Effects of lipid oxidation initiators and antioxidants on the total antioxidant capacity of milk and oxidation products during storage

by

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ABSTRACT

Oxidized flavor is a major problem currently affecting the dairy industry. The objectives of this research were to determine the effects of light (2300 Lux) and/or cupric sulfate (0, 0.5, 1 mg/kg) as pro-oxidative factors in milk during an 11-day refrigerated storage period. The effects of added vitamin A (2113 IU/L) and vitamin E (100 mg/kg milk fat) as potential inhibitors of oxidation in the presence of light (2300 Lux) and light with cupric sulfate (0.05 mg/kg) during a 7-day refrigerated storage were also investigated. The presence of pro-oxidants significantly decreased the total antioxidant capacity (TAC) and increased the oxidation products in milk during storage. Light had a greater effect in the decrease of TAC, while cupric sulfate in the presence of light significantly increased oxidation products. Added vitamin A and E showed a significant (p<0.05) increase in TAC when samples were exposed to light on day 0. On day 1, only vitamin A significantly increased TAC when samples were exposed to light and cupric sulfate. Oxidation products were not affected by the presence of either vitamin A or E. Sensory results showed that the presence of light and cupric sulfate significantly increase oxidized flavor score from the control, but no significant difference in oxidized flavor intensity was detected between milk with added vitamins and milk without added vitamins.
CHAPTER 1

GENERAL INTRODUCTION

A common defect affecting commercial bovine milk is the development of oxidized flavor. This flavor defect can be described as cardboardy, painty, fatty and metallic (Alvarez 2009) and can be a major cause for rejection of milk products by consumers. In 1995, a New Zealand research study determined that one-third of females surveyed did not consume milk due to dislike of the taste (Howard et al. 1995). This research was followed by Porubcan and Vickers (2005) who reported that among people who disliked milk, they do so because of its aftertaste. It was also reported that light-induced oxidized samples were perceived as having a sour and fatty coating aftertaste that lingered longer than for all other samples presented. This sample also received the lowest flavor score and was ranked as the 2nd highest “would not drink” sample.

The development of oxidized flavor has been linked to the imbalance of pro-oxidative and antioxidative factors in milk (Nielsen et al. 2002). Lipid oxidation has been a major contributor to this flavor defect and the effects of common pro-oxidants such as light and metals have been extensively researched (O’Connor and O’Brien 2006). The prevalence of oxidized flavor has been attributed to the use of translucent packaging and illuminated dairy cases (Mestdagh et al. 2005). Chapman et al. (2002) reported that milk remained in the dairy case for at least 8 hr, and untrained consumers could detect differences between light-exposed samples (2000 Lux) and non-illuminated samples after 54 min to 2 hr of exposure.

Due to the many factors that affect the oxidative stability of milk, inconsistent results have been reported in the use of antioxidants as inhibitors of oxidized flavor. Depending on the
conditions, some of the antioxidants commonly found in milk, such as α-tocopherol, carotenoids, retinol and ascorbic acid, can exhibit varying levels of antioxidant activity or even pro-oxidant activity.

The objective of this research was to investigate the effects of cupric sulfate and/or light as lipid oxidation initiators and determine if the addition of vitamin A or E in the presence of these pro-oxidants can inhibit oxidation in milk during refrigerated storage. Understanding the interactions of antioxidative and pro-oxidative factors in milk will help prevent the development of oxidized flavor in milk and allow dairy producers to provide a quality product to consumers.
Chapter 2
LITERATURE REVIEW

Bovine milk has been part of the human diet for centuries. The consumption of milk and other dairy products varies depending on sex, age, ethnicity and other cultural factors. The United States and Canada are considered high consumers of dairy, and this is attributed to the many nutritional benefits as well as the sensory attributes of these products (MacDonald 2010).

**Milk Composition**

Whole milk is composed of 88% water, 3.3% fat, 3.3% protein, 4.8% carbohydrate, and some other minor constituents such as vitamins and minerals (USDA 2014); however, its composition can vary due to lactation, genetics, season and feed (Heck and others 2009; Juhlin and others 2010a).

The majority of lipids found in milk are in the form of triacylglycerols, which play a major role in the density and melting characteristics of milk fat. Other lipids, which include diacylglycerols, monoacylglycerols and free fatty acids, are likely intermediate products of fatty acid synthesis. Phospholipids and cholesterol, although found in small amounts, play a major structural role in the lipid system (Table 1) (MacGibbon and Taylor 2006).

Milk fat is normally present in the form of droplets or globules ranging from 0.2 to 10 µm in diameter. These globules are surrounded by the milk fat globule membrane (MFGM), which is composed of highly unsaturated polar phospholipids and proteins. The role of the MFGM is to protect the globules from coalescing with each other and from disruption from outside forces (Jensen 2002).
Table 1. Lipids in milk\(^1\)

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Concentration in the United States (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
<td>0.02-1.00</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.42</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>97.00-98.00</td>
</tr>
<tr>
<td>1,2 Diacylglycerol</td>
<td>0.28-0.59</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.10-0.44</td>
</tr>
<tr>
<td>Monoacylglycerol</td>
<td>0.16-0.38</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>Trace</td>
</tr>
</tbody>
</table>

\(^1\)Jensen 2002

Due to the presence of microorganisms in the rumen, over 400 fatty acids have been found in bovine milk (Fox 2003). The most abundant fatty acids in milk are palmitic and oleic acid (Table 2). Unusual fatty acids with beneficial health effects and uncommon functional properties have also been reported in milk, such as short chain, medium chain, branched chain and odd chain fatty acids and conjugated linoleic acid (Chilliard and others 2000; Vlaeminck and others 2006).

Table 2. Composition of the major fatty acids in milk fat\(^1\)

<table>
<thead>
<tr>
<th>Lipid acid carbon number</th>
<th>Fatty acid common name</th>
<th>Average range (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:0</td>
<td>Butyric</td>
<td>2-5</td>
</tr>
<tr>
<td>6:0</td>
<td>Caproic</td>
<td>1-5</td>
</tr>
<tr>
<td>8:0</td>
<td>Caprylic</td>
<td>1-3</td>
</tr>
<tr>
<td>10:0</td>
<td>Capric</td>
<td>2-4</td>
</tr>
<tr>
<td>12:0</td>
<td>Lauric</td>
<td>2-5</td>
</tr>
<tr>
<td>14:0</td>
<td>Myristic</td>
<td>8-14</td>
</tr>
<tr>
<td>15:0</td>
<td>Pentadecanoic</td>
<td>1-2</td>
</tr>
<tr>
<td>16:0</td>
<td>Palmitic</td>
<td>22-35</td>
</tr>
<tr>
<td>16:1</td>
<td>Palmitoleic</td>
<td>1-3</td>
</tr>
<tr>
<td>17:0</td>
<td>Margaric</td>
<td>0.5-1.5</td>
</tr>
<tr>
<td>18:0</td>
<td>Stearic</td>
<td>9-14</td>
</tr>
<tr>
<td>18:1</td>
<td>Oleic</td>
<td>20-30</td>
</tr>
<tr>
<td>18:2</td>
<td>Linoleic</td>
<td>1-3</td>
</tr>
<tr>
<td>18:3</td>
<td>Linolenic</td>
<td>0.5-2</td>
</tr>
</tbody>
</table>

\(^1\)Jensen 2002
There are six major proteins found in bovine milk. These proteins, are classified as casein and whey proteins, and account for approximately 82% and 18% of all proteins in milk, respectively. The casein proteins include αs1-casein, αs2-casein, β-casein and κ-casein. The whey proteins include β-lactoglobulin and α-lactalbumin. Casein proteins are insoluble at pH 4.6, while whey proteins are soluble in these conditions. This property allows the precipitation of casein and the production of other dairy products such as acid-catalyzed cheeses, caseinates and fermented milk products (Fox 2003).

The main carbohydrate in milk is lactose, a reducing disaccharide comprised of glucose and galactose (Fox 2003). Lactose is responsible for the mild sweet taste of milk and can participate in Maillard reactions that provide a desirable brown color to food products (Yang and Silva 1995). Some undesirable properties of lactose include low digestibility and intolerance for some individuals, but many dairy products like butter, certain cheeses and yogurt only contain negligible amounts of this carbohydrate (MacDonald 2010).

The total mineral content of bovine milk is 7.3 g/L and it includes both macrominerals and trace elements. Macrominerals are defined as being present in the human body at 0.01% by weight or greater and these include sodium, potassium, calcium, chloride, magnesium and phosphorus (Flynn and Cashman 1997). Dairy products are the food group with the highest calcium content in a highly absorbable form and they provide about 60% of the calcium in western diets (de Vrese and others 2010). Some of the essential trace elements found in milk are iron, zinc, copper, manganese, selenium and iodine (Flynn and Cashman 1997).

Milk and other dairy products are good sources of water-soluble and lipid-soluble vitamins. Riboflavin is one of the most important water-soluble vitamins in milk because of its
contribution to the overall riboflavin status of the United States population. The average
content of riboflavin in milk ranges from 80 to 250 µg/100 g (Oste and others 1997). Boisvert
and others (1993) found that a high correlation exists between frequency of milk intake and
riboflavin status. Milk is also a good source of thiamin, niacin, pantothenic acid, folic acid,
pyridoxine and biotin (MacDonald 2010). Ascorbic acid is present in milk at low concentrations
(2.11 mg/100g) compared with other water soluble vitamins. Large variations in ascorbic acid
can be explained by the difference in storage and handling of milk and the susceptibility of
ascorbic acid to oxidation (Oste and others 1997).

Dairy products are good contributors of lipid-soluble vitamins in the American diet. Milk
is an especially a good source of vitamin A (50 µg/100 g) (MacDonald 2010). Thus, due to the fat
removal in reduced-fat and fat-free milk, vitamin A content is lowered. All reduced-fat milk is
required to be fortified with vitamin A to a concentration of 2000-3000 international units (IU)
per quart (Newcomer and Murphy 2001). There are eight naturally occurring forms of vitamin E
with biological activity. These are classified as tocopherols and tocotrienols (Oste and others
1997). The main form of vitamin E found in milk is α-tocopherol at about 13-30 mg/kg milk fat
(Van Aardt and others 2005a). Vitamin E is the only vitamin found in the MFGM and it plays a
major role as an antioxidant (Jensen and Nielsen 1996). Lipid-soluble vitamins are more
dependent on the feed of the cow, so, large variations can occur during different seasons
(Butler and others 2008). Although milk is not a good source of vitamin D, fortification of milk
(400-600 IU per quart) is helping consumers meet their daily vitamin D needs (Calvo and others
2004).
Milk Flavor

Fresh milk has a bland and delicate flavor. It has a slightly sweet and salty taste due to lactose and salts, and no unpleasant aftertaste (O’Connor and O’Brien 2006). Because of its bland taste, off-flavors in milk are easily noticeable and the compounds responsible for these flavor defects are either transmitted or generated from hydrolysis, oxidation, enzymatic or microbial activity in milk (Nursten 1997).

Off-flavors can be transmitted to milk through cow feed. Weed flavors like garlic, onions and peppergrass can also be transmitted to milk if cows are milked within 4-5 hr after ingesting odorous weeds (Bassette and others 1986). Cowy and barny flavors are transmitted to milk if cows are housed in a non-ventilated area (Jeon 1996). Lipolized flavors arise from enzymatic activity of endogenous lipases or exogenous lipases of microbial origin. Lipases from psychrotrophic bacteria are common microbial lipases that can give rise to lipolized flavors (Goff and Hill 1993). The native lipase in milk responsible for lipolized flavor is lipoprotein lipase (Deeth and Fitz-Gerald 2006). This enzyme is only activated when the MFGM is damaged (Wedding and Deeth 2009). Enzymatic activity products include acids, lactones, aldehydes and ketones that can have low flavor thresholds (Fitz and others 1999). Although pasteurization usually contributes to the inactivation of enzymes present in milk, enzymatic reactions can still occur before the heat treatments or towards the end of shelf life (Deeth and Fitz-Gerald 2006).

Oxidized Flavor

Oxidized flavor in milk continues to be a common problem affecting the dairy industry (Visscher 2001). Although oxidized flavor is a general term for this type of flavor defect, it can be classified as light-induced, metal-induced and spontaneous oxidized flavor.
Light-induced oxidized flavor is described as burnt hair, plastic, burnt feathers and cabbage-like. It can also be called light-activated, sunlight or sunshine flavor. This type of off-flavor has a specific smell that is described as “wet cardboard” or “wet paper” and it is quite different from the smell of other types of oxidation in milk (Alvarez 2009). Light-induced oxidized flavor is developed in the presence of light and a photosensitizer such as riboflavin, which can act as a pro-oxidant under light (Choe and others 2005). Cadwallader and Howard (1998) identified the development of hexanal, pentanal and dimethyl sulfide in milk after 18 hr of light exposure at 2150 Lux. Heptanal, dimethyl disulfide and lipid derived alcohols such as 1-hexanol and 1-heptanol have also been reported in light exposed milk (Webster and others 2009; Jung and others 1998a; Van Aardt and others 2005b).

Metal-induced oxidized flavor is mostly characterized as a metallic and cardboard-like flavor (O’Connor and O’Brien 2006); these descriptors have been linked to the presence of 1-octen-3-one and octanal, respectively (Hammond and Seals 1972). A puckering mouthfeel is also associated with this flavor, predominantly after expectoration or swallowing (Alvarez 2009). Metal-induced oxidized flavor develops mainly due to the presence of naturally occurring metals in milk, which can participate in the initiation of auto-oxidation reactions of dairy lipids (Jeon 1996). Other compounds detected in milk containing high concentrations of copper are pentanal, hexanal, heptanal, nonanal, 2-octenal and 2-nonenal (Marsili 1999; Jenq and others 1988).

Although the development of spontaneous oxidized flavor is not well understood, certain factors such as genetic predisposition (Juhlin and others 2010a) and the balance of antioxidative and oxidative factors have been linked to this flavor (Granelli and others 1998).
Parks and others (1963) indicated that 2, 4 decadienal, an alkenal associated with oily and deep fried flavor, was linked to spontaneous oxidized flavor in milk (O’Connor and O’Brien 2006).

**Lipid Oxidation in Milk**

Lipid oxidation is one of the most basic chemical reactions in food. It can be responsible for the desirable flavor of fried foods, but it can also be responsible for the development of off-flavors of many other food products (Zhang and others 1994). Lipid oxidation in milk and other dairy products has been extensively reviewed (O’Connor and O’Brien 2006; Sattar and others 1975). The presence of volatile compounds produced from the decomposition of primary oxidation products can cause milk to become unpalatable and be rejected by consumers (Vazquez-Landaverde and others 2005; Hough and others 2002).

Aside from the sensory impact of lipid oxidation products, nutritional impacts are also of concern when consuming products with a high level of oxidation. Although lipid oxidation can lead to a decrease of the essential polyunsaturated fatty acids present, this effect is negligible compared to the amount of lipids present. A bigger effect is the interaction of lipid oxidation products with other essential nutrients such as vitamins (Wasowicz and others 2004). Light exposure, which can initiate photo-oxidation reactions in milk, has been linked to a decrease of retinyl palmitate, α-tocopherol, ascorbic acid and riboflavin (Gaylord and others 1986; Havemose and others 2004).

Lipid oxidation reactions can occur by photo-oxidation and autoxidation pathways (El-Beltagi and Mohamed 2013). A bigger focus is given to non-enzymatic oxidation reactions in dairy products because these reactions are major contributors to oxidized flavor development.
Photo-oxidation Reactions involving Milk Lipids

Photo-oxidation reactions require the presence of light, a photosensitizer and triplet state oxygen. Photosensitizers include chlorophyll, heme, flavonoids, vitamin K and riboflavin (Webster and others 2007). Photo-oxidation in milk mostly involves riboflavin as the main photosensitizer due to its high concentrations in milk; other photosensitizers include chlorophyll a and b (Wold and others 2005). In the presence of light, a photosensitizer absorbs the light energy, leading to the shift of one of its electrons to a higher energy state. This change in energy state transforms the ground state photosensitizer to an unstable excited singlet state photosensitizer. Singlet state photosensitizers can then either lose the energy as heat/light or go through intersystem crossing. The latter pathway converts the photosensitizer from an excited singlet state to an excited triplet state (Kim and Min 2003).

Triplet state photosensitizers can undergo two mechanisms: type I and type II. The type I pathway is the direct interaction of the excited triplet state sensitizer with a substrate. The reduction potential of triplet state riboflavin was found to be 1.7 V in a neutral pH, which allowed it to remove electrons or hydrogen atoms from polyunsaturated fatty acids, ascorbic acid or tocopherols, resulting in the production of free radicals (Van Dyck 2010). An excited triplet state sensitizer can also react with triplet oxygen to produce a superoxide anion, but this reaction only occurs in less than 1% of the total reactions of this molecule (Kim and Min 2003).

The type II pathway involves the energy transfer from the excited triplet state photosensitizer to triplet oxygen via triplet-triplet annihilation. The reaction rate of triplet state riboflavin with triplet oxygen is $9 \times 10^8 / M/s$ (Choe and others 2005). This energy transfer results in the formation of singlet state oxygen and a low energy ground state singlet sensitizer (Kim...
and Min 2003). Singlet oxygen is an electrophilic and highly reactive molecule capable of interacting directly with the double bonds of unsaturated molecules through “ene” reactions. The reaction of singlet oxygen with linoleic and linolenic acids through “ene” type addition leads to both conjugated and nonconjugated diene hydroperoxides, which differs from the triplet oxygen reactions that only produce conjugated diene hydroperoxides (Min and Boff 2002).

The use of type I or type II pathways is dependent on the type of sensitizer, concentrations of substrate, and concentration and solubility of triplet oxygen. A highly nonpolar environment such as an oil or a high lipid food will favor the type II pathway because of the high solubility of oxygen in these types of systems (Kim and Min 2003). Although, water-based systems like milk are likely to favor type I pathways, singlet oxygen formation (Type II pathway) has been reported in skim milk exposed to light (Bradley and others 2003; Jung and others 1998a).

**Autoxidation Reactions Involving Milk Lipids**

Autoxidation is a free radical chain reaction involving molecular oxygen, also called triplet state oxygen, leading to the formation of hydroperoxides as primary oxidation products. This reaction consists of initiation, propagation and termination steps. (Figure 1).
The initiation step begins by the formation of lipid radicals from the action of external energy sources such as light, heat or metals (Kamal-Eldin and others 2003). Matsushita and Terao (1984) reported that products formed from photo-oxidation reactions of fatty acid esters can lead to the initiation of autoxidation reactions. Hydroxyl radicals have also been reported to act as autoxidation chain initiators in lipid oxidation (Choe 2003). Lipid radicals or alkyl radicals are formed from the abstraction of hydrogen atoms of lipid molecules. The hydrogens from monoallylic and bisallylic carbons are usually abstracted because of the low bond dissociation energy needed for removal. The bond dissociation energy for monoallylic and bisallylic carbon positions are 88 kcal/mol and 75-80 kcal/mol, respectively; therefore, highly unsaturated lipids are more susceptible to hydrogen removal (Wagner and others 1994).

In the propagation step, triplet state oxygen reacts with alkyl radicals to form lipid peroxyl radicals. The type of lipid will determine the composition of lipid peroxyl radicals formed (Kamal-Eldin and Pokorny 2005). Peroxyl radicals have a 1.0 V standard reduction
potential (Choe 2003), which allows them to easily abstract a hydrogen from other lipid molecules. This reaction leads to the accumulation of lipid hydroperoxides, a primary oxidation product, and more fatty acid radicals that can continue this cycle. The termination step involves the formation of non-radical stable products (Kamal-Eldin and Pokorny 2005).

Hydroperoxides are unstable, flavorless and odorless compounds, but their decomposition leads to the development of off-flavors (Shahidi 2000). Metal ions play a major role in the decomposition of hydroperoxides and the further oxidation of secondary products (Kochhar 1996). The decomposition of hydroperoxides involves the formation of alkoxyl radicals which are precursors of secondary oxidation products such as aldehydes, ketones and alcohols (Kamal-Eldin and others 2003). Further oxidation of these secondary products leads to the formation of tertiary oxidation products such as shorter chains compounds or low molecular weight fatty acids (Kamal-Eldin and Pokorny 2005).

Secondary oxidation products, especially aldehydes, are the most important volatile compounds contributing to off-flavors in foods. This is due to their low sensory threshold, which ranges from 0.002 to 1.50 mg/kg in milk (Badings 1984). The type of aldehyde formed will be dependent on the scission route utilized during homolytic cleavage of the lipid hydroperoxide (Kochhar 1996). The flavor of saturated aldehydes are described as fatty, pungent, green and tallowy. Unsaturated aldehydes contribute sweet, fruity, green and fatty flavors (Badings 1984; Hamilton 1994). Ketones also contribute to the undesirable flavors of oxidized foods; their sensory threshold ranges from 0.4 to 80 mg/kg of milk (Badings 1984). Ketones are mostly responsible for the metallic and musty/mushroom-like flavors in oxidized milk (Kochhar 1996). Alcohols are minor contributors to off-flavors in milk because of higher
sensory thresholds than aldehydes and ketones. Alcohols contribute grassy, green, fatty, rancid and stale flavors (Hamilton 1994; Kochhar 1996).

**Factors that Affect Lipid Oxidation in Milk**

The imbalance of pro-oxidative and antioxidative factors in milk lead to the development of lipid oxidation reactions and oxidized flavor. Some of the pro-oxidative factors include oxygen, light, metals and unsaturated lipids. Antioxidative factors primarily include vitamins with antioxidant activity such as α-tocopherol, ascorbic acid, carotenoids and retinol (Nielsen and others 2002).

**Oxygen**

Oxygen is required for the oxidation of milk lipids, whether it be through autoxidation or photo-oxidation. In autoxidation reactions, triplet state oxygen reacts with allylic or bisallylic carbons. In photo-oxidation reactions, excited singlet state oxygen directly reacts with double bonds in lipids (Kim and Min 2003). Because of the high energy of singlet oxygen (93.6 KJ above the energy of triplet state oxygen), the reaction rates of fatty acids with singlet oxygen are significantly higher than the reaction rates with triple state oxygen (Table 3) (Choe and Min 2009).

Table 3. Relative rates (M⁻¹ s⁻¹) of oxidation by triplet (autoxidation) and singlet (photo-oxidation) oxygen¹

<table>
<thead>
<tr>
<th>Oxygen</th>
<th>C18:1</th>
<th>C18:2</th>
<th>C18:3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triplet</td>
<td>1</td>
<td>27</td>
<td>77</td>
</tr>
<tr>
<td>Singlet</td>
<td>3 X 10⁴</td>
<td>4 X 10⁴</td>
<td>7 X 10⁴</td>
</tr>
</tbody>
</table>

¹ Shahidi 2000

The amount of dissolved oxygen in milk can influence the development of oxidized flavor. Oxygen-depleted milk exposed to light developed less light oxidized flavor than milk with
normal oxygen level, but copper induced oxidation was not affected by the decrease in oxygen (Shroder 1982). High levels of oxygen transfer through different types of packaging correlated with an increase in oxidized flavor formation in Ultra High Temperature pasteurized (UHT) milk exposed to light (Rysstad and others 1998).

**Light**

The combination of packaging and light exposure is a major problem for the dairy industry. Light exposure can initiate photo-oxidation reactions because of the presence of photosensitizers in milk, leading to the development of oxidized flavor (Webster and others 2007). In the United States, 68% of milk purchased is packaged in clear high density polyethylene containers (HDPE) (Keoleian and Spitzley 1999) and remains in the dairy case for at least 8 hr (Chapman and others 2002). Light intensities in supermarkets range from 750 to 6460 Lux, with a median light intensity of 2000 Lux (Chapman and others 2002). By comparing milk exposed to 1000 and 2000 Lux, Whited and others (2002) found that the intensity of light that milk is exposed to can significantly affect the intensity of the oxidized flavor developed.

Light can also deactivate or degrade vitamins present in milk known to have antioxidative activity; these include retinol, carotenoids, ascorbic acid and tocopherols (Sattar and others 1975). As these compounds are degraded, they lose their antioxidant activity making milk more susceptible to oxidation. Retinyl palmitate, a synthetic vitamin A supplement added to milk is degraded in the presence of light through isomerization to form 9 and 13 cis isomers, that have reduced vitamin A activity (Murphy and others 1998). Light exposure can accelerate the conversion of ascorbic acid to dehydroascorbic acid and further oxidation of this compound forms diketogulonic acid, an inactive form of vitamin C (Duncan and Webster 2010;
Sattar and others 1975). Chemical quenching of singlet oxygen by α-tocopherol leads to the development of intermediate hydroperoxides that can decompose to tocopherol quinone and tocopherol quinone epoxides, which do not have vitamin E activity (Choe and Min 2009; Clough and others 1978)

The effect of packaging and its use in protecting milk from light exposure has been extensively studied (Webster and others 2009; Rysstad and others 1998). Mestdagh and others (2005) exposed UHT milk to light (2500 Lux) continuously for 2 months in three different types of packaging: a complete light barrier bottle, a transparent 3-layered bottle with an oxygen scavenger and a transparent bottle with a UV absorbing additive. The complete light barrier bottle was the only packaging that offered enough protection against light-induced oxidation reactions and vitamin degradation. Packaging studies have also focused on blocking certain wavelengths to decrease sensitization of riboflavin, but found that even when all riboflavin excitation wavelengths were blocked (385-580 nm), light oxidized flavor was still produced (Webster and others 2009). This was attributed to the presence of other photosensitizers in milk such as chlorophyll a and b (Wold and others 2005).

Metals

Many metals are naturally present in milk; these include iron and copper. Although iron is considered a stronger oxidizing agent than copper, in milk, copper has a more significant pro-oxidative effect (O’Connor and O’Brien 2006). The association of iron with caseins, whey proteins and lactoferrin are likely to reduce their availability for oxidation reactions (Flynn and Cashman 1997). Naturally occurring copper in milk can range from 20 to 500 µg/L (Jenq and others 1988) and about 10-35% can be associated with the MFGM (O’Connor and O’Brien
2006), with a ratio of one metal ion per 24 polyunsaturated fatty acids (Richardson and Guss 1965). Additionally, copper ions, found in milk can also originate from contamination through processing equipment or water (Jeon 1996). The susceptibility of milk to metal-induced oxidation can be lowered by homogenization. Homogenization can help disperse copper ions throughout the milk system and allow proteins to be incorporated into the MFGM, which can help bind metals and prevent them from reacting with lipids (Hegenauer and others 1979).

Copper can have several roles in milk lipid autoxidation. It can react directly with triplet state oxygen and phenol compounds to generate reactive oxygen species (Kanner 2010). The pro-oxidant effect of α-tocopherol in the presence of copper has been previously reported by Slots and others (2007). Copper is also able to react with ascorbic acid to form ascorbyl radicals or copper-ascorbate complexes (Kanner 2010; Rysstad and others 1998). Copper also plays a major role in the decomposition of hydroperoxides formed in the propagation step of autoxidation (Kochhar 1996). Juhlin and others (2010b) reported an increase in susceptibility to spontaneous oxidized flavor in milk with an increase in copper concentration. Spontaneous oxidized flavor was also found to increase in milk with high levels of both polyunsaturated fatty acids and copper (Timmons and others 2001).

**Lipid composition**

The lipid composition of milk is affected by factors such as breed, stage of lactation, feed, season variation and supplements (Jensen 2002). The current interest of increasing concentrations of polyunsaturated fatty acids (PUFA) in milk for health purposes has led to a decrease in oxidative stability in milk (Liu and others 2010). Unsaturation plays a major role in the susceptibility of milk lipids to oxidation. The rate of oxidation by both triplet and singlet
oxygen increases with an increase in unsaturation of triacylglycerols (Table 3). Havemose and others (2004) proposed that an increase in linolenic acid contributed to the accumulation of lipid peroxides in milk when exposed to 2000 Lux for up to 24 hr. A significant increase in hexanal content was detected in milk with high levels of linolenic acid when exposed to 10 µM Cu²⁺ (Hedegaard and others 2006). Phospholipids, located in the MFGM, are also highly susceptible to oxidation due to their unsaturation and their close proximity to membrane-bound copper (Jensen and Nielsen 1996; Deeth 1997). Phospholipids have been proposed as the initial fatty acids that are oxidized in autoxidation reactions in milk (Walstra and Jenness 1984).

**Antioxidants**

The major antioxidants in milk can be classified as either lipid-soluble or water-soluble. Lipid-soluble antioxidants include carotenoids, retinol and α-tocopherol. The major water-soluble antioxidant is ascorbic acid (Lindmark-Mansson and Akesson 2000). Antioxidants can function as preventative or chain breaking antioxidants depending on their location and mode of action. Preventative antioxidants act as scavengers for reactive singlet oxygen and excited state photosensitizers that can initiate oxidation. Some preventative antioxidants are also involved in the chelation of metal. Chain breaking antioxidants will directly participate in stopping the chain reaction once oxidation has begun by intercepting compounds active in the propagation step (Laguerre and others 2007).

α-Tocopherol can be considered one of the most important lipid-soluble antioxidants in milk due to its location in the MFGM (Jensen and Nielsen 1996). It can act as both a preventative and chain breaking antioxidant in milk (O’Connor and O’Brien 2006). α-Tocopherol
is capable of quenching singlet oxygen through physical and chemical quenching, although the quenching efficiency was only reported as one-fiftieth of that of β-carotene (Hosaka and others 2005). Physical quenching is the major mechanism used by α-tocopherol, deactivating 120 singlet oxygen molecules before degrading to non-active compounds (Yang and Min 1994). α-Tocopherol can donate a hydrogen to radicals because of its low reduction potential (500 mV) in relation to radical compounds with reduction potentials ranging from 940 to 2300 mV. The tocopheryl radical formed is stabilized by the resonance stability of the phenol ring (Choe and Min 2009). As a chain-breaking antioxidant, α-tocopherol can induce a lag phase in which substrate is not oxidized (Laguerre and others 2007).

Al-Mabruk and others (2004) supplemented red clover silage (RCS) and grass silage (GS) feed for lactating dairy cows with 20.7 and 17.1 mg/kg dry matter of α-tocopherol, respectively. They reported only a transfer of 2.7 to 5.2% α-tocopherol when feeding RCS and GC, respectively. The final concentrations of α-tocopherol in RCS and GC were 0.268 and 0.368 µg/mL. Milk from cows fed RCS had 30% higher linoleic acid and 50% higher linolenic acid than the milk from GS-fed cows. The authors attributed the lower α-tocopherol final concentrations on the high levels of polyunsaturated fatty acids in the RCS diet. No significant differences were found in the oxidative stability of both milks as measured by thiobarbituric acid-reactive species (TBARS) during storage. The similarities in oxidative stability was attributed to the supplementation of vitamin E and its antioxidant effects even in the presence of high levels of PUFA. At concentrations above 2.7 mg/kg milk and in the presence of pro-oxidants, α-tocopherol can also have a pro-oxidative effect (Jacobsen and others 2008; Slots and others
Van Aardt (2005a) found that an addition of 0.05% α-tocopherol to milk increased the hexanal peak area after exposed to light (1200 Lux for 10 hr).

The principal mode of action of β-carotene and retinol is as preventative antioxidants by quenching singlet oxygen (Donnelly and Robinson 1995). One molecule of β-carotene can quench 250 to 1000 molecules of singlet oxygen (Yang and Min 1994). β-Carotene can also physically quench excited triple state sensitizer by acquiring the energy from the excited molecule and dissipating it as heat (Kim and Min 2003).

Ascorbic acid is the major water-soluble antioxidant in milk and can act as a radical scavenger due to its low action potential (330 mV) (Choe and Min 2009). Ascorbic acid has been reported to scavenge superoxide anion radicals (Hosaka and others 2005), alkoxy radicals (O’Connor and O’Brien) and singlet oxygen (Choe and Min 2009). Low concentrations of ascorbic acid can also promote lipid oxidation because they can reduce transition metals or form complexes that promote the formation of singlet oxygen (Barrefors and others 1995; Rysstad and others 1998). Huang and others (2004) reported a 78.3% reduction in the degradation of riboflavin in a dairy cream solution exposed to 1000 Lux for 96 hr in the presence of ascorbic acid (160 mM).

Ascorbic acid can reduce tocopheryl radicals generated during oxidation (Laguerre and others 2007; Nielsen and others 2002). This contributes to the suppression of the potential pro-oxidative effect of α-tocopherol radicals in the presence of fatty acids (Niki 1987). Addition of a single dose of 100 mg α-tocopherol/kg milk fat and 100 mg
ascorbyl palmitate/kg milk fat to UHT milk prior to light exposure (1200 Lux for 12 hr/days and 6 weeks) decreased hexanal content over the first 4 weeks of storage (Van Aardt and others 2005b). Ascorbic acid also protected the degradation of retinyl palmitate in skim milk exposed to light (3300 Lux) (Jung and others 1998b). Matsushita and Terao (1984) reported the protection of methyl esters from photo-oxidation when both β-carotene and α-tocopherol were present together.

**Measuring Oxidative Stability of Milk**

Lipid oxidation in milk is a result of many interactive factors, and because of this there are many ways to measure oxidative changes in milk. A common way of determining oxidation in milk is by using instrumental methods capable of detecting and measuring specific compounds that are produced from oxidation reactions. Sensory methods are also very often used in the food industry because they correlate to quality and consumer acceptance. Measuring the antioxidant activity using total antioxidant capacity (TAC) assays can also be a useful way of determining the oxidative stability of a product.

**Measurement of Lipid Oxidation**

*Peroxide Value (PV)*

Peroxide value determination is a method to quantify the amount of peroxides that are produced from lipid oxidation. This primary oxidation product can serve as a guide to the quality of the product and its oxidative status. This method utilizes ammonium thiocyanate and ferric chloride, which react with peroxides, to form colorful complexes that can be analyzed spectrophotometrically (Liang 2000). Due to the high temperatures used in this method, peroxides can degrade during the procedure causing inaccurate results (Rossell 1994). Peroxide
value has been extensively used to measure peroxides as well as the breakdown of peroxides to secondary oxidation products in milk (Mestdagh and others 2005), cheese (Mortensen and others 2002), butter and ice cream (Gonzalez and others 2003).

_Thiobarbituric Acid (TBA) Test_

The thiobarbituric acid test (TBA) has been used for decades to quantify secondary oxidation products. TBA primarily reacts with malondialdehyde (MDA), a common secondary oxidation product, as well as with other reactive species (TBARS) such as sugars, urea, ketones, acids and oxidized proteins (Rossell 1994). Although large variations in MDA content have been observed with different versions of the method (Fenaille and others 2001), good correlations between TBA values and oxidized flavor in milk have been reported (Guillen-Sans and Guzman-Chozas 1998).

_SafTest™_

The use of rapid test kits to determine oxidation are in growing demand because they are easy to use, give fast results and are relatively inexpensive when compared with other laboratory equipment. The SafTest™ system is a rapid method composed of automated analytical test kits that can measure different chemical indices of oil, fats and oil-containing products such as jerky snacks (Kong and others 2011) and pet food (Osawa and others 2008). Research has not been published on the use of the SafTest™ system with dairy products. These kits are AldeSafe, PeroxySafe, FaSafe or AciSafe and AlkalSafe and are meant to replace official methods for measuring TBA, PV, % free fatty acids and p-anisidine values respectively. All test kits in The SafTest™ system are quick and easy to perform, uses less toxic reagents, generate
less waste and are capable of analyzing many samples in less time than with American Oil Chemist’s Society (AOCS) methods.

The test kits are divided by sensitivity and application: Standard Application (STD), Matrix Special Application (MSA) and High Sensitivity (HSY). STD is applicable to pressed oils, frying fats and simple matrices, whereas MSA is applicable with complex matrices such as food products that contain oil and are likely to have smaller values. HSY has a larger range of detection than the other kits and is mostly used to obtain a quick analysis that does not require much precision (Osawa and others 2008). The detection range of the AldeSafe MSA kit is 0 to 0.320 mg/kg sample. AldeSafe, a kit specific for measuring malondialdehyde, has shown good correlation to AOCS methods during frying oil stability studies (Bansal and others 2010).

Gas Chromatography

Gas chromatography is a useful method for separating, identifying and quantifying volatile compounds such as secondary oxidation products. Gas chromatography is composed of two phases: a mobile phase in the form of a gas and a stationary phase in the form of a solid or an immobilized liquid that remains on the column (Ahuja 2003). Prior to separation in a gas chromatograph, volatiles in a sample need to be isolated or extracted from the matrix. Oxidation products in milk are commonly extracted using headspace techniques. Headspace techniques take advantage of the volatility of compounds for their extraction. These techniques use the space above the entrapped liquid or solid sample to monitor its quality. Some of the advantages of using these methods include lower risk of destroying the sample and ease of sample preparation (Cadwallader 2010). These techniques include static or equilibrium, dynamic-purge and trap, and solid phase microextraction (SPME).
Static or equilibrium method is the simplest headspace extraction method. A syringe is used to remove a small sample of the headspace after equilibration. Different sizes of container, temperature and matrix will result in noticeable result variations (Cadwallader 2010). Dynamic-purge and trap uses an inert gas to purge the volatiles from the food matrix and concentrates them into traps, which are then desorbed at high temperatures and cryofocused with liquid nitrogen prior to injection into a gas chromatograph (Pillonel and others 2002). Carry over effects and sample degradation during desorption have been reported (Park and Goins 1992; Marsili 1999), but this method was effective at extracting small molecular weight compounds in milk (Contarini and Povolo 2002).

In solid phase microextraction, volatiles are diffused from the matrix into the headspace of a sealed container and absorbed by a SPME fiber which is later desorbed into a gas chromatograph. SPME fibers can be composed of liquid, solid porous particles or a combination of liquid and solid particles. Liquid polymers include polydimethylsiloxane (PDMS), polyacrylate (PA) and carbowax (CW), while solid porous particles refer to divinylbenzene (DVB) (Shirey 1999). A combination of DVB/carboxen/PDMS can facilitate the extraction of a wider range of molecules due to the different coating layers in the fiber (Pillonel and others 2002). During extraction, samples are usually heated to 40-50°C and constantly stirred; this facilitates the extraction from the matrix to the headspace and shortens extraction times (Wercinski and Pawliszyn 1999). SPME is one of the extraction methods most commonly used in dairy research and has proven to be effective in the extraction of oxidation products in milk (Marsili 1999; Havemose and others 2007; Vazquez-Landaverde and others 2005; Lee and Min 2009) and other dairy products (Kim and Lee 2003; Panseri and others 2011).
Following extraction, separation is done using a gas chromatograph and an inert carrier gas such as helium to carry the volatiles from the injection port through a capillary column to a detector. Samples can be injected in split or splitless mode, to prevent overload of the column. In the case of SPME, the fiber is introduced into the inlet port and thermally desorbed at high temperatures (150-330°C) depending on the fiber coating used (Hubschmann 2009). The order of desorption is dictated by the boiling point of compounds and their interaction with the SPME fiber coating. Individual compounds will travel through the column and their interaction with the stationary phase in the column will determine their travel time. As compounds exit the column they are recognized by a detector. The most commonly used detector is a flame ionization detector (FID). This detector uses an oxygen-hydrogen flame to form ions; these ions produce a signal which is recorded as a peak at a given retention time. The area of the peak will be proportional to its concentration (Ahuja 2003). Analytical standards are commonly used to identify unknown compounds. A mass spectrometric detector can also be used to simultaneously identify and quantify compounds.

*Sensory Methods*

Sensory evaluation is a useful tool not only for determining likeness and acceptability of a product, but also to obtain objective data that can correlate well to instrumental values. Discrimination tests can be used to determine whether two samples are different, and are often use in dairy research to reformulate products, determine the effect of a processing change, or to determine threshold values. This method can also be used to determine flavor defects in milk exposed to light (Chapman 2010; Chapman and others 2002)
Descriptive tests are often used to quantify the descriptive analysis of specific characteristics. This quantification method is usually done through scaling techniques (Meilgaard and others 1999). A well trained sensory panel can be used to determine if oxidation is present in a sample and its intensity based on anchors used in training. Claassen and Lawless (1992) used descriptive tests to characterize the intensity of light and metal oxidized milk samples. Descriptive tests have also been used to characterize the aftertaste of milk and the ability of light-induced oxidation in affecting the perception of aftertaste (Porubcan and Vickers 2005).

Sensory methods are often coupled with instrumental methods to more accurately detect odor active compounds and characterize them. In Gas Chromatography-Olfactory (GC-O) samples are injected into the gas chromatograph in split mode, splitting the effluent between the detector and the sniffing port. As the compounds are detected, they are released and able to be perceived by the human nose. These descriptors and intensity ratings can then be matched to the chromatogram and used to characterize specific compounds (Maarse and Grosch 1996). GC-O has been used to identify light-induced oxidation aromas in antioxidant treated milk (Van Aardt and others 2005a).

**Determining Total Antioxidant Capacity (TAC)**

Antioxidant capacity assays are useful in measuring the overall antioxidant activity in foods. This is due to the multiple factors that affect the oxidative status and the possible interactions that can occur between each individual antioxidant present. Antioxidant capacity assays can be categorized into hydrogen atom transfer based assays (HAT) and electron-transfer based assays (ET) (Huang and others 2005). One HAT assay used in dairy research is the
oxygen radical absorbance capacity (ORAC). Zulueta and others (2009) reported that this assay measured antioxidant activity from amino acids in milk that could act as hydrogen donors.

A common ET assay used in dairy research is the ferric reducing antioxidant power assay (FRAP). This method, originally proposed by Benzie and Strain (1996), was based on the development of an intense blue color due to the reduction of a ferric tripyridyltriazine complex to its ferrous form at low pH. The FRAP method can measure reactions with a reduction potential below that of the Fe$^{3+}$/Fe$^{2+}$ half reactions, which include reactions involving ascorbic acid, α-tocopherol, carotenes and uric acid (Smet and others 2008, Mueller and Boehm 2011). The antioxidant activity of amino acids was not detected with this assay, and unfractioned milk was reported to be difficult to measure due to the precipitation of the proteins at a low pH, creating a turbid and unclear sample (Chen and others 2003). A new modification to the method was published by Amamcharla and Metzger (2014), correcting the turbidity issue with a filtration step.

The development of off-flavors in milk, especially oxidized flavor, can lead to the rejection of products by consumers. Lipid oxidation reactions are responsible for the formation of oxidation products in milk and can be affected by a variety of factors. Oxygen, light, metals and unsaturated lipids are common pro-oxidative factors in milk that decrease the oxidative stability of milk. Ascorbic acid, tocopherols, beta carotene and retinol act as antioxidative factors that can help improve the oxidative stability of milk and inhibit oxidation reactions. Understanding the mechanisms of lipid oxidation and the role of pro-oxidative and antioxidative factors in milk can help dairy producers with the prevention of oxidized off-flavor development in milk.
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Chapter 3

EFFECTS OF LIPID OXIDATION INITIATORS ON THE TOTAL ANTIOXIDANT CAPACITY AND OXIDATION PRODUCTS IN FLUID MILK DURING STORAGE

Abstract

The formation of oxidized off-flavors in milk continues to be a problem for the dairy industry. The precise mechanism for the oxidation of milk lipids is unknown, though copper and light have been proposed as contributors. The objective of the research was to determine the effect of cupric sulfate and light as catalysts of lipid oxidation and off-flavor formation in milk. The effect of cupric sulfate (0, 0.5 and 1.0 mg/kg) and/or light (2300 Lux) on milk stored for up to 11 days in refrigerated conditions (3.3°C) on the total antioxidant capacity (TAC) and the development of oxidation products was determined. TAC was measured by ferric reducing antioxidant power assay, and oxidation products were measured by AldeSafe™ Matrix Specific Application Kit (MSA) and solid phase microextraction-gas chromatography (SPME-GC). With storage, TAC decreased and oxidation products increased. Hexanal was the major aldehyde formed in all oxidized samples. The effect of cupric sulfate on oxidation products was only significant in the presence of light (p<0.05), whereas its effect on TAC was only significant in the absence of light (p<0.05). Light had a significant effect on the formation of malondialdehyde measured by AldeSafe™ MSA. Our research suggests that, while both cupric sulfate and light exposure affected TAC degradation and oxidation product formation, light had a greater effect. Further understanding of the factors that affect the formation of oxidized off-flavors in milk will allow producers and processors to improve the quality and consumer acceptability of milk.
Introduction

Oxidized flavor is one of the most common defects affecting milk and other dairy products. This flavor is often described as cardboardy, metallic, oily, stale, painty and fishy (Jeon 1996). The development of oxidized flavor is frequently attributed to lipid oxidation (Shahidi 2000; Juhlin and others 2010a; Park and Goins 1992) and promoted by the imbalance of antioxidative and pro-oxidative factors. The major pro-oxidants in milk are light, metals and unsaturated lipids (Rysstad and others 1998; Juhlin and others 2010b; Timmons and others 2001). The antioxidative factors of milk include ascorbic acid, α-tocopherol, and carotenoids (Hall and others 2010; St-Laurent and others 1990; Laguerre and others 2007).

In the United States, 68% of milk purchased is packaged in clear high density polyethylene (HDPE) containers (Keoleian and Spitzley 1999) and remains in the dairy case for at least 8 hr (Chapman and others 2002). Chapman and others (2002) reported that a trained panel could detect oxidized flavor in milk within 15-30 min of light exposure at 2000 Lux and consumers could detect differences from control within 54 min to 2 hr of light exposure. Light-induced oxidation in milk involves the degradation of lipids via photosensitizers such as riboflavin, which is readily present in milk (170 µg/100 g) (Oste and others 1997). Photosensitized riboflavin can play a role in the formation of oxidized flavor through direct reaction with fatty acids, leading to the formation of free radicals (type I mechanism) or through interaction with molecular oxygen and the generation of singlet oxygen (type II mechanism) (Kim and Min 2003).

Copper present in milk has also been proven to contribute to the development of off-flavors (Jenq and others 1988). The amount of copper in milk ranges from 20 to 500 µg/L (Jenq...
and others 1998). This wide range has been attributed to the effects of season, feed and genetic factors (Juhlin and others 2010b). Spontaneous oxidized flavor in milk, a type of oxidized flavor with unidentified origin, has been linked to the presence of high copper concentrations (Juhlin and others 2010a; Timmons and others 2001). Copper, as a transition metal, can initiate or participate in oxidation reactions through interaction with α-tocopherol (Slots and others 2007), ascorbic acid (Rysstad and others 1998) and polyunsaturated fatty acids (Timmons and others 2001).

The total antioxidant capacity (TAC) of milk provides an overall picture of the antioxidant activity present rather than the activity of each individual antioxidant. The ferric reducing antioxidant power (FRAP) assay is a method to measure the TAC of milk (Smet and others 2007; Amancharla and Metzger 2014). Due to their volatility, secondary oxidation products produced in milk during oxidation can be detected with solid phase microextraction-gas chromatography (SPME-GC) (Mestdagh and others 2005; Havemose and others 2007; Contarini and Povolo 2002; Vazquez-Landaverde and others 2005). SPME is a simple, sensitive, solvent free method which can reduce the analysis time compared to other extraction methods. (Spietelun and others 2010).

The objectives of this study were to assess the effects of cupric sulfate and/or light exposure on the formation of oxidation products and total antioxidant capacity of fluid milk over time and to determine if an interaction effect exists when cupric sulfate and light are present together. We hypothesize that both cupric sulfate and light will act individually as pro-oxidants in milk, and that the effect that either has will be independent of the presence of the other.
Materials and Methods

Milk Collection, Processing and Storage

Milk was collected at four different times from the Iowa State University (ISU) Dairy Farm between September and October 2013. All supplies and containers used for milk collection were washed and sanitized using Ecolab® Oasis Enforce and Ecolab® MikroKlene® sanitizer (St. Paul, MN) respectively. Milk was collected in stainless steel milk cans and transported to the Center for Crop Utilization Research (CCUR) Pilot Plant (Iowa State University) for processing.

Milk was then filtered through layered cheesecloth to remove any rocks, hairs, and other unwanted material. Milk was separated using a Type LWA 205 Westfalia Separator (219 rpm in 2.5 dial setting, Düsseldorf, Germany). The final fat percentage was standardized to range between 3.02 and 3.34%. After separation, milk was HTST pasteurized at 74°C with a flow rate of 4 mL/min to give a holding time of 15 s (UHT/HTST Lab Electric Model 25HV Hybrid Pasteurizer MicroThermics®, Raleigh, NC). The pasteurized milk was collected in a one gallon HDPE container (Dean Foods Dallas, TX). The milk collected was stored in the CCUR walk-in cooler at 3.3°C for 72 hr prior to application of treatments.

Experimental Design

Milk was divided into 6 equal parts: three levels of cupric sulfate (0, 0.5, 1 mg/kg) (Enartis Vinquiry Windsor, CA), each of which was exposed to two levels of light (light protected, light exposed at 2300 Lux). Each of the six treatments were stored in individual HDPE bottles (60 mL 44X72 mm Fisher Scientific, Fair lawn, NJ) for each storage time.
Light-exposed samples were placed in the walk-in cooler (3.3°C) on a shelf cart with attached fluorescent light bulbs (32 W, Sylvania, Danvers, MA) and light intensity was recorded with a light meter (General Electric type 213 Cleveland, OH). The samples were continuously illuminated during the storage period. The control and light-protected cupric sulfate samples were placed inside a black plastic bag in a cardboard box located on the same shelf of the cart. The samples were stored in the walk-in cooler for a total of 11 days and sampling was done on days 0 (1 hour after treatment was applied), 1, 2, 3, 4, 7, 9 and 11.

**Malondialdehyde Content Analysis**

The secondary oxidation products were evaluated using the SafTest™ AldeSafe Matrix Special Application (MSA) Kit (MP Biomedicals, Solon, OH) that measures the malondialdehyde content present in samples. To prepare milk samples, 1 mL of milk was transferred into a 15 mL conical tube, 3 mL of SafTest™ Standard Preparation Reagent and 1 mL of hexane (Fisher Scientific, Fair Lawn, NJ) was added to the milk sample and vortexed (Vortex Genie 2, Fisher Scientific, Fair Lawn, NJ) for 1 min on dial 8. The mixed samples were placed in a Dri-Bath heat block (type 17600 Barnstead/Thermolyne, Dubuque, IA) set at 40°C for 10 min. The samples were then vortexed for another 30 s and placed back in the heat block for 5 additional min. Samples were then vortexed for 5 s and filtered through a membrane filter on the SafTest™ Vacuum Filtration Unit. The clear filtrate was used for analysis.

Malondialdehyde content analysis was performed following the AldeSafe™ MSA Kit protocol. The filtrate was vortexed for 5 s and 400 µL was transferred to 12 mm test tubes in duplicates. One aliquot each of AldeSafe™ Reagent A and AldeSafe™ Reagent B was added to the test tubes. The capped test tubes were vortexed for 30 s and placed on the Labquake tube
rocker (Barnstead/Thermolyne Dubuque, IA) for 90 min. A set of 5 calibrators with known concentrations ranging from 0-0.32 mg/kg were used to construct a standard curve ($r^2<0.990$). After 90 min, the absorbance of the milk samples and calibrators were recorded using a 550/690 filter on the Microchem II analyzer (BI Source Scientific, Garden Grove, CA). The malondialdehyde concentration of each sample was calculated from the standard curve constructed by the 5 calibrators. The final results were adjusted to account for the dilution factor (1:5).

**Total Antioxidant Capacity (TAC)**

The total antioxidant capacity of milk was measured by the ferric reducing antioxidant power (FRAP) assay as described by Amamcharla and Metzger (2014). A daily FRAP working reagent was prepared with a 1:1:10 mixture of 300 mM Acetate buffer (pH 3.6, Fisher Scientific, Fair Lawn, NJ), 10 mM 2,4,6-tripyridyl-s-triazine (Acros Organic, New Jersey, USA), 20 mM FeCl$_3$ (Fisher Scientific, Fair Lawn, NJ). To measure the FRAP value, 0.3 mL of milk was placed in a test tube and mixed with 4.5 mL of the FRAP working reagent. The analysis was done in triplicates. The samples were vortexed using a Type 37600 Mixer (Thermolyne, Dubuque IA) for 5 s on high speed and placed in a water bath (Isotemp11 Fisher, Fair Lawn, NJ) at 37°C for 4 min. The sample was filtered through a 0.45 µm syringe filter (28 mm, Surfactant-Free Cellulose Acetate membrane, Corning, Corning, NY). The absorbance of the sample was measured with a spectrophotometer (Model 4001/4 Genesis 20 Thermo Spectronic Waltham, MA) at 593 nm using the FRAP working reagent as a blank. A standard curve was constructed by a series of FeSO$_4$.7H$_2$O aqueous solutions ranging from 50-600 µM and used to quantify the FRAP value of each sample, which was expressed as µmol/L FeSO$_4$.7H$_2$O of milk.
Volatile Analysis

Solid Phase Microextraction

25 g of milk were placed in a 125 mL Wheaton™ Crim-top serum bottle (Fisher Scientific, Fair Lawn, NJ) with a magnetic stir bar and sealed with an aluminum cap with a pierceable septum center (Polytetrafluoroethylene/silicone liner 20 mm, Supelco, Bellefonte, PA). The sealed bottle was then placed in a waterbath (Thermolyne SPA1025B Dubuque, IA) at 40°C with continuous stirring on dial 3. A SPME fiber (Supelco 2 cm-50/30 µm DVB Carboxen™/PDMS Stableflex™, Bellefonte, PA) was inserted into the headspace of the vial and left to equilibrate for 45 min.

Analysis of Volatiles by Gas Chromatography (GC)

Analysis of the volatiles absorbed to the SPME fiber was carried out by gas chromatography using a Hewlett Packard Agilent 6890 series gas chromatograph (Palo Alto, CA) equipped with a flame ionization detector and a 30 m x 0.25 mm x 0.25 µm SPB™-5 fused silica capillary column (Supelco, Bellefonte, PA). The fiber was desorbed in the injection port at 220°C for 3 min in splitless mode. Helium was used as a carrier gas with a constant inlet pressure of 124 kPa to the column and a 1.9 mL/min flow. Hydrogen gas and air were used to ignite the flame ionization detector with flow rates of 30 mL/min and 400 mL/min, respectively. The initial column temperature was 30°C, which was held for 3 min; the temperature was then increased by 5°C/min to 180°C with a hold time of 5 min, followed by an increase of 10°C/min to 200°C with a hold of 15 min. The total run time was 38 min.
Identification of volatile compounds

Identification of volatile compounds was performed by the combination of mass spectra and gas chromatographic analysis of standard compounds of interest.

Gas Chromatography-Mass Spectroscopy (GC-MS)

A 7890B Agilent gas chromatograph equipped with a 5977A mass spectroscopy detector was used for compound identification. Separation was completed on a 60 m x 0.25 mm x 0.25 µm SPB™-5 fused silica capillary column (Supelco, Bellefonte, PA). Program temperature and conditions were the same as mentioned above. MassHunter Workstation Software was utilized to find compounds using chromatogram deconvolution and the National Institute of Standards (NIST) database (Version 2.0, 2011 Gaithersburg, MD) was used for identification of compounds based on mass spectra.

Analytical standards

Identification of compounds was based on retention times. Standard (2 µL) was mixed with 2 mL of hexane (Fisher Scientific, Fair Lawn, NJ). Diluted standard (1 µL) was then directly injected into the GC in split mode (10:1) at 220°C. The same oven condition and temperatures were followed as in the GC analysis of volatiles. Kovats Retention indices calculated from the retention times of compounds of interest were compared with published values found on Flavornet (Acree and Arn 2004), Pherobase (El-Sayed 2014) and the National Institute of Standards and Technology (U.S. Dept. of Commerce 2014) databases and were used to confirm identification. 2-Hexanone, hexanal, 1-hexanol, heptanal, 1-heptanol, 2-nonanone and nonanal were purchased from Accustandard (New Haven, Ct). 1-octen-3-ol, t-2-octenal, 1-octanol, t-2-nonenal were purchased from Sigma Aldrich (St. Louis, MO)
**Statistical Analysis**

All data analysis was performed using SAS 9.2 (Cary, NC). For both AldeSafe™ MSA Kit and FRAP assays, data were analyzed using a linear mixed means model (Glimmix) and Tukey-Kramer multiple pairwise comparisons adjustment. Light, cupric sulfate and their interactions were treated as main effects. Period, treatment by period and a time-correlated daily effect for all samples within treatment by period were treated as random effects. A daily analysis using the same model, but excluding the correlated daily effect was used to assess the significance of main effects on individual days.

For volatile analysis, a linear regression model was used to calculate slopes of each treatment. The slopes were analyzed using two-way ANOVA with light and cupric sulfate as main effects and Tukey-Kramer multiple pairwise comparisons adjustments. Significant differences were defined as having a p<0.05.

**Results and Discussion**

**Determination of Malondialdehyde content in milk**

The formation of malondialdehyde in each treatment over time is presented in Figure 1. Malondialdehyde is an aldehyde derived from hydroperoxiepidioxides and bicycloendoperoxides formed from linolenic acid and it is a common indicator of lipid oxidation (Guillen-Sans and Guzman-Chozas 1998). Although no set trend is detected in Figure 1, data pattern shows that after day 4, the light-exposed samples had a higher malondialdehyde content than other treatment samples.

Jenq and others (1988) reported that for oxidized flavor to be noticeable in milk, the malondialdehyde content needs to be above 0.055 mg/kg. The only value that exceeded this
baseline was 0.075 mg/kg, measured in the light/cupric sulfate (1 mg/kg) treatment on day 4. This would suggest that on all other days and treatments oxidized flavor would not noticeable by consumers.

Figure 1. Malondialdehyde content in milk samples exposed to lipid oxidation initiators during storage (n=8)

Exposure to light significantly increased (p<0.05) the malondialdehyde content in milk, while the addition of cupric sulfate and day effect did not prove to be a significant factor for the development of this aldehyde. Table 1 gives a summary of the average malondialdehyde content in all treatments over the entire storage period. Even though the AldeSafe test kit was meant to replace the 2-Thiobarbituric acid test (TBA), an AOCS official methods for detecting aldehydes, this test kit is only specific to the detection of malondialdehyde (Foo and others 2006).
Table 1. Average\textsuperscript{1} malondialdehyde content in milk samples exposed to lipid oxidation initiators during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Malondialdehyde (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.014\textsuperscript{a}</td>
</tr>
<tr>
<td>Cs\textsuperscript{2} (0.5 mg/kg)</td>
<td>0.010\textsuperscript{a}</td>
</tr>
<tr>
<td>Cs (1 mg/kg)</td>
<td>0.009\textsuperscript{a}</td>
</tr>
<tr>
<td>Light</td>
<td>0.028\textsuperscript{b}</td>
</tr>
<tr>
<td>Light/cs (0.5 mg/kg)</td>
<td>0.028\textsuperscript{b}</td>
</tr>
<tr>
<td>Light/cs (1 mg/kg)</td>
<td>0.036\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Data for periods 1, 2, 3 and 4. 2 repetitions per period. \textsuperscript{2}cs-cupric sulfate. Means not sharing a common superscript a-b are significantly different (p<0.05)

Most of the AldeSafe™ values determined were in the low end of the detection range (0-0.32 mg/kg) and had very high coefficients of variation (<10). Turbidity in the some of the samples, caused by protein precipitation, could not be removed even after multiple filtration step and likely affected the spectrophotometric readings. Mestdagh and others (2005) reported that TBA values correlated poorly to sensory scores of oxidized flavor in milk exposed to light (2500 Lux) for 2 months The lack of correlation with other volatile compounds measured in our study, suggests that this test kit might not be an appropriate test to measure the aldehyde content in milk due to the low concentrations of linolenic acid commonly present in dairy products (0.5-2%) and the lack of instructions specifically for milk samples (Jensen 2002).

**Determination of the total antioxidant capacity of milk using FRAP method**

The FRAP method is a viable tool for measuring the ferric-reducing capacity of reactions with a reduction potential below that of the Fe\textsuperscript{3+}/Fe\textsuperscript{2+} half reactions (0.7V); these include α-tocopherol, ascorbic acid, uric acid and carotenoids reactions found in milk (Smet and others
2007, Chen and others 2003, Mueller and Boehm 2011). Figure 2 presents the FRAP values of milk as a function of time for the different treatments. All FRAP values decreased over time regardless of treatment. The control curve shows a significant increase on day 1 followed by a more gradual decline, whereas the other treatment curves show a much steeper decline on day 1.

![Figure 2. Ferric reducing antioxidant power value (µmol Fe^{2+}/L milk) of milk exposed to lipid oxidation treatments during storage (n=12)](image)

From Figure 2, all treatments on day 3, with the exception of the control were below 190 µmol Fe^{2+}/L milk. Amamcharla and Metzger (2014) reported that for a FRAP value to be considered low it needed to be below 190 µmol Fe^{2+}/L milk. The authors stated that at this level, the low antioxidant activity would not be adequate to limit oxidative damage to the milk. This indicates that after day 3, the antioxidant activity of all treatments were already too low to prevent oxidation.
Table 2 shows the p-values for the main effects and their interaction on the FRAP values by day. The presence of cupric sulfate has a significant effect on days 0, 1, 2 and 3, while the presence of light was significant on day 1 until the end of storage at day 11. Interactions between cupric sulfate and light are seen on days 1, 2, 3, 4, and 7, where levels of cupric sulfate behaved differently in the two levels of light.

Table 2. Summary of p-values of main effects on the ferric reducing antioxidant power values by day

<table>
<thead>
<tr>
<th>Main effect</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Cs</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Light</td>
<td>NS</td>
</tr>
<tr>
<td>Light*cs</td>
<td>NS</td>
</tr>
</tbody>
</table>

Separation between the dark and light samples can be noticed on the second week of storage (Figure 2). Results on table 2 suggests that light was the main driving factor in the decrease of FRAP values especially towards the end of the storage period, whereas cupric sulfate was only a factor during the early days of storage. The average FRAP values of milk exposed to different lipid oxidation treatments during storage are shown on Table 3. The FRAP values were also evaluated as an average over the storage period because it provided an overall picture of what occurs to the antioxidant capacity over time as lipid oxidation initiators are present.
Table 3. Average\(^1\) ferric reducing antioxidant power values of milk samples exposed to lipid oxidation initiators during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FRAP Value (µmol Fe(^{2+})/L milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>216.71 (^a)</td>
</tr>
<tr>
<td>Cs(^2) (0.5 mg/kg)</td>
<td>177.83 (^b)</td>
</tr>
<tr>
<td>Cs (1 mg/kg)</td>
<td>181.70 (^b)</td>
</tr>
<tr>
<td>Light</td>
<td>141.01 (^d)</td>
</tr>
<tr>
<td>Light/cs (0.5 mg/kg)</td>
<td>143.08 (^d)</td>
</tr>
<tr>
<td>Light/cs (1 mg/kg)</td>
<td>153.06 (^c)</td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1, 2, 3 and 4. \(^2\)cs-cupric sulfate. Means not sharing a common superscript \(^a-d\) are significantly different (p<0.05)

When averaging the treatment effects over the entire storage period, Light (p<0.05) and cupric sulfate (p<0.05) significantly decreased the FRAP values. A day effect (p<0.05) and a treatment by day effect (p<0.05) was also detected since there was a decrease over time, and this decrease was related to the treatment applied. The control had the highest FRAP value and it was significantly different from all treatments. In the dark, the addition of cupric sulfate decreased the FRAP value significantly, but no significant difference was found between 0.5 and 1 mg/kg of cupric sulfate levels. In the presence of light, cupric sulfate did not further decrease the FRAP value. However, the average FRAP value of the light/cupric sulfate (1 mg/kg) samples was significantly higher than values of the light/cupric sulfate (0.5 mg/kg) samples. The average FRAP value for the light only samples was not significantly different than the values of the light/cupric sulfate (0.5 mg/kg) samples.

When focusing on the average FRAP values over the total storage period, in the absence of light, the addition of cupric sulfate decreased the FRAP value, but the same effect was not seen in the presence of light. This effect could be due to the light-induced degradation or
deactivation of the antioxidants present in milk and the interaction of these antioxidants with cupric sulfate in the absence of light. When focusing on each individual day, the presence of cupric sulfate was only significant during the first week of storage. During this time, cupric sulfate might have been able to interact with the antioxidants present in milk before they were deactivated by light. The formation of a copper-ascorbate complex, capable of generating singlet oxygen has been reported by Rysstad and others (1998). When this complex is formed, copper is reduced from Cu$^{2+}$ to Cu$. The Cu$^+$ is able to reduce triplet oxygen to superoxide anion or hydrogen peroxide, which can later react to form singlet oxygen.

Slots and others (2007) reported that α-tocopherol degraded in milk when exposed to light due to its chemical quenching of singlet oxygen and decomposing to tocopherols quinone and tocopherols quinone epoxides (Choe and Min 2009; Clough and others 1978). Retinyl palmitate was reported to be degraded in milk through isomerization in the presence of light forming 9-cis retinyl palmitate and 13-cis-retinyl palmitate, which have reduced vitamin A activity (Murphy and others 1998). Our research suggests that once antioxidants are deactivated by light and oxidation reactions are uninhibited, cupric sulfate does not have the power to lower the FRAP values any further. On the other hand, when cupric sulfate is present in the absence of light, it is able to act as the main pro-oxidant, thus lowering the FRAP values significantly.

The effect of cupric sulfate in the absence of light could also be partly attributed to the pro-oxidative effect of α-tocopherol (Nicholson and St. Laurent 199; Wade and others 1986). Tocopherol radicals, which are formed from the oxidation of tocopherols, can sometimes have a pro-oxidative effect if present at high concentrations. Tocopherol radicals can abstract
hydrogens from lipids (Choe and Min 2009). Slots and others (2007) reported that in milk, \( \alpha \)-tocopherol acted as a pro-oxidant in the presence of copper by initiating new reaction chains of oxidation but this pro-oxidative effect seems to decrease in the presence of ascorbic acid (Niki 1987). Because \( \alpha \)-tocopherol is deactivated in the presence of light, the effect of cupric sulfate could be decreased under light exposure, if the interaction between copper and \( \alpha \)-tocopherol produced significant oxidation reactions.

**Determination of Volatile Compounds using SPME-GC**

Table 4 lists the identified compounds that were identified in milk samples. Other unidentified compounds can also be associated with oxidized off-flavors in milk but are not shown on Table 4. The light only and light/cupric sulfate samples had the most volatile compounds present. Cupric sulfate samples in the absence of light did not produce many of the oxidation products identified. None of the volatile compounds were found in the control samples.
Table 4. Volatile compounds identified in milk by solid phase Microextraction-gas chromatography

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kovats index</th>
<th>Treatments</th>
<th>Control</th>
<th>Cs (0.5mg/kg)</th>
<th>Cs (1 mg/kg)</th>
<th>Light</th>
<th>Light/Cs (0.5mg/kg)</th>
<th>Light/Cs (1mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Hexanone</td>
<td>789</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Hexanal</td>
<td>800</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>873</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Heptanal</td>
<td>902</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>1-Heptanol</td>
<td>973</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>1-Octen-3-one</td>
<td>975</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>981</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>t-2-Octenal</td>
<td>1059</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>1073</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2-Nonanone</td>
<td>1093</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Nonanal</td>
<td>1104</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>t-2-Nonenal</td>
<td>1160</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>t-2-Decenal</td>
<td>1261</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>


Changes of the peak areas of selected volatile compounds over time were analyzed, and slopes of these volatile compounds are reported in Table 5. These compounds were selected due to their repeatability, duration of their occurrence and peak area. Hexanal, heptanal and nonanal are saturated aldehydes and t-2 octenal is an unsaturated aldehyde. Aldehydes greatly contribute to the off-flavor in milk due to their low sensory threshold values compared to other oxidation products (Badings 1984).
Table 5. Slopes (pA*s/day) of volatile compounds’ peak areas identified in milk samples exposed to lipid oxidation initiators

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slope of Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexanal</td>
</tr>
<tr>
<td>Control</td>
<td>-0.024 a</td>
</tr>
<tr>
<td>Cs² (0.5mg/kg)</td>
<td>0.013 a</td>
</tr>
<tr>
<td>Cs (1mg/kg)</td>
<td>0.259 a</td>
</tr>
<tr>
<td>light</td>
<td>1.722 b</td>
</tr>
<tr>
<td>light/cs (0.5mg/kg)</td>
<td>3.741 c</td>
</tr>
<tr>
<td>light/cs (1mg/kg)</td>
<td>3.850 c</td>
</tr>
</tbody>
</table>

¹Data for periods 1,2,3 and 4. 1 repetition per period. ²cs-cupric sulfate. ³nd-not detected. Slopes not sharing a common superscript a-c are significantly different (p<0.05).

Hexanal and nonanal were the only aldehydes present in all oxidized treatments.

Hexanal has been described as a cut-grass smell, while nonanal has a waxy, painty odor, with a sensory threshold value of 0.07 and 0.20 mg/kg in milk, respectively (Van Aardt and others 2005; Fenaille and others 2003; Badings 1984). Hexanal, derived from linolenic acid, was the volatile compound with the largest peak area detected in this study. Hexanal was reported to be the major aldehyde generated during lipid oxidation of human milk and bovine milk (Elisia and Kitts 2011; Mestdagh and others 2005). Both hexanal and nonanal can be produced through reactions of unsaturated fatty acids with singlet state oxygen (photo-oxidation) and triplet state oxygen (autoxidation), but a higher proportion is attributed to singlet state oxygen reactions (Shahidi 2000).

Hexanal and nonanal were detected within 24 hr of applied treatment. Light (p<0.05) and cupric sulfate (p<0.05) had a significant effect on the area of both of these compounds, but
a significant interaction between these effects was also found \( (p<0.05) \). In the absence of light, cupric sulfate did not significantly increase the peak area, but, in the presence of light, cupric sulfate significantly increased the peak area, although no significant difference was detected between the 0.5 and 1 mg/kg levels. In the hexanal data, the light only sample was significantly higher than the control but lower than the light/cupric sulfate mixtures. In the nonanal data the control was not significantly different than the light only sample. This was likely due to the smaller size of the peak, where noise in the signal could have had a larger impact on the data.

Heptanal and t-2-octenal were only present in all light exposed samples. Heptanal, an aldehyde derived from oleic acid, has a green-fish oil odor with a sensory threshold value of 0.10 mg/kg in milk (Van Aardt and others 2005; Badings 1984; SHahidi 2000). t-2-Octenal, an unsaturated aldehyde derived from 3-nonenal, has a green and waxy odor (Warner and Neff 2001; Acree and Arn 2004). The 0.5 mg/kg cupric sulfate sample was not significantly different than the light only sample. The copper effect \( (p<0.05) \) was only evident in the 1 mg/kg cupric sulfate sample. This sample had a significantly larger peak area than the light only sample but not the 0.5 mg/kg cupric sulfate sample. The presence of cupric sulfate significantly increased \( (p<0.05) \) the peak area of t-2-octenal and no significant difference was found between 0.5 and 1 mg/kg levels.

Results suggest that, even though cupric sulfate had a significant effect in all the volatile peak areas in the presence of light, this effect was not significant in the dark, meaning that the light exposure played a major role in the development of the volatile compounds. The generation of volatile compounds was accelerated by the presence of cupric sulfate, but cupric sulfate did not play a role in its initiation.
The formation of oxidation products in milk due to light has been investigated in previous studies. Lee and Min (2009) measured the increase of pentanal, hexanal, heptanal and dimethyl disulfide under 8 hr of light exposure (1000 Lux) at different levels of added riboflavin. Samples with no added riboflavin developed pentanal and hexanal, while samples with added riboflavin (10 ppm) developed pentanal, hexanal, heptanal and dimethyl disulfide. UHT pasteurized milk is also susceptible to light-induced oxidation. Van Aardt and others (2005) reported the formation of pentanal, dimethyl disulfide, hexanal, 1-hexanol, heptanal, 1-heptanol, 3-octen-3-ol and nonanal in UHT milk exposed to 1200 Lux for 12 hr a day for 6 weeks.

These results found in this study could be due to the reaction rates of the different oxygen species used in oxidation reactions and the mode of action of the pro-oxidants. Both singlet and triplet oxygen can form the hydroperoxides that lead to the generation of volatile compounds. The reaction rate ratio of singlet oxygen generated during photo-oxidation compared to triplet oxygen used in autoxidation reactions with oleic and linoleic acids are 3000:1 and 40000: 27, respectively (Shahidi 2000). The higher reaction rate of singlet oxygen compared to triplet oxygen allows for a much rapid accumulation of lipid peroxides during photo-oxidation. Triplet oxygen, utilized during the propagation step of autoxidation, has a much slower rate of reaction with lipids. This leads to a lower accumulation of lipid peroxides, which generates a lower concentration of oxidation products, as seen in cupric sulfate samples in the absence of light when compared to cupric sulfate samples in the presence of light.

The higher reaction rate of singlet oxygen with fatty acids and the free radical-generating photo-oxidation reactions (type I mechanism) (Kim and Min 2003) can accumulate a
high concentration of lipid peroxides and initiate autoxidation reactions. Metals, such as cupric sulfate, play a major role in the decomposition of hydroperoxides (Kochhar 1996). Together, photo-oxidation and autoxidation can lead to a greater accumulation of volatile compounds.

The interaction effect between light and cupric sulfate found in the formation of oxidation products suggest that cupric sulfate might enhance light catalyzed oxidation. The breakdown of peroxides by metal catalyzed reactions have been previously reported and can be a driving force for the generation of volatile compounds (Jacobsen and others 2008; Antolovich and others 2002). These reactions, coupled with photo-oxidation reactions, could help form larger amounts of oxidation products over time. This interaction differs from the interaction found in the FRAP data, where cupric sulfate did not decrease the FRAP value any further in the presence of light. Antioxidant assays are only meant to measure early changes of oxidative stability (Smet and others 2009). Analysis of the FRAP assay data only found the addition of cupric sulfate significant during the first week of storage and on average in the absence of light, while the effect of light was significant after day 1 until the end of the storage period. Our research suggests that the FRAP assay might not be a good indicator of existing oxidation but rather of susceptibility to oxidation due to changes in antioxidant activity, which are highly affected by the presence of light.

**Conclusion**

Results of this study demonstrated the pro-oxidative effects of light and/or cupric sulfate in milk. An interactions between cupric sulfate and light, when present together, was demonstrated in the TAC and volatile compounds data, which illustrated the complex nature of lipid oxidation in milk. The presence of cupric sulfate and light, individually, decreased the TAC
of milk, especially during the first week of storage, but the effect of cupric sulfate was not significant in the presence of light. Light had the most significant effect on the formation of all volatile compounds but cupric sulfate enhanced the effects of light catalyzed oxidation product formation.

Hexanal and nonanal were the only two common volatiles present in all oxidized samples, and hexanal was the major aldehyde formed. No significant differences were detected between copper levels (0.5 and 1 mg/kg) meaning that at these levels, the amount added was not as important as its presence. Further research on the effects of common pro-oxidants in milk can help prevent oxidized flavor formation and improve the sensory qualities of milk and consumer acceptability.

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Chapter 4

VITAMINS A AND E AS POTENTIAL ANTIOXIDANTS AND INHIBITORS OF THE TOTAL ANTIOXIDANT CAPACITY DEGRADATION AND OXIDATIVE OFF-FLAVOR DEVELOPMENT IN FLUID MILK WHEN EXPOSED TO LIPID OXIDATION INITIATORS

Abstract

Lipid oxidation leads to the development of oxidized flavors in milk. The oxidative stability of milk is influenced by the balance of pro-oxidative and antioxidative factors. The objective of this research was to determine the effects of added vitamin A (retinyl palmitate) and vitamin E (tocopheryl acetate) as potential inhibitors of the total antioxidant capacity (TAC) degradation and formation of oxidation products and oxidized flavor. Vitamin A (2113 IU/L) or vitamin E (100 mg/kg milk fat) were added to milk exposed to light (2300 Lux) or a combination of light (2300 Lux) and cupric sulfate (0.5 mg/kg) and stored for up to 7 days in refrigerated storage (3.3°C). TAC was measured by the ferric reducing antioxidant power assay. Solid phase microextraction-gas chromatography (SPME-GC) was used to detect oxidation products and a trained sensory panel was used to measure oxidized flavor. On day 0, vitamins A and E significantly increased (P<0.05) TAC in samples exposed to light compared to samples exposed to light without added vitamins. On day 1, only vitamin A in the presence of both light and cupric sulfate had a significantly higher (p<0.05) TAC compared with light and cupric samples with no added vitamins. The peak areas of hexanal and heptanal were not affected by the presence of either vitamin A or E. The decrease in TAC and increase in oxidation products was more gradual in all light-only samples. The light/cs samples showed a more rapid decrease in TAC and increase in oxidation products during the first days of storage. Sensory results did
not show a significant vitamin effect in the intensity of oxidized flavor, but samples with both light and cupric sulfate received significantly higher scores than light-only on day 1 of exposure.

**Introduction**

Lipid oxidation in milk is attributed to exposure to pro-oxidants such as light and metals (Mestdagh and others 2005; Juhlin and others 2010). Lipid oxidation leads to the development of oxidized flavor, a common flavor defect affecting the dairy industry today (Visscher 2001). There are three types of oxidized flavors that can develop in milk: light-induced, metal-induced and spontaneous (O’Connor and O’Brien 2006). Light-induced oxidation requires the presence of light and a photosensitizer (Sattar and others 1975). Packaging milk in clear high density polyethylene (HDPE) containers and storing it in illuminated dairy cases are the major causes of light-induced oxidized flavor development (Webster and others 2007). Metal-induced oxidation usually involved high concentrations of copper ions that can participate in autoxidation reactions (Jeon 1996). The origins of spontaneous oxidized flavor have not been identified in milk, but its occurrence has been linked to an imbalance of pro-oxidative and antioxidative factors, as well as genetic disposition (Granelli and others 1998; Juhlin and others 2010).

Antioxidants can play a major role in the protection of milk from oxidative degradation. Because of the many possible interactions between antioxidants in milk, total antioxidant capacity (TAC) assays are useful in providing an overall assessment of the oxidative stability of milk (Smet and others 2007). Some of the most essential lipid-soluble antioxidants in milk are α-tocopherol and carotenoids (O’Connor and O’Brien 2006). Major importance has been given to these lipid-soluble antioxidants because their concentration can be manipulated through diet since they depend on the lipid content of the feed (Jensen and others 1999).
In milk, α-tocopherol is found in concentrations between 13-30 mg/kg milk fat and is the only antioxidant present in the milk fat globule membrane (MFGM) (Van Aardt and others 2005a; Jensen and Nielsen 1996). Its location allows it to protect the unsaturated phospholipids that make up the MFGM and prevent access to the internal neutral lipid core of the globule. The role of α-tocopherol is primarily as a free radical scavenger (Laguerre and others 2007), but it is also capable of quenching singlet oxygen generated during photo-oxidation (Yan and Min 1994).

β-Carotene and retinol are found in milk at concentrations of 1-17 µg/g and 1-12 µg/g, respectively (Noziere and others 2006). These antioxidants are found in the neutral lipid core of the fat globule (Jensen and Nielsen 1996; Barrefors and others 1995). Both of these compounds function as preventative antioxidants by quenching singlet oxygen and triplet state photosensitizers that form during light exposure (Donnelly and Robinson 1995; Kim and Min 2003). During processing and cream separation, vitamin A is removed from milk, as it is located in the cream portion of the milk. To counteract this effect, the dairy industry fortifies fat-free and low-fat milk with retinyl palmitate, a synthetic form of vitamin A (Newcomer and Murphy 2001).

Due to the complexities of the antioxidant system in milk, inconsistencies in the efficiency of antioxidants as lipid oxidation inhibitors have been reported. Slots and others (2007) reported that milk from cows that were supplemented with 2600 IU and 3400 IU per day per cow only had an increase in α-tocopherol of 37 and 29%, respectively. The hexanal accumulation of milk from the 2600 IU supplementation was not significantly different than the control milk (no supplementation) when exposed to light (2200Lux), but the milk from the 3400
IU supplementation had a higher accumulation of hexanal when exposed to light than non-supplemented cow milk.

Additional research is needed to understand the effects of antioxidants in the presence of lipid oxidation initiators. The objective of this study is to determine the potential of vitamin A (retinyl palmitate) and vitamin E (tocopheryl acetate) in inhibiting total antioxidant capacity degradation and oxidative off-flavor development of fluid milk when exposed to lipid oxidation initiators during storage. We hypothesize that vitamin A and vitamin E will inhibit off-flavor formation and reduce the effects of lipid oxidation initiators on the degradation of the total antioxidant capacity of milk.

Materials and Methods

Milk Collection, processing and storage

Milk was collected at three different times from the Iowa State University (ISU) Dairy Farm’s bulk tank between February and March, 2014. All supplies and containers used for milk collection were washed and sanitized using Ecolab® Oasis Enforce and Ecolab® MikroKlene® sanitizer (St. Paul, MN), respectively. Milk was collected in stainless steel milk cans and transported to the Center for Crop Utilization Research (CCUR) Pilot Plant (Iowa State University) for processing.

Milk was separated and pasteurized using the same procedure outlined in chapter II. The final fat percentage ranged between 3.24 and 3.31%. After pasteurization the collected milk was stored in the CCUR walk-in cooler at 3.3C for 24 hr prior to application of treatments.
**Experimental Design**

Milk was divided into 7 equal parts: a control (light-protected, no antioxidants or cupric sulfate added) and 6 treatments. The treatments were divided into 2 groups: samples exposed only to light (2300 Lux) and samples exposed to light (2300 Lux) and cupric sulfate (0.5 mg/kg) (Enartis Vinquiry Windsor, CA) simultaneously. Within each group, one set of samples did not have vitamins added (light0 or light/cs0), the other two sets of samples were spiked with either 2113 IU/L milk of vitamin A (lightA, light/csA) (retinyl palmitate; Dairy House, Fenton, MO) or 100 mg/kg milk fat (5.02 IU/L) of vitamin E (lightE, light/csE) (tocopheryl acetate Diary House, Fenton, MO). In lieu of a homogenizer, vitamins were added by mixing the samples at high speed (dial 8) for 1 min using a stirrer plate and a sanitized stir bar (Corning PC-420, Corning, NY). Each of the six treatments and the controls were stored in individual HDPE containers (32 oz., Quality Environmental Containers, Beaver, WV) for each storage time.

Light-exposed samples were placed in a walk-in cooler (3.3°C) on a shelf cart with attached fluorescent light bulbs (32 W, Sylvania, Danvers, MA) and light intensity was recorded with a light meter (General Electric type 213 Cleveland, OH). The samples were continuously illuminated during the storage period. The control (light-protected) samples were placed inside a black plastic bag in a cardboard box located on the same shelf of the cart. The samples were stored in the walk-in cooler for a total of 7 days and sampling was done on days 0 (1-3 hour after treatment was applied), 1, 3 and 7.

**Total antioxidant capacity (TAC)**

The total antioxidant capacity was measured by the ferric reducing antioxidant power assay (FRAP) using the same method outlined in chapter III.
Volatile analysis

The analysis of volatiles was performed using solid phase microextraction-gas chromatography as detailed in chapter III.

Sensory Evaluation – Descriptive Analysis

The Institutional Review Board of Iowa State University approved the use of human subjects for the sensory panel in this study on January 2014. Subjects with previous milk tasting experience (participation in other milk sensory panels or as part of the Dairy Products Evaluation Team) were recruited through email. The panelists were required to be over 18 years old, consume milk at least once a week and have an interest in sensory evaluation. A total of 8 panelists were selected as official panelists consisting of students and staff at ISU. Each panelist was given $5 compensation for each training or tasting session attended.

Panelist training

Panelists received a total of 9 hr of training, 3 one-hour sessions for two weeks, followed by 2 one-hour session for 1 week and an additional one-hour review session between the first two repetitions of the experiment. The off-flavors used in this sensory study included: feed, foreign, lacks freshness, light oxidized, metal oxidized and total oxidized. These off-flavors were selected because they were likely to contribute to the off-flavor formation in the milk presented in this study. Off-flavors, their description and their intensities, are shown in Table 1.
Table 1. Off-flavors and descriptors used to train sensory panel

<table>
<thead>
<tr>
<th>Off-flavor</th>
<th>Description</th>
<th>Preparation by intensity (700 mL milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td>Grassy, hay-like</td>
<td>S-2 mL of alfalfa tea added 2 hr prior to tasting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D- 4 mL of alfalfa tea added 2 hr prior to tasting</td>
</tr>
<tr>
<td>Foreign</td>
<td>Any other flavor not commonly found in milk (sanitizer, vitamin taste)</td>
<td>S-0.2 mL of vitamin E added 30 minutes prior to tasting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-0.5 mL of vitamin E added 30 minutes prior to tasting</td>
</tr>
<tr>
<td>Lacks Freshness</td>
<td>Old milk, tastes like the fridge</td>
<td>S- milk opened a couple days prior to tasting and left in the fridge</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D- milk close to expiration date, left unsealed in the fridge</td>
</tr>
<tr>
<td>Light Oxidized</td>
<td>Wet cardboard or paper towels smell, mouth drying sensation</td>
<td>S- 1 hour light exposure (2300 Lux) in translucent container</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D- 24 hour light exposure (2300 Lux) in translucent container</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P- 48 hour light exposure (2300 Lux) in translucent container</td>
</tr>
<tr>
<td>Metal Oxidized</td>
<td>Metallic taste and smell, penny-like taste, numbing in the back of mouth and sides of tongue,</td>
<td>S- 0.7 mL of 2 mg/mL cupric sulfate solution added 2 days prior to tasting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-0.7 mL of 5 mg/mL cupric sulfate solution added 2 days prior to tasting</td>
</tr>
<tr>
<td>Total Oxidized</td>
<td>Total oxidized flavor perceived, an overall assessment on the intensity of light and/or metal oxidized off-flavors (additive effect if both present simultaneously)</td>
<td></td>
</tr>
</tbody>
</table>

S, D, P represent flavor intensity using store bought milk. S-slight, D-definite, P-pronounced

The training began by introducing the panelists to the off-flavors and reintroducing the technique used for milk tasting. They poured milk samples into a clean cup (3 oz. Solo Cup company, Lake Forest, IL), filling it only at about ¼ of capacity. They placed one hand on top of the sample cup, covering the cup, while holding the bottom of the cup with another hand to warm up the milk. They swirled the cups for about 20 seconds to entrap the volatiles in the headspace. They lifted up their hand from the top of the cup and smelled the sample prior to
tasting the milk sample. Panelists were encouraged to expectorate samples and then take in deep breaths though the mouth and exhale through the nose to assess any aromas detected retronasally. Panelists were also instructed to consider the aftertaste and sensations remaining after samples were expectorated in their overall assessment of flavor intensity.

During the first week of training, panelists were familiarized with all off-flavor descriptors used in the study and the ballot used during sample evaluations (Appendix A). The second and third week of training focused primarily on training the panelists with oxidized samples (light and metal). Panelists tasted an average of 6-8 samples each session. The ballot used a 15 cm line scale to score the intensity of each off-flavor descriptor. Different levels of off-flavors in milk (slight, definite, pronounced) were prepared following the guidelines in Table 1. A slight, definite and pronounced score represented a score of 3, 7.5 and 12 cm on the ballot. Only slight and definite anchors were prepared for feed, foreign and lacks freshness samples because high levels of these attributes were not expected and a larger focus was given to oxidized off-flavors. Only slight and definite levels of metal oxidized were prepared because higher level could not be achieved even after experimentation with higher concentrations of cupric sulfate and longer exposure times.

In each training session, panelists were presented with unidentified milk samples. After tasting each sample, panelists engaged in discussion about the off-flavor and its intensity. After discussion, the identity of the sample was revealed and a consensus on its intensity was reached with all the panelists. Following discussion, panelists were encouraged to re-taste samples as needed. In other occasions, a group of 2-4 unidentified samples was given to the panelists to identify and rank in order of intensity. After the exercise, the identity of the off-
flavor and intensity of each sample was revealed and discussed to reach an agreement with all the panelists. Towards the end of training, panelists were taken to the sensory booths attached to the CCUR sensory kitchen to evaluate samples and to familiarize themselves with a sensory booth environment.

Sample evaluation

Sensory evaluation of experimental milk was done in the CCUR sensory booths. Each panelist was given a one-time identifying panelist number to use during evaluations. Booths were set up with unsalted crackers and water to use between sample tastings as palate cleansers. To prepare samples, 40 mL of experimental milk was poured into serving container (2 oz. Solo Cup Company, Lake Forrest, IL) and covered with tight fitted lids. Samples were left at room temperature and protected from light for 20 min prior to being evaluated by panelists. Each panelist received a “no defect sample” whole milk (light block container, Land O’Lakes, Arden Hills MN) at the beginning of each sensory evaluation session as a reference sample. Samples were served in randomized order and one at a time; panelists were not able to return to previous samples once ballots were submitted.

Statistical Analysis

All data analysis was performed using SAS 9.2 (Cary, NC). A mixed model (Glimmix) using treatment and day as fixed effects and period as a random effect was used for TAC and volatile compounds data analysis. A mixed model (Glimmix) using treatment and day as fixed effects and period and panelist as random effects was used for sensory data analysis. Tukey-Kramer multiple pairwise comparisons adjustment was utilized for each main effect. Significant differences were defined as having a p<0.05.
A significant period effect was found in all data with the exclusion of the sensory data. Period 3 was significantly different than periods 1 and 2 and was considered an outlier in this study due to possible microbial contamination during processing. Average data including all 3 periods are shown on Appendix C, D and E.

**Results and Discussion**

**Effect of added vitamins to the total antioxidant capacity of milk using FRAP method**

Table 2 presents the FRAP values of each treatment by day. On day 0, the presence of lipid oxidation treatments (light0 and light/cs0) lowered the TAC, but this effect was not significantly different from the control. The additions of vitamin A and E significantly increased (p<0.05) the TAC of light-only samples compared to light0. An increase in TAC in the light/csA and light/csE was also observed, but this effect was not significant. On day 1, the addition of cupric sulfate significantly lowered TAC when compared to the control and all the light samples (0, A, E). TAC of light/csA and light/csE were higher than that of Light/cs0, but only the addition of vitamin A had a significant effect on this day. On this day, lightA and lightE were not significantly different from light0, meaning that vitamin additions did not increase TAC under light-only exposure. On day 3, all treatments were significantly lower than the control and no vitamin effect was seen in ether the light-only or light/cs groups. An increase in TAC in the light/csA and light/csE compared to light/cs0 was observed, but this was not significant. On day 7, all treatments were also significantly lower than the control, but no significant difference were observed between treatments.

The day effect was significant in all treatments, meaning that the FRAP value changed by day. In the control, a significant decrease in TAC was observed between day 0-1 and 3-7 with a
total decrease of 45% from day 0 by the end of storage. This is likely due to the natural
decrease in antioxidant activity. Amamcharla and Metzger (2014) reported a 27% reduction in
TAC after 48 hr in control samples of raw milk. For all the light-only treatments (0,A,E) and
light/csA and light/csE, a significant decrease in TAC began on day 1, and this steadily decreased
significantly on day 3 and day 7. For light/cs0, the decrease in TAC began on day 1 and
continued until the end of storage, but days 3 and 7 were not significantly different from each
other. Light/cs samples had a much larger initial reduction on day 1 (36-43%) than all the light
samples. (18-30%).

Table 2. Average ferric reducing antioxidant power values (µmol Fe²⁺/L milk) of milk exposed to
lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day (% decrease from day 0)</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>318.59±28.0</td>
<td>276.55(13)</td>
<td>288.60(9)</td>
<td>173.96(45)</td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>296.27±12.6</td>
<td>244.38(18)</td>
<td>151.71(49)</td>
<td>85.88(71)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>338.96±13.8</td>
<td>236.32(30)</td>
<td>129.86(62)</td>
<td>87.65(74)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>329.95±18.2</td>
<td>249.05(24)</td>
<td>139.36(58)</td>
<td>91.75(72)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light*cs²</td>
<td>0</td>
<td>293.75±5.8</td>
<td>167.39(43)</td>
<td>109.83(63)</td>
<td>105.70(64)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>319.03±21.3</td>
<td>203.29(36)</td>
<td>122.83(61)</td>
<td>96.90(70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>319.28±11.8</td>
<td>195.16(39)</td>
<td>150.45(53)</td>
<td>101.22(68)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Data for periods 1 and 2. 3 repetitions per period. ²cs-cupric sulfate. Superscripts a-d within
each column correspond to the treatment effect by day. Superscripts w-z within each row
correspond to the day effect by treatment. Means not sharing a common superscript are
significantly different (p<0.05)

The antioxidative effect of the added vitamins was only seen in the early stages of
storage. This is likely due to the inactivation or degradation of antioxidants in the presence of
light towards the end of storage. Kristensen and others (2004) reported that oxidation of
buttermilk began in the serum phase, where a significant decrease of water-soluble antioxidant
activity was observed during storage. Ascorbic acid has proven to be play an important role in
the regeneration of α-tocopherol in different biological and food systems (Niki 1987) and can also prevent the light-induced degradation of retinyl palmitate (Jung and others 1998). Ascorbic acid, a water-soluble antioxidant, is present in milk at low levels (2.11 mg/100 g) (Oste and others 1997). The synergism between ascorbic acid and α-tocopherol is possible due to a reduction potential of the ascorbate radical/ascorbate system (0.28 V) above that of α-tocopherol radical/α-tocopherol (0.5 V) (Nielsen and others 2002). Although this effect has not been fully established in milk, its occurrence has been suggested by Timmons and others (2001). With less available ascorbic acid, α-tocopherol would not be able to be regenerate from its oxidized form, leading to the accumulation of inactive forms of α-tocopherol and no increase in TAC would be observed during this time.

Light exposure can also have a direct effect on vitamins A and vitamin E. Vitamin A can go through photoisomerization in the presence of light, leading to the formation cis isomers with reduced antioxidant activity (Murphy and others 1988). The tocopheryl radicals formed from α-tocopherol oxidation can decompose to tocopherol quinone and tocopherol quinone epoxides, which are compounds with no antioxidant activity (Choe and Min 2009; Clough and others 1978).

An adequate fortification method could have increased the protection of vitamins and allowed them to continue exerting their antioxidative effects throughout storage. In this study, vitamins were directly added to unhomogenized milk and mixed for 1 min. This method did not allow the vitamins to get incorporated into the milk fat and migrate to the locations where they would naturally be present. Vitamin A is naturally found in the neutral lipid core of the milk fat
globules, while α-tocopherol can be found in the membrane (Jensen and Nielsen 1996; Barrefors and others 1995)

Bartholomew and Ogden (1990) fortified milk with high a concentration of vitamin A (4210 IU/L) in a carrier emulsifier without homogenizing it into milk and compared it to vitamin A fortification followed by homogenization. Adding vitamin A without a homogenization step led to an increase in vitamin A degradation, and this effect was attributed to the inability of the carrier emulsifier to coalesce with the milk fat present, which prevented the association of the added vitamin to the milk fat. Whited and others (2002) reported on the protective effect of milk fat by exposing whole milk, 2% and skim milk with added vitamin A to light (2000 Lux). A better protective effect of vitamin A was found in whole milk than the other types of milk. Since the added vitamins used in our study were not homogenized into the milk, they are did not have enough contact with milk fat and were more exposed to degradation than natural occurring antioxidants.

Cupric sulfate significantly decreased TAC only on day 1 (Table 2). During this day, light in the presence of cupric sulfate did not have the same damaging effect on antioxidants as light in the absence of cupric sulfate. Singlet oxygen accumulates during photo-oxidation reactions, while radical compounds accumulate due to autoxidation. Although both vitamin A and E can quench singlet oxygen, the chemical and physical quenching of singlet oxygen can lead to the loss of antioxidant activity (Matsushita and Terao 1980; Yang and Min 1994).

Our research suggests that when both light and cupric sulfate are present, a possible decrease in singlet oxygen might occur, because even though both light/csA and light/csE increased TAC, the effect was only significant in light/csA. Amamcharla and Metzger (2014),
reported that the addition of 7.9 IU/L vitamin E to raw milk with 0.1 mg/kg cupric sulfate did not significantly increase TAC after 24 or 48 hr storage. The major role of vitamin A is as a singlet oxygen quencher, but vitamin E can have a dual role as a singlet oxygen quencher and a radical scavenger. The possible decrease in singlet oxygen, but increase in radical compounds due to metal-induced autoxidation can explain why vitamin A can significantly increase TAC but this same effect is not seen with vitamin E. It is also possible that vitamin A is more stable than vitamin E in the presence of light. Whited and others (2002) reported a 10% loss of vitamin A in whole milk after 16 hr of light exposure at 2000 Lux, while Havemose and others (2006) reported a 71% loss of vitamin E in whole milk after 12 hr of light exposure at the same light intensity.

**Effect of added vitamins to volatile compounds in milk using GC-SPME**

Hexanal was the largest peak area in milk from all oxidized treatments. The hexanal peak areas over the seven days of storage are shown on Table 3. Exposure to light increased the peak area of hexanal starting on day 1 until the end of storage, while the presence of cupric sulfate significantly increased the hexanal peak area on all days of storage. No vitamin effect was observed in any of the storage days, but a trend is observed in light/csA samples during day 0, 1 and 3, where the hexanal peak area was lower than light/cs0. A day effect is observed for all treatments, but not for the control. The hexanal peak area of lightE significantly increased from day 1-3 but remained unchanged from day 3-7. The hexanal peak areas of light0 and lightA had a more gradual increase over the storage period. The hexanal peak areas of light/cs0 and light/csE significantly increased every day of storage except between days 3 and 7, while
light/csA slowly increased on in the earlier part of storage but the most significant increase is seen between days 3 and 7.

Table 3. Average\(^1\) hexanal peak areas\(^1\) of milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.24</td>
<td>1.62</td>
<td>3.37</td>
<td>3.68</td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>2.05</td>
<td>8.57</td>
<td>37.10</td>
<td>46.43</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.56</td>
<td>9.87</td>
<td>29.24</td>
<td>39.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2.82</td>
<td>10.25</td>
<td>47.67</td>
<td>49.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>5.86</td>
<td>92.56</td>
<td>127.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>5.24</td>
<td>76.84</td>
<td>94.48</td>
<td>155.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>7.41</td>
<td>85.48</td>
<td>130.04</td>
<td>141.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1 and 2. \(^2\)cs-cupric sulfate. Superscripts a-d within each column correspond to the treatment effect by day. Superscripts x-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)

The heptanal peak areas over the seven days of storage are shown in Table 4. Heptanal was not detected on day 0 in any of the samples. This volatile compound was also never present in any of the control samples throughout the storage period. The vitamin addition did not have a significant effect on the heptanal peak area of treatments. On days 1, 3 and 7, cupric sulfate increased the area of this volatile, but this effect was only significant on day 1. The presence of light significantly increased the heptanal peak area only on days 3 and 7 since heptanal was not detected in light samples on days 0 and 1. A significant increase in heptanal was seen with an increase in storage time for all light/cs samples. A significant increase was found in the heptanal peak areas of light/cs0 and ligt/csE between all days, but light/csA was only significantly higher between days 3 and 7, similar to the hexanal data.
Table 4. Average\(^1\) heptanal peak areas of milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
<td>1.89(^{b,x})±0.3</td>
<td>3.32(^{ab,x})±0.2</td>
<td></td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>A</td>
<td>nd</td>
<td>nd</td>
<td>1.48(^{b,x})±0.1</td>
<td>2.85(^{b,x})±0.6</td>
<td></td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>E</td>
<td>nd</td>
<td>nd</td>
<td>2.31(^{b,x})±0.4</td>
<td>3.38(^{ab,x})±0.2</td>
<td></td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>nd</td>
<td>2.77(^{a,x})±0.5</td>
<td>4.67(^{a,y})±0.4</td>
<td>8.23(^{ab,z})±1.8</td>
<td></td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>A</td>
<td>nd</td>
<td>2.31(^{a,x})±0.1</td>
<td>3.73(^{ab,x})±0.4</td>
<td>8.86(^{a,y})±1.9</td>
<td></td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>E</td>
<td>nd</td>
<td>2.45(^{a,y})±0.1</td>
<td>5.53(^{a,y})±0.8</td>
<td>7.94(^{ab,z})±0.4</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1 and 2. 1 repetition per period. \(^2\)cs-cupric sulfate. Superscripts a-b within each column correspond to the treatment effect by day. Superscripts x-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)

Hexanal and heptanal are two of the major aldehydes responsible for oxidized flavor, but other secondary oxidation products can also be associated with the development of this flavor. Their sensory thresholds are 0.07 and 0.1 mg/kg milk, respectively (Badings 1984).

Adding vitamins A or E did not inhibit the formation of oxidation products, but a trend showed that vitamin A, in the presence of light/cs, decreased the peak areas of both hexanal and heptanal, although this effect was not significant. This effect of light/csA could be related to the significant increase observed in TAC on day 1, meaning that an increase in antioxidant capacity might be able to decrease the accumulation of oxidation products.

Vitamin A is not typically added for the purpose of serving as an antioxidant in milk, instead it is added for nutritional purposes (Newcomer and Murphy 2001). Research has been focused on examining the effects of β-carotene on the oxidative stability of milk instead of vitamin A. Both vitamin A and β-carotene can quench singlet oxygen but β-carotene has a higher quenching rate than vitamin A (Li and others 2000). Havemose and others (2004)
supplemented grass silage (GS) and corn silage (CS) with β-carotene and α-tocopherol. The final concentrations in GS-fed bovine milk was 697 µg/L of β-carotene and 854 µg/L of α-tocopherol, while the final concentrations in CS-fed bovine milk was 223 µg/L of β-carotene and 375 µg/L of α-tocopherol. Both milks were exposed to light (2000 Lux). GS-fed bovine milk has significantly higher lipid oxidation products, but CS-fed bovine milk has significantly higher protein oxidation products. α-Tocopherol in GS-fed bovine milk significantly decreased during the 24 hr of storage, but β-carotene remained unchanged.

Charmley and Nicholson (1994) reported that a 20% increase in α-tocopherol concentration was not sufficient at protecting milk from developing spontaneous oxidized flavor. Van Aardt and others (2005) exposed milk samples with either 0.05% α-tocopherol or a combination of 0.025% α-tocopherol and 0.025% ascorbic acid to light (1200 Lux) and found that even though the peak areas of oxidation products increased in both groups, panelists did not detect an oxidized flavor in the α-tocopherol samples.

**Sensory analysis of oxidized flavor**

The main objective of sensory analysis was to determine if the addition of vitamins significantly affected the level of oxidized flavor, but other flavor attributes were also evaluated. The addition of vitamin A or E did not have a significant effect any of the flavor attributes measured. The scores for metal oxidized, lacks freshness, feed and foreign flavor attributes were not significantly different among the treatments and control (Appendix B).

The presence of cupric sulfate significantly increased the score of light oxidized and total oxidized flavor, but no effect was seen in any of the other flavor attributes measured. Table 5 shows the light oxidized flavor scores.
Table 5. Light oxidized flavor score\(^1\) for milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>2.0(^{a,x}) ±2.9</td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>1.6(^{a,x}) ±2.2</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>1.5(^{a,x}) ±3.0</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>2.1(^{a,x}) ±2.8</td>
</tr>
<tr>
<td>Light(^*)cs(^2)</td>
<td>0</td>
<td>2.4(^{a,x}) ±2.8</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>2.3(^{a,x}) ±2.9</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>2.1(^{a,x}) ±2.7</td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1 and 2. 8 panelists on a 15 cm scale. \(^2\)cs-cupric sulfate. Superscripts a-d within each column correspond to the treatment effect by day. Superscripts x-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)

As expected, no significant differences were found in the light-oxidized flavor scores between samples on day 0. On days 1, 3 and 7, only the presence of cupric sulfate significantly increased light-oxidized flavor scores compared with the control, but no significant differences were found between light/cs treatments (0,A,E). Only on day 1, the light-oxidized flavor scores were significantly higher for all light/cs samples than for all the light-only samples. The light-oxidized score of the light-only treatments increased throughout storage, but they were not significantly different than the control on any of the sampled days. The only exception was found on day 7, where light0 was significantly higher than the control. Large standard deviations in the light-oxidized score data could have accounted for a lack of significant difference between light-exposed samples and the control.

A day effect was observed in the light oxidized flavor score for all the treatments, with the exception of the control, which remained unchanged throughout storage. All light-only treatments (O, A, E) had a more gradual increase throughout storage, whereas the light/cs
samples only has a significant increase on day 1. No further increase was reported for light/cs0 or light/csA after day 1, but the light oxidized score for light/csE significantly increased from day 3 to day 7, although this score was not significantly different than light/cs0.

Table 6 shows the total oxidized flavor scores. The total oxidized flavor attribute accounts for all oxidized flavor present, including metal and light-induced oxidized flavor.

Table 6. Total oxidized flavor score\(^1\) for milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>2.3(x\pm3.0)</td>
<td>1.6(b, x\pm2.0)</td>
<td>1.4(b, x\pm0.4)</td>
<td>1.4(c,x\pm1.3)</td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>2.0(a,x\pm2.2)</td>
<td>2.7(b,x\pm2.9)</td>
<td>4.4(a,y\pm3.0)</td>
<td>6.8(b, x\pm3.9)</td>
<td></td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>A</td>
<td>1.9(a,x\pm2.9)</td>
<td>2.9(b,xy\pm2.4)</td>
<td>4.5(a,y\pm2.5)</td>
<td>5.3(b,y\pm3.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>2.2(a,x\pm2.7)</td>
<td>2.5(b,x\pm2.3)</td>
<td>4.6(a,y\pm2.2)</td>
<td>5.2(b,y\pm2.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>3.0(a,x\pm2.4)</td>
<td>7.2(b,y\pm3.2)</td>
<td>7.1(a,y\pm3.3)</td>
<td>6.9(ab,y\pm4.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>3.7(a,x\pm3.3)</td>
<td>6.3(a,y\pm3.8)</td>
<td>5.9(a,y\pm2.6)</td>
<td>7.8(ab,y\pm2.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>3.3(a,x\pm3.1)</td>
<td>6.6(a,y\pm3.7)</td>
<td>7.3(a,y\pm2.8)</td>
<td>8.8(a,y\pm3.2)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1 and 2. 8 panelists on a 15 cm scale. \(^2\)cs-cupric sulfate. Superscripts a-c within each column correspond to the treatment effect by day. Superscripts x-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)

On day 1, no significant differences were found between treatments and control. By day 1, the addition of cupric sulfate significantly increased the total oxidized flavor score compared to all the light-only treatments and the control. On day 3 and 7, all treatments were significantly different than the control, but no significant differences were observed between treatments, with the exception of light/csE, which was significantly higher than all the light-only treatments. A day effect was also observed for all light-only samples and all light/cs samples but not for the control, showing the effectiveness of protection from light exposure. Similarly to the light oxidized scores, the total oxidized scores for the light-only samples steadily significantly
increased over time. The scores for the light/cs samples had a significant increase only on day 1 and remain unchanged throughout storage although a significant increase was observed on the total oxidized flavor score for light/csE from day 1 to day 7.

The addition of vitamins did not protect milk from oxidation, and vitamin E in the presence of light/cs had higher scores in both light and total oxidized flavor, but this effect was not significant. This trend was not observed with vitamin E in the presence of only light. Amamcharla and Metzger (2014) reported that oxidized flavor of raw milk with added vitamin E (7.9 IU/L) and cupric sulfate (0.1 mg/kg) was not significantly different from copper-only samples. Slots and others (2007) reported that the accumulation of hexanal increased in milk from cows supplemented with 3400 IU of all-rac-α-tocopherols per day per cow when exposed to light (2200 Lux) or to cupric sulfate (25 µM). Further analysis showed that the ascorbyl radical formed by copper catalysis was more pronounced in samples with higher tocopherol content. The pro-oxidant effect of α-tocopherol in milk can be observed when levels reach above 2.7 mg/kg of milk (Jacobsen and others 2008). In our study, 100 mg/kg milk fat of α-tocopherol was added to samples, which is equivalent to 3.28 mg/kg milk. It is possible that vitamin E already present in the milk could have increased the total α-tocopherol content to levels higher than 3.28 mg/kg. The pro-oxidative effect of α-tocopherol occurs when α-tocopherol donates a hydrogen to a radical and it becomes a tocopheryl radical. This radical can abstract hydrogens from unsaturated lipids if present at high concentrations (Choe and Min 2009).

Panelists gave higher scores to light and total oxidized flavor attributes when samples had added cupric sulfate, but metal oxidized flavor scores did not increase. Panelists did not
associate the increase in oxidized flavor to metal-induced oxidation; instead, they associated it with light-induced oxidation. Lawless and others (2004) reported that copper sulfate has some bitter and astringent properties that could be felt as a tactile sensation in the tongue, which distinguishes it from light-oxidized flavor. At the levels used in this study, it is possible that metal-induced oxidation products and sensations were overpowered by light-induced oxidation products. During training, only a definite level of metal-oxidized flavor was achieved in samples and panelists were never exposed to pronounced levels of this attribute. It is also likely that panelists were not very confident in their assessment of metal oxidized flavor, because the milk used in this study was unhomogenized and tasted different from the commercial milk used for training.

Conclusion

The addition of vitamins A and E at the levels used in this study and in the presence of lipid oxidation initiators only had a significant effect on the TAC of milk initially but no effect was seen by the end of storage. Even though TAC was significantly higher on day 0 with the additions of vitamins A or E in the presence of light, and on day 1 with the addition of vitamin A in the presence of light/cs, this was not enough to decrease the peak areas of the oxidation products measured in this study. Trained panelists were not able to detect significant differences in oxidized flavor development between samples with added vitamins and samples without added vitamins. Future research on the effects of vitamins as potential antioxidants in milk can help explain some of the interactions that are likely to occur when milk is exposed to different levels and types of pro-oxidative factors. Understanding these antioxidant
mechanisms can aid in the development of adequate supplemented feed that can increase oxidative stability and decrease the incidence of oxidized flavor in milk.

References


http://agromedia.ca/ADM_Articles/content/oxmilk.pdf

degradation of foods and beverages due to photo-oxidation. Polymer Preprints 48(2):724.


CHAPTER 5

GENERAL CONCLUSION

The overall objective of this research was to understand the effects of light and cupric sulfate as lipid oxidation initiators and determine if vitamins A or E can inhibit TAC degradation, oxidized product formation and oxidized flavor development. The first study investigated the effects of light and/or cupric sulfate as pro-oxidants in milk. Results showed that both light and cupric sulfate significantly decreased TAC when present alone. In the presence of light, the effect of cupric sulfate was not significant. Light had the most significant effect on the formation of all volatile compounds, but cupric sulfate in the presence of light significantly increased the accumulation of oxidation products.

The second study evaluated the potential of vitamins A and E as inhibitors of oxidation in milk. A significant increase in TAC was detected on day 0, when milk with added vitamin A or E was exposed to light. Only vitamin A significantly increased TAC when exposed to light and cupric sulfate simultaneously. This increase in TAC was not sufficient at inhibiting oxidation product formation or oxidized flavor as detected by a trained sensory panel.

Overall, this research demonstrated that the pro-oxidative effect of light or cupric sulfate can be affected by the presence of the other, and increasing levels of vitamin A and E in the feed might have a beneficial effect on the total antioxidant capacity of milk. Understanding the interactions between factors affecting oxidized flavor in milk will allow dairy producers to identify potential causes of oxidized flavor and take action in preventing its development.
Future Work

Future research should focus on increasing the antioxidant levels of milk by supplementing cows’ feed. This would allow for better incorporation of antioxidants into the milk system, which would place the antioxidants in their natural location and help protect them from degradation. Direct measurement of each antioxidant as well as the measurement of the total antioxidant capacity (TAC) of samples, would shed light into how each antioxidant contributes to the TAC. Since synergistic relationships between milk antioxidants can occur, it would be beneficial to evaluate the effects on oxidized flavor when one antioxidant is present at different levels of another antioxidant, such as α-tocopherol in the presence of ascorbic acid. When evaluating the effects of pro-oxidative factors in milk such as light, research should focus on exposing milk for only about 8 hr, since this more accurately represents what consumers experience.

The use of Electron Spin Resonance (EPR) could be beneficial for assessing the initial oxidative changes that occur prior to oxidized flavor development and oxidation product formation. Measuring oxygen depletion with varying types of antioxidants and pro-oxidants can also help determine how oxygen might play a role in lipid oxidation when photo-oxidation and autoxidation occur simultaneously in milk. Having a deeper understanding of the different stages of oxidation and the factors that lead to the development of oxidized flavor would greatly benefit the dairy industry. Milk producers would become more aware about how proper handling practices, supplementing feed and milk packaging can influence the quality of milk and hopefully help minimize the incidence of oxidized flavor.
APPENDIX A. SENSORY EVALUATION BALLOT

Sample #_________                                              Panelist number_____

Evaluation of Milk Flavor

Feed

________________________________________
slight   definite   pronounced

Foreign (describe ________________________________________)

________________________________________
slight   definite   pronounced

Lacks freshness (describe ____________________________________)

________________________________________
slight   definite   pronounced

Light Oxidized

________________________________________
slight   definite   pronounced

Metal Oxidized

________________________________________
slight   definite   pronounced

Total Oxidized

________________________________________
slight   definite   pronounced
APPENDIX B. AVERAGE SENSORY SCORES FOR MILK EXPOSED TO LIPID OXIDATION TREATMENTS AND VITAMINS DURING STORAGE (PERIODS 1 AND 2)

APPENDIX B1. Metal oxidized flavor score\(^1\) for milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.2(\text{a,x}\pm0.6)</td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>0.5(\text{a,x}\pm0.8)</td>
</tr>
<tr>
<td>A</td>
<td>0.3(\text{a,x}\pm0.7)</td>
<td>1.4(\text{a,x}\pm2.2)</td>
</tr>
<tr>
<td>E</td>
<td>0.1(\text{a,x}\pm0.4)</td>
<td>0.9(\text{a,x}\pm1.4)</td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>0.7(\text{a,x}\pm1.1)</td>
</tr>
<tr>
<td>A</td>
<td>1.4(\text{a,x}\pm2.9)</td>
<td>0.4(\text{a,x}\pm1.4)</td>
</tr>
<tr>
<td>E</td>
<td>1.4(\text{a,x}\pm2.5)</td>
<td>0.5(\text{a,x}\pm1.2)</td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1 and 2. 8 panelists on a 15 cm scale. \(^2\)cs-cupric sulfate. Superscripts a within each column correspond to the treatment effect by day. Superscripts x within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)

APPENDIX B2. Feed flavor score\(^1\) for milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.0(\text{a,x}\pm0.0)</td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>0.1(\text{a,x}\pm0.2)</td>
</tr>
<tr>
<td>A</td>
<td>0.0(\text{a,x}\pm0.0)</td>
<td>0.0(\text{a,x}\pm0.0)</td>
</tr>
<tr>
<td>E</td>
<td>0.0(\text{a,x}\pm0.0)</td>
<td>0.1(\text{a,x}\pm0.3)</td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>0.0(\text{a,x}\pm0.0)</td>
</tr>
<tr>
<td>A</td>
<td>0.0(\text{a,x}\pm0.0)</td>
<td>0.0(\text{a,x}\pm0.0)</td>
</tr>
<tr>
<td>E</td>
<td>0.0(\text{a,x}\pm0.0)</td>
<td>0.0(\text{a,x}\pm0.0)</td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1 and 2. 8 panelists on a 15 cm scale. \(^2\)cs-cupric sulfate. Superscripts a within each column correspond to the treatment effect by day. Superscripts x within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)
APPENDIX B3. Foreign flavor score\(^1\) for milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.0(^{a,x}\pm0.0)</td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>0.2(^{a,x}\pm0.7)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.3(^{a,x}\pm0.8)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0.1(^{a,x}\pm0.5)</td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>0.0(^{a,x}\pm0.1)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.0(^{a,x}\pm0.0)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0.1(^{a,x}\pm0.2)</td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1 and 2. 8 panelists on a 15 cm scale. \(^2\)CS-cupric sulfate. Superscripts \(^a\) within each column correspond to the treatment effect by day. Superscripts \(^x\) within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)

APPENDIX B4. Lacks freshness flavor score\(^1\) for milk exposed to lipid oxidation treatments and vitamins during storage (n=16)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.5(^{a,x}\pm0.6)</td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>1.0(^{a,x}\pm1.8)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>1.1(^{a,x}\pm1.7)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>1.0(^{a,x}\pm1.4)</td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>0.7(^{a,x}\pm1.0)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.4(^{a,x}\pm0.8)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0.4(^{a,x}\pm0.6)</td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1 and 2. 8 panelists on a 15 cm scale. \(^2\)CS-cupric sulfate. Superscripts \(^a\) within each column correspond to the treatment effect by day. Superscripts \(^x\) within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)
APPENDIX C. Average\(^1\) ferric reducing antioxidant power values (µmol Fe\(^{2+}\)/L milk) of milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day (%decrease from day 0)</th>
<th>vitamin</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0</td>
<td>293.03(^{a\ldots}\pm43.0)</td>
<td>286.13(2)(^{a\ldots}\pm17.5)</td>
<td>281.39(4)(^{a\ldots}\pm11.2)</td>
<td>177.17(40)(^{a\ldots}\pm7.4)</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td>0</td>
<td>295.59(^{a\ldots}\pm12.3)</td>
<td>234.92(21)(^{b\ldots}\pm23.7)</td>
<td>145.32(51)(^{b\ldots}\pm14.1)</td>
<td>89.54(70)(^{b\ldots}\pm11.7)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td></td>
<td>306.83(^{a\ldots}\pm46.9)</td>
<td>234.30(24)(^{b\ldots}\pm10.9)</td>
<td>121.10(61)(^{b\ldots}\pm16.8)</td>
<td>86.03(72)(^{b\ldots}\pm21.0)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td></td>
<td>305.66(^{a\ldots}\pm35.1)</td>
<td>222.69(27)(^{b\ldots}\pm39.9)</td>
<td>130.63(57)(^{b\ldots}\pm19.1)</td>
<td>90.28(70)(^{b\ldots}\pm15.2)</td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td></td>
<td>0</td>
<td>279.47(^{a\ldots}\pm21.9)</td>
<td>157.31(44)(^{b\ldots}\pm22.4)</td>
<td>110.64(60)(^{b\ldots}\pm13.8)</td>
<td>102.46(63)(^{b\ldots}\pm14.8)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td></td>
<td>293.98(^{a\ldots}\pm39.5)</td>
<td>190.62(35)(^{c\ldots}\pm22.3)</td>
<td>97.66(67)(^{c\ldots}\pm46.9)</td>
<td>79.58(73)(^{b\ldots}\pm31.6)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td></td>
<td>298.03(^{a\ldots}\pm32.2)</td>
<td>183.64(38)(^{d\ldots}\pm17.9)</td>
<td>129.64(57)(^{b\ldots}\pm30.0)</td>
<td>93.92(68)(^{b\ldots}\pm35.3)</td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1, 2 and 3. 3 repetitions per period. \(^2\)cs-cupric sulfate. Superscripts a-d within each column correspond to the treatment effect by day. Superscripts w-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)
APPENDIX D. AVERAGE PEAK AREAS OF VOLATILE COMPOUNDS IN MILK EXPOSED TO LIPID OXIDATION TREATMENTS AND VITAMINS DURING STORAGE (PERIODS 1, 2 AND 3)

APPENDIX D1. Average\(^1\) hexanal peak areas for milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vitamin</th>
<th>Day</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>2.14(^{b,c,w})±0.8</td>
<td>1.08(^{b,w})±0.8</td>
<td>2.24(^{c,w})±2.0</td>
<td>4.13(^{b,w})±0.8</td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>1.37(^{d,w})±1.0</td>
<td>7.27(^{b,w})±2.1</td>
<td>34.15(^{b,c,xy})±4.6</td>
<td>40.64(^{b,y})±8.3</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>1.71(^{c,d,w})±1.2</td>
<td>8.68(^{b,w})±1.7</td>
<td>28.07(^{b,c,w})±2.3</td>
<td>35.28(^{b,x})±10.4</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>1.88(^{b,c,d,w})±1.3</td>
<td>10.00(^{b,w})±0.6</td>
<td>39.58(^{b,c,xy})±13.6</td>
<td>41.80(^{b,y})±11.5</td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>4.61(^{a,b,c,w})±1.9</td>
<td>74.16(^{a,x})±30.7</td>
<td>102.52(^{a,xyz})±35.4</td>
<td>123.28(^{a,z})±40.2</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>4.90(^{a,b,w})±0.8</td>
<td>65.44(^{a,x})±16.1</td>
<td>81.05(^{a,b,x})±20.4</td>
<td>132.74(^{a,y})±41.4</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>6.81(^{a,w})±0.9</td>
<td>72.32(^{a,x})±18.7</td>
<td>103.21(^{a,xyz})±39.2</td>
<td>118.26(^{a,z})±32.6</td>
</tr>
</tbody>
</table>

\(^1\)Data for periods 1, 2 and 3. 1 repetition per period. \(^2\)cs-cupric sulfate. Superscripts a-d within each column correspond to the treatment effect by day. Superscripts w-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)

APPENDIX D2. Average\(^4\) heptanal peak areas for milk exposed to lipid oxidation treatments and vitamins during storage

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Day</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Light</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
<td>1.73(^{c,x})±0.4</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>nd</td>
<td>nd</td>
<td>1.51(^{c})±0.1</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>nd</td>
<td>nd</td>
<td>1.97(^{b,c,x})±0.6</td>
</tr>
<tr>
<td>Light*cs(^2)</td>
<td>0</td>
<td>nd</td>
<td>2.35(^{a,x})±0.7</td>
<td>3.99(^{ab,y})±1.0</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>nd</td>
<td>2.16(^{a,x})±0.2</td>
<td>3.40(^{abc,xy})±0.6</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>nd</td>
<td>2.27(^{a,x})±0.3</td>
<td>4.47(^{a,y})±1.6</td>
</tr>
</tbody>
</table>

\(^4\)Data for periods 1, 2 and 3. 1 repetition per period. \(^2\)cs-cupric sulfate. Superscripts a-c within each column correspond to the treatment effect by day. Superscripts x-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)
APPENDIX E. AVERAGE LIGHT AND TOTAL OXIDIZED FLAVOR SCORES FOR MILK EXPOSED TO LIPID OXIDATION TREATMENTS AND VITAMINS DURING STORAGE (PERIODS 1, 2 AND 3)

APPENDIX E1. Light oxidized flavor scores\(^1\) for milk exposed to lipid oxidation treatments and vitamins during storage

\[
\begin{array}{ccc|ccc}
\text{Treatment} & \text{Vitamin} & \text{Day} & 0 & 1 & 3 & 7 \\
\hline
\text{Control} & 0 & 1.67^{a,x} \pm 2.6 & 1.74^{c,x} \pm 3.1 & 1.27^{d,x} \pm 2.0 & 1.21^{c,x} \pm 1.7 \\
\text{Light} & 0 & 1.69^{b,x} \pm 2.6 & 2.38^{bc,x} \pm 3.1 & 3.24^{cd,y} \pm 2.5 & 4.47^{ab,y} \pm 3.6 \\
& A & 1.61^{a,x} \pm 3.2 & 2.08^{bc,xy} \pm 2.5 & 3.31^{cd,y} \pm 2.4 & 4.07^{ab,x} \pm 3.4 \\
& E & 1.89^{a,x} \pm 2.8 & 1.91^{c,x} \pm 2.6 & 3.68^{bcd,x} \pm 2.6 & 3.72^{bc,x} \pm 3.5 \\
\text{Light*cs}^2 & 0 & 2.12^{a,x} \pm 2.6 & 6.10^{b,y} \pm 3.7 & 6.49^{a,y} \pm 4.0 & 6.66^{ab,y} \pm 4.3 \\
& A & 1.86^{a,x} \pm 2.6 & 4.96^{ab,y} \pm 3.6 & 5.49^{abc,y} \pm 3.1 & 7.01^{a,z} \pm 4.3 \\
& E & 1.87^{a,x} \pm 2.5 & 5.76^{b,y} \pm 4.2 & 6.14^{ab,y} \pm 4.0 & 7.06^{a,y} \pm 3.5 \\
\end{array}
\]

\(^1\)Data for periods 1, 2 and 3. 8 panelists on a 15 cm scale. \(^2\)cs-cupric sulfate. Superscripts a-d within each column correspond to the treatment effect by day. Superscripts x-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)

APPENDIX E2. Total oxidized flavor scores\(^1\) for milk exposed to lipid oxidation treatments and vitamins during storage

\[
\begin{array}{ccc|ccc}
\text{Treatment} & \text{Vitamin} & \text{Day} & 0 & 1 & 3 & 7 \\
\hline
\text{Control} & 0 & 1.90^{a,x} \pm 2.6 & 2.37^{c,x} \pm 3.1 & 1.63^{d,x} \pm 2.0 & 2.36^{b,y} \pm 2.4 \\
\text{Light} & 0 & 2.30^{a,x} \pm 2.9 & 3.65^{bc,xy} \pm 3.4 & 4.63^{bc,y} \pm 3.0 & 7.07^{a,z} \pm 4.0 \\
& A & 2.24^{a,x} \pm 3.2 & 3.48^{bc,xy} \pm 2.7 & 4.89^{bc,y} \pm 0.8 & 5.76^{a,y} \pm 3.3 \\
& E & 2.36^{a,x} \pm 3.0 & 2.80^{bc,x} \pm 2.5 & 4.94^{bc,y} \pm 2.9 & 5.69^{a,y} \pm 3.2 \\
\text{Light*cs}^2 & 0 & 2.83^{a,x} \pm 2.3 & 7.23^{a,y} \pm 3.2 & 7.59^{ab,y} \pm 3.2 & 7.39^{a,y} \pm 4.0 \\
& A & 3.59^{a,x} \pm 3.6 & 6.13^{a,b,y} \pm 3.9 & 6.49^{abc,y} \pm 2.9 & 8.32^{a,z} \pm 3.0 \\
& E & 2.97^{a,x} \pm 3.0 & 6.30^{ab,y} \pm 3.8 & 7.20^{ab,y} \pm 3.1 & 8.62^{a,z} \pm 3.2 \\
\end{array}
\]

\(^1\)Data for periods 1, 2 and 3. 8 panelists on a 15 cm scale. \(^2\)cs-cupric sulfate. Superscripts a-d within each column correspond to the treatment effect by day. Superscripts x-z within each row correspond to the day effect by treatment. Means not sharing a common superscript are significantly different (p<0.05)