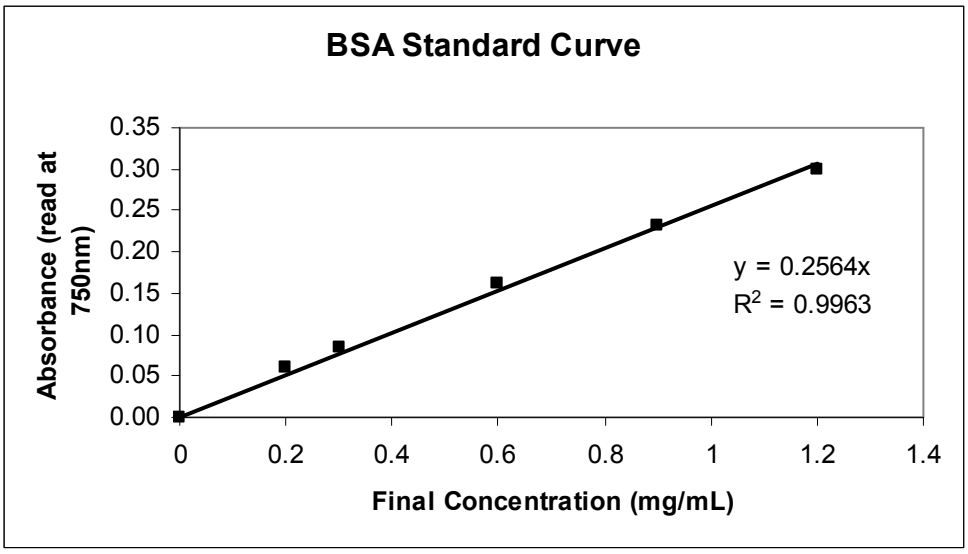

Figure B1. BSA standard curve prepared by measuring absorbance of BSA protein standards at 0, 0.2, 0.3, 0.6, 0.9 and 1.2 mg/mL

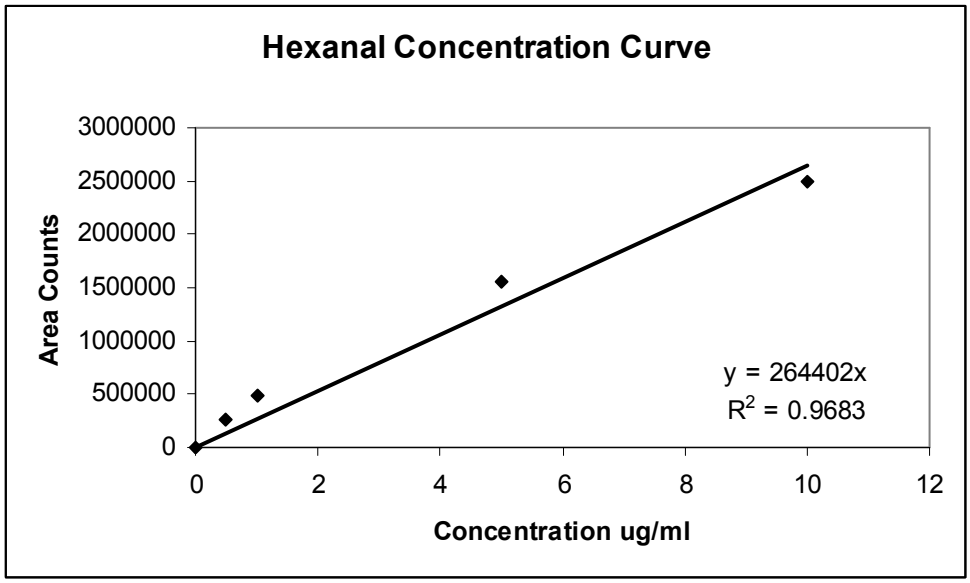

Figure B2: **Hexanal concentration curve prepared by measuring the peak area counts by hexanal concentration (µg/mL).**

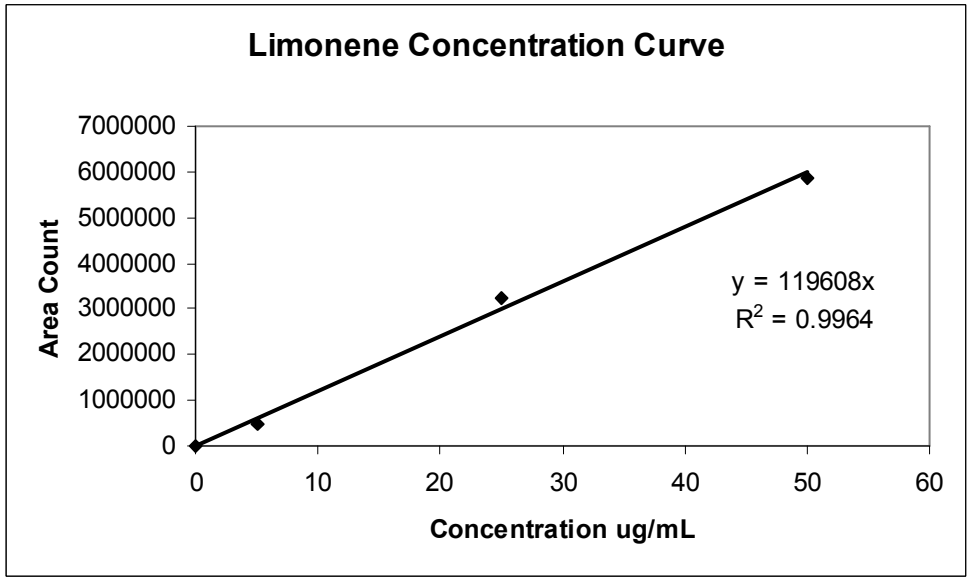

Figure B3. Limonene concentration curve prepared by measuring peak area counts by limonene concentration (µg/mL)

CURRICULUM VITAE

Mark Kline

Education

Virginia Tech — Blacksburg, VA
Masters Degree in Food Science & Technology — December 2006

Indiana University of Pennsylvania (IUP) — Indiana, PA
Bachelor of Science in Nutrition — December 2004
Minor in Chemistry — December 2004

IUP Academy of Culinary Arts — Punxsutawney, PA
Certificate of Completion in Culinary Arts — December 2001

Work experience

Masterfoods USA. — Elizabethtown, PA
Product Development Scientist — Anticip Jan 2006

Hagelin Flavors — Branchburg, NJ — May 2006-August 2006
Intern

- Tested beverage, candy, health care and other product formulations to match or improve flavor for customers
- Prepared and conducted sensory tests to determine effects of flavor reformulations on quality of products
- Conducted flavor descriptor sessions to improve and expand flavor library

Virginia Tech — Blacksburg, VA
Teaching Assistant for Dairy Products Sensory Evaluation — Fall 2006

- Prepared and administered quizzes and exams
- Trained students in evaluation of milk, cottage cheese, ice cream, butter, cheddar cheese, Swiss-style strawberry yogurt for various defects

Teaching Assistant for Food Product Development — Fall 2005

- Administered exam and answered questions

Teaching Assistant for Wines and Vines — Spring 2005

- Administered exams and calculated grades
- Planned and prepared wine tasting sensory evaluations

Learning Enhancement Center Indiana, PA

Workshop Leader for College Undergraduate Success Program (CUSP)
First week Fall 2003 & 2004

- Prepared and conducted workshops on college life

Supplemental Instructor for Cell Biology Spring 2003 & Fall 2003

- Attended classes and took notes
- Prepared and conducted two hour-long weekly group tutoring sessions
- Met with SI Supervisor and other SI leaders

Tutor for Cell Biology Spring 2003

- Helped students in a one-on-one atmosphere

Professional membership

President of Dairy Products Evaluation Team Club	(2006)
Institute of Food Technologists	(October 2004-Present)
Food Science Club Member	(Fall 2005 – Fall 2006)
Phi Eta Sigma National Honor Society	(Fall 2003 – Present)

Volunteer experience

IFT oral presentation at 2006 IFT convention	(2006)
IFT Product Development Team Captain	(2005-2006)
Danisco Product Development Team Captain 2nd place win with Sweetzza Concept	(2006)
Competed in Collegiate Dairy Products Eval Contest	(2005)
Danisco Product Development Team Captain	(2005)