Controlled Release of Antioxidants via Biodegradable Polymer Films into Milk and Dry Milk Products

Marleen van Aardt

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Susan E. Duncan, Chair
Timothy E. Long
Joseph E. Marcy
Sean F. O'Keefe
Susan R. Nielsen-Sims

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(ABSTRACT)

Thermogravimetric analysis (TGA) was used to determine the oxidative stability of edible oils (olive oil, milkfat) and triacylglycerides (triolein, trilinolein), while the effect of natural (α-tocopherol, ascorbic acid) and synthetic antioxidants (butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ)) were evaluated on stability of trilinolein. Trilinolein was oxidatively less stable than triolein, olive oil, and milkfat, while triolein was less stable than olive oil and milkfat. When comparing effectiveness of antioxidants, a combination of 0.01% BHA and 0.01% BHT increased trilinolein stability the most.

Sensory testing showed that the addition of a combination of 0.025% α-tocopherol and 0.025% ascorbic acid to milk did not influence milk flavor. However, when adding α-tocopherol and ascorbic acid to milk at these levels, light-induced oxidation off-flavor was significantly reduced in comparison to unspiked milk after 10 h of light exposure (1100-1300 lx).

The effect of antioxidants (added in a single initial dose, and in weekly additions) on light-exposed extended shelf-life milk over six weeks of light-exposed storage was measured by volatile analysis. An initial single addition of α-tocopherol and ascorbyl palmitate significantly reduced hexanal content in light exposed milk throughout the first four weeks of storage, whereas the weekly addition of a combination of BHA and BHT reduced heptanal content of milk to such a degree that no significant difference was observed when compared with light-protected control milk.

Poly(lactide-*co*-glycolide) (50:50) films loaded with 2% α-tocopherol and a combination of 1% BHT and 1% BHA were used in an antioxidant release study in water and Miglyol 812® at 4°C

and 25°C, as well as a study on the effect of these films on dry whole milk and dry buttermilk stability. BHT was released through the hydrolytic degradation of the polymer when stored in water at room temperature for eight weeks. However, it was expected that hydrolytic polymer degradation would not take place when antioxidant-loaded films were stored in dry whole milk (3.01% moisture) and dry buttermilk (4.60% moisture). α-Tocopherol, BHA, and BHT migrated into whole milk powder through diffusion over four weeks of storage at 25°C, and significantly reduced light-induced oxidation compounds.

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CHAPTER I

INTRODUCTION

Oxidation reactions in fluid and dry milk products are common mechanisms for off-flavor development, resulting in decreased shelf-life. There is a trend towards increasing shelf-life of milk for packaging as a convenience food through ultra-high temperature (UHT) processing. This convenience packaging, using clear single-serve packages, provides increased opportunity for light-induced oxidation of milk components thereby reducing the quality and value of the product. Naturally occurring antioxidants in milk help prevent oxidation, but are depleted during processing and storage. Spontaneous oxidation (autoxidation) of lipids is a natural phenomenon in biological and food systems. Lipid oxidation also can be induced by catalytic systems such as light, temperature, enzymes, metals, metalloproteins and microorganisms. All oxidation reactions involve oxygen species, and most of these reactions involve free radicals.

In biological systems, various biochemical defense mechanisms involving trace minerals and antioxidant vitamins, protect cellular components from oxidative damage (Madhavi *et al*., 1996; Verellotti *et al*., 1992). In food systems, naturally occurring antioxidants also can protect lipids against oxidation. Milk naturally contains low concentrations of antioxidants (α-tocopherol: 25-35 $\mu g.g^{-1}$ milkfat; ascorbic acid: < 20 $mg.l^{-1}$) (Nath *et al*., 1992, Rosenthal *et al*., 1993). However, processing and storage deplete these natural resources, resulting in decreased protection of lipid oxidation. Lipids oxidize in the presence of oxygen and initiators such as light, to form reactive species such as hydroperoxides that eventually lead to the formation of unwanted flavor compounds (Cadwallader and Howard, 1998).

Antioxidants such as α-tocopherol, ascorbic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBQH) inhibit the formation of hydroperoxides, inhibiting production of oxidation derived off-flavors. Indirect addition of α-tocopherol to milk through supplementation in cow diets and intramuscular injections yield low levels or short duration of antioxidant levels in milk (Focant *et al*., 1998). A limitation to adding antioxidant directly to food in one large initial dose is rapid depletion of the antioxidants, as well

as very high initial concentrations required (Jung *et al*., 1998). Indirect addition of antioxidants to food through diffusion from packaging also shows depletion of antioxidant content over time, but not as rapidly as initial single additions (Wessling *et al.*, 2000). BHT, BHA, and TBHQ are currently used in direct and indirect additions for prevention of oxidative reactions in dry food products and oils. The effectiveness of a combination of α-tocopherol and ascorbic acid, and a combination of BHA and BHT, in limiting lipid oxidation and protecting light sensitive vitamins is well known (Bandarra et al., 1999, Smith and Berge, 1997, Frankel, 1998). In the United States BHA, BHT, α-tocopherol, and ascorbic acid are approved for human consumption, with maximum addition of BHA and BHT allowed at 200 ppm in fat as specified in the US Code of Federal Regulation for food additives (CFR, 2001).

Consistent levels of antioxidants in food might be achieved by controlled release of antioxidants from biodegradable plastic films, such as polylactide (PLA), polyglycolide (PGA), and copolymers such as poly(lactide-*co*-glycolide) (PLGA). These polymers are widely used in the biomedical field for sustained-release preparations (Cheng *et al*., 1998). Degradation of PLA, PGA, and PLGA occurs by a mechanism of hydrolytic splitting, and is therefore best suitable in an oil-in-water emulsion environment such as milk (Anderson and Shive, 1997). The diffusion-controlled release of BHA and BHT from food package liners into dry food products, such as cereal, is well researched (Miltz *et al*., 1995). However, the migration of these antioxidants from biodegradable polymers into liquid and dry foods has not received much attention. Potential use of biodegradable polymers as unique active packaging options for sustained delivery of antioxidants could be a great benefit to the dairy industry by limiting oxidation of some dairy products.

The objectives of this study are to:

1. Determine the extent of oxidation in model oxidation systems (triolein, trilinolein, olive oil, and milkfat) with and without various antioxidants (α-tocopherol, ascorbic acid, BHA, and BHT), using thermogravimetric analysis (TGA);
2. Compare volatile flavor compounds of light-exposed extended shelf-life milk when dosed with initial single additions and constant weekly additions of (α-tocopherol, ascorbic acid, ascorbyl palmitate, BHA, BHT, and combinations thereof);

3. Determine antioxidant release characteristics of antioxidant-loaded PLA and PLGA films when exposed to model systems;
4. Compare volatile flavor compounds of dry whole milk and dry buttermilk when exposed to antioxidant-loaded PLGA films.

CHAPTER II

LITERATURE REVIEW

Milk is a complex biological system that consists of approximately 87% water, 4% fat, 3.5% proteins, lactose, minerals, and other solids. The distinctive fresh milk flavor is attributed to many flavor compounds. The natural balance of these flavor compounds can easily be disturbed by pre-harvest, process, storage, and packaging conditions. Exposure to oxygen and light can cause oxidation of key milk components, which subsequently results in the development of off-flavors frequently described as cardboard, metallic, mushroom, and cooked vegetable (Cadwallader and Howard, 1998). In this section, the effects of storage conditions, packaging, and antioxidants on causing and inhibiting oxidative changes related to altered milk flavor will be discussed.

2.1 LIPID OXIDATION

During storage of milk and dried milk products, oxygen dependent reactions, such as oxidation, progress. Oxidation of unsaturated lipids is the major cause of the development of off-flavor compounds and oxidative rancidity, as well as a number of other reactions, with the subsequent formation of secondary products, which reduce the shelf-life, nutritive value, and product safety. Pre- and post-harvest technologies have been employed to increase levels of unsaturated fatty acids in an effort to make milk more healthful, and butter more spreadable (Focant *et al*., 1998). A major disadvantage of milk that it contains mono- and polyunsaturated fatty acids that increase susceptibility to oxidation. Storage at low temperatures efficiently preserves saturated fats but is not sufficient to protect mono- and polyunsaturated fats against oxidation (Madhavi *et al*., 1996). The potential for oxidation of lipids also increase in milk products with an increased shelf-life such as extended shelf-life milk (ESL), ultra high temperature (UHT) processed milk, and powdered dairy products. Two major mechanisms for lipid oxidation include the free radical autoxidation of unsaturated lipids with triplet oxygen, and the oxidation of unsaturated lipids with singlet oxygen that occur at a significantly greater rate than with normal triplet oxygen (Frankel, 1998).

High levels of unsaturated fatty acids make lipids susceptible to oxygen attack, and subsequently autoxidation. Oxygen behaves as a biradical ($^{\bullet}O\text{-}O^{\bullet}$) in its ground state, also called its triplet state ($^3O_2$). The autoxidation of lipids proceeds by a three-stage free radical process.

2.1.1 Initiation reactions

Initiation reactions take place in the presence of an initiator (I) either by the abstraction of a hydrogen radical ($H^{\bullet}$) from an allylic methylene group (-C**H**$_2$-CH=CH-) of an unsaturated fatty acid or by the addition of a radical to a double bond. Initiators can produce radicals by different mechanisms (heat, metals, and light).

Generic:	$I^{\bullet} + LH \rightarrow IH + L^{\bullet}$
Heat:	$LOOH \rightarrow LO^{\bullet} + {}^{\bullet}OH$
Metals:	$LOOH + M^{2+} \rightarrow LO^{\bullet} + OH^{-} + M^{3+}$
	$LOOH + M^{3+} \rightarrow LOO^{\bullet} + H^{+} + M^{2+}$
Light:	$RCOR + h\nu \rightarrow RCO^{\bullet} + R^{\bullet}$

2.1.2 Propagation reactions

During propagation reactions, free radicals ($L^{\bullet}$, $LO^{\bullet}$, $^{\bullet}OH$, $LOO^{\bullet}$, $RCO^{\bullet}$) are converted to other radicals via a chain reaction that consumes molecular oxygen and yields new free radical species (peroxyl radicals, $LOO^{\bullet}$). This is a much faster step than the subsequent hydrogen transfer reaction between unsaturated lipids and peroxyl radicals to form primary oxidation products - hydroperoxides (LOOH). The chain-propagation reactions are a continuous process as long as unsaturated lipid or fatty acid molecules are available (Gordon, 1990; Jadhav *et al*., 1996). Since the formation of hydroperoxides is a slow reaction, hydrogen abstraction from unsaturated lipids becomes selective for the most weakly bound hydrogen. Therefore, the susceptibility of lipids to oxidize depends on the availability of allylic hydrogens, and their ease to bind with peroxyl radicals.

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$
$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

2.1.3 Termination reactions

Termination reactions occur when there is a critical reduction in the amount of unsaturated lipids or fatty acids. Radicals bind to one another, forming stable non-radical compounds.

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow \text{non-radical products}$$

Although the lipid hydroperoxides formed during lipid oxidation are flavorless, thermal degradation of hydroperoxides generates peroxyl and alkoxyl radicals ($LO^{\bullet}$). Alkoxyl radicals can react with unsaturated lipid to form alcohols, or undergo fragmentation into unsaturated aldehydes.

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet}$$
$$LO^{\bullet} \rightarrow RCHO + L^{\bullet}$$

Termination reactions can also include condensation of peroxyl, alkoxyl, or alkyl radicals to form odorous ether-containing dimers, carbon linked dimers, and carbonyl compounds as oxidation-fragmentation products (Frankel, 1998; Cadwallader and Howard, 1998; Gordon, 1990; Jadhav *et al*., 1996).

$$2LOO^{\bullet} \rightarrow LOOL + O_2$$
$$LO^{\bullet} + L^{\bullet} \rightarrow LOL$$
$$2L^{\bullet} \rightarrow L\text{-}L$$

Flavor deterioration of food lipids is caused mainly by volatile lipid oxidation products, often at concentration below 1 ppm. The main volatile decomposition products from oleate, linoleate, and linolenate are carbonyl compounds, alcohols, and hydrocarbons. These compounds form

when alkoxyl radicals, a primary oxidation product of hydroperoxides of autoxidized and photosensitized oxidized fatty esters, are cleaved. In addition to the decomposition of hydroperoxides, unsaturated aldehydes and ketones undergo autoxidation to provide additional volatile compounds that will influence flavor (Frankel, 1998).

2.2 PROTEIN OXIDATION

Traditionally, oxidation in milk has focused primarily on lipid oxidation. However, proteins, peptides, and amino acids are also susceptible to oxidative changes caused by free radicals (Davies and Dean, 1997; Ostdal *et al*., 2000). The most significant effect of protein oxidation is changes in rheological properties of a food. This is mainly due to protein cross-linkage, breakdown of protein primary structure, and conformational changes (Dean *et al*., 1997). To a lesser extent, protein oxidation leads to flavor changes (Jadhav *et al*., 1996). As is the case with lipid oxidation, protein oxidation can be initiated by factors including light, gamma irradiation, peroxidizing lipids, metal ions, the products of enzymatic and non-enzymatic browning reactions, and certain food additives (Macrae *et al*., 1993).

Some oxidative changes in proteins and amino acids that result in the development of off-flavors are summarized here: *(i)* the oxidation of histidine, cysteine, methionine, tryptophan, and tyrosine by superoxide ion, hydrogen peroxide, and singlet oxygen can occur when food containing photosensitizers such as riboflavin is exposed to light. The attach of peroxides on proteins lead to the formation of methionine sulfoxide, which is further oxidized to methionine sulfones (Jadhav *et al*., 1996); *(ii)* free amino acids and amino acid residues undergo substantial oxidation in the presence of peroxidizing lipids. Methionine, cysteine, histidine, and lysine are the most susceptible amino acids or amino acid residues. Compounds formed from amino acids in reaction with peroxidized lipids include imidazole, lactic acid, hydrogen sulfide, methionine sulfoxide, and diaminopentane (Jadhav *et al*., 1996; Macrae *et al.,* 1993).

Ostdal *et al.* (2000) studied the effects of lactoperoxidase (LPO), a milk enzyme, on the stability of milk proteins. Lactoperoxidase is present in raw milk and is added to yogurt during production to suppress acid production. It readily induces milk lipid oxidation through a radical

reaction that involves H_2O_2. They used freeze-quench electron spin resonance to prove that milk proteins are also oxidized by H_2O_2-activated LPO.

2.3 PHOTO-OXIDATION OF MILK COMPONENTS

Exposure of milk to light accelerates the oxidation of unsaturated fats. It results in the development of off-flavors and causes the destruction of several key nutrients such as riboflavin (Vitamin B_2) (Figure 2.1), ascorbic acid (Vitamin C) and the essential amino acid methionine. Direct photo-oxidation occurs as a result of free radicals produced by ultraviolet (UV) and visible light irradiation which catalyses the decomposition of hydroperoxides, peroxides, carbonyl compounds, or other oxygen complexes of unsaturated lipids. After catalysis, lipid oxidation progresses by normal free radical autoxidation as described above in Section 2.1.1.

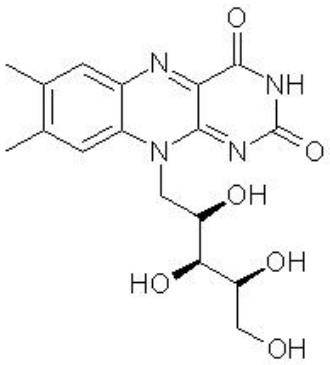

Figure 2.1. Chemical structure of riboflavin.

Both visible (380-780 nm) and UV light (UV A, 380-320 nm; UV B 320-280 nm) can lead to the degradation of food. Most lipid, protein, and sugar fractions in food do not absorb light in the visible region. However, in milk, riboflavin absorbs into the visible region (Figure 2.2) and acts as a photosensitizer by transferring energy into highly reactive forms of oxygen, which will in turn cause lipid oxidation to occur (Borle *et al.*, 2001). Fluorescent lights, which are regularly used in dairy display cases, have spectral power distributions from 250 to 750 nm that contain maximum mercury emission lines at approximately 254, 313, 365, 405, 436, 546, and 587 nm. The intensity of these lines varies and will depend upon the amount of mercury vapor (the conducting gas) (Piechocki, 1998).

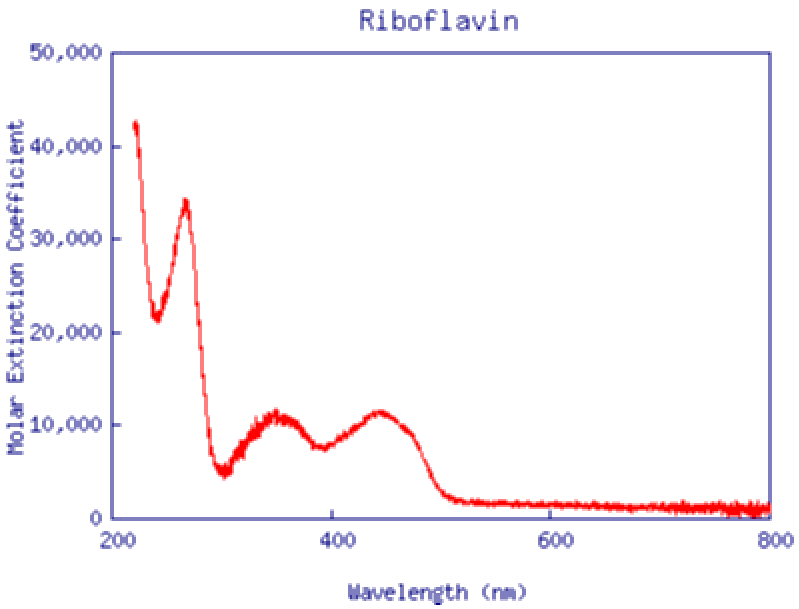

Figure 2.2. Absorbance spectrum of riboflavin.

Sensitizers, such as riboflavin, have two excited states, the singlet (^{1}Sens) and the triplet (^{3}Sens) state, with the latter having a longer life-time, which in turn, can initiate photosensitized oxidation. Two types of sensitizers exist. Type I sensitizers, such as riboflavin, serve as photochemically activated free radical initiators. The sensitizer in its triplet state reacts with the lipid by hydrogen atom or electron transfer to form radicals, which can react with oxygen. Riboflavin has three absorption bands, in the UV B, UV A, and in the visible region (blue to green). The latter is the main band responsible for the photo-oxidation of milk (Frankel, 1998).

$$^3\text{Sens} + \text{LH} + h\nu \rightarrow [\text{intermediate}] + O_2 \rightarrow \text{LOOH} + \text{Sens}$$

Type II sensitizers interact with oxygen by energy transfer to give singlet oxygen ($^1O_2$). Singlet oxygen is highly electrophilic and reacts very rapidly with unsaturated fats. In contrast to free radical oxidation, photosensitized reactions are not inhibited by chain-breaking antioxidants.

$$^3\text{Sens} + O_2 + h\nu \rightarrow [\text{intermediate}] + {}^1\text{Sens} \rightarrow {}^1O_2\ (+\ \text{LH}) \rightarrow \text{LOOH}$$

Many researchers have described off-flavors in milk due to light oxidation. Skibsted (2000) recently reviewed light-induced changes in dairy products and identified two types of chemical

reactions involved. (i) Reactions of singlet oxygen with protein, lipid, and vitamins give rise to the destruction of riboflavin and the formation of oxidation products that include unpleasant off-flavors such as a "burnt feather" flavor. (ii) Reactions of free radicals induce a cascade of reactions leading to oxidation products, which are responsible for the deterioration of food flavor to produce compounds such as "cardboard" flavor. Cadwallader and Howard (1998) described two components that are involved in the development of "light-activated" flavor in milk. They reported that initially a burnt, activated sunlight flavor develops and predominates for about two or three days. They attributed this flavor to photoreduction of riboflavin in milk that leads to the Strecker degradation of methionine to form odorant methional, and also leads to the photogeneration of superoxide anion. The second component is attributed to superoxide anion that undergoes dismutation to form singlet oxygen and other activated oxygen species which can initiate free radical lipid oxidation to form numerous volatile carbonyl compounds. This off-flavor, often characterized as metallic or cardboardy, usually develops after two days and does not dissipate (Jadhav *et al*., 1996; Marsili, 1999). Analyses of volatile components found in the headspace of milk exposed to fluorescent light over 12, 24, and 48 h have shown a steady increase in methional, hexanal, pentanal, and dimethyl disulfide, which are directly related to the amount of oxygen available (Kim and Morr, 1996) Likewise, cream powders stored at 30^{o}C under fluorescent lights for 35 weeks showed an increase in hexanal concentration, which was strongly influenced by the exposure to light and the presence of oxygen in the headspace (Andersson and Lingnert, 1998).

Li and Min (1998) conducted kinetic studies on the depletion of oxygen in headspace over model systems (water/acetone) when riboflavin, and protective agents such as α-tocopherol (Vitamin E) and ascorbic acid were present and where the model systems were exposed to light (4000 lx). They found that the amount of oxygen present in the headspace did not change in the absence of light, but decreased in the presence of light, thus confirming the action of riboflavin.

A review on the influence of light on water-soluble vitamins (Vitamin C and B_2) in milk was recently published (Sharma and Lal, 1998). The loss of riboflavin (Vitamin B_2) is highly correlated with the formation and reactions of singlet oxygen. Riboflavin-sensitized photo-

oxidation of ascorbic acid (Vitamin C) involves the addition reactions of electrophilic singlet oxygen and is affected by amino acids such as tryptophan and tyrosine.

2.4 PACKAGING MATERIALS AS PHOTO-PROTECTORS

Packaging could play a significant role in the protection of milk against light-induced oxidation. The ideal package would not transmit any light or oxygen to limit light-induced and autoxidation maximally. Christi *et al*. (1981) reported that clear polyethylene (PE) pouches transmit light in both UV and visible regions of the spectrum while pouches covered with a black/white PE over-wrap transmitted less than 3% light in the UV and visible regions. Milk packaged in the clear PE pouches produced an oxidized off-flavor after only 24 h exposure at 1600 lx, while milk packaged in the pouches covered with a pigmented over-wrap did not suffer from oxidation off-flavor even after 10 d of exposure to light (1600 lx). The development of new polymer packaging materials has led to several studies concerning light transmittance. Polyethylene terephthalate (PETE) has an advantage over high-density poly(ethylene) (HDPE), the polymer currently used for the larger sizes of milk packaging, since the oxygen transmission rate at 4^{o}C, 50% relative humidity, and 21% oxygen of a commercial one-pint PETE bottle is 19 $\mu l.d^{-1}$ compared to 390 - 460 $\mu l.d^{-1}$ for a commercial one-pint HDPE bottle (Van Aardt *et al.*, 2001). However, translucent HDPE has an advantage over clear PETE in that it blocks approximately 40% light between 300 nm and 700 nm, whereas clear PETE only blocks approximately 20% light in the same range (Van Aardt *et al.*, 2001)

Van Aardt *et al.* (2001) studied the effects of light exposure of 1300 lx on milk packaged in pigmented and UV-protected clear PETE containers for three weeks and concluded that amber PETE showed significantly lower levels of hexanal, a typical lipid oxidation product, than clear PETE, or PETE with UV-block. Amber PETE completely blocked light between 300 and 400 nm, and partially blocked light between 400 nm and 700 nm, while PETE with UV-block completely blocked light between 300 nm and 350 nm and transmitted almost all light between 400 and 700 nm. Cladman *et al.* (1998) compared several translucent green PETE packages with UV blockers for their protection of lipid oxidation and vitamin A degradation in milk stored at 4^{o}C. Green PETE afforded better light protection against lipid oxidation and loss of vitamin A in

milk, as compared to clear PETE. They reported that UV-blockers absorbed 95% of UV radiation, while green PETE bottles blocked radiation in the visible range and some in the UV range.

2.5 MEASUREMENT OF OXIDATIVE STABILITY

Primary products of lipid oxidation are odorless and tasteless lipid hydroperoxides, which degrade rapidly into odorous secondary oxidation products such as carbonyls and hydrocarbon compounds. The only quantitative indicator of oxidation reactions is oxygen consumption. Since it is hard to determine oxygen consumption in food or biological systems, alternative measures include chemical, spectrophotometric, chromatographic, and sensory methods of primary or secondary oxidation products. Chemical methods include peroxide value, Kreiss test, thiobarbituric acid (TBA/TBARS) test, anisidine value, and carbonyl value. Spectrophotometric methods include UV absorption, electron spin resonance (ESR) spectroscopy, and chemiluminescence. Chromatographic methods are used most widely for the measurement of volatile compounds either by headspace analysis or by direct injection of the product. A combination of gas chromatography and mass spectroscopy (GC/MS) offers high sensitivity for the identification and quantification of off-flavor compounds. High-performance liquid chromatography (HPLC) is a very useful technique to measure peroxides, hydroperoxides and secondary oxidation products (Rajalakshmi and Narasimhan, 1996).

2.5.1 Peroxide and Hydroperoxide Analysis

Determining the extent of oxidation is routinely done by many different methods of varying effectiveness. The oldest and most commonly used measurement for oxidation of oils is the peroxide value. The results of peroxide value determinations vary according to the procedures as a result of interference from oxygen in the air, exposure to light, and slow absorption of iodine by unsaturated fatty acids in the oil. Another limitation to this technique is that during autoxidation, peroxides reach a maximum followed by a decrease at more advanced stages. Conditions also influence the rate of decomposition of hydroperoxides. Hydroperoxides are

rapidly decomposed during oxidation at temperatures above 100°C, or in the presence of methionine, or when exposed to light (Frankel, 1998). The use of HPLC for separation of lipid hydroperoxides has increased in popularity. The determination of conjugated dienes at 234 nm has been used to quantify phospholipids hydroperoxides (Crawford *et al.*, 1980), while the separation of phosphatidylcholine and phosphatidylethanolamine by normal phase HPLC and detection of corresponding hydroperoxides with a spectrophotometric iron thiocyanate post-column assay has been used (Müllertz *et al*., 1990).

2.5.2 Carbonyl Analysis

Total carbonyl content also is measured routinely. During autoxidation of unsaturated lipids, the carbonyl content increases slowly, followed by a rapid increase after hydroperoxides reach a maximum corresponding to their decomposition into aldehydes and carbonyl products. The anisidine value provides useful information on non-volatile carbonyl compounds (α-, β-alkenals) formed in oils during oxidation. The TBARS test is also an old and popular method used to measure carbonyl oxidation products in biological systems. It is based on color absorbance at 532-535 nm formed between TBARS and oxidation products of polyunsaturated lipids. However, the TBARS test is not specific and produces color reactions by a large number of secondary oxidation products (Frankel, 1998). Other components present in foods such as browning reaction products, amino acids, nucleic acid, nitrites, and metals (sometimes present in natural antioxidants in lipids) interfere with the formation of the TBARS color complex (Frankel, 1998).

2.5.3 Oxygen Absorption Methods

Oxygen absorption methods that monitor the uptake of atmospheric oxygen and an increase in the weight of the sample with a Warburg manometer also are used in the food industry. Oxygen absorption methods are based on the fact that the oxidation of products is accompanied by an uptake of atmospheric oxygen and an increase in the weight of the samples. An estimation of oil

resistance to oxidation therefore is obtained by measuring weight gain percent due to oxidation (Rajalakshmi and Narasimhan, 1996). The method of thermogravimetric analysis (TGA) is used extensively in the polymer chemistry industry for the measurement of oxidative stability of polymers. TGA continuously monitors changes in sample weight while the sample is temperature programmed in a modified atmosphere environment. Recently Gennaro *et al*. (1998) successfully used TGA for evaluating the effect of antioxidants on oxidative stability of virgin olive oil in an oxygen environment.

2.5.4 Chromatographic Methods

Gas chromatographic methods are used to determine volatile oxidation products that are either directly responsible for or serve as markers of flavor development in oxidation of lipids. GC analyses for volatile compounds not only correlate with flavor scores by sensory analysis, but also provide sensitive methods to detect low levels of oxidation in oils and food lipids. Generally, volatile flavor components of milk are present in trace amounts and require isolation and concentration for GC analysis. Different analytical approaches have been applied for isolation and studying of volatile components. These include simultaneous distillation-extraction, molecular distillation, solvent extraction, dynamic headspace sampling, static headspace methods, membrane dialysis, conventional solid-phase extraction and purge-and-trap sampling. Among conventional GC sample preparation methods, the static headspace appears to be the least costly and simplest. It also provides a volatile profile similar to the aroma perceived by the nose. The other methods generally require solvents or special apparatus, and are relatively time-consuming (Chin *et al*., 1996; Yang and Peppard, 1994).

Solid phase microextracion (SPME) initially was developed by Arthur and Pawliszyn (1990). Arthur *et al*. (1992) applied the technique for the extraction of volatile and semi-volatile compounds from waste water samples. Since then SPME has been used extensively for the extraction of volatiles from many foods and beverages. Marsili (1999) used SPME-GC/MS for the analysis of light-induced lipid oxidation products in milk and Yang and Peppard (1994) compared liquid and headspace SPME sampling. Yang and Peppard (1994) examined liquid and

headspace SPME sampling in a test solution comprising 25 common flavor components and applied this technique to the analysis of authentic food, beverage and flavor samples. A detection limit of the order of 0.1-10 ppb was estimated for most of the components. Van Aardt *et al.* (2001) measured light-induced lipid oxidation in milk as a function of hexanal, pentanal, and dimethyl disulfide content using SPME-GC.

The addition of antioxidants to foods has shown to reduce oxidation and the off-flavors associated with it. In the next section, natural and synthetic antioxidants used in the food industry today will be discussed.

2.6 FOOD ANTIOXIDANTS

It is well known that unsaturated lipids in foods can oxidize when in contact with important factors such as oxygen, light, and trace metals. The role of antioxidants in preserving polyunsaturated lipids is of considerable practical importance. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) are efficient and relatively cheap, and are commonly used in the food industry to retard lipid oxidation. A worldwide trend toward natural and healthy includes avoiding or minimizing the use of synthetic food additives has drawn special attention to natural antioxidants, such as tocopherols and ascorbic acid. The relative effectiveness of antioxidants is dependent on the lipid substrates, test system, concentration, oxidation time, and method used to determine lipid oxidation. Based on their function, food antioxidants are classified as primary or chain-breaking antioxidants, synergists, or secondary antioxidants. In this review, only primary and synergistic antioxidants will be discussed, since antioxidants used in this study fell under these two categories.

2.6.1 Primary Antioxidants

Primary antioxidants, such as BHA, BHT, TBHQ, and tocopherols (Figure 1) terminate free-radical chain reactions by donating hydrogen atoms or electrons to free radicals and converting them to more stable products. Antioxidant radicals ($A^{\bullet}$) are stabilized by electron delocalization

of their phenoxyl structures. The effectiveness of the phenolic antioxidant, such as BHA, BHT, and α-tocopherol, is directly related to the resonance stabilization of the phenoxy radicals. Bulky alkyl substituents on phenolic antioxidant also make for more effective chain-breaking capabilities since they produce stable antioxidant radicals (Frankel, 1998).

$$LH \rightarrow L^{\bullet} + I^{\bullet}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$$

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

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet}$$

$$A^{\bullet} + LOO^{\bullet} \rightarrow \text{non-radical products}$$

$$A^{\bullet} + A^{\bullet} \rightarrow \text{non-radical products}$$

BHA and BHT are fat soluble antioxidants that are more effective in less unsaturated animal fats than polyunsaturated vegetable oils. Tocopherols are also fat-soluble and increase in antioxidant activity from α-tocopherol to δ-tocopherol, although vitamin activity increases from δ-tocopherol to α-tocopherol. Tocopherols also can quench singlet oxygen. Chemical quenching rates for α-, γ-, and δ- tocopherol are 6.6×10^6, 2.6×10^6, and 0.7×10^6, respectively (Frankel, 1998). Under certain conditions, tocopherols are less effective because of their tendency to act as a chain-carrier and become a pro-oxidant, when the antioxidant radical undergoes transfer with the lipid substrate and regenerates peroxyl radicals.

2.6.2 Synergistic Antioxidants

Synergistic antioxidants can be broadly classified as oxygen scavengers and chelators. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant, or they may react with free oxygen and remove it in a closed system. Ascorbic acid acts as a synergist with primary antioxidants, especially tocopherols, thus allowing the primary antioxidant to be used at lower levels (Gordon, 1990; Jadhav *et al*., 1996). Another example of synergistic antioxidants is between two primary antioxidant, BHA and BHT. These antioxidants

both act as hydrogen donors to the phenoxy radical, to regenerating each other. Bandarra *et al*. (1999) and Deeth (1997) studied the antioxidative synergism between phospholipids in milk and α-tocopherol.

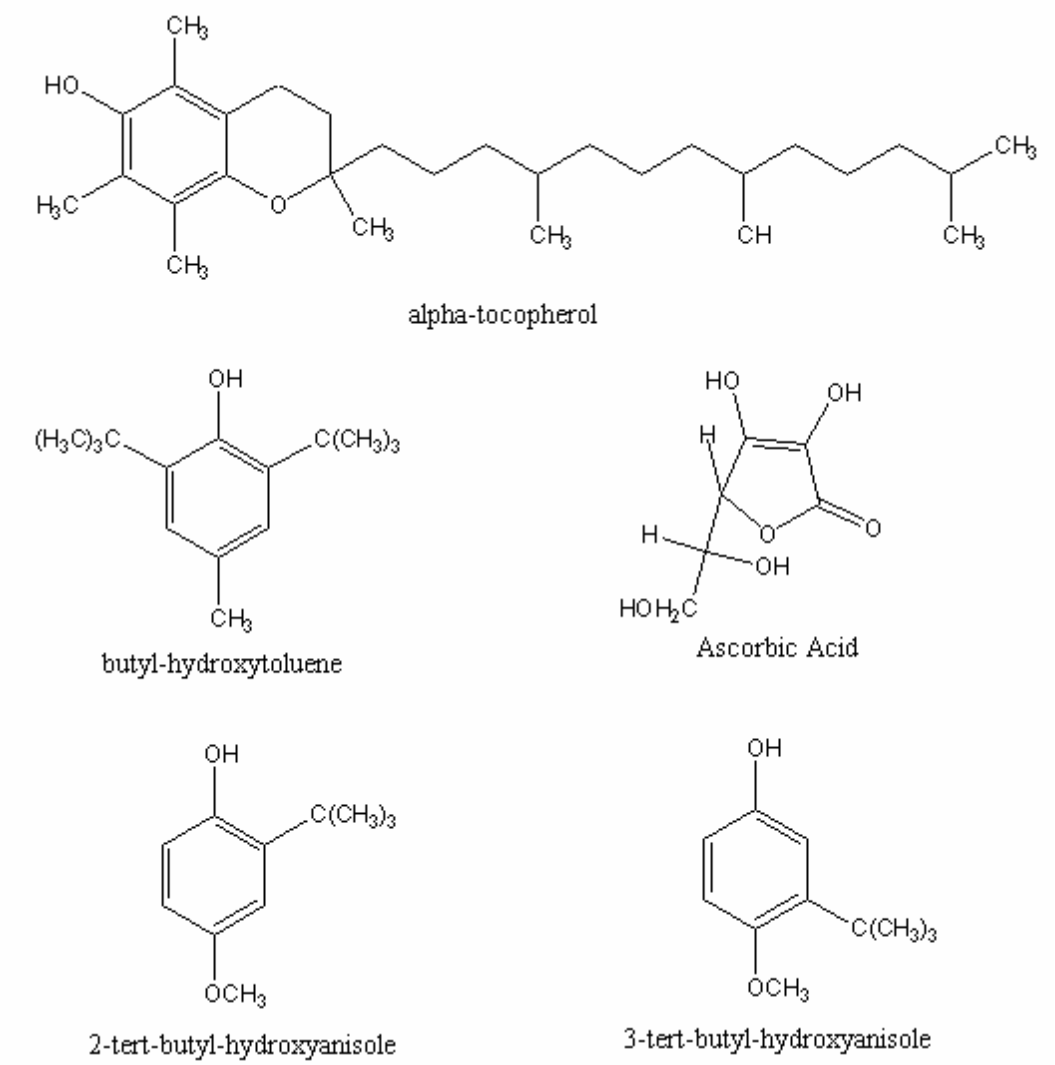

Figure 2.3. Structures of major synthetic and natural antioxidants.

2.6.3 Antioxidants in Milk

Although milk contains low concentrations of natural antioxidants, such as α-tocopherol (13-30 $\mu g.g^{-1}$ milkfat) and ascorbic acid (<20 $mg.kg^{-1}$), processing and storage deplete these natural resources (Jensen and Nielsen, 1996; Rosenthal *et al*., 1993). Various studies have attempted to

control oxidized flavor in milk by either injecting antioxidants in the muscles of dairy cows, adding antioxidants to the feed of dairy cows, or directly adding antioxidants to dairy products (Charmley and Nicholson, 1993; Focant *et al*., 1998; Jung *et al*., 1998).

Jung *et al*. (1998) concluded that dimethyl disulfide was mainly responsible for the light-induced off-flavor in skim milk and that increasing levels of ascorbic acid (from 200 – 1000 ppm) lowered the formation of dimethyl disulfide and off-flavor in skim milk. Yang (1994) reported that ascorbic acid chemical quenching rate of singlet oxygen was 1.53×10^8 at pH 4.0, 1.86×10^8 at pH 5.6, and 1.19×10^8 $M^{-1}sec^{-1}$ at pH 7.0. By supplementing cow diets with α-tocopherol, Focant *et al.* (1998) successfully increased milk α-tocopherol levels from 45 $mg.kg^{-1}$ milkfat to 78 $mg.kg^{-1}$ milkfat. However, this substantial vitamin E increase in milk showed a decrease in resistance to oxidation, as measured with the Rancimat apparatus, indicating a possible pro-oxidant effect. The Rancimat method has been developed as an automated version of the very demanding AOM method (active oxygen method). In this method highly volatile organic acids produced by autoxidation are absorbed in water and used to indicate the induction time of fats and oils. Charmley and Nicholson (1993) used intramuscular injections of α-tocopherol to control spontaneous oxidized flavor in milk from dairy cows. They found that 5% of injected dose was excreted in milk over seven days following injection, but that oral supplementation can prolong these benefits. Phospholipids in milk have also shown to be an effective synergistic antioxidant with α-tocopherol (Deeth, 1997). Phospholipids of the milkfat globule membrane consist of approximately 40-60% unsaturated fatty acids of which one third are polyunsaturated, which might indicate a high susceptibility to oxidation. However, Deeth (1997) and Bandarra *et al*. (1999) studied the mechanism responsible for the observed synergy of tocopherols and various phospholipids against milkfat and fish oil oxidation, respectively. They concluded that the highest inhibitory effect against oxidation was shown by the synergy between α-tocopherol and phosphatidylethanolamine.

The biggest limitations to adding antioxidants to milk via injection into muscles or addition through diet are low yield and a short duration of elevated antioxidant levels in milk. A limitation to adding antioxidants directly to milk in one large dose is rapid depletion of the antioxidants (Charmley and Nicholson, 1993; Focant *et al*., 1998; Jung *et al*., 1998).

BHA, BHT, and TBHQ are widely used in the food industry as direct food additives but also are used in indirect addition of antioxidants to food products through diffusion from polymeric films. BHA and BHT have been shown to be effective in controlling oxidized flavor in dry food products such as breakfast cereal and crackers (Hoojjat *et al*., 1987; Jadhav *et al*., 1996). Byrd (2001) suggested that BHA and BHT are excellent for use in packaging materials for cereal products. The large surface area of most breakfast cereal products not only increases exposure to oxygen and increases the possibility of oxidation, but also allow for BHT and BHT to come into contact with the food. Miltz *et al.* (1995) and Sharma *et al*. (1990) reported that storage stability was increased when oatmeal cereal was stored in antioxidant-impregnated plastic films. They also attributed the stability of the food to the volatilization of the antioxidant from the packaging surface, with subsequent diffusion into the food. Wessling *et al.* (1998, 1999; 2000) did extensive work on the ability of antioxidants to diffuse from plastic packaging into fatty food and food simulating liquids, and the effect on lipid oxidation. The least variation in volatile profile between treated and control samples, as determined by GC-MS and electronic nose analysis, were seen in oatmeal samples stored for 10 weeks in the presence of BHT-impregnated low density polyethylene (LDPE) films. However, α-tocopherol-impregnated LDPE films did not prolong shelf-life of oatmeal. Immersion of α-tocopherol-impregnated LDPE films into linoleic acid emulsion significantly reduced the rate of oxidation at 6^{o}C, when measuring conjugated diene content. In general, polypropylene (PP) film exhibited excellent retention of α-tocopherol when in contact with ethanol, white wine, tap water, orange juice, milk, linoleic acid emulsion, cream or mayonnaise. However, losses of α-tocopherol were observed from LDPE film when in contact with the mentioned media.

2.6.4 Legislative Aspects Concerning Food Antioxidants

By adding antioxidants to milk, whether by direct addition or by controlled release from the container, antioxidants will be considered an additive. The Code of Federal Regulations specifies how much of an additive can be added to food, whether by direct or indirect addition, in Title 21, Volume 3 Part 172 (CFR, 2001). In general, the use of synthetic primary antioxidants such as BHA, BHT, or TBHQ for the stabilization of fats and oils is limited to 200 ppm in fat.

Natural antioxidants such as tocopherols and ascorbic acid are generally recognized as safe when used in accordance with good manufacturing practice and therefore not limited (CFR, 2001; Rajalakshmi and Narasimhan, 1996). The International Dairy Foods Association (IDFA) permits the addition of α-tocopoherol, ascorbic acid, and ascorbyl pamitate to milk. The presence of suhbstances must be noted on the label. If worlds such as "enriched" or "fortified" are used, vitamin content must be at least 10% (personal communications, M.Albee Mattow, IDFA).

Toxicological studies are crucial in determining the safety of an antioxidant for food use and also in determining the acceptable daily intake (ADI) levels. ADI levels are allocated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). ADI levels for antioxidants in foods are: *(i)* α-tocopherol, 0.15-2 $mg.kg^{-1}$ body weight (bw); *(ii)* L-ascorbic acid and sodium ascorbate, not limited; *(iii)* BHA, 0-0.5 $mg.kg^{-1}$ bw; *(iv)* BHT, 0-0.125 $mg.kg^{-1}$ bw; and *(v)* TBHQ, 0-0.2 $mg.kg^{-1}$ bw (Madhavi and Salunkhe, 1996).

2.7 POLY(LACTIDE-*co*-GLYCOLIDE) IN THE FOOD INDUSTRY

Often, in the synthesis of polymers, antioxidants are added as stabilizers. The main function of these antioxidants is to protect the polymer against oxidative degradation. However, these antioxidants may have a dual function; (i) to protect the polymer from oxidative degradation during processing, and (ii) to delay the onset of oxidation of the packaged foodstuff during storage. BHT is one of the most commonly used synthetic antioxidants and has long been known to inhibit oxidation processes in both food products and polymers. Diffusion parameters of antioxidants from polymeric packaging into food are influence by factors such as hydrophobicity/hydrophilicity, molecular weight, temperature, to name but a few. To achieve a controlled release of additives from polymer films, biodegradable polymers have been used extensively in the biomedical industry. Biodegradable polymers such as poly(lactic acid) PLA and poly(glycolic acid) (PGA) (Figure 2.4) could potentially be used in the food industry, since polymer degradation products include lactic and glycolic acids, the former of which is commonly found in dairy foods, while the latter is a common alpha hydroxy acid found in fruit.

PLA, PGA, and copolymers (poly(lactide-co-glycolide)) (PLGA) thereof, are thermoplastic polyesters that are chemically synthesized by polycondensation of free lactic- or glycolic acid or by catalytic ring-opening polymerization of lactide or glycolide (dilactone and –glycones of lactic- and glycolic acid) (Gümüsderelioglu and Deniz, 2000). PLA, PGA, and their copolymers are the most widely used and studied class of synthetic biodegradable polymers because of their good histocompatibility and biodegradability (Okada, and Toguchi, 1995). They have been widely exploited for the microencapsulation of drugs, e.g., narcotic antagonists, fertility controlling agents, anticancer agents, local anesthetics, antibiotics and vaccines, to obtain sustained or controlled release of the therapeutic agent (Cheng *et al*., 1998). Although PLA and PGA in the past have been considered primarily for medical implants and drug delivery, they meet many requirements as packaging thermoplastics and are suggested as commodity resins for general packaging applications (Sinclair, 1996). PLA, PGA, and its copolymers have good thermoplasticity, processability, and physical properties such as high strength and high modulus that are similar to conventional packaging resins (Jun, 2000; Sinclair, 1996). These polymers have good biocompatibility in that they slowly degrade by hydrolysis in a slightly moist environment over a period of several months to a year into environmentally friendly products, such as lactic acid. In turn, lactic acid biodegrades to carbon dioxide, methane, and water. An attractive feature of PLA is the potential relatively low cost of the monomer, lactic acid, which can be derived from biomass (fermentation), coal, petroleum, or natural gas (Brody and Marsh, 1997). PLA and PGA are clear, colorless thermoplastics when quenched from the melt and is similar in many respects to polystyrene. However, they are brittle under tensile and bending loads and will develop serious physical aging during applications (Jun, 2000). PLA and PGA can be plasticized by its own monomers to increase flexibility to compare with polyvinylchloride (PVC), LDPE, linear low-density polyethylene (LLDPE), polypropylene (PP), and polystyrene (PS) (Sinclair, 1996).

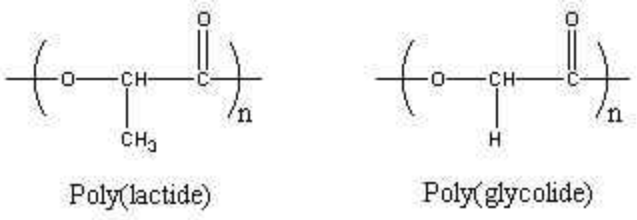

Figure 2.4. Chemical structures of polylactide and polyglycolide repeat units.

2.7.1 Synthesis

The homo- and copolymers of lactic- and glycolic acids can be synthesized by the direct condensation of the acid or by the ring-opening melt condensation of the cyclic dimers, lactide and glycolide (Figure 2.5) (Lewis, 1990, Sinclair, 1996). The direct condensation of the acids yields low molecular weight polymers. An alternative process is through the intermediate monomers, lactide and glycolide. The cyclic dimers can be prepared by a tedious cracking procedure, or by a much easier process that uses dimers and trimers of the acids (Sinclair and Preston, 1993). Due to the asymmetrical β-carbon of lactic acid, D and L stereoisomers exist, and the resulting polymer can be D, L, or racemic DL. The polymerizations are usually conducted over a period of 2-6 h at about 175^{o}C in the melt. Organotin catalysts are normally utilized with stannous chloride and stannous sulfonic acid and have been successfully employed on a limited basis. Lauryl alcohol is often added to control molecular weight during synthesis. As with most polymerizations, monomer purity is highly critical in the synthesis of PLA and PGA. Differential scanning calorimetry (DSC) purity of 99.9% or greater is usually required with the starting lactide and glycolide materials. Low monomer acidity is also a critical parameter. Free acid of 0.05% or less is normally required for achieving a high molecular weight polymer. Of equal importance, however, are the environmental conditions, particularly humidity or high monomer acidity (Lewis, 1990; Gümüsderelioglu and Deniz, 2000).

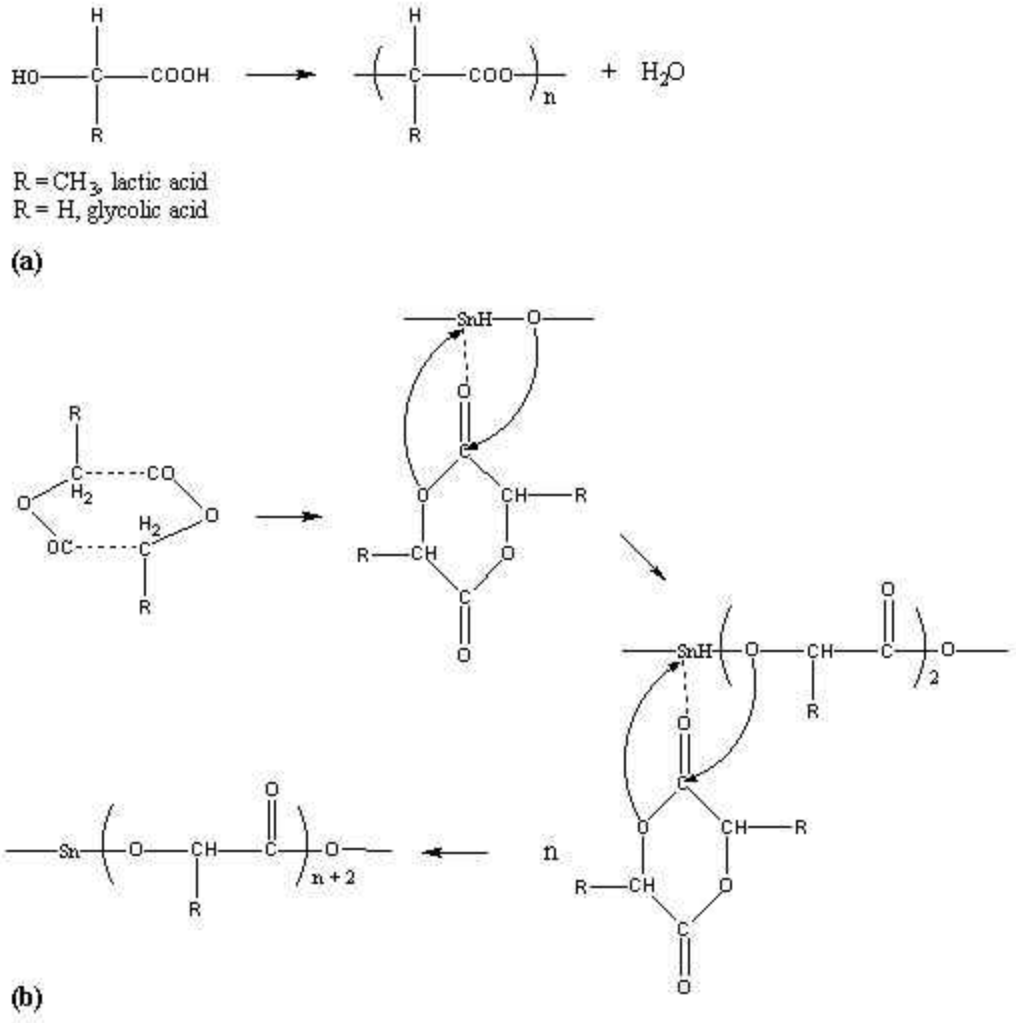

Figure 2.5. Production of polylactide and polyglycolide via (a) direct condensation of the acids and (b) ring-opening melt condensation of the cyclic dimers, lactide and glycolide.

2.7.2 Polymer Characteristics

Among the many kinds of biodegradable polymers described, the system of linear aliphatic polyesters is one of the few that can possess very high molecular weight (thus good melt processability and mechanical strength) and excellent degradation properties because of its extremely hydrolysable backbones (Zong *et al*., 1999). L-PLA has a high melting temperature (T_m) and poor processing properties. It crazes easily because of its high spherulitic crystallinity.

It is difficult to keep transparent, even when quenched from the melt. D,L-PLA is an amorphous polymer with a glass transition temperature (T_g) of 60°C, which is low for many packaging uses. The 90/10 copolymer of L-/D,L-lactide is a better composition for volume packaging resins, since it is melt processible and quenches from the melt with transparency (Sinclair, 1995; Sinclair, 1996). Poly(L-lactide-*co*-D,L-lactide) has a T_g of 60°C but it is easily orientable and heat set to provide a transparent and ordered composition that is form-stable at elevated temperatures, overcoming the weakness of a low T_g (Sinclair and Preston, 1990). Amorphous 90/10 poly(L-lactide-*co*-D,L-lactide) has a tensile strength of approximately 6800 psi (47 MPa) when the residual lactide is 5%, and 8000psi (55MPa) at 0 to 2% lactide (Sinclair, 1996). To produce flexible PLA formulations, lactide or oligomeric lactic acid is added as plasticizer. With increasing oligomeric lactic acid or lactide plasticizer, T_gs of the melt blends are lowered so that a well-plasticized PLA (15 to 20% lactide, or 30 to 40% oligomeric lactic acid) can have a T_g as low as 32 to 40°C (Sinclair, 1996).

2.7.3 Degradation

Some of the most cited studies on the biodegradation of synthetic polymers showed that, among high-molecular-weight polymers, only aliphatic polyesters are biodegradable (Brody and Marsh, 1997; Sinclair, 1996). Other biodegradable materials include starch-based materials; cellulose; chitin and chitosan; pullulan; proteins; polyesters; polyamids; polyurethanes; poly(vinyl alcohol) and its copolymers; poly(ethylene-*co*-vinyl alcohol); and poly(ethylene oxide) (Brody and Marsh, 1997).

Anderson (1994, 1995) and Anderson and Shive (1997) studied the biodegradation and biocompatibility as it relates to biodegradable polymers in controlled release systems. It is generally considered that degradation of aliphatic polyesters occurs via hydrolysis. Degradation proceeds first by diffusion of water into the material (initially into the more amorphous zones), followed by random hydrolysis, fragmentation of the material, and finally a more extensive hydrolysis accompanied by phagocytosis, diffusion and metabolism (when ingested). Hydrolysis of a biodegradable polymer is affected by a large number of factors (Anderson and Shive, 1997):

1. Water permeability and solubility (hydrophilicity/hydrophobicity)
2. Chemical composition
3. Mechanism of hydrolysis (noncatalytic, autocatalytic, enzymatic)
4. Additives (acidic, basic, monomers, solvents, drugs)
5. Morphology (crystalline, amorphous)
6. Porosity
7. Dimensions (film thickness)
8. Glass transition temperature (glassy, rubbery)
9. Molecular weight and molecular weight distribution
10. Physico-chemical factors (ion exchange, ionic strength, pH)
11. Sterilization

In general, the degradation time will be shorter for low-molecular-weight polymers, more hydrophilic polymers, more amorphous polymers and copolymers higher in glycolide content. The acidic or basic nature of additives as well as loading level markedly affect degradation rate of PLA. Maulding *et al*. (1986) reported on the acceleration of microsphere degradation rates by incorporating a tertiary amino compound. The nitrogen of the tertiary amine does not react as a nucleophile but acts as a base that participate in the degradation of ester bonds, thus accelerating polymer degradation. On the other hand, Maulding *et al*. (1986) also found that appropriate amounts of basic compounds can neutralize carboxyl endgroups and thus decrease the rate of degradation. Pistner *et al.* (1993) confirmed that semicrystalline PLA permits release of low molecular weight degradation products whereas the degradation of amorphous PLA did not permit the release of low molecular weight degradation products. The latter showed an autocatalytic mechanism of hydrolysis. In general, molecular weight is a critical factor in the degradation process. A large molecular weight distribution would indicate relatively large numbers of carboxylic end groups, which can facilitate the autocatalytic degradation of the polymer chains, as well as decrase pH (Maulding *et al*., 1986). Below critical numbers, oligomers of many polymers might be biodegradable. For example, below 5000 $g.mol^{-1}$, oligomers of polyethylene are biodegradable while those of polystyrene are not (Brody and Marsh, 1997).

Once hydrolyzed, the products of hydrolysis of PLA and PGA can be metabolized or excreted. The lactic- and glycolic acids generated by the hydrolytic degradation of PLA and PGA, respectively, becomes incorporated into the tricarboxylic acid (Krebbs) cycle and are excreted as carbon dioxide and water. Vert and coworkers (Grizzi *et al*., 1995; Vert *et al*., 1994, Vert *et al*., 1995) did extensive work on hydrolytic degradation of microspheres in *in vitro* and *in vivo* systems.

Biodegradation studies of poly(D,L-lactide) and poly(D,L-lactide-*co*-glycolide) concluded that a higher glycolide concentration yields shorter degradation times. Visscher *et al.* (1988) found that poly(D,L-lactide) microspheres showed extensive erosion and breakdown at 360 days, and at 480 days, only residual particulate was noted in the histological studies. In contrast, poly(D,L-lactide-*co*-glycolide) microspheres demonstrated extensive erosion and breakdown at 56 days with complete degradation at 63 days. Molecular weight loss studies demonstrated that 50:50 poly(D,L-lactide-*co*-glycolide) microspheres had a half-life (50% loss of molecular weight) of 15 days.

Studies have shown that biodegradability varies dramatically with chemical composition. Poly(L-lactide) and poly(D-lactide) degrade slowly, while copolymers and PGA degrade much faster. Huang *et al*. (1998) studied the crystallization kinetics, microstructure, and equilibrium melting behavior of random copolymers of melt-crystallized poly(L-lactide-*co*-meso-lactide)s. They found that the T_m decreased significantly with increasing *meso*-lactide concentration and that spherulitic growth rates were strongly dependent on *meso* content. They also found that copolymers contained significant interfibrillar regions whose concentration increased with higher comonomer content.

As mentioned before, degradation proceeds through two separate stages with different degradation rates, which correlates well with the amorphous and crystalline regions in the polymer sample, respectively. Zong *et al*. (1999) found that both stages involve chain scission. The first stage is through the amorphous gaps between lamellar stacks, where the scission of the amorphous chains can take place by the diffusion of water molecules. As the chain scission increases, chain mobility increases, facilitating the second crystallization process producing

thinner lamellar stacks with lower values of long period and lamellar thickness. The second pathway is through the scission of interlamellar amorphous layers between the adjacent lamellae within the stacks. As water molecules can eventually diffuse into this region, chain scission can lead to the collapse of lamellar stacks, causing a slight decrease in amorphous layer thickness, resulting in the rapid decrease of polydispersity.

Synclair (1996) studied the degradation of PLA films upon standing during ambient conditions and exposures to seawater. Unplasticized compression molded L-PLA films showed changes in physical properties in approximately 12 weeks. Films plasticized with oligomeric lactic acid and lactide showed changes in physical properties in 9 and 1 weeks, respectively. They also found that low lactide-containing PLA showed loss of strength and transparency in about nine weeks of warm seawater exposure, or about 1 year in ambient air and humidity.

2.7.4 Controlled Release

The use of PLA and PGA for the controlled release of drugs has been extensively studied (Cheng *et al.*, 1998; Anderson and Shive, 1997). Drugs can be incorporated in either a dissolved or dispersed form within the polymer matrices, which degrade in biological fluids with a progressive release of immobilized drug. Since polymer selection affects their biodegradation rate, a system can be designed for short or long release periods.

Gümüsderelioglu and Deniz (2000) studied the degradation and release of mitomycin-C, a drug that inhibits DNA synthesis and fibroblast proliferation, from homo- and copolymers of PLA and PGA films with different drug loading capacities and thicknesses. They found that intrinsic viscosities decreased continuously at a steady rate after being exposed to phosphate buffered saline at 37^{o}C. Mass loss showed sigmoidal curve characteristics of bulk hydrolysis, indicating no significant mass loss of the matrix during the initial lab time period, then rapid degradation with time. Gümüsderelioglu and Deniz (2000) found that the lag time depends on the molecular weight of polymer e.g., 100 days for PLA $[\eta] = 0.50 dl.g^{-1}$ and 130 days for PLA $[\eta] = 0.61 dl.g^{-1}$.

Polymer intrinsic viscosities also played a role in the release of mitomycin-C from PLA films. Gümüsderelioglu and Deniz (2000) confirmed that increasing glycolide content causes a substantial increase in release rate and released amount. This is due to the fact that increasing glycolide content increases polymer hydrophilicity, decreases crystallinity, as well as decrease degradation rate.

Gümüsderelioglu and Deniz (2000) reported that drug particles connect together to form two types of clusters when they are distributed in the polymer matrix system. The one type of cluster is connected to the surface of the film or device, therefore making it easily releasable. The other type of cluster is surrounded by intact polymer. A water-soluble drug will therefore not be released from a hydrophobic polymer such as PLA or PGA, unless the physical structure of the film changes during hydrolysis. The physical loss of an antioxidant which is soluble in a polymer involves two distinct processes: *(i)* the removal of antioxidant from the surface by osmosis, and *(ii)* the replacement of antioxidant in the surface layer by diffusion from the bulk polymer (Rajalakshmi and Narasimhan, 1996; Hoojjat *et al*., 1987; Wessling *et al*., 1999). Gümüsderelioglu and Deniz (2000) confirmed Zhang *et al*.'s (1993) findings that drug release from biodegradable matrices is diffusion controlled, osmotic controlled, degradation controlled, or a combination thereof.

2.7.5 Diffusion Parameters

Since oxygen initiates reactions on the food surface, treating packaging material with an antioxidant can help protect the product from oxidation and rancidity. This technique of intentionally adding antioxidants to food products via migration from packaging materials has been used in the breakfast cereal industry for many years. Incorporation of the additive into the polymer reduces the total amount of antioxidants consumed with products by putting less into the product and more into the packaging material. The advantages of indirect addition of antioxidants to food products through gradual migration from polymeric films versus direct addition are: *(i)* the total amount of antioxidant consumed with the product is reduced substantially; *(ii)* a single large dose is depleted rapidly, and *(iii)* oxidative reactions can be

inhibited for a longer period of time through controlled addition (Ahvenainen and Hurme, 1997; Rajalakshmi and Narasimhan, 1996). The main mechanism driving such a migration is diffusion.

The knowledge of additive diffusion allows manufacturers to design products specific to consumer demand. All that is needed for a reasonable prediction of migration in many practical cases is the availability of data for two fundamental constants: the partition coefficient $K_{P/L}$ of a migrating solute between the plastic P and the foodstuff or simulating liquid L and the diffusion coefficient D_P of the solute in P. The diffusion coefficient is influenced by a number of factors including the molecular size and physical state of the diffusant (Foldes, 1993), the morphology of the polymer, the compatibility or solubility limit of the additive within the polymer matrix (Joshi *et al*., 1996; Quijada-Garrido *et al*., 1996;), the volatility or extractability of the additive (Quijada-Garrido *et al*., 1996; Rawls, 1997), and the associated surface or interfacial energies of the monolayer and multilayer films, respectively (Foldes, 1993).

2.7.6 Polymer Safety

PLA and PGA were declared as "Generally Recognized As Safe" for the intended uses as a polymer for fabricated containers that will hold and/or package food (Conn *et al*., 1996). Limited migration of food grade substances was not found to be of significant risk.

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CHAPTER III

Effect of Antioxidants on Oxidative Stability of Edible Fats and Oils: Thermogravimetric Analysis

M. van Aardt,[*] S. E. Duncan,[*] J. E. Marcy,[*] T. E. Long,[†] S. F. O'Keefe,[*] and S. R. Nielsen-Sims[‡]

[*]Department of Food Science and Technology and

[†]Department of Chemistry,

Virginia Polytechnic Institute and State University, Blacksburg 24061

[‡]Eastman Chemical Co., Kingsport 37662-5125

Corresponding Author: Marleen van Aardt

27F FST Building, Virginia Tech, Blacksburg, VA 24061

Phone: (540) 231-8675

Fax: (540) 231-9293

E-mail: mvanaard@vt.edu

ABSTRACT

Thermogravimetric analysis (TGA) was used to determine the oxidative stability of various edible oils (olive oil, milkfat) and triacylglycerides (triolein, trilinolein, and tristearin), while the effect of natural (α-tocopherol, ascorbic acid) and synthetic antioxidants (butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroquinone (TBHQ)) on the oxidative stability of trilinolein was evaluated. Oil resistance to oxidation was obtained by measuring the increase in sample weight due to the uptake of molecular oxygen, the temperature at maximum sample weight, and the temperature at the onset of oxidation. When comparing sample weight increase, trilinolein proved to be oxidatively less stable than triolein, olive oil, and milkfat, while triolein was less stable than olive oil and milkfat. Tristearin, a saturated triglyceride, showed no increase in sample weight. Olive oil showed significantly higher stability than milkfat when comparing the temperature at the onset of oxidation. When comparing effectiveness of antioxidants, a combination of 0.01% BHA and 0.01% BHT increased trilinolein stability the most.

Key words: "oxidative stability; oils, milkfat; antioxidants; thermogravimetric analysis (TGA)"

Abbreviations used: TGA = thermogravimetric analysis, **BHT** = butylated hydroxytoluene, **BHA** = butylated hydroxyanisole, **TBHQ** = tertiary butyl hydroquinone, **ASC** = ascorbic acid, **Toc** = tocopherol

INTRODUCTION

Oxidation of unsaturated lipids is one of the major causes of the development of off-flavor compounds and the reduction in nutritive value of food products (Cadwallader and Howard, 1998). Although lipid oxidation can be induced by catalytic systems such as light, temperature, enzymes, metals, metalloproteins and microorganisms, the reactions involve free radical and/or active oxygen species (Jadhav *et al.*, 1996; Gordon, 1990). Triplet oxygen lipid oxidation, a free radical process, has been extensively studied during the past 70 years. However, triplet oxygen oxidation does not fully explain the initiation step of lipid oxidation. Singlet oxygen is involved in the initiation of triplet oxygen lipid oxidation because singlet oxygen can react directly with double bonds without the formation of free radicals (Min and Boff, 2002). During the last 30 years, increased attention has been given to singlet oxygen oxidation of foods, since (i) the rate of singlet oxygen oxidation is much greater than that of triplet oxygen oxidation, and (ii) singlet oxygen oxidation produces compounds absent in triplet oxygen oxidation due to the different reaction mechanisms (Min and Boff, 2002). Interaction with light, sensitizers, and oxygen is mainly responsible for singlet oxygen formation in food (Bradley and Min, 1992).

In food systems, naturally occurring antioxidants, such as tocopherols and ascorbic acid, protect lipids against oxidation by either quenching free radical reactions or by scavenging oxygen. However, natural antioxidants are often lost during processing or storage, necessitating the addition of exogenous antioxidants that will effectively retard the onset of lipid oxidation (Madhavi *et al.*, 1996;). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ) are synthetic antioxidants widely used in the food industry as direct food additives or as indirect additives through diffusion from plastic packaging (Jadhav *et al.*, 1996; Hoojjat *et al.*, 1987).

Direct quantification of oxidation - by measuring oxygen consumption - is often difficult in food or biological systems. Alternative measures for determining extent of oxidation include chemical methods (peroxide value, thiobarbituric acid test, anisidine value, and carbonyl value), spectrophotometric (ultraviolet absorption, electron spin resonance spectroscopy, and chemiluminescence), chromatographic, and sensory methods. Gas chromatography combined

with mass spectroscopy (GC-MS) is most widely used for the measurement of flavor compounds resulting from oxidation either by headspace analysis or by direct injection of the product. High-performance liquid chromatography (HPLC) is a very useful technique to measure peroxides, hydroperoxides and secondary oxidation products (Rajalakshmi and Narasimhan, 1996).

The Warburg manometer is an example of a method that does direct quantification of oxidation by measuring the uptake of atmospheric oxygen as an increase in sample weight. Thermogravimetric analysis (TGA) is another oxygen absorption method used extensively in the polymer chemistry industry for the measurement of oxidative stability of polymers. TGA continuously monitors changes in sample weight while the sample is subjected to controlled increases in temperature in a modified atmosphere environment. An estimation of oil resistance to oxidation is obtained by measuring percent weight gain due to oxidation (Rajalakshmi and Narasimhan, 1996). The use of TGA for food applications has received limited attention. Gennaro *et al.* (1998) used TGA for evaluating the effect of antioxidants on oxidative stability of virgin olive oil in an oxygen environment, while Rudnik *et al.* (2001) and Biswas and Staff (2001) studied the oxidative stability of linseed oil and distilled grains, respectively. TGA shows high reproducibility. It requires a small sample amount (~10 mg) and it takes a relatively short time of analysis (~ 20 min per sample).

The first objective of this study was to determine the sensitivity of TGA in measuring oxidative stability of various edible oils, fats, and triacylglycerides. The second objective was to measure the effect of various antioxidants on trilinolein stability. Since the Code of Federal Regulations specifies the maximum addition of antioxidants such as BHT, BHA, and TBHQ to fats and oils as 200 ppm (FDA, 2001), this level was not exceeded in our study. Natural antioxidants such as tocopherols and ascorbic acid are generally recognized as safe when used in accordance with good manufacturing practice and therefore are not limited (Rajalakshmi and Narasimhan, 1996). Since literature shows prooxidant effect of tocopherol at high concentrations (Cillard and Cillard, 1986; Koskas *et al*., 1984), α-tocopherol was tested at 100 ppm (0.01%) and 200 ppm (0.02%).

MATERIALS AND METHODS

Milkfat, olive oil, trilinolein, triolein, and tristearin were evaluated for oxidative stability using TGA with a temperature program where initial temperature was constant at 100°C for 5 min. In a separate experiment, trilinolein stability was evaluated in the presence of various antioxidants: (i) 0.01% α-tocopherol; (ii) 0.02% α-tocopherol; (iii) 0.01% ascorbic acid and 0.01% α-tocopherol; (iv) 0.01% BHA and 0.01% BHT; and (v) 0.01% TBHQ, where initial temperature was constant at 70°C for 5 min. The different temperature programs are a result of either having to evaporate water or solvent from the system.

Materials and Sample Preparation

Trilinolein (C18:2 Δ9*cis*, Δ12*cis*) (50 mg in amber glass ampules), triolein (C18:1 Δ9*cis*) (100 mg in amber glass ampules), and tristearin (C18:0) (1 g in amber glass ampules) were purchased from Sigma (Saint Louis, MO), and stored at -5°C. Virgin olive oil was purchased in a 250-ml clear glass container from a local grocery store in Blacksburg, VA. Olive oil aliquots (5-ml) were stored under nitrogen gas at 4°C in a dark environment until further use. Fresh raw milk was obtained locally from the Virginia Tech dairy farm within approximately 2 hours of milking. Fat was extracted within 12 hours according to the Bligh and Dyer lipid extraction procedure (Bligh and Dyer, 1959). Milkfat was then stored under nitrogen at 4°C in a dark environment until further use. BHA, BHT, TBHQ, α-tocopherol, and ascorbic acid were purchased from Aldrich (Milwaukee, WI). Weighed quantities of antioxidants were dissolved in a 1:9 (v/v) mixture of methanol/chloroform, and then added to weighed trilinolein samples.

Fatty Acid Profiles

Fatty acid contents of milkfat, olive oil, trilinolein, and triolein were determined. Extracted milkfat and oils were methylated by *in situ* transesterification with 0.5 N NaOH in methanol

followed by 14% boron trifluoride in methanol. Undecenoate (Nu-Check Prep, Elysian, MN) was used as the internal standard. Samples were injected by using an auto-sampler into a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Sunnyvale, CA). Methyl esters were separated on a 100 m × 0.25 mm i.d. fused silica capillary column (CP-Sil 88, Chrompack, Middleburg, The Netherlands). Pure methyl ester standards (Nu-Check Prep, Elysian, MN; Supelco Inc., Bellefonte, PA) were used to identify peaks and determine response factors for individual fatty acids. An 80 to 1 split ratio was used for injection of 0.5 µL hexane containing methyl esters of all samples. The carrier gas was ultra-pure hydrogen, and inlet pressure was maintained at 23.1 psi linear flow. Injector temperature was maintained at 250°C, and detector temperature was maintained at 255°C. The initial oven temperature was 70°C (held for 1 min), increased 5°C.min^{-1} to 100°C, (held for 2 min), increased 0°C.min^{-1} to 175°C (held for 40 min), and increased 5°C.min^{-1} to 225°C (held for 15 min) (Park and Goins, 1994).

Thermogravimetric Analysis

A thermobalance (TGA 2950, TA Instruments, New Castle, DE) was used. The TGA was calibrated with "alumel" alloy and nickel for temperature settings and with a 100-mg standard for weight accuracy. Approximately 9 mg of sample was placed on a tared aluminum balance pan. The pan was placed in a room temperature furnace, and the exact sample weight was determined. The temperature program for oil samples without antioxidants consisted of raising the temperature at a rate of 10°C.min^{-1} to 100°C, then holding this temperature constant for 5 min to remove all traces of moisture. The sample was then heated to 250°C at a rate of 2°C.min^{-1} to detect the oxidation peak. Since trilinolein samples containing added antioxidants also contained trace amounts of solvent, the temperature program differed in that the temperature was held constant at 70°C for 5 min, rather than at 100°C as previously described. Another reason for holding temperature constant at 70°C and not at 100°C was due to the fact that oxidation of trilinolein was observed as early as 70°C. O_2 was used as the purge gas to establish a suitable environment for the oxidation process (flow of 50 cm^3.min^{-1}) (Gennaro *et al.*, 1998), while N_2 was used throughout as control. All samples were analyzed in triplicate.

Statistical Analysis

Analysis of variance tested the null hypothesis that sample means were equal, and the alternative hypothesis that means were not equal. Fisher's protected least significant difference was the mean separation method used. A significance level of $p < 0.05$ was established to detect statistical differences. Analysis was performed using SAS Version 7, (SAS Institute, Inc., Cary, NC) (SAS, 1998).

RESULTS AND DISCUSSION

TGA makes it possible to *(i)* estimate a product's resistance to oxidation by measuring weight gain percent as a function of oxygen uptake by a sample, and *(ii)* determine the temperature at maximum oxygen uptake (Figure 1). Table 1 shows the weight increases in edible oil samples due to oxidation, as well as the temperature at maximum oxidation (T_{max}), while Table 2 reports on the effect of various antioxidants on trilinolein stability.

When comparing the oxidative stability of edible oils – compounds were subjected to an oxygen environment - in the absence of exogenous antioxidants, trilinolein proved to be significantly more unstable than triolein, tristearin, milkfat, and olive oil. The respective sample weight increases was 2.3250% (trilinolein), not detectable (tristearin), 0.8870% (triolein), 0.2337% (milkfat), and 0.2260% (olive oil) (Table 1). Fatty acid composition explains to some degree why trilinolein was oxidatively the least stable. Trilinolein consists of nearly 100% linoleic acid (C18:2) (Table 3), which provides the highest number of potential sites for oxidation (6 double bonds per molecule). When comparing that with triolein, which consists of approximately 100% oleic acid (C18:1), one would expect triolein to oxidize at half the rate of trilinolein, due to half the amount of potential oxidation sites, which is not the case. However, double allylic hydrogen atoms are expected to oxidize ten times faster than single allylic hydrogens.

Doleiden *et al.* (1974) reported reaction rates of singlet oxygen with oleic, linoleic, and linolenic acids of 0.74, 1.3, and 1.9 x 10^5 $M^{-1}s^{-1}$, respectively, which is relatively proportional to the number of double bonds in the molecules. On the other hand, triplet oxygen reacts with unsaturated fatty acids by abstracting allylic hydrogens. Once a hydrogen is removed from a polyunsatured system, a pentadienyl radical intermediate is formed. The energy required for the removal of hydrogens at different carbons is quite different. Min and Boff (2002) reported that the relative reaction ratio of triplet oxygen with oleic, linoleic, and linolenic acid for hydroperoxide formation is 1:12:25, which is dependent on the relative difficulty for the radical formation in the molecule. The reaction rate of triplet oxygen with linolenic acid is approximately twice as fast as that of linoleic acid because linolenic acid has two pentadienyl groups in the molecule, compared with the linoleic acid with one pentadienyl group (Min and

Boff, 2002). The classic mechanism for free radical oxidation of unsaturated fatty acids involves hydrogen abstraction at the allylic carbons to produce delocalized three-carbon allylic radicals (Park and Goins, 1994) (Figure 2). Various other proposed mechanisms also exist that explain the role of allylic hydrogens in free radical oxidation (Min and Boff, 2002).

Although tristearin was not evaluated for fatty acid content, it is expected to contain approximately 100% stearic acid (C18:0) (Sigma, Saint Louis, MO), which correlates well with the fact that no oxidation was observed. The estimation of values such as (i) the average number of double bonds per molecule, (ii) the average number of allylic hydrogens per molecule, and (iii) the energy required for the removal of hydrogens at different carbons are difficult for complex fats such as milkfat and olive oil.

Subsequently, triolein showed lower oxidative stability than milkfat and olive oil. As mentioned before, triolein consists of approximately 100% oleic acid, while milkfat and olive oil consist of lower percentages of unsaturated fatty acids (Table 3). The major differences in fatty acid composition between milkfat and olive oil are that milkfat contains a larger amount of short chain saturated fatty acids and that olive oil contains approximately 71.7% oleic acid in comparison to 24.6% in milkfat. Sample weight increases did not indicate differences in olive oil and milkfat stability. However, the temperature at which a weight increase first was detected (T_{onset}, Table 1) indicated that olive oil oxidized at a higher temperature than milkfat. In a similar study done by Gennaro *et al.* (1998) on the stability of olive oil, similar values were observed for increases in olive oil sample weight (0.22 ± 0.02) when exposed to oxygen. Since olive oil contains many natural phenolic antioxidants such as caffeic acid, p-hydroxy-phenylethanol, 3,4-dihydroxyphenylethanol, and elenolic acid linked to 3,4-dihydroxy-phenylethanol, a certain degree of oil stability was expected (Gennaro *et al*. 1998).

A substantial difference in fatty acid composition and the presence of natural antioxidants in olive oil and milk might also contribute to the differences in T_{onset} (Table 1). In general, vegetable oils are high in tocopherols. While the tocopherol content of olive oil used in this study is unknown, table 4 shows the variety of naturally occurring tocopherols in olive and other food oils (Padley *et al*., 1994). Milk naturally contains low concentrations of α-tocopherol (25-

35 $\mu g.g^{-1}$ fat) and ascorbic acid (<20 $mg.L^{-1}$) (Nath and Usha, 1992; Rosenthal *et al.*, 1993). Tocopherol levels in milk vary according to the diet of the cows, while pasteurization and skimming results in a reduction in the tocopherol content of milk (Focant et al., 1998; Crawley, 1993). Phospholipids in milk have also shown to be an effective antioxidant, either alone or in synergism with α-tocopherol (Bandarra *et al.*, 1999; Deeth, 1997). The phospholipids of the milkfat globule membrane consist of approximately 40-60% unsaturated fatty acids of which one third are polyunsaturated, which might indicate a high susceptibility to oxidation. It therefore appears that the role of milk phospholipids in milkfat oxidation is complex and depends on the nature of the medium and the oxidation condition (Deeth, 1997).

It is well known that the mechanism of antioxidation varies from antioxidant to antioxidant. Primary or chain-breaking antioxidants such as BHA, BHT, TBHQ, and tocopherols inhibit or retard lipid oxidation by interfering with either chain propagation or initiation by readily donating hydrogen atoms to lipid peroxyl radicals. These antioxidants are effective because (i) they produce stable and relatively unreactive antioxidant radicals, and (ii) they are able to compete with the lipid substrate (Frankel *et al.*, 1994). Ascorbic acid is known for its multifunctional effects. In foods, it can scavenge oxygen, shift the redox potential of food systems to the reducing range, act synergistically with chelators, and regenerate primary antioxidants. The synergistic effect between a free radical acceptor such as α-tocopherol and ascorbic acid is well recognized. The effect is explained by the regeneration and recycling of the tocopheroxyl radical intermediate ($TOC^{\bullet}$) to the parent phenol, α-tocopherol (Frankel et al., 1994).

$$TOC^{\bullet} + ASC^{-} = TOC = ASC^{\bullet}$$

When comparing the effect of antioxidants on the oxidative stability of trilinolein, only a combination of 0.01% BHA and 0.01% BHT significantly retarded oxidation, whereas tocopherol, alone, or in combination with ascorbic acid did not significantly improve trilinolein stability (Table 2). Phenolic antioxidants such as BHA, BHT, TBHQ, and α-tocopherol can stop the reaction of two chain-carrying peroxyl radicals and thus break two kinetic chains per molecule. The equivalent efficiency of these antioxidants might play a role in antioxidant

efficacy. BHA, BHT, and α-tocopherol each have one hydroxyl group that participates in the donation of protons to free radicals to convert them to more stable products. However, the molecular weight of α-tocopherol (430.72 $g.mol^{-1}$) is approximately double that of BHA (180.2 $g.mol^{-1}$) and BHT (220.4 $g.mol^{-1}$). By adding similar weights of each of these antioxidants, approximately double the amount of hydroxyl groups is added in the case of BHA and BHT. This might partially explain the reduced efficacy of α-tocopherol versus BHA and BHT, but does not explain why differences have been seen in the antioxidant activity of the various tocopherol isomers, which have similar molecular weights. Antioxidant activity of tocopherols increase from the α through the δ-isomers, while vitamin activity decreases from the α through the δ-isomers (Kamal-Elden and Appelqvist, 1996). Gennaro and coworkers (1998) found that 100 $mg.kg^{-1}$ BHT was as effective in stabilizing olive oil against oxidation as was 50 $mg.kg^{-1}$ of natural polyphenols such as 3,4-dihydroxyphenylethanol. BHA and BHT are fairly volatile antioxidants which also makes them useful in dried food applications. BHA has a melting point of 48-65°C while BHT has a melting point of 69°C (Aldrich Chemicals, Milwaukee, WI).

Table 2 does not indicate a significant difference between the effect of α-tocopherol alone or in combination with ascorbic acid on trilinolein stability. Even though ascorbic acid is mostly soluble in water, Frankel et al. (1998) observed that hydrophilic ascorbic acid was a more effective antioxidant in bulk corn oil than in emulsified corn oil. In contrast, the lipophilic ascorbyl palmitate was a more effective antioxidant in a corn oil emulsion than in bulk corn oil, due to its orientation in the oil-water interface. The ability of ascorbic acid to scavenge oxygen is also a well-known effect. One ascorbic acid molecule reacts easily with atmospheric oxygen and behaves as a two-electron donor. The most probable reason for not observing an improved stability of trilinolein when ascorbic acid was present, is due to the fact that the TGA flow of pure oxygen (50 $cm^3.min^{-1}$) exceeded the theoretical maximum consumption level of headspace of ascorbic acid (3.3 $mg.cm^{-1}$) (Frankel *et al*., 1994).

All antioxidants were evaluated alone in the presence of oxygen and nitrogen (Table 5) to ensure that when added to trilinolein, initial weight decreases was not attributed to antioxidant weight decrease but rather to the instability of the oil. T_d is the measure of initial weight loss of the sample, and does not indicate that a sample is necessarily stable over the temperature range in

which a sample maintains a consistent weight. A significant difference might have been observed between α-tocopherol alone and in combination with ascorbic acid if T_{onset} could have been measured. However, in this study the combination of the temperature and solvent evaporation temperature caused all T_{onset} values to fall within the 5 min of isothermal treatment at 70°C. Rudnik and coworkers (2001) compared T_{onset} of linseed oil in the presence of BHA and a combination of α-tocopherol, ascorbyl palmitate, ascorbic acid, and ethoxylated ethylene glycol. They found that a combination of natural antioxidants at 0.05% proved more effective as antioxidant than the addition of 0.01 or 0.02% BHA.

From Table 2 it seems that the addition of 0.02% TBHQ had a prooxidant effect. This might be partially due to the fact that TBHQ has two hydroxyl groups that are available to donate protons to reactive free radicals and a comparatively lower formula weight of 166.22 $g.mol^{-1}$.

CONCLUSION

TGA is a valuable technique for evaluating oxidative stability differences between different oils and fats, as well as evaluating small differences in oxidative stability that occur upon addition of antioxidants to oils. It is reproducible, requires a small sample weight, and takes a relatively short time to analyze each sample. However, further research need to be done on factors such as the effect of load on activity, etc. Altogether, this technique may be a viable option for use as a quality control measure in the food industry.

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TABLES

Table 1. Thermogravimetric analysis of various edible oils: oxidative stability as a function of weight increase due to oxygen consumption.

Edible Oils	Weight increase (%) ± *S*	T_{onset} (^oC ± *S*)	T_{max} (^oC) ± *S*
Olive Oil	0.23^c ± 0.003	149.4^a ± 0.80	162.1^a ± 0.24
Milkfat	0.23^c ± 0.019	100.0^b* ± 0.00	161.9^a ± 2.58
Triolein	0.89^b ± 0.015	101.1^b ± 0.60	151.4^b ± 1.63
Trilinolein	2.33^a ± 0.069	70.4^c ± 1.56	126.4^c ± 0.66
Tristearin	0.00^d ± 0.000	0.00^d ± 0.000	0.00^d ± 0.000

T_{onset}: Temperature at which sample weight increase is first detected

T_{max}: Temperature at which sample weight increase is at its maximum

* Onset of oxidation occurred within the 5-min period at a constant temperature of 100^oC

$^{a, b, c}$ Means within a column with different superscript letters indicate significant differences at $p < 0.05$

Table 2. Thermogravimetric analysis of trilinolein in the presence of natural and synthetic antioxidants: Oxidative stability as a function of weight increase due to oxygen consumption.

Antioxidants added to pure trilinolein	Weight increase (%) ± *S*	T_{max} (^{o}C) ± *S*
No antioxidants (control)	$2.21^{b} \pm 0.222$	$125.1^{ab} \pm 4.27$
0.01% α-tocopherol	$2.28^{b} \pm 0.080$	$117.3^{c} \pm 1.28$
0.02% α-tocopherol	$2.34^{b} \pm 0.196$	$119.9^{bc} \pm 6.79$
0.01% α-tocopherol + 0.01% ascorbic acid	$2.24^{b} \pm 0.075$	$121.2^{bc} \pm 3.21$
0.01% BHA + 0.01% BHT	$1.88^{c} \pm 0.108$	$131.8^{a} \pm 5.81$
0.02% TBHQ	$2.68^{a} \pm 0.056$	$116.5^{c} \pm 1.82$

T_{onset}: Temperature at which samp le weight increase is first detected

T_{max}: Temperature at which sample weight increase is at its maximum

$^{a, b, c}$ Means within a column with different superscript letters indicate significant differences at $p < 0.05$

Table 3. Fatty acid content (% of total fatty acid) of various edible oils determined by gas chromatography (GC)

Sample	4:0	6:0	10:0	12:0	14:0	15:0	16:0	16:1	18:0	18:1	18:2
Milkfat *	2.2	1.3	2.6	2.7	11.1	1.1	33.0	2.3	12.5	24.6	2.8
Olive Oil*							11.4		2.9	71.8	11.9
Trilinolein											99.9
Triolein										99.9	

* GC-results obtained for extracted milkfat and virgin olive oil used in this study

Table 4. Natural tocopherol content of various food oils ($mg.kg^{-1}$)*.

Oil	α-Tocopherol ($mg.kg^{-1}$)	β-Tocopherol ($mg.kg^{-1}$)	γ-Tocopherol ($mg.kg^{-1}$)	δ-Tocopherol ($mg.kg^{-1}$)
Canola	210.0	1.0	42.0	0.4
Coconut	5.0	-	-	6.0
Corn	112.0	50.0	602.0	18.9
Cottonseed	389.0	-	387.0	-
Olive	119.0	-	7.0	-
Palm	256.0	-	316.0	70.0
Peanut	130.0	-	214.0	21.0
Safflower	342.0	-	71.0	-
Sesame	136.0	-	290.0	-
Soybean	75.0	15.0	797.0	266.0
Sunflower	487.0	-	51.0	8.0
Walnut	563.0	-	595.0	450.0

* Adapted from Padley et al. (1974)

- no value, or trace amounts

Table 5. Weight loss temperatures of antioxidants under nitrogen and oxygen.

Antioxidants	T_d (Nitrogen) (^oC)	T_d (Oxygen) (^oC)	Weight increase (Oxygen) (%)
α-tocopherol	264.4	226.6	0.378
ascorbic acid	195.9	188.5	0.027
BHA	119.7	113.5	n/d
BHT	119.5	105.5	n/d
TBHQ	157.5	131.1	n/d

T_d: Weight loss temperature associated with a change in chemical structure.

n/d, not detected

FIGURES

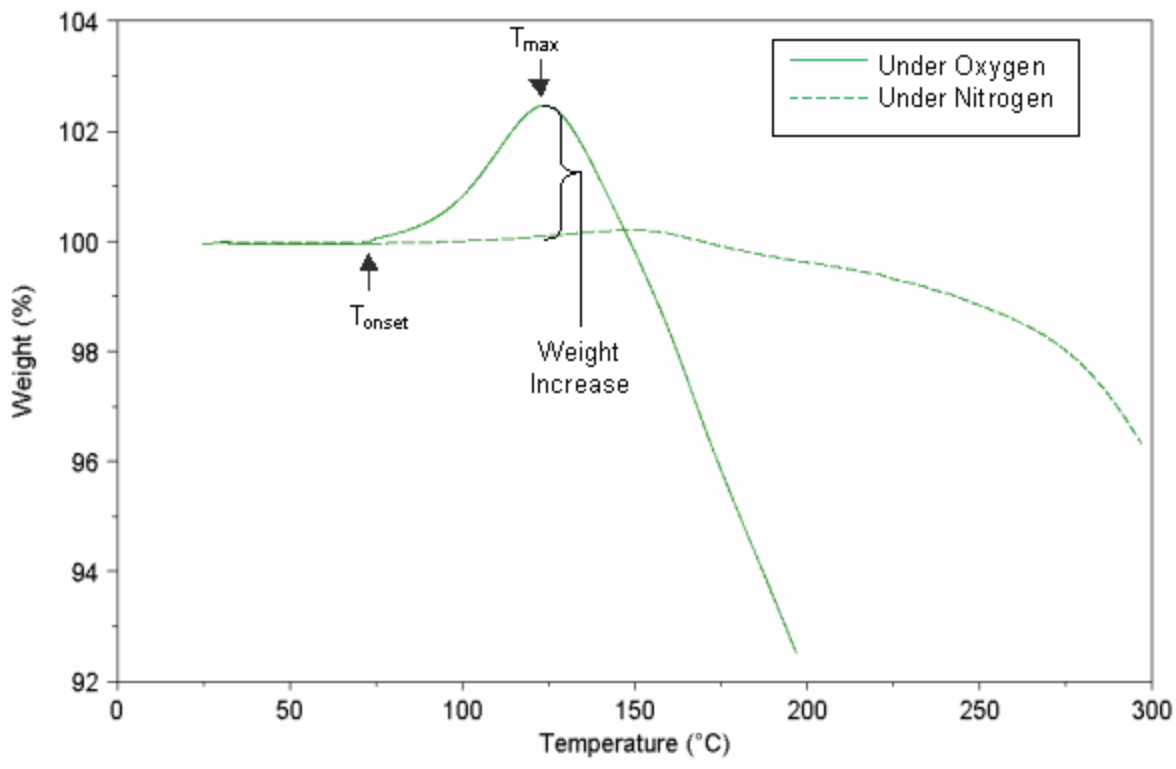

Figure 1. Typical TGA graph of weight loss* and oxidation of trilinolein under nitrogen and oxygen, respectively (Temperature Program: * $10^{o}C.min^{-1}$ to $70^{o}C$, $70^{o}C$ for 5 min, $2^{o}C.min^{-1}$ to $200^{o}C$; ** $10\ ^{o}C.min^{-1}$ to $300^{o}C$).**

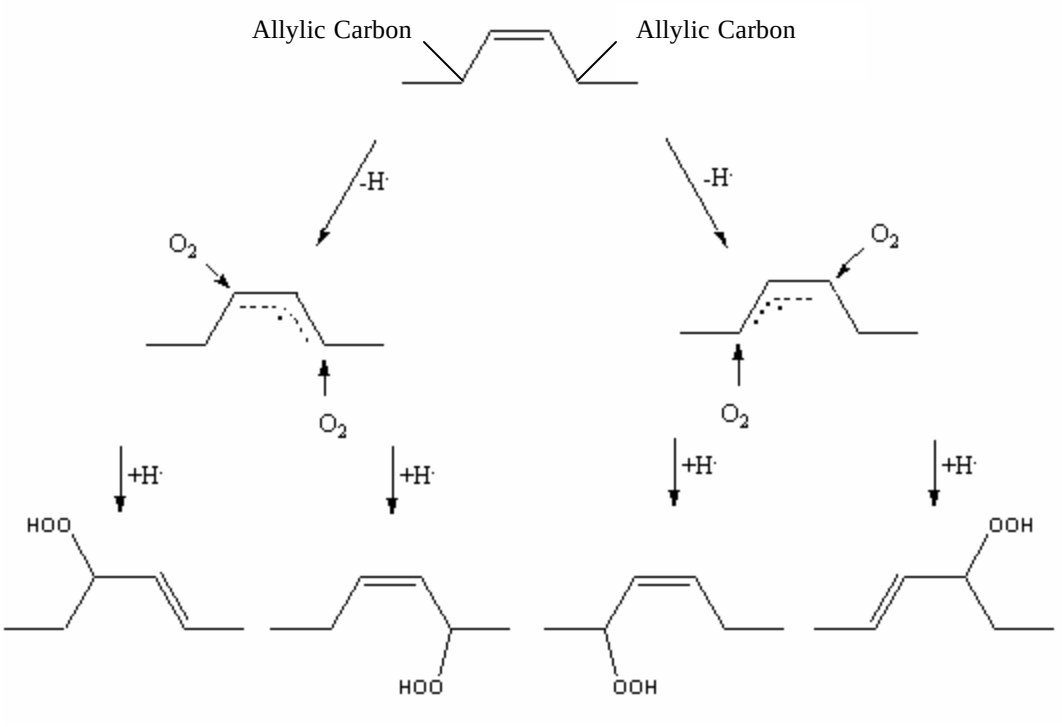

Figure 2. Hydrogen abstraction at the allylic carbons of unsaturated bonds in free radical oxidation of fatty acids (modified from Park and Goins, 1994).

CHAPTER IV

Light-Induced Flavor of Milk Fortified with a-Tocopherol and Ascorbic Acid

M. van Aardt,[*] S. E. Duncan,[*] J. E. Marcy,[*] T. E. Long,[†] S. F. O'Keefe,[*] and S. R. Nielsen-Sims[‡]

[*]Department of Food Science and Technology and

[†]Department of Chemistry,

Virginia Polytechnic Institute and State University, Blacksburg 24061

[‡]Eastman Chemical Co., Kingsport 37662-5125

Corresponding Author: Marleen van Aardt

27F FST Building, Virginia Tech, Blacksburg, VA 24061

Phone: (540) 231-8675

Fax: (540) 231-9293

E-mail: mvanaard@vt.edu

ABSTRACT

The effectiveness of added antioxidants against oxidation off-flavor development in light-exposed milk was evaluated using sensory and chemical analysis. Sensory testing for similarity showed no perceivable difference between control milk and milk with added *(i)* α-tocopherol (0.05%) and *(ii)* α-tocopherol (0.025%) and ascorbic acid (0.025%), but did demonstrate a perceivable difference when adding ascorbic acid alone (0.05 %) ($n = 30$, $\beta = 0.05$, $\alpha = 0.30$). Subsequently, sensory testing for difference showed a significant difference in oxidation off-flavor between light-exposed control milk and light-exposed milk with added α-tocopherol (0.025%) and ascorbic acid (0.025%), while addition of α-tocopherol (0.05%) alone showed no significant difference ($n = 24$, $\beta = 0.40$, $\alpha = 0.05$). Gas chromatography-olfactometry showed that more aroma-active flavor compounds were observed in light-exposed milk treated with 0.05% α-tocopherol, and a combination of 0.025% α-tocopherol and 0.025% ascorbic acid than light-exposed milk with no added antioxidants. The thiobarbituric acid (TBARS) test verified chemically the extent of oxidation in control and antioxidant-treated milk samples. Milk that was exposed to light for 10 hours showed a significantly higher TBARS value (0.92 ± 0.093 $mg.kg^{-1}$) than milk that was protected from light (0.59 ± 0.184 $mg.kg^{-1}$), or milk that was treated with a combination of 0.025% α-tocopherol and 0.025% ascorbic acid (0.26 ± 0.092 $mg.kg^{-1}$).

Key words: "light-induced flavor, lipid oxidation, milk, antioxidant, α-tocopherol, ascorbic acid"

Abbreviation key: TBARS = thiobarbituric acid reactive substances, **HP** = Hewlett Packard, **AA** = Ascorbic acid, **Toc** = α-Tocopherol, **SPME** = solid phase microextraction, **GC-O** = gas chromatography-olfactometry, **GC-MS** = gas chromatography-mass spectrometry, **PDMS** = poly(dimethyl siloxane), **FID** = flame ionization detector, **LSD** = least significant difference, $\mathbf{H_o}$ = null hypothesis, $\mathbf{H_1}$ = alternative hypothesis

INTRODUCTION

Oxidative reactions in milk are detrimental because these reactions reduce the nutritional quality of milk and also contribute significantly to the deterioration and reduction in shelf-life of the product. Milk oxidation can be catalyzed by certain metals, light exposure or it may occur spontaneously. Both milkfat and proteins may undergo oxidation reactions. Whole milk contains approximately 3.25% fat and 4% proteins. Milkfat consists of approximately 70% saturated fatty acids, 27% monounsaturated fatty acids (palmitoleic and oleic acid), and 4% polyunsaturated fatty acids (linoleic acid). Phospholipids surround milkfat globules in milk and consist of approximately 40-60% unsaturated fatty acids of which one third are polyunsaturated (Deeth, 1997). Unsaturated lipids undergo autoxidation, as well as light-induced oxidation, which cause the destruction of several key nutrients such as riboflavin and ascorbic acid. Oxidative changes in proteins and amino acids result in the development of off-flavors. Histidine, cysteine, methionine, tryptophan, and tyrosine oxidize in the presence of light, riboflavin (photosensitizer) and superoxide ion, hydrogen peroxide, and singlet oxygen to form various odorous compounds such as methionine sulfoxide and dimethyl disulfide (Jadhav *et al.*, 1996; Cadwallader and Howard, 1998).

Various researchers have reported on the specific compounds responsible for oxidation off-flavors in milk. Cadwallader and Howard (1998) identified dimethyl sulfide, 2-methylpropanal, n-pentanal, n-hexanal, dimethyltrisulfide, and 1-octen-3-one as odorous oxidation compounds by using gas chromatography-olfactometry. The respective odor descriptions for these compounds were “canned corn”, “dark chocolate”, “sour cut-grass”, “green cut-grass”, “cooked cabbage”, and “earthy mushroom”. Marsili (1999) reported the same flavor compounds as Cadwallader and Howard (1998) and attributed the development of these flavors as a result of oxidative breakdown of unsaturated fatty acids, particularly those present in the phospholipids. Jung *et al.* (1998) suggested that dimethyl disulfide was mainly responsible for the light-induced off-flavor in skim milk, as supported by sensory testing.

Antioxidants, such as α-tocopherol and ascorbic acid, play a crucial role in preventing or delaying oxidation and have attracted much attention as food additives (Madhavi *et al.*, 1995).

The former is a primary antioxidant functioning by terminating free-radical chain reactions by donating hydrogen or electrons to free radicals and converting them to more stable products. Ascorbic acid is a secondary antioxidant that can be broadly classified as an oxygen scavenger/singlet oxygen quencher, reacting with free oxygen and removing it in a closed system (Madhavi *et al*., 1995). Some primary and secondary antioxidants, such as tocopherols and ascorbic acid, show synergistic action against oxidation. These antioxidants reduce the rate of initiation reactions in the free-radical chain reaction and function at very low concentrations, 0.01% or less (Madhavi *et al*., 1995). Although milk contains low concentrations of natural antioxidants, such as α-tocopherol (13-30 $\mu g.g^{-1}$ milkfat) and ascorbic acid (< 20 $mg.L^{-1}$), processing and storage deplete these natural resources (Jensen and Nielsen, 1996; Rosenthal *et al*., 1993).

Various studies targeting the control of oxidized flavor in milk have included preharvest approaches, such as injecting α-tocopherol in the muscles of dairy cows or adding α-tocopherol to the feed, and processing approaches that include the addition of ascorbic acid to milk (Charmley and Nicholson, 1993; Focant *et al*., 1998; and Jung *et al*., 1998). Jung *et al*. (1998) concluded that dimethyl disulfide was mainly responsible for the light-induced off-flavor in skim milk and that increasing levels of ascorbic acid (from 200 – 1000 ppm) lowered the formation of dimethyl disulfide and off-flavor in skim milk. Yang (1994) reported that ascorbic acid chemical quenching rate of singlet oxygen was 1.53×10^8 at pH 4.0, 1.86×10^8 at pH 5.6, and 1.19×10^8 $M^{-1}sec^{-1}$ at pH 7.0. Bandarra *et al*. (1999) studied the mechanism responsible for the observed synergy of tocopherols and various phospholipids against fish oil oxidation, and concluded that the highest inhibitory effect against oxidation was shown by the synergy between α-tocopherol and phospatidylethanolamine. Studies that research the effect of tocopherol on the stability of milkfat and off-flavor in milk evaluated the degree of oxidation with sensory evaluation or chemical analysis such as the Rancimat method (Charmley and Nicholson, 1993; Focant *et al*., 1998). Various studies report on the influence of antioxidants on lipid oxidation products in model oils or oil-in-water systems by looking at specific flavor compounds (Wessling *et al*., 1999; Mei *et al*., 1999; Huang *et al*., 1996). However, further research is necessary to show the effect of antioxidants on milkfat oxidation, in terms of specific flavor compounds such as pentanal, hexanal, and 1-octen-3-ol.

The objectives of this study are:

1. to determine if panelists can distinguish between control milk (freshly pasteurized lowfat milk) and (a) milk with 0.05 % α-tocopherol; (b) milk with 0.05 % ascorbic acid; and (c) milk with a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid under the conditions of sensory testing for similarity;
2. to determine if panelists can distinguish between control milk (light-exposed freshly pasteurized lowfat milk) and (a) lowfat milk with 0.05 % α-tocopherol and exposed to light; (b) lowfat milk with 0.05 % ascorbic acid and exposed to light; and (c) lowfat milk with a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid and exposed to light under the conditions of sensory testing for difference;
3. to qualify aroma-active flavor compounds and rate intensities using gas chromatography-olfactometry (GC-O), as well as evaluate the extent of oxidation using the thiobarbituric acid reactive substances (TBARS) test.

MATERIALS AND METHODS

Milk Processing

Fresh raw milk was obtained from the Virginia Tech dairy farm and processed within 24 h. Milk was pre-warmed to 55^{o}C and separated into cream and skim milk using a pilot plant separator (Elecrem separator, model 1G, 6400 rpm, Bonanza Industries, Inc., Calgary, Canada). Milk was standardized at 2% fat content by adding cream to skim milk in appropriate proportions, homogenized at 2000 psi (1500 psi – 1st stage; 500 psi – 2nd stage) on a two-stage homogenizer (Type DX, Cherry Burrel Corp. Delavan, WI), and pasteurized at 63.3^{o}C for 30 min in a vat pasteurizer (Creamery Package, P50.8770, MFG Co., Chicago, IL) and cooled to 25^{o}C. Milk was stored at 4^{o}C in 10 gallon stainless steel containers until needed.

Two separate batches of milk were processed on different days for sensory testing and chemical analysis, respectively. Sensory analysis and GC-O tests were not replicated, while TBARS analysis was done in triplicate. Triplicate samples were obtained by spiking antioxidants in three separate aliquots from a single processed milk batch.

Preparation of Antioxidant-Spiked Samples

α-Tocopherol and ascorbic acid were obtained from Fisher Scientific (Cincinnati, OH). For sensory analysis, lowfat milk was spiked with *(i)* 0.05% α-tocopherol, *(ii)* 0.05% ascorbic acid, and *(iii)* a combination of 0.025% α-tocopherol and 0.025% ascorbic acid respectively and stored in appropriate stainless steel containers at 4^{o}C in a Tonka refrigeration unit (Hopkins, Minnesota) until sensory testing. Milk was used for sensory analysis within two days of processing. Sensory testing for similarity was done within 24 hours of processing, since no light treatment was applied. A lag time of two days existed between processing and sensory testing for difference due to the time it took to expose milk samples to 10 h of light as well as sample preparation for sensory analysis.

For chemical analysis, one batch of milk was subdivided into three aliquots for triplicate analysis. Each aliquot was spiked with *(i)* 0.05% α-tocopherol, *(ii)* 0.05% ascorbic acid, and *(iii)* a combination of 0.025% α-tocopherol and 0.025% ascorbic acid respectively.

Milk Storage and Handling

For the triangle test for similarity aliquots (10-ml) of both control (unspiked) and antioxidant-treated milk samples were poured into 20-ml plastic cups with plastic lids and stored at 4°C until sensory testing. For the triangle test for difference, both control and antioxidant-treated milk samples were stored in 1-L clear glass containers at 4°C, while undergoing exposure to fluorescent light. Samples were positioned approximately 15 cm below a row of fluorescent Econ-o-watt lights in a Tonka refrigeration unit (Hopkins, Minnesota). Light exposure was regulated at a light intensity of 1100 - 1300 lx, as measured at the top of sample bottles, for 10 h. After light exposure, 10-ml aliquots were poured into 20-ml plastic soufflé cups with plastic lids for sensory testing. Milk samples intended for GC-O and the TBARS test were pipetted (21-ml aliquots) into 40-ml clear glass bottles fitted with Teflon-coated septa (Supelco, Bellefonte, PA). After packaging, samples were exposed to light conditions as described for sensory testing above. Light-protected control milk was obtained by covering sample bottles with aluminum foil to prevent any light exposure.

Sensory Analysis

A triangle test for similarity was used to determine the effect of antioxidant addition on milk flavor. Similarity was tested by comparing control milk (unspiked) and treated milks (*(i)* 0.05% α-tocopherol, *(ii)* 0.05% ascorbic acid, and *(iii)* a combination of 0.025% α-tocopherol and 0.025% ascorbic acid). The second test was a triangle test for difference, determining the differences in oxidation off-flavor intensity between light-exposed control milk to light-exposed

treated milks (*(i)* 0.05% α-tocopherol, *(ii)* 0.05% ascorbic acid, and *(iii)* a combination of 0.025% α-tocopherol and 0.025% ascorbic acid).

Triangle Test for Similarity

A panel of 30 volunteers from among students, staff, and faculty at the Department of Food Science and Technology at Virginia Tech (Blacksburg, VA) participated in the test. Testing was done in the sensory laboratory within the Department. Panelists were seated in individual sensory booths under white lighting. Each panelist was requested to complete a human subject's consent form (Human subject's consent form - Appendix A) prior to testing. Each panelist was presented with three 3-sample sets on one tray and informed to choose the sample that tasted "different" within each three-sample set (Scorecard – Appendix B) (Lawless and Heymann, 1998). Each sample set contained a different order of samples e.g. AAB, BBA, ABA, BAA, BAB, and ABB, where A and B represent control and treated samples, respectively (Worksheet – Appendix C). Panelists were instructed to rinse with room-temperature spring water between each sample set. Each three-sample set included either two samples of unspiked lowfat milk and one antioxidant-spiked sample or two antioxidant-spiked samples and one unspiked lowfat milk sample. Each sample cup was coded with a random three-digit number. The position of the "odd" sample within the three-sample set was randomized to remove positional bias. Samples were presented at approximately 7^{o}C.

Triangle Test for Difference

For this test a panel of 24 volunteers from among students, staff, and faculty at the Department of Food Science and Technology at Virginia Tech participated in the test. Testing was done in the same venue, under the same conditions and according to the same procedure as mentioned for similarity testing.

Chemical Analysis

The TBARS test was done in triplicate for all treatments, with one replication also used for GC-O analysis.

Gas Chromatography Olfactometry

Extraction and concentration of volatile compounds were done with solid phase microextraction (SPME). A 75-µm carboxen poly(dimethyl siloxane) (PDMS) coated SPME fiber (Supelco, Bellefonte, PA) was exposed to the milk headspace with the end of the fiber approximately 1 cm above the milk surface for 22 min at 45°C with magnetic stirring of the sample. Volatile compounds were desorbed in the injector port of a gas chromatograph-olfactometer system consisting of a HP 5890A GC (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame ionization detector (FID) and a sniffing port (ODOII; SGE, Inc. Austin, TX). The injector temperature was 280°C, and all injections were made in the splitless mode. Separation was completed on a 30-m x 0.25-mm i.d. x 0.25-µm film thickness capillary column (DB-5ms; J&W Scientific, Folson, CA) with helium carrier gas flow rates of 1.0 $ml.min^{-1}$. Column eluent was split 1:1 between FID and sniffing port using deactivated fused silica capillaries (1-m length x 0.32-µm i.d.). Chromatograms were graphed on a HP integrator (HP 3396A, Hewlett-Packard Co., Palo Alto, CA). The GC oven temperature was programmed from 35°C to 180°C at a rate of $15°C.min^{-1}$, and from 180°C to 260°C at a rate of $20°C.min^{-1}$ with initial, intermediate, and final hold times of 0.5 min. The FID and sniffing port were maintained at 300°C. The sniffing port was supplied with humidified nitrogen at 10-15 $ml.min^{-1}$. Gas chromatography-mass spectrometry (GC-MS) (HP 6890, 5973 Mass Selective Detector, Hewlett-Packard Co., Palo Alto, CA) was done to identify volatile compounds in light-exposed milk samples. Separation was completed on a 15-m x 0.25-mm i.d. x 0.25-µm film thickness capillary column (HP-5; Hewlett-Packard Co., Palo Alto, CA). Oven temperature program and conditions were the same as mentioned above.

Training of Panelists for GC-O

Six people, who regularly participate in GC-O studies and consisting of students and staff at the Department of Food Science and Technology at Virginia Tech in Blacksburg, VA were trained in four 20-min sessions prior to GC-O analysis of treated samples. Two aroma training kits were obtained: (I) Beer Aroma Recognition kit, (ii) Beer Taint Recognition kit (Brewing Research International, UK). These kits contained a variety of chemical compounds representing odors commonly associated with lipid oxidation (Table 1). During training, panelists were asked to

sniff each aroma, practice placing a verbal descriptor with the aroma, and then compare to the identified aroma for each reference standard. Intensities were rated on a scale of 1 (slight odor) to 5 (extreme odor). One panelist was chosen who consistently correctly identified aroma compounds to participate in GC-O of samples.

Thiobarbituric Acid Reactive Substances Test (TBARS)

The TBARS test used was modified from Spanier and Traylor (1991). The direct chemical/extraction method was followed rather than the distillation method. Modifications include: (i) A 10% (w/v) dilution was made by blending 3.0g milk with 84 ml water, 9.5 ml of Solution III, and 1 ml of 10% SDS. No homogenization was done before 1-ml aliquots were combined with 4 ml of solution I. After incubation in a 95^{o}C water bath for 60 min, 5 ml of Solution II were sodium dodecyl sulfate added to each sample. The TBARS value for samples were calculated by the following equation, where A = absorbance of sample, Wt = exact sample weight, and Ks = constant obtained from the slope of the standard curve.

$$\text{Sample TBARS} = Ks \times A \times 5/Wt$$

Statistical Analysis

For both similarity and difference tests, data was analyzed by counting the number of correct responses (correctly identified "different" sample) and the number of total responses. These numbers was compared with values found in Table T8 in Meilgaard *et al*. (1999) to obtain significant differences. For the similarity test significance of differences were defined at $\alpha = 0.3$ and $\beta = 0.05$, while differences for the second test was defined at $\alpha = 0.05$ and $\beta = 0.40$. Similarity testing is done at a lower β-value to reduce the risk of a Type II error (failing to reject H_0 when in fact it is false).

Volatile flavor compounds were identified and their intensities rated after comparing retention times with known retention times of flavor compounds from preliminary GC-mass spectrometry results of light exposed milk samples. TBARS values were determined by calculating means (n = 3) and standard deviations. Analysis of variance was used to test the H_0 (H_0: $\mu_{control-light}$ =

$\mu_{tocopherol(0.05\%)} = \mu_{tocopherol(0.025\%),\ ascorbic\ acid(0.025\%)}$), while Tukey's least significant difference (LSD) was used to compare means. Significant differences were defined at $P < 0.05$ (SAS, 1998).

RESULTS AND DISCUSSION

Exposure of milk to light leads to the development of a typical off-flavor, previously described as "metallic", "cardboard", and "light-activated flavor" (Cadwallader and Howard, 1998; Dimick, 1982). GC-O and TBARS results supported sensory results in that specific odorous flavor compounds were identified and intensities rated, as well as the extent of oxidation was quantified.

Sensory Analysis

Similarity testing was done to ensure that the levels of added antioxidant to lowfat milk in this study were not significantly perceivable by sensory analysis. A low β-value was chosen to reduce the risk of a Type II error (failing to reject H_o when in fact it is true). In this case a high α-value is acceptable to keep the number of assessors within reasonable limits. Correct identification of "odd" samples in each triangle set is shown in Table 1. The critical number of correct responses in a triangle test is 12 for a total of 30 observations at $\alpha = 0.40$, $\beta = 0.05$, and $p_d = 30$ %. The p_d refers to the maximum allowable proportion of distinguishers in the population (Meilgaard *et al*., 1999). In this study it was assumed that the sensory difference would be moderate, thus justifying the $p_d = 30\%$.

The general null hypothesis for triangle sensory tests states that the probability of making a correct selection when there is no perceptible difference between the samples is one in three. The alternative hypothesis states that the probability that the underlying population will make the correct decision when they perceive a difference between the samples will be larger than one in three. This is a one-sided alternative hypothesis, and the test is one-tailed (Lawless and Heymann, 1998). The specific null (H_0) and alternative (H_1) hypotheses for the sensory similarity and difference tests are tabulated in Table 1.

Similarity testing showed that only eight and five correct selections out of 30 were made when comparing milk flavor when treated with (i) 0.05 % α-tocopherol and (ii) addition of a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid, respectively. The upper 99%

one-sided confidence limit on the proportion of distinguishers for (i) and (ii) are 18% and 10%, respectively, while the lower 80% one-sided confidence limit falls at 0% for both (i) and (ii). We can conclude that we are 99% sure that the true proportion of the population that can distinguish the control and treatment (i) samples is no greater than 18% and may be as low as 0%, while we are 99% sure that the true proportion of the population that can distinguish the control and treatment (ii) samples is no greater than 10% and may be as low as 0%. Therefore we conclude that control milk and milk spiked with 0.05 % α-tocopherol or a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid are sufficiently similar to be used interchangeably (Table 3). However, 13 correct responses out of 30 were made when comparing control milk flavor and milk with 0.05 % ascorbic acid added, indicating with 95% confidence that the proportion of the population who can perceive this difference is more than 30% and was therefore not meaningful for use in the difference test.

Difference testing was done to determine if there is a perceivable sensory difference in oxidation off-flavor between light-exposed lowfat milk with added antioxidants and light-exposed lowfat milk with no antioxidants added. Here a low α-value is important to reduce the risk of a Type I error (failing to reject H_0 when in fact it is false). Table 3 shows the number of correct responses for difference testing. The critical number of correct responses in a triangle test is 13 for a total of 24 observations at an α-level of 0.05 (Meilgaard *et al*., 1999). The number of correct responses (correctly identified odd samples) was compared to the critical number of correct responses to indicate significant differences. A significant difference was observed between light-exposed control milk flavor and milk with 0.025 % α-tocopherol and 0.025 % ascorbic acid added. These results indicate that significantly lower light-activated flavor developed in control milk than in milk treated with a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid. Since the addition of 0.05 % ascorbic acid to milk showed a perceivable difference in fresh milk flavor (Similarity test, Table 3), the further testing of this treatment was not conducted.

General remarks on scoresheets from panelists that correctly identified the "odd" samples indicated that lowfat milk treated with a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid showed more fresh milk flavor character than light-exposed milk without added antioxidant. This supports the theory that the synergistic action of α-tocopherol and ascorbic

acid showed greater inhibition of oxidation than each independent antioxidant (Madhavi *et al*., 1995).

Preharvest technologies aiming to increase natural antioxidant levels in milk as a way of limiting oxidation have received much attention. Charmley and Nicholson (1993) showed a decrease in oxidation flavor in milk from 27.5 to 8.3 on a 140-point scale (140 - extremely oxidized) with increased α-tocopherol levels in milk. A combination of oral and intramuscular injections increased the level of α-tocopherol from 17.6 $\mu g.g^{-1}$ to 37.8 $\mu g.g^{-1}$ milkfat over two weeks. Focant *et al*. (1998) supplemented cow diets with α-tocopherol and oilseeds and, although α-tocopherol levels in milk increased from 44.37 $mg.kg^{-1}$ to 84.59 $mg.kg^{-1}$ fat, observed an increase in milkfat oxidation, as measured with the Rancimat apparatus. They attributed this to the increase in unsaturated fatty acids in milk as a result of a high oilseed diet. However, it might be that α-tocopherol reached a pro-oxidant level. The Rancimat method is an automated version of the extremely demanding AOM method (active oxygen method). The highly volatile organic acids produced by autoxidation are absorbed in water and used to indicate the induction time. Post-harvest fortification of milk with a single addition of antioxidants has also seen limited success. Rosenthal *et al*. (1993) added 100% ascorbic acid (1000 $mg.ml^{-1}$) to whole milk (approximately 3.5% milkfat) to evaluate milkfat stability. Not only were these levels of ascorbic acid sensory "objectionable", no difference in oxidation were observed in fortified milk (TBARS value = 0.61 $mg.kg^{-1}$), as compared to control milk (TBARS value = 0.71 $mg.kg^{-1}$) over seven weeks of storage in the dark. In our study, the highest addition of ascorbic acid to milk was at 0.05% (0.5 $mg.ml^{-1}$), which also showed differences in milk flavor to that of unspiked milk.

Chemical Analysis

The TBARS test was done to determine the extent of oxidation. This test measures malondialdehyde, a secondary oxidation product, and reports results as mg malondialdehyde per kg sample. TBARS results support sensory data. Milk that was exposed to light for 10 h had a significantly higher TBARS value (0.92 ± 0.93 $mg.kg^{-1}$) than milk that was protected from light (0.59 ± 0.184 $mg.kg^{-1}$) or milk that was treated with a combination of 0.025% ascorbic acid and

0.025% α-tocopherol (0.26 ± 0.092 mg.kg^{-1}) (Table 4). It is interesting to see that milk treated with antioxidants showed a significantly lower level of oxidation than milk protected from light. Rosenthal *et al.* (1993) reported comparable levels of TBARS (0.61 mg.kg^{-1}) for whole milk that was stored in the dark for seven days. Angulo *et al*. (1997) evaluated lipid oxidation in whole milk powder and skim milk powder after 1 year of storage in the dark at 32^{o}C and 55^{o}C, and under oxygen or nitrogen. TBARS values for skim milk powder at 32^{o}C under nitrogen and whole milk powder at 55^{o}C under air were 0.29 mg.kg^{-1} and 3.69 mg.kg^{-1}, respectively. Since whole milk powder consists of approximately 26% fat, a high TBARS value is expected in comparison to skim milk powder with a fat content of <0.5% and lowfat milk with a fat content of 2%. Fenaille *et al*. (2001) evaluated malondialdehyde concentration as a measure of oxidation in 4 commercial milk powders. TBARS values ranged from 1.02 to 1.31 mg.kg^{-1}.

GC-O was used for identifying and rating intensities of odor-active compounds that contribute to milk flavor and light-activated flavor. Compounds such as hexanal, heptanal, and 1-octene-3-one are common light-activated flavors that impart grassy, cabbage, and mushroom odors, respectively (Cadwallader and Howard, 1998). In this study, it is interesting to note that both light-exposed antioxidant-treated milk samples showed more odor-active compounds than in the light treated milk with no additional antioxidants added (Table 5). This could be due to odors associated with the antioxidants itself, although sensory testing did not find significant differences between light-protected control milk and light-protected milk that was spiked with a combination of 0.025% ascorbic acid and 0.025% α-tocopherol, and 0.05% α-tocopherol. It also is interesting to note that, with light exposure, the addition of α-tocopherol at 0.05% seemed to increase the intensities of the aroma-active compounds, which could be an indication of a pro-oxidant effect of the antioxidant. Figure 1 shows that relative peak areas for hexanal were substantially higher when milk was treated with 0.05% α-tocopherol as compared to control milk and milk treated with a combination of 0.025% α-tocopherol and 0.025% ascorbic acid.

Many studies have been done to determine odorous oxidation compounds in control and light-treated dairy products by GC-O. However, few, if any GC-O data is available to show the effect of antioxidants on control and light-treated milk. Cadwallader and Howard (1998) evaluated control and light-activated flavored milk by GC-O and sensory analysis and found that green cut-

grass (hexanal), mushroom (1-octen-3-one and 1-nonen-3-one), and plastic (1-hexen-3-one) were terms predominant in light-activated flavor after 48 h light exposure at 2200 lux. Although the aroma of light-activated flavor in milk is impacted mostly by fat level, protein oxidation also plays a limited role to produce odorous compounds such as dimethyl disulfide. Freidrich and Acree (1998) studied the effects on heating, which accelerated lipid autoxidation, on milk. Milk that was pasteurized and UHT processed showed high levels of hexanal, 2-nonanone (grassy-herbal, green-fruity), benzothiozole (quinoline, rubbery), and δ-decalactone (coconut). Jung *et al*. (1998) reported that the reaction of singlet oxygen with sulfur containing proteins and amino acids is able to produce sulfur containing volatile compounds such as dimethyl disulfide and identified that compound as the agent responsible for the off-flavor of light-exposed milk. Kim and Morr (1996) and Marsili (1999) stated that the "sunlight" or burnt feather" flavor occurring after an exposure of milk to visible or ultraviolet light was related to the oxidative degradation of its sulfur containing protein fraction, and the "card board" flavor, which appears after a more prolonged exposure to the light, was related to the oxidation of the lipid fraction. In our study, hexanal, a typical lipid oxidation product, did not show up in control milk and milk treated with low antioxidant levels. One would expect oxidative protein oxidation to have a bigger effect on milk samples exposed to only 10 h of light at 1300 lux. However, dimethyl disulfide, a protein oxidation product, was observed in control milk and milk treated with a possible pro-oxidant whereas the addition of 0.025% ascorbic acid and 0.025% α-tocopherol to milk did significantly decrease dimethyl disulfide.

Although GC-O data suggested the presence of substantially more odorous flavor compounds in antioxidant treated light exposed milk, these compounds could be below human threshold in the sample, which might indicate why sensory results did not indicate an increased light oxidation flavor.

CONCLUSION

Sensory analysis showed that a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid was the only treatment that did not negatively impact fresh milk flavor and did limit light-induced flavor in milk after 10 h of light exposure. On the other hand, addition of 0.05% tocopherol to milk also did not negatively impact fresh milk flavor, but it acted as a pro-oxidant when milk was exposed to 10 h of light. Since light activated flavor in milk is comprised of lipid and protein oxidation products, it is important to know the stage during light exposure that odorous flavor compounds increase to levels above human threshold. In this study, volatile compounds associated with protein oxidation were mostly measured since milk was exposed for a short period of time (10 h). This study shows that the addition of antioxidants to milk, whether directly added or through indirect diffusion from packaging, could protect milk flavor and may have value for further exploration.

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TABLES

Table 1. Chemical compounds used in gas chromatography-olfactometry (GC-O) training.

Aroma	Chemical Compound
Floral	trans-2-hexenal
Citrus	nonanal
Spicy	eugenol
Grassy	hexanal
Mushroom	1-octen-3-ol
Green	heptanal
Mushroom/metallic	1-octen-3-one
Fatty, citrus	octanal
Diacetyl	2,3-butanedione
Rancid	Butyric acid
Cheesy	Isovaleric acid
Cardboard	trans 2-nonenal
Papery	5-methyl furfural
Cooked vegetable	Dimethyl disulfide
Onion	n-Propanethiol

Table 2. The null (H_0) and alternative (H_1) hypotheses for the sensory similarity and difference tests.

Sensory Test	Hypothesis	Result
Similarity testing	H_0: [1]$P_{\text{correct response}} < 12/30$[2]	(no significant difference)
	H_1: [1]$P_{\text{correct response}} \geq 12/30$[2]	(significant difference)
Difference testing	H_0: [1]$P_{\text{correct response}} < 13/24$[3]	(no significant difference)
	H_1: [1]$P_{\text{correct response}} \geq 13/24$[3]	(significant difference)

[1] $P_{\text{correct response}}$: Proportion of correct identification of the "different" sample in each triangle set of samples

[2] n = 30 independent observations with $\alpha = 0.30$, $\beta = 0.05$, and $p_d = 30$; Treatments: Milk with (a) 0.05 % α-tocopherol, (b) 0.05 % ascorbic acid, and (c) a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid

[3] n = 24 independent observations with $\alpha = 0.05$, $\beta = 0.4$, and $p_d = 30$; Treatments: Light exposed milk with (a) 0.05 % α-tocopherol, (b) 0.05 % ascorbic acid, and (c) a combination of 0.025 % α-tocopherol and 0.025 % ascorbic acid

Table 3. Sensory triangle test responses for antioxidant-spiked lowfat milk compared to control (unspiked) milk.

	Similarity Test[1] (No Light Exposure)		Difference Test[2] (Light Exposure)	
Treatment	**# of Correct Responses**	**Confidence limits (P_{max}[4], P_{min}[5])**	**# of Correct Responses**	**Confidence limits (P_{min}[5])**
0.05 % α-tocopherol	8	18%, 0%	6	
0.05 % ascorbic acid	13[3]			
0.025 % α-tocopherol,	5	10%, 0%	14[3]	25 %
0.025 % ascorbic acid				

[1] Total observations = 30; critical response # = 12; $\alpha = 30$, $\beta = 0.05$; $p_d = 30\%$

[2] Total observations = 24; critical response # = 12; $\alpha = 0.05$, $\beta = 0.40$; $p_d = 30\%$

[3] Significantly different from control

[4] P_{max}, Upper 99% one-sided confidence limit

[5] P_{min}, Lower 80% one-sided confidence limit

Table 4. Thiobarbituric acid reactive substances test (TBARS) values for light-activated and light-protected control and antioxidant-treated milk.

Treatment	**TBARS value ($mg.kg^{-1}$)**
Milk (light-protected)	$0.59^a \pm 0.184$
Milk (light exposed)	$0.92^b \pm 0.093$
Milk + 0.025% AA[1], 0.025% Toc[1] (light exposed)	$0.26^c \pm 0.092$

[1]AA, Ascorbic acid; Toc, α-Tocopherol

[a-c]Means (n = 3) with different superscripts are significantly different ($P < 0.05$)

Table 5. Odor-active compounds detected by gas chromatography-mass spectrometry (GC-MS) of control and antioxidant-treated milk exposed to light for 10 h.

Compound[1]	Retention Index[2]	Odor Description	Treatment		
			Control	0.05% Toc[3]	0.025% AA[3] 0.025% Toc[3]
dimethyl disulfide	4.42	cooked milk	++	++	
hexanal	4.72	grass		+++	
2-heptanone	6.10	nutty		++	+
n-heptanal	6.82	rancid/cabbage	+	++	+
1-octene-3-ol	7.30	nut/mushroom	+		++
1-octanol	8.47	cooked/green/grassy		+++	+
nonanal	8.65	plastic/sweet,		++	+

[1] Compound identified by comparison of its mass spectrum.

[2] Retention indices from GC-O data.

[3] Toc, α-Tocopherol; AA, Ascorbic acid

Odor intensities: + (0-1), ++ (2-3), +++ (4-5)

FIGURES

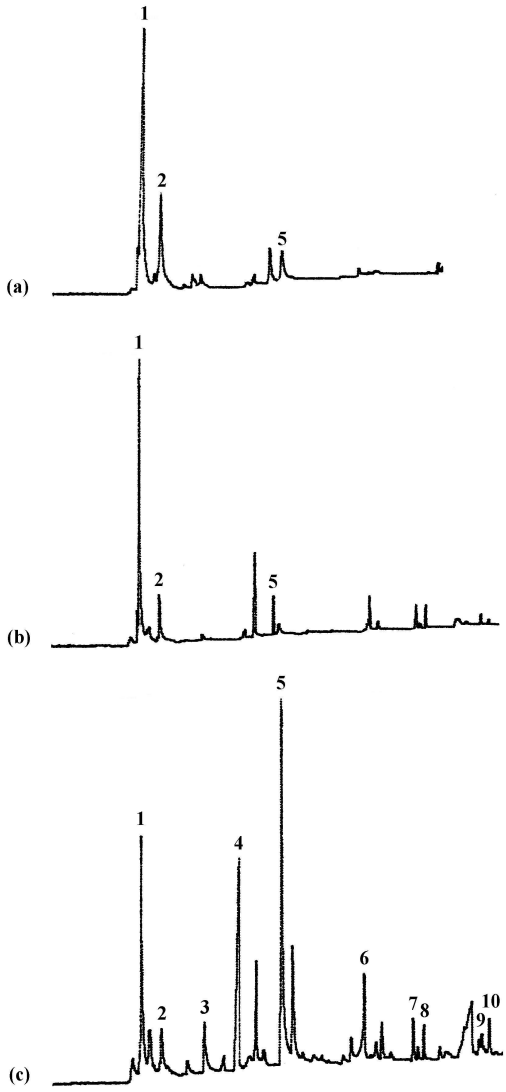

Figure 1. Volatile compounds detected by gas chromatography-mass spectrometry of (a) control milk, (b) milk with 0.025% ascorbic acid and 0.025% a-tocopherol, and (c) milk with 0.05% a-tocopherol added. 10 h light exposure (1300 lx) was applied. (1, 2-propanone; 2, 2-butanone; 3, pentanal; 4, dimethyl disulfide; 5, n-hexanal; 6, 2-heptanone; 7, n-heptanal; 8, 1-oceten-3-ol; 9, octanal; 10, nonanal).

CHAPTER V

Aroma Analysis of Light-Exposed Milk Stored With and Without Natural and Synthetic Antioxidants

M. van Aardt,[*] S. E. Duncan,[*] J. E. Marcy,[*] T. E. Long,[†] S. F. O'Keefe,[*] and S. R. Nielsen-Sims[‡]

[*]Department of Food Science and Technology and

[†]Department of Chemistry,

Virginia Polytechnic Institute and State University, Blacksburg 24061

[‡]Eastman Chemical Co., Kingsport 37662-5125

Corresponding Author: Marleen van Aardt

27F FST Building, Virginia Tech, Blacksburg, VA 24061

Phone: (540) 231-8675

Fax: (540) 231-9293

E-mail: mvanaard@vt.edu

ABSTRACT

The effect of antioxidants, added in a single initial dose or in weekly additions, to extended shelf-life milk was evaluated over six weeks of storage. Light-induced oxidation was measured by determining pentanal, hexanal, heptanal, and 1-octen-3-ol content. A weekly addition of a combination of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (100 $mg.kg^{-1}$ milkfat each) controlled heptanal content of milk to levels comparable to light-protected milk, whereas an initial single addition of α-tocopherol significantly decreased hexanal content over the first four weeks of storage. Odor-active compounds associated with light-induced oxidation included 2,3-butanedione, pentanal, dimethyl disulfide, hexanal, 1-hexanol, heptanal, 1-heptanol, and nonanal. Different antioxidant treatments showed the decrease or increase of different odors. The addition of BHA and BHT in a single initial addition showed decreases in pentanal and hexanal odor, but not in heptanal and 1-heptanol odor, while the addition of α-tocopherol and ascorbyl palmitate decreased pentanal and heptanol odor, but not hexanal and heptanal odor.

Key words: "light-oxidized flavor, lipid oxidation, milk, antioxidant, α-tocopherol, ascorbic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT)"

Abbreviation key: HP = Hewlett Packard, **Asc** = Ascorbyl Palmitate, **Toc** = α-Tocopherol, **BHA** = butylated hydroxyanisole, **BHT** = butylated hydroxytoluene, **HDPE** = high-density polyethylene, **PETE** = poly(ethylene terephthalate), **ESL** = extended shelf-life, **SPME** = solid phase microextraction, **GC-O** = gas chromatography-olfactometry, **GC-MS** = gas chromatography-mass spectrometry, **HPLC** = high-performance liquid chromatography, **IDFA** = International Dairy Foods Association, **ANOVA** = analysis of variance, **LSD** = least significant difference

INTRODUCTION

Lipid oxidation is a common cause of off-flavor development in food that contains unsaturated fatty acids. Milkfat contains approximately 2.3% palmitoleic acid (C16:1), 24.6% oleic acid (C18:1), and 2.8% linoleic acid (C18:2) which are very susceptible to lipid oxidation. Factors such as light exposure and the presence of oxygen increase the potential for lipid oxidation. Milk is sold commercially (in clear, translucent, or opaque containers) in dairy display cases under rows of fluorescent lights. Clear glass, poly(ethylene terephthalate) (PETE), and translucent high-density polyethylene (HDPE) containers allow light to reach the product and initiate lipid oxidation.

Aroma-active compounds in light-activated milk were described as green/fish oil, sour grass, sweet, mushroom, cut-grass, boiled potato, cheesy, pungent, and sulfurous (Friedrich and Acree, 1998). These odors relate to heptanal, pentanal, heptanol, 1-octene-3-ol, hexanal, dimethyl disulfide, 2,3-butanedione, and other sulfur containing compounds, respectively (Kim and Morr, 1996; Cadwallader and Howard, 1998). Light-activated odors in milk can be limited by proper storage, such as packaging in light- and oxygen-impermeable packaging, or possibly by adding antioxidants.

Pre-harvest approaches, such as injecting α-tocopherol in the muscles of dairy cows or adding α-tocopherol to the feed, have been employed in an attempt to reduce lipid oxidation in milk. Charmley and Nicholson (1993) showed with sensory analysis that oxidation flavor in milk decreased significantly when increasing α-tocopherol levels in milk from 17.6 $\mu g.g^{-1}$ to 37.8 $\mu g.g^{-1}$ milkfat after two weeks of oral and intramuscular injections. Post-harvest approaches also have been employed. Jung *et al*. (1998) added ascorbic acid (from 200 to 1000 ppm) directly to milk and concluded that dimethyl disulfide decreased as a result. Ahvenainen and Hurme (1997) and Rajalakshmi and Narasimhan (1996) listed the advantages of indirect addition of antioxidants to food products through gradual migration from polymeric films versus direct addition as: *(i)* the total amount of antioxidant consumed with the product is reduced

substantially; *(ii)* a single large dose is depleted rapidly, and *(iii)* oxidative reactions can be inhibited for a longer period of time through controlled addition.

Antioxidants in food may limit oxidation of its components. However, natural antioxidants are often depleted during processing and storage, necessitating the addition of external antioxidants (Jensen and Nielsen, 1996). Synthetic antioxidants commonly used in the food industry include butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). These antioxidants are lipid soluble and terminate free-radical chain reactions by donating hydrogen atoms or electrons to free radicals and converting them to more stable structures (Frankel, 1998). The legal limit for the addition of BHA and BHT to foods is 200 $mg.kg^{-1}$ fat. When added in combination, a total of 200 $mg.kg^{-1}$ fat is allowed (CFR, 2001). However, the addition of BHA or BHT to milk is not allowed (personal communication, M. Albee Mattow, International Dairy Foods Association (IDFA)).

A worldwide trend to eat and live healthier and more naturally, results in the avoidance or minimization of the use of synthetic food additives. This trend has drawn special attention to natural antioxidants, such as α-tocopherol and ascorbic acid. α-Tocopherol is also fat-soluble and limits free radical oxidation reactions by the same mechanism as BHA and BHT. Ascorbic acid is water soluble and acts as a synergist with α-tocopherol by (i) acting as a hydrogen donor to the phenoxy radical of tocopherol, thereby regenerating it, or (ii) reacting with free oxygen and removing it in a closed system. Ascorbyl palmitate, a fat-soluble analogue of ascorbic acid, also is used widely.

Raw milk contains α-tocopherol at approximately 13-30 $mg.kg^{-1}$ milkfat and ascorbic acid at <20 $mg.kg^{-1}$. The addition of natural antioxidants to milk has received much attention. Natural antioxidants are generally recognized as safe when used in accordance with food manufacturing practices and therefore not limited (CFR, 2001). The addition of a-tocopherol, ascorbic acid, and ascorbyl palmitate to milk is permitted. The presence of these substances must be noted on the label. If words such as “enriched” or “fortified” are used, vitamin content must be at least 10% of recommended dairy intake (personal communication, M. Albee Mattow, IDFA).

Although no legal limit exists for the addition of α-tocopherol and ascorbic acid, care should be taken when adding these antioxidants to food since high concentrations can cause pro-oxidation (Frankel, 1998). Nath *et al*. (1992) found that α-tocopherol at 1 $g.kg^{-1}$ (1000 ppm) milkfat caused autoxidation to occur at a faster rate as compared to milkfat with no additional α-tocopherol added.

Although many studies have evaluated the profile of odor-active compounds in light-exposed milk, not much research has been conducted to relate the effect of antioxidants on odor-active compounds associated with light-induced oxidation in milk, especially over an extended shelf-life. More research is necessary to show the effect of antioxidants on milkfat oxidation, in terms of specific aromas and flavor compounds such as pentanal, hexanal, heptanal, dimethyl disulfide, and 1-octen-3-ol.

The objectives of this study are:

1. To compare aroma profile of light-exposed- and light-protected extended shelf-life milk (ESL) for eight weeks when treated with a single addition of: (i) 100 $mg.kg^{-1}$ milkfat of α-tocopherol and 100 $mg.kg^{-1}$ milkfat of ascorbyl palmitate, and (ii) 100 $mg.kg^{-1}$ milkfat of BHA and 100 $mg.kg^{-1}$ milkfat of BHT; and a weekly addition of: (i) 100 $mg.kg^{-1}$ milkfat of α-tocopherol and 100 $mg.kg^{-1}$ milkfat of ascorbyl palmitate, and (ii) 100 $mg.kg^{-1}$ milkfat of BHA and 100 $mg.kg^{-1}$ milkfat of BHT.
2. To describe the extent of oxidation between control milk and milk treated with antioxidants (as described in Objective 1) by comparing pentanal, hexanal, heptanal, and 1-octen-3-ol concentration.
3. To evaluate weekly the antioxidant content of milk, to continuously document the amount of antioxidants left in the milk throughout the six weeks of storage.

MATERIALS AND METHODS

Lowfat ESL milk was treated with an initial single addition or weekly additions of antioxidants, exposed to light for eight weeks and evaluated for volatile flavor content (solid phase micro-extraction coupled with gas chromatography (SPME-GC), aroma analysis, (SPME coupled with gas chromatography-olfactometry (GC-O)), and antioxidant content (high performance liquid chromatography (HPLC)).

Milk Processing

Fresh raw milk (80 L) was obtained from the Virginia Tech dairy farm. Milk was pre-warmed to 55^{o}C and separated into cream and skim milk using a pilot plant separator (Elecrem separator, model 1G., 292xG, Bonanza Industries, Inc., Calgary, Canada). Lowfat milk was standardized at 2% milkfat by adding cream and skim milk in appropriate proportions. Milk was separated into seven aliquots. Approximately 32 L was used as control (unspiked) milk, while a combination of BHA and BHT was added to three aliquots (8 L each) to represent three replications, and a combination of α-tocopherol and ascorbyl palmitate was added to the remaining three aliquots (8 L each) to yield three replications. Milk aliquots were homogenized and pasteurized separately. Homogenization was done at 2000 psi (1500 psi – 1st stage; 500 psi – 2nd stage) on a two-stage homogenizer (Type DX, Cherry Burrel Corp., Delavan, WI). Milk was pasteurized at 128^{o}C for 2 s using a UHT tubular heat exchanger (UHT/HTST Lab 25 HV, Microthermics Inc., Raleigh, NC). Milk was subsequently cooled to less than 25^{o}C and stored at 4^{o}C in sterile glass containers covered with aluminum foil to prevent any light exposure until sample bottles were filled.

Preparation of Antioxidant-spiked Milk Samples

Food grade BHA, BHT, α-tocopherol, and ascorbyl palmitate were obtained from Fisher Scientific (Cincinnati, OH). Four antioxidant treatments included (i) an initial single addition of 100 mg.kg^{-1} milkfat of α-tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate, (ii) an initial single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT, (iii) a weekly

addition of 100 mg.kg^{-1} milkfat of α-tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate, and (iv) a weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT. Antioxidant treatments that included one single initial dose were spiked during processing on d 0. Treatments (iii) and (iv) were spiked with the antioxidant doses on wk 1, 2, 3, 4, and 5 Weekly 100-ml lowfat UHT milk (Parmalat, Wallington, NJ, obtained from a local grocery store), was used to make a concentrated antioxidant solution for each antioxidant combination and for each replication. From each treated sample to be spiked, 1 ml of milk was withdrawn and discarded. A subsequent addition of 1 ml of the concentrated antioxidant solution was added to achieve an addition concentration of 100 mg.kg^{-1} milkfat of α-tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate for treatment (iii) and 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT for treatment (iv).

Storage and Light Exposure

Aliquots (38 ml) were poured into 40-ml clear glass bottles also fitted with Teflon-coated septa (Supelco, Bellefonte, PA). One half of the antioxidant treated samples within each treatment group were randomly distributed within control (unspiked) milk samples on the shelf. Control milk samples were exposed to two levels (light-protected, light-exposed) of light. Light-protected sample bottles were covered with aluminum foil to prevent any light exposure whereas light-exposed samples were not covered with foil. All samples were exposed to light of 1100-1300 lx, as measured at the top of sample bottles, for 12 h per day, every day. All samples were stored at 4^{o}C for six weeks in a refrigeration unit (Tonka, Hopkins, Minnesota). One individual sample of each treatment and each replication was randomly chosen for analysis on d 0, wk 1, 2, 3, 4, 5, and 6 of storage.

Microbiological Analysis

In order to determine that milk used in the experiment was of similar microbiological quality and to verify that the type of antioxidant addition did not affect the microbiological shelf-life of the product, all milk samples were evaluated for standard plate count and coliform bacteria count

according to standard methods using Petrifilm (3M, St Paul, MN) on d 0, wk 1, 2, 3, 4, 5, and 6 of storage. When necessary, ten-fold dilutions were prepared in peptone water. Determinations were made according to standard methods (Marshall, 1993).

Volatile Analysis

After drawing an appropriate amount of sample for microbial analysis, a 21-ml aliquot of milk was pipetted into separate 40-ml clear glass bottles and fitted with teflon septa (Supelco, Bellefonte, PA). Volatiles were extracted and concentrated using solid phase microextraction (SPME) (as described in Chapter IV) (Van Aardt *et al*., 2001). Pentanal, hexanal, heptanal, and 1-octen-3-ol concentrations, determined in triplicate, were calculated by comparing area counts to those of a calibration curve (McNair and Miller, 1997). Stock solutions of these compounds in water consisted of concentration levels of 0.01 $mg.kg^{-1}$, 0.1 $mg.kg^{-1}$, 1 $mg.kg^{-1}$, 10 $mg.kg^{-1}$, 100 $mg.kg^{-1}$, and 10,000 $mg.kg^{-1}$. An external standard of 3-methyl-3-heptanone in water was used to determine an absolute calibration factor for each day of analysis (Marsili, 1999). This was done by adjusting external standard and subsequently volatile compound area counts from wk 1 to 6 according to the external standard area counts from d 0.

GC-O was done weekly on only one replication per treatment. Three trained panelists randomly evaluated samples. Panelist training and GC-O conditions were the same as described in Chapter IV. Gas chromatography-mass spectrometry (GC-MS) was done to identify volatile compounds in light-exposed milk samples according to the procedure in Chapter IV.

Lipid Extraction and HPLC Analysis of Antioxidants

After 21-ml milk samples were analyzed for volatile concentration, milk from the same sample vials was ultra centrifuged (Beckman L2-65B, Beckman Instruments, Inc. Palo Alto, CA) for 30 min at 15,000 rpm under refrigeration ($10^{o}C$ to $15^{o}C$). The top fat layer was extracted with the Bligh and Dyer fat extraction method to obtain pure milkfat (Kates, 1972). Pure milkfat was combined with 1 ml HPLC-grade methanol in a 2-ml clear glass crimp vial (HP 5181-3375, Hewlett-Packard Co., Palo Alto, CA) for further analysis on HPLC. The methanol extracts were

placed in an Agilent G1313A (Agilent, Palo Alto, CA) auto sampler and analyzed on an Agilent 1100 Series HPLC system using a Zorbax Eclipse XDB-C8 column (5 µm, 4.6 mm i.d. x 15 cm (Agilent, Palo Alto, CA), a Diode Array Detector, and peak detection at 295 nm (band width 20 nm). The mobile phase consisted of 95:5 of methanol:water at a flow rate of 1 $ml.min^{-1}$. The thermostat temperature was $50^{o}C$.

Statistical Analysis

Volatile compounds were quantified by calculating means (n = 3) and standard deviations. Two-way analysis of variance (ANOVA) was used to test the H_0 (H_0: $\mu_{control-light} = \mu_{control-no\ light} = \mu_{tocopherol,\ ascorbyl\ palmitate\ (single\ addition)} = \mu_{tocopherol,\ ascorbyl\ palmitate\ (weekly\ addition)} = \mu_{BHA,\ BHT\ (single\ addition)} = \mu_{BHA,\ BHT\ (weekly\ addition)}$) for each week of storage, with 147 degrees of freedom (n-1) and six treatment level as mentioned in the H_0. Tukey's least significant difference (LSD) was used to compare means. Significant differences were defined at $P < 0.05$ (SAS, 1998).

Volatile odor compounds were identified and their intensities rated after comparing retention times with known retention times of flavor compounds from preliminary GC-MS results of light exposed milk samples. Intensities were rated on a 5-point scale (1 - slight odor, to 5 extreme odor), and tabulated as "+" (odor intensities 0-1), "++" (odor intensities 2-3), and "+++" (odor intensities 4-5).

RESULTS AND DISCUSSION

The addition of antioxidants to milk could potentially limit light-induced lipid oxidation and the off-flavors that are associated with it (Friedrich and Acree, 1998; Van Aardt *et al*., 2003). In this study, combinations of natural and synthetic antioxidants in a single initial addition, and in constant weekly additions were added to milk to observe flavor changes. Hexanal, pentanal, heptanal, and 1-octen-3-ol concentration was monitored as indicators of lipid oxidation, while GC-O was done to observe odor changes in milk. Antioxidant content of milk was determined to observe antioxidant stability.

Microbiological Analysis

The rate of microbiological growth in ESL milk was monitored to ensure that addition of antioxidant treatments did not cause a microbial contamination, as well as to ensure that the milk quality never dropped below a level that was fit for human consumption. All samples were of comparable bacterial quality and within normal range on wk 6 of storage. Aerobic bacteria counts ranged between 0 - 33 $CFU.ml^{-1}$, while coliform counts stayed at 0 $CFU.ml^{-1}$ throughout six weeks of storage (Appendix D). These results fall within FDA specifications (FDA, 1989).

Extent of Lipid Oxidation: Volatile Content

Hexanal, pentanal, heptanal, and 1-octen-3-ol were used to measure the extent of light-induced oxidation of antioxidant-treated milk (Cadwallader and Howard, 1998). In general, pentanal content of control- and antioxidant-treated milk did not differ significantly when exposed to light (1100 to 1300 lx) for 12 h per day throughout six weeks of storage (Figure 1, Table 1). The only reduction in pentanal content was observed at wk 3 and 6 when a combination of BHA (100 $mg.kg^{-1}$) and BHT (100 $mg.kg^{-1}$) were added weekly.

Figure 2 and Table 2 show the increase in hexanal concentration, as a function of lipid oxidation, for milk treated with antioxidants and exposed to light for six weeks. From as early as one week

of storage under fluorescent lights, significant differences existed in hexanal content of milk protected from- and milk exposed to light. An initial single addition of a combination of α-tocopherol (100 $mg.kg^{-1}$) and ascorbyl palmitate (100 $mg.kg^{-1}$) significantly reduced hexanal content in milk throughout the first four weeks of storage. However, the constant weekly addition of these two antioxidants resulted in hexanal levels similar to that of light-exposed control milk throughout storage. The single- and weekly addition of BHA and BHT also reduced hexanal content to some degree over the first four weeks of storage.

Heptanal content of milk increased in light-exposed control- and antioxidant-treated milk over six weeks of storage (Figure 3, Table 3). However, the weekly addition of a combination of BHA and BHT reduced heptanal content of milk to such a degree that, except for week five, no significant difference was observed when compared with light-protected control milk. No other antioxidant treatment evaluated in this study yielded that degree of protection against the effect of light. The initial single dose of a combination of BHA and BHT or a combination of α-tocopherol and ascorbyl palmitate limited light-induced oxidation for the first four weeks of storage, but not thereafter. The observed increase in heptanal content at week five might indicate that the antioxidant content dropped below a critical value for the stability of milkfat. No significant reduction in heptanal content was observed when adding a combination of α-tocopherol and ascorbyl palmitate on a weekly basis.

1-Octen-3-ol content in light-exposed control milk increased only after two weeks of storage (Figure 4, Table 4). Once again, the weekly addition of a combination of BHA and BHT significantly reduced 1-octen-3-ol content of milk when compared to light-exposed control milk. No other antioxidant treatment evaluated in this study yielded that degree of protection against the effect of light. Single doses of a combination of BHA and BHT and a combination of α-tocopherol and ascorbyl palmitate to milk, at the beginning of storage, reduced 1-octen-3-ol content of milk from 3 to 5 - and 3 to 4 weeks, respectively. The weekly addition of α-tocopherol and ascorbyl palmitate did not decrease 1-octen-3-ol content of milk at all.

Overall it seems that a weekly addition of a combination of BHA and BHT to milk protected milkfat against light-induced oxidation when evaluating pentanal, hexanal, heptanal, and 1-

octen-3-ol content of milk. Limited research has been done on the effect of antioxidant on milkfat stability, especially on its effect on specific oxidation products, such as the compounds mentioned above. Nath *et al*. (1992) found that a large addition of 100 $g.kg^{-1}$ α-tocopherol to milkfat increased the rate of oxidation significantly when stored at 60°C for 12 days, as measured by peroxide values. Huang *et al*. (1996) evaluated the stability of bulk oils and oil-in-water emulsions in the presence of α-tocopherol. α-Tocopherol at 150 $mg.kg^{-1}$ and 300 $mg.kg^{-1}$ significantly stabilized bulk linoleic acid and 10% emulsified linoleic acid after three days at 37°C, when comparing hexanal concentrations. Bulk corn oil and 10% emulsified corn oil showed an induction period of approximately 25 days before oxidation commenced, whereas α-tocopherol significantly stabilized these media up to 35 days at 37°C.

Smith and Berge (1997) suggested that primary antioxidants, such as BHA, BHT, and α-tocopherol, that normally act as free-radical terminators are relatively ineffective in inhibiting photodegradation of milkfat, as opposed to autoxidation of milkfat. Photochemical oxidation of milkfat produces large amounts of unconjugated hydroperoxides while autoxidation of milkfat produce conjugated hydroperoxides. There is a migration of the double bond in unconjugated hydroperoxides when oxygen abstracts a hydrogen atom, which yields BHA, BHT, and α-tocopherol ineffective in inhibiting light-induced oxidation. However, in our study a constant addition of BHA and BHT significantly reduced light-induced oxidation of milkfat throughout six weeks of storage.

Lipid Oxidation Odors

The chemical composition of flavor in milk and other dairy products is complicated due to the heterogeneous nature of milk. GC-O provides an avenue for evaluating odorous compounds that comprise milk flavor. Table 5 and Appendix E lists the odorous volatile compounds associated with light-exposed control- and antioxidant treated milk. It is important to note that although compounds might be detected as peaks on a chromatogram, these compounds might not be odor-active, or human thresholds for these odors could be below the detected concentrations. The opposite also could be true. Compounds with strong odors even at low concentrations, might not

yield significant peaks on a chromatogram. Figure 5 shows a chromatogram of aroma-active compounds in light-exposed control milk after five weeks of refrigerated storage.

Strong odors associated with light-exposed control milk include sour- and green cut-grass, fish oil, and floral which can be associated with pentanal, hexanal, heptanal, and heptanol, respectively (Table 5). Although Cadwallader and Howard (1998) observed a combination of similar odor-active compounds when exposing lowfat milk to light for a maximum of 48 h, compounds with highest intensities included dimethyl disulfide, 2-methylpropanal, 1-hexen-3-one, and 1-octen-3-one. Jadhav *et al*. (1996) described two components that are involved in the development of "light-activated" flavor in milk. Initially a burnt, activated sunlight flavor develops and predominates for about two or three days. This burnt flavor can be contributed to photoreduction of riboflavin in milk that leads to the Strecker degradation of methionine to form odorant methional and other sulfur-containing compounds. The second component is attributed to superoxide anion that undergoes dismutation to form singlet oxygen and other activated oxygen species which can initiate free radical lipid oxidation to form numerous volatile carbonyl compounds. This off-flavor, often characterized as metallic or cardboardy, usually develops after two days and does not dissipate. This might indicate why Cadwallader and Howard (1998) observed slightly different odors in milk exposed to light for 48 h (due to protein oxidation), versus our study which observed lipid oxidation odors after 6 weeks of light-exposed storage.

GC-O observations on milk samples with initial single- and weekly additions of BHA and BHT, and a weekly addition of α-tocopherol and ascorbyl palmitate suggest a decrease in the amount of odorous compounds (Table 5). However, GC-O data shows that a single addition of α-tocopherol and ascorbyl palmitate caused increased oxidation, when compared to light-exposed control milk. Although there was no difference in odor intensities that represent pentanal, hexanal, and heptanal, odors that represent acetone, 2,3-butanedione, dimethyl disulfide, 1-hexanol, 2-heptanone, nonanal increased from light-exposed control milk to light-exposed milk treated with a single dose of α-tocopherol and ascorbyl palmitate. The apparent pro-oxidant effect might be due to the antioxidants being consumed with the subsequent effect being zero ability to stabilize milkfat.

Furthermore, it seems that different antioxidant treatments decreased and increased different odors. The addition of BHA and BHT in a single initial addition showed decreases in hexanal odor, but not in heptanal and 1-heptanol odor, while the addition of α-tocopherol and ascorbyl palmitate decreased pentanal and heptanol odor, but not hexanal and heptanal odor. Sensory analysis showed an overall protection against light-induced flavor of milk after 10 h of light exposure when an initial single dose of 250 $mg.kg^{-1}$ α-tocopherol and 250 $mg.kg^{-1}$ ascorbic acid was added (Van Aardt *et al.*, 2003). Odor-active compounds associated with light-induced changes in milk after 10 h included 2-heptanone, n-heptanal, 1-octene-3-ol, octanal, and nonanal and ranged in intensities from zero to three. Since light-oxidized flavor in milk is comprised of lipid and protein oxidation products, it is important to know the stage during light exposure that odorous flavor compounds increase to levels above human threshold. In Chapter III volatile compounds associated with protein oxidation (dimethyl disulfide) were mostly measured since milk was exposed for a short period of time (10 h). In this study off-odors can be associated with lipid oxidation, since evaluations were done after weeks of storage.

Antioxidant Content in Control- and Antioxidant-treated Milk

In general, liquid chromatographic analysis of antioxidants in milk did not show significant increases, decreases, or trends in BHT and ascorbyl palmitate content of milk for antioxidant-treated milks (Appendix F). Maximum extracted amounts did not exceed 8 $mg.kg^{-1}$ and 5 $mg.kg^{-1}$ milkfat, respectively. However, BHA content of milk with an initial single addition and before weekly addition ranged between 7.4 $mg.kg^{-1}$ and 20.4 $mg.kg^{-1}$ milkfat. Analysis of BHA content of milk right after weekly addition showed levels of up to 75.0 $mg.kg^{-1}$ milkfat (wk 1), which was lower than the spiked 100 $mg.kg^{-1}$ milkfat. α-Tocopherol content of milk spiked with an initial single addition of 100 $mg.kg^{-1}$ milkfat stayed below 50.3 $mg.kg^{-1}$ milkfat throughout storage. When spiked weekly, α-tocopherol levels increased to a maximum of 123.5 $mg.kg^{-1}$ milkfat after five weeks of storage.

However, standard curves for individual antioxidants in methanol resulted in high correlation coefficients ($R^2 > 0.99$) of spiked and retrieved amounts, which might indicate that the Bligh and Dyer fat extraction method, commonly used to extract dairy fats, did not sufficiently extract the

fat-soluble antioxidants used in this study. Antioxidants most likely dissolved in the discarded methanol:water (1:1) wash solution. The Bligh and Dyer lipid extraction method was specifically chosen since it does not involve heating or drying under elevated temperatures (Bligh and Dyer, 1959). A methanol extraction method that extracts antioxidants directly from milk potentially could have worked better (Huang *et al*., 1996). Also, since extraction occurred on milk samples that were previously heated for SPME analysis, it might be possible that BHA and BHT were volatilized and lost, or that thermal degradation of antioxidants occured.

CONCLUSION

The addition of antioxidant to milk was effective in controlling or limiting the development of increased concentrations of hexanal, pentanal, and 1-octen-3-ol, compounds used to monitor oxidation of milk. Antioxidant addition to milk may help protect milk flavor from oxidative changes resulting from protein and lipid oxidation over six weeks of light-exposed storage. Typical protein oxidation products that develop after only a few days of light-exposed storage were decreased during the first few weeks of storage with the single- or weekly additions of BHA and BHT, or α-tocopherol and ascorbyl palmitate. Typical lipid oxidation products such as heptanal and 1-octen-3-ol were significantly decreased for six weeks of light-exposed storage with a constant weekly addition of a combination of BHA and BHT. In conclusion, this study showed that a constant timed addition of BHA and BHT to milk protect milk flavor significantly more than initial single additions of BHA and BHT or α-tocopherol and ascorbyl palmitate. Timed addition could potentially be achieved by controlled migration of antioxidants from polymeric packaging. Further research on the addition of antioxidants to polymeric packaging for intentional migration into milk might yield significant results, which could benefit the dairy industry.

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TABLES

Table 1. Pentanal content (mg.kg^{-1}) of control- and antioxidant-treated milk after light exposure (1100 – 1300 lx) for 12 h per day for 6 weeks.

		Week											
		1[1]		2		3		4		5		6	
Treatment[2]	**Light**	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***
Control	Yes	18.69^{a}	± 3.79	35.39^{a}	± 4.85	30.05^{a}	± 3.35	28.51^{a}	± 3.99	47.81^{a}	± 1.10	37.28^{a}	± 4.12
Control	No	14.47^{a}	± 5.98	29.83^{ab}	± 2.81	19.25^{b}	± 2.57	24.63^{ab}	± 2.89	31.60^{a}	± 6.22	22.54^{bc}	± 2.52
Control (weekly)	Yes	18.69^{a}	± 3.79	31.26^{a}	± 2.18	27.12^{ab}	± 1.21	31.77^{a}	± 3.42	40.99^{a}	± 8.35	45.47^{a}	± 1.27
Control (weekly)	No	14.47^{a}	± 5.98	18.49^{b}	± 5.24	17.47^{b}	± 1.84	17.78^{b}	± 3.84	25.17^{a}	± 3.28	19.69^{c}	± 3.33
BHA/BHT (single)	Yes	8.21^{a}	± 5.98	29.29^{ab}	± 2.14	24.98^{ab}	± 1.10	30.85^{a}	± 1.38	40.07^{a}	±13.8	34.71^{ab}	± 1.18
Toc/Asc (single)	Yes	21.85^{a}	± 1.35	27.00^{ab}	± 6.49	23.29^{ab}	± 0.14	29.18^{a}	± 3.36	28.05^{a}	±12.1	37.28^{a}	± 4.12
BHA/BHT (weekly)	Yes	8.21^{a}	± 5.98	28.82^{ab}	± 3.67	19.65^{b}	± 2.11	25.08^{ab}	± 2.56	40.41^{a}	±5.13	22.55^{bc}	± 1.00
Toc/Asc (weekly)	Yes	21.85^{a}	± 1.35	31.14^{a}	± 1.28	24.41^{ab}	± 1.83	29.03^{a}	± 3.87	28.05^{a}	±12.1	33.17^{abc}	± 3.16

[1] Pentanal content on d 0 was 0 mg.kg^{-1} for all samples.

[2] Treatments: Control milk (unspiked); Control milk with UHT milk added at the same levels as antioxidant samples weekly spiked; Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in 1 ml of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in 1 ml of UHT milk.

[a-e] Means (n = 3) in the same column with different superscripts are significantly different ($P < 0.05$)

Table 2. Hexanal content (mg.kg^{-1}) of control- and antioxidant-treated milk after light exposure (1100 – 1300 lx) for 12 h per day for 6 weeks.

		Time (wk)											
		1[1]		2		3		4		5		6	
Treatment[2]	**Light**	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***
Control	Yes	13.81^{a}	± 0.57	18.64^{ab}	± 2.48	36.75^{bc}	± 3.80	31.69^{b}	± 3.93	28.50^{b}	± 4.09	32.41^{a}	± 2.62
Control	No	4.42^{c}	± 0.37	6.48^{c}	± 0.21	4.06^{e}	± 0.00	6.43^{d}	± 0.07	5.70^{c}	± 1.64	4.06^{b}	± 0.00
Control (weekly)	Yes	13.81^{a}	± 0.57	23.91^{a}	± 1.97	45.00^{a}	± 0.72	40.31^{a}	± 3.53	41.73^{a}	± 4.98	50.11^{a}	± 5.77
Control (weekly)	No	5.07^{c}	± 0.58	5.22^{c}	± 0.60	6.31^{e}	± 1.14	4.70^{d}	± 0.65	4.06^{c}	± 0.00	4.06^{b}	± 0.00
BHA/BHT (single)	Yes	10.62^{b}	± 0.85	15.80^{b}	± 3.23	30.36^{c}	± 5.26	22.94^{c}	± 1.15	27.29^{b}	± 1.97	30.96^{ab}	± 1.12
Toc/Asc (single)	Yes	9.79^{b}	± 1.17	16.36^{b}	± 2.59	21.34^{d}	± 2.41	21.60^{c}	± 1.73	38.47^{a}	± 0.37	32.41^{a}	± 2.62
BHA/BHT (weekly)	Yes	10.62^{b}	± 0.85	18.05^{ab}	± 3.47	29.55^{c}	± 1.99	19.48^{c}	± 2.83	21.32^{b}	± 3.14	38.12^{a}	± 25.4
Toc/Asc (weekly)	Yes	9.79^{b}	± 1.17	21.43^{ab}	± 0.84	39.21^{ab}	± 2.21	37.77^{ab}	± 3.10	38.47^{a}	± 0.37	46.64^{a}	± 3.90

[1] Hexanal content on d 0 was 0 mg.kg^{-1} for all samples.

[2] Treatments: Control milk (unspiked); Control milk with UHT milk added at the same levels as antioxidant samples weekly spiked; Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in 1 ml of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in 1 ml of UHT milk.

[a-e] Means (n = 3) in the same column with different superscripts are significantly different ($P < 0.05$)

Table 3. Heptanal content (mg.kg^{-1}) of control- and antioxidant-treated milk after light exposure (1100 – 1300 lx) for 12 h per day
for 6 weeks.

		Time (wk)											
		1[1]		2		3		4		5		6	
Treatment[2]	**Light**	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***
Control	Yes	58.1^{a}	± 2.6	87.1^{b}	± 25.2	274.3^{ab}	± 36.5	291.8^{a}	± 36.1	334.7^{ab}	± 28.9	342.8^{a}	± 66.3
Control	No	33.2^{bc}	± 0.3	34.8^{d}	± 0.4	32.2^{d}	± 0.0	32.2^{d}	± 0.0	34.5^{d}	± 2.3	32.2^{b}	± 0.0
Control (weekly)	Yes	58.1^{a}	± 2.6	143.1^{a}	± 21.9	284.6^{a}	± 21.8	312.4^{a}	± 40.2	359.5^{a}	± 29.4	346.8^{a}	± 99.7
Control (weekly)	No	32.2^{c}	± 0.0	34.9^{d}	± 1.2	32.2^{d}	± 0.0	32.2^{d}	± 0.0	32.2^{d}	± 0.0	32.2^{b}	± 0.0
BHA/BHT (single)	Yes	38.3^{bc}	± 0.9	57.3^{cd}	± 9.6	108.8^{c}	± 24.8	117.8^{bc}	± 11.3	187.5^{bc}	± 18.9	261.6^{a}	± 25.9
Toc/Asc (single)	Yes	42.2^{b}	± 5.8	69.3^{cd}	± 12.9	126.1^{c}	± 23.7	166.5^{b}	± 15.9	271.6^{b}	± 15.4	318.0^{a}	± 29.2
BHA/BHT (weekly)	Yes	38.3^{bc}	± 0.9	51.8^{cd}	± 9.8	92.8^{cd}	± 9.3	71.1^{cd}	± 6.9	69.2^{c}	± 8.2	52.9^{b}	± 12.4
Toc/Asc (weekly)	Yes	42.2^{b}	± 5.8	116.6^{ab}	± 12.5	214.8^{b}	± 23.5	260.2^{a}	± 39.3	285.2^{b}	± 39.1	349.1^{a}	± 25.7

[1] Heptanal content on d 0 was 37.7 mg.kg^{-1} for all samples.

[2] Treatments: Control milk (unspiked); Control milk with UHT milk added at the same levels as antioxidant samples weekly spiked; Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in 1 ml of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in 1 ml of UHT milk.

[a-e] Means (n = 3) in the same column with different superscripts are significantly different ($P < 0.05$)

Table 4. 1-Octen-3-ol content (mg.kg^{-1}) of control- and antioxidant-treated milk after light exposure (1100 – 1300 lx) for 12 h per day for 6 weeks.

		Time (wk)									
		2[1]		3		4		5		6	
Treatment[2]	**Light**	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***	**mean**	**± *s***
Control	Yes	7.5^{bc}	± 6.5	92.7^{ab}	± 19.5	100.4^{a}	± 10.6	107.4^{bc}	± 15.3	96.0^{b}	± 22.0
Control	No	0.0^{c}	± 0.0	0.0^{d}	± 0.0	0.0^{c}	± 0.0	0.0^{f}	± 0.0	0.0^{c}	± 0.0
Control (weekly)	Yes	39.2^{a}	±12.0	118.8^{a}	± 7.7	120.1^{a}	± 9.7	140.9^{ab}	± 25.5	166.4^{a}	± 23.1
Control (weekly)	No	0.0^{c}	± 0.0	0.0^{d}	± 0.0	0.0^{c}	± 0.0	0.0^{f}	± 0.0	0.0^{c}	± 0.0
BHA/BHT (single)	Yes	0.4^{c}	± 0.2	34.8^{cd}	± 27.7	15.6^{bc}	± 3.4	51.2^{de}	± 11.4	76.7^{b}	± 9.5
Toc/Asc (single)	Yes	5.6^{c}	± 3.7	21.4^{cd}	± 10.2	35.0^{b}	± 5.9	82.9^{cd}	± 3.7	89.0^{b}	± 8.1
BHA/BHT (weekly)	Yes	2.3^{c}	± 1.3	25.6^{cd}	± 6.4	15.4^{bc}	± 2.5	27.4^{e}	± 5.2	21.2^{c}	± 5.0
Toc/Asc (weekly)	Yes	23.4^{ab}	± 6.2	62.8^{bc}	± 31.1	105.4^{a}	± 21.7	158.7^{a}	± 10.2	151.8^{a}	± 7.8

[1] 1-Octen-3-ol content on d 0 and wk 1 was 0 mg.kg^{-1} for all samples.

[2] Treatments: Control milk (unspiked); Control milk with UHT milk added at the same levels as antioxidant samples weekly spiked; Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in 1 ml of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in 1 ml of UHT milk.

[a-e] Means (n = 3) in the same column with different superscripts are significantly different ($P < 0.05$)

Table 5. Odor-active compounds detected by gas chromatography-olfactometry of control and antioxidant-treated milk exposed to light for 12 h per day for 5 weeks (Odor intensities: + (0-1), ++ (2-3), +++ (4-5).

			Antioxidant Treatment[3]					
Compound[1]	**Retention Index[2]**	**Odor Description**	**Contr ol**	**Control (Light-protected)**	**BHA/BHT (single)**	**Toc/Asc (single)**	**BHA/BHT (weekly)**	**Toc/Asc (weekly)**
1 (2-propanone)	0290	Sweet, perfume	+		++	++		
2	0321	Butter, cooked milk	+	++	++	++		+
3 (2,3-butanedione)	0347	Diacetyl, butter	+		+			+
4	0408	Roasted, manure	++				+	
5 (pentanal)	0440	Sour, cut-grass	++	+		++		
6 (dimethyl disulfide)	0503	Cooked vegetable, grain	+			++		
7 (hexanal)	0567	Cut-grass	+++		+	+++	++	+++
8 (1-hexanol)	0639	Grass, earth	+			++	+	
9 (2-heptanone)	0687				++	++		+
10 (n-heptanal)	0702	Green, fish oil	+++	+	+++	+++	+++	+++
11 (1-heptanol)	0792	Plastic, floral	++		++			
12 (1-octene-3-ol)	0805	Mushroom				+++	++	++
13	0836	Burnt floral	+			++		
14	0907	Cooked			++	+++	++	
15 (nonanal)	0962	Soapy, floral	+			+++		

[1] Compound identified by comparison of its mass spectrum

[2] Retention indices from GC-O data

[3] Treatments: Control milk (unspiked); Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in 1 ml of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in 1 ml of UHT milk.

FIGURES

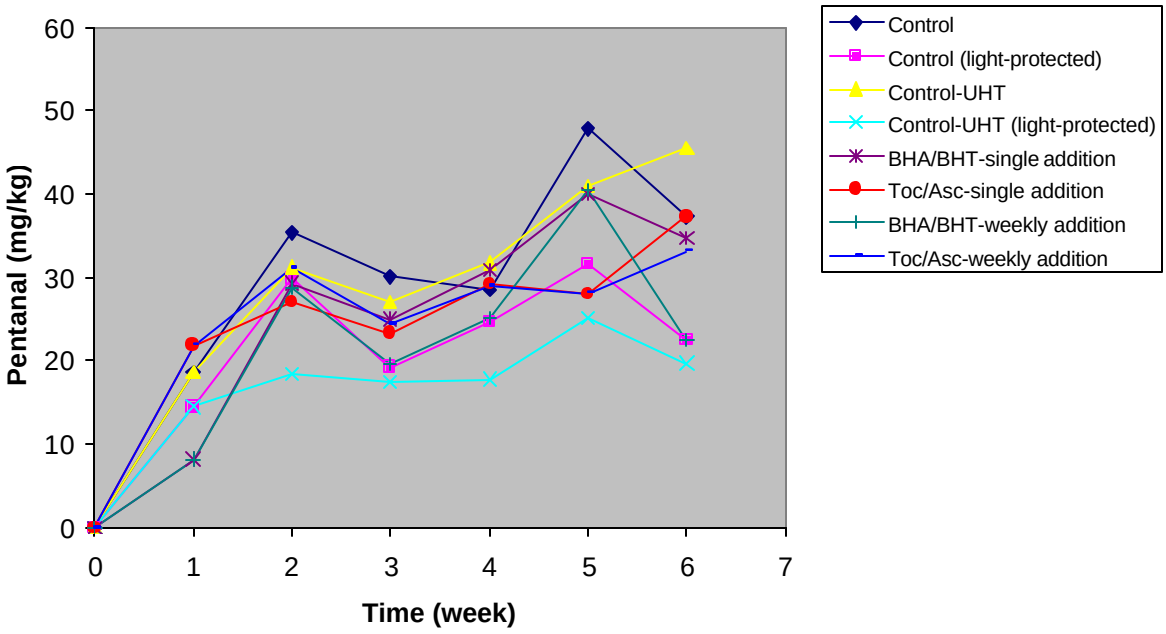

Figure 1. Pentanal content of control- and antioxidant treated milk after 12 h of light exposure per day (1100 – 1300 lx) for 6 weeks. Treatments included: Control milk (unspiked); Control milk with UHT milk added at the same amounts as antioxidant samples spiked weekly; Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in a small quantity of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in a small quantity of UHT milk.

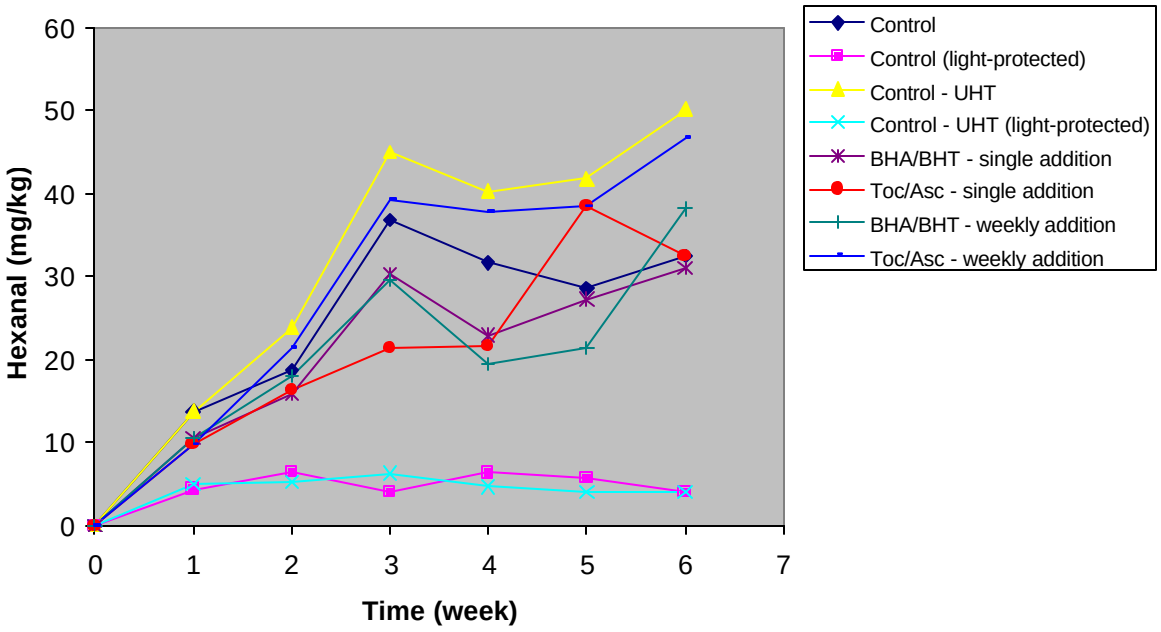

Figure 2. Hexanal content of control- and antioxidant treated milk after 21 h of light exposure per day (1100 – 1300 lx) for 6 weeks. Treatments included: Control milk (unspiked); Control milk with UHT milk added at the same amounts as antioxidant samples spiked weekly; Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in a small quantity of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in a small quantity of UHT milk.

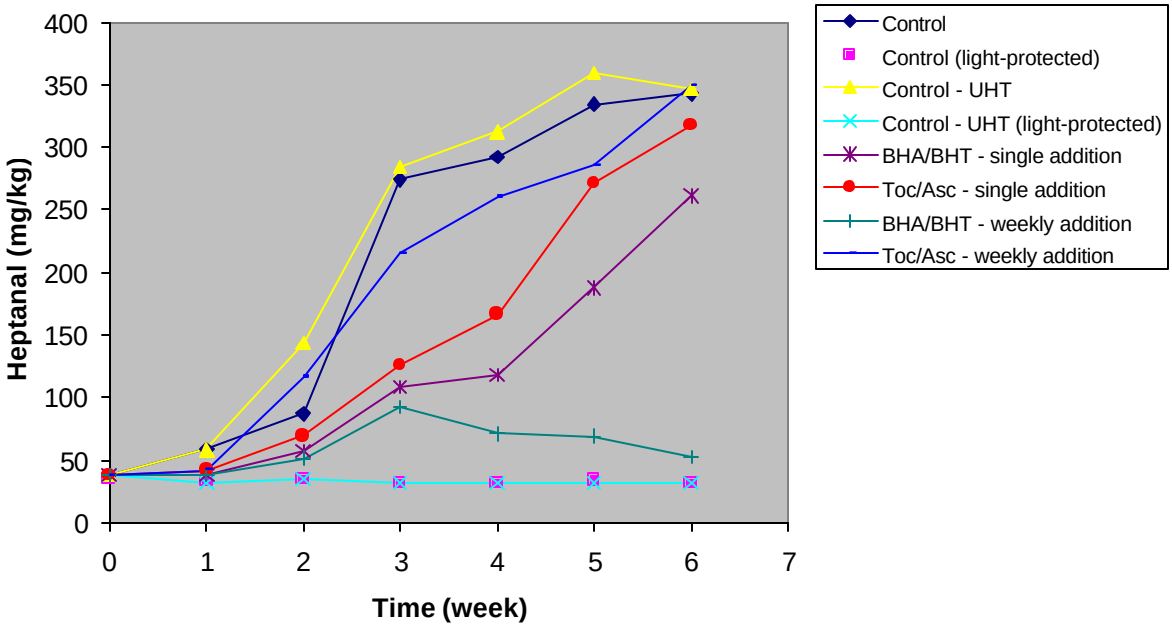

Figure 3. Heptanal content of control- and antioxidant treated milk after 12 h of light exposure per day (1100 – 1300 lx) for 6 weeks. Treatments included: Control milk (unspiked); Control milk with UHT milk added at the same amounts as antioxidant samples spiked weekly; Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in a small quantity of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in a small quantity of UHT milk.

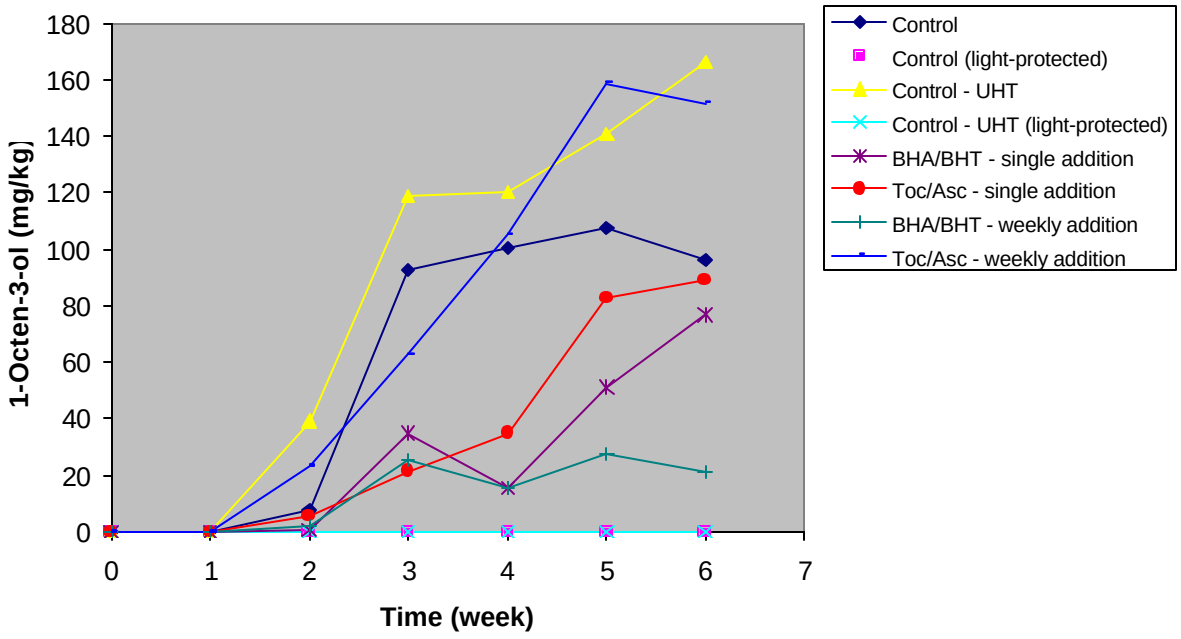

Figure 4. 1-Octen-3-ol content of control- and antioxidant treated milk after 12 h of light exposure per day (1100 – 1300 lx) for 6 weeks. Treatments included: Control milk (unspiked); Control milk with UHT milk added at the same amounts as antioxidant samples spiked weekly; Single addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; Weekly addition of 100 mg.kg^{-1} milkfat of BHA and 100 mg.kg^{-1} milkfat of BHT dissolved in a small quantity of UHT milk; Weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in a small quantity of UHT milk.

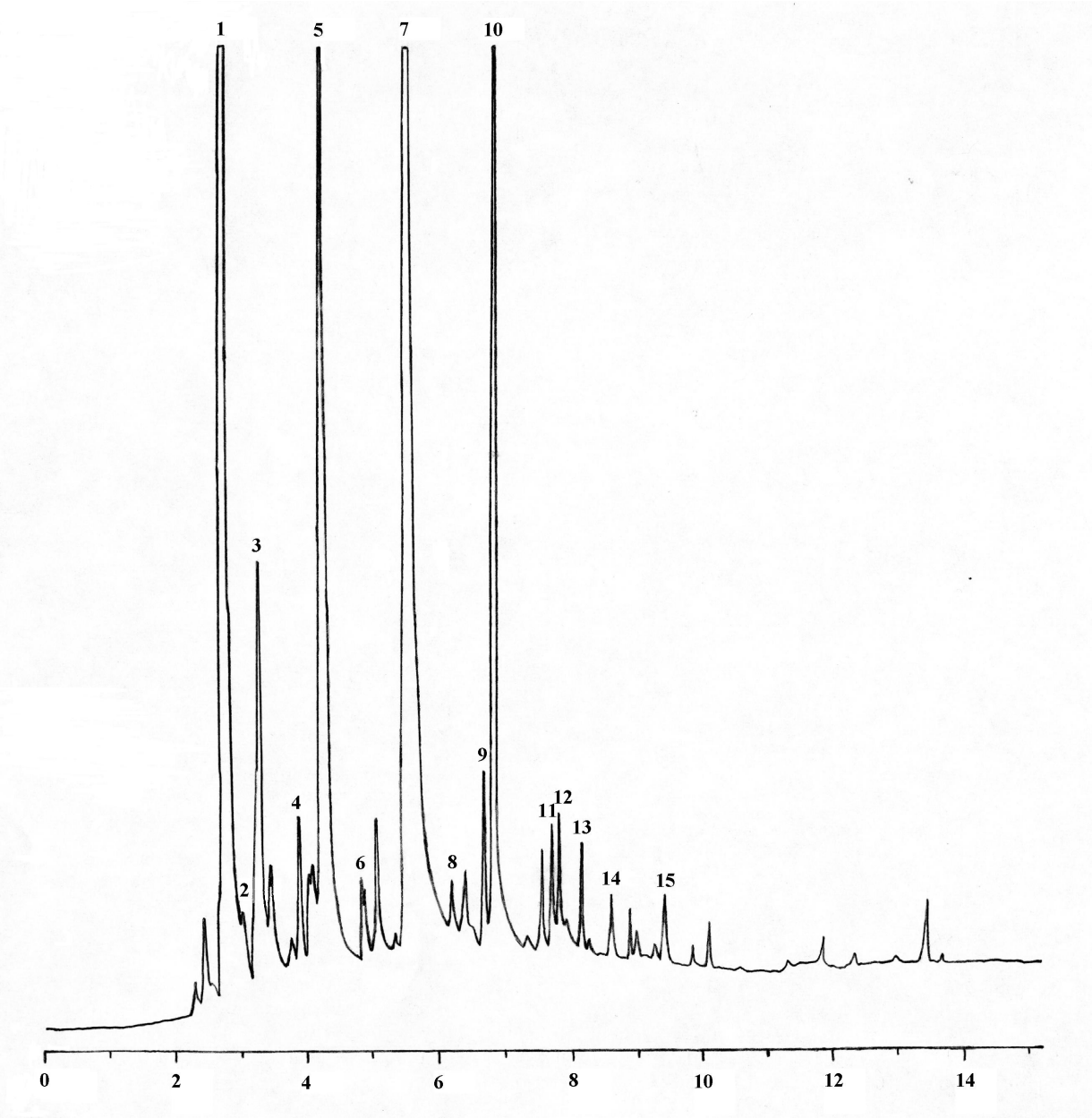

Figure 5. Gas chromatogram of milk exposed to light (1100-1300 lx) for 12 h per day for 6 weeks. Aroma-active compounds include (1, 2-propanone; 2, unknown; 3, 2-butanone; 4, unknown; 5, pentanal; 6, dimethyl disulfide; 7, hexanal; 8, 1-hexanol; 9, 2-heptanone; 10, n-heptanal; 11, 1-heptanol; 12, 1-octen-3-ol; 13, unknown; 14, 1-octanol; 15, nonanal).

CHAPTER VI

Antioxidant-Loaded Biodegradable Films: Controlled Release of Antioxidants into Dry Milk Products and Food Simulating Liquids

M. van Aardt,[*] S. E. Duncan,[*] J. E. Marcy,[*] T. E. Long,[†]

S. F. O'Keefe,[*] and S. R. Nielsen-Sims[‡]

[*]Department of Food Science and Technology and

[†]Department of Chemistry,

Virginia Polytechnic Institute and State University, Blacksburg 24061

[‡]Eastman Chemical Co., Kingsport 37662-5125

Corresponding Author: Marleen van Aardt

27F FST Building, Virginia Tech, Blacksburg, VA 24061

Phone: (540) 231-8675

Fax: (540) 231-9293

E-mail: mvanaard@vt.edu

ABSTRACT

Poly(lactide-*co*-glycolide) (50:50) films loaded with antioxidants (i) 2% α-tocopherol, and (ii) a combination of 1% butylated hydroxytoluene (BHT) and 1% butylated hydroxyanisole (BHA), were used in an antioxidant release study in water and Miglyol 812® at 4°C and 25°C, as well as a study on the effect of these films on dry whole milk and dry buttermilk stability. BHT was released through the hydrolytic degradation of the polymer when stored in water at room temperature for eight weeks. As expected, polymer degradation did not take place when antioxidant-loaded films were stored in whole milk powder (3.01% moisture) and buttermilk powder (4.60% moisture). However, α-tocopherol, BHA, and BHT were released through diffusion from 0 $mg.kg^{-1}$ to 21.9, 60.0, 192.0 $mg.kg^{-1}$ milkfat, respectively, in whole milk powder after four weeks of storage at 25°C. The mechanism of diffusion (surface vs. bulk) could potentially be evaluated by scanning electron microscopy. Buttermilk powder did not show increasing antioxidant content, which might be due to a much lower fat content (2.35%), as compared to whole milk powder (24.48%). Milkfat stability was measured by determining hexanal, pentanal, and heptanal content. Although limited reductions were observed in these volatiles in control- and antioxidant-treated powders, pentanal content was significantly decreased for the first three weeks of storage when buttermilk powders were packaged in contact with BHA/BHT-loaded PLGA films.

Key words: "biodegradable polymers, antioxidants, poly(lactic acid), poly(glycolic acid), lipid oxidation, milk powder, α-tocopherol, BHA, BHT"

Abbreviation key: BHA = butylated hydroxyanisole, **BHT** = butylated hydroxytoluene, **LDPE** = low-density polyethylene, **PLA** = polylactide, **PGA** = polyglycolide, **PLGA** = poly(lactide-*co*-glycolide), **GRAS** = Generally Recognized As Safe, **PTFE** = polytetrafluoroethylene (Teflon), **NMR** = nuclear magnetic resonance spectroscopy, **GPC** = gel permeation chromatography, **SEC** = size exclusion chromatography, **SEM** = scanning electron microscopy, **HP** = Hewlett Packard, **SPME** = solid phase microextraction, **GC** = gas chromatography, **MS** = mass spectrometry,

LSD = least significant difference, **ANOVA** = analysis of variance, $\mathbf{H_o}$ = null hypothesis, $\mathbf{H_1}$ = alternative hypothesis

INTRODUCTION

Lipid oxidation in whole milk powder and other milk powders high in fat is a major cause of deterioration during processing and storage (McCluskey *et al*., 1997). Flavor of dry milk products is very important since it plays an important role in the flavor of the products in which the powder is used. Bodyfelt *et al*. (1988) described the ideal flavor of dry whole milk as "clean, rich, sweet, fresh, and pleasant, not unlike that of fine pastry", and the ideal flavor of dry buttermilk as "clean, sweet, and pleasant". Dry milk products that are high in fat, such as whole milk powder, are very susceptible to lipid oxidation reactions. The off-flavor associated with the oxidation of whole milk powder is "oxidized/tallowy" as specified by Bodyfelt *et al*. (1988). This off-flavor is attributed to compounds such as 2-methyl propanal, 2-methyl butanal, 3-methyl butanal, hexanal, 2-butanone, 2-heptanone, 2-furanmethanol, and pentane (Lee and Morr, 1994).

The addition of antioxidants to fat-containing food to protect flavor is a common practice. As early as the 1950s and 1960s researchers experimented with the decrease in oxidation of dry milk products in the presence of antioxidants such as dodecyl gallate, propyl gallate, nordihydroguaretic acid, thiodipropionic acid, and butylated hydroxytoluene (Busch *et al.,* 1952; Tamsma *et al.,* 1963, Hill *et al.,* 1969, Hammond, 1970). However, a single initial dose might not be as effective in preventing oxidative degradation as a continuous controlled addition over the long storage duration of milk powders. Antioxidants have been added to food packaging for the intentional purpose of migration into food. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), when added to plastic food packaging, have proven to be effective in controlling oxidized flavor in dry food products such as breakfast cereal and crackers (Hoojjat *et al*., 1987; Jadhav *et al.*, 1996). Miltz *et al*. (1995) and Sharma *et al*. (1990) reported that storage stability was increased when oatmeal cereal was stored in antioxidant-impregnated plastic films. They attributed the stability of the food to the volatilization of the antioxidant from the packaging surface, with subsequent diffusion into the food. Wessling *et al*. (1999; 2000) studied the ability of antioxidants to diffuse from various plastic packaging materials into fatty food and food simulating liquids, and the effect on lipid oxidation. Oatmeal cereal stored in BHT-impregnated low-density polyethylene (LDPE) film underwent the least change in volatile

profile as compared to fresh control powders. However, a-tocopherol-impregnated LDPE film did not appear to prolong the shelf-life of oatmeal over 10 weeks of storage at 20°C. High levels of a-tocopherol in LDPE film was found to inhibit oxidation of a linoleic acid emulsion stored in contact with the film at 6°C for 25 days of storage.

A limitation to adding antioxidants directly to food in one large initial dose is rapid depletion of the antioxidants, as well as very high initial concentrations (Jung *et al*., 1998). Indirect addition of antioxidants to food through diffusion from packaging also shows a steady decrease in antioxidant content over storage time as the added antioxidants are consumed or degraded, but not as rapidly as with an initial single addition (Wessling *et al*., 1998). However, a consistent level of antioxidant in food might be achieved with the use of an antioxidant-loaded biodegradable polymer, possibly as a liner or coating in the food packaging. Polylactide (PLA) and copolymers of PLA and polyglycolide (PGA) are biodegradable polymers commonly used in the biomedical industry for sustained release preparations. A higher glycolide content increases the degradation rate of the copolymer. The mechanism of degradation for aliphatic polyesters occurs via hydrolysis. Degradation proceeds first by diffusion of water into the material (initially into the more amorphous zones), followed by random hydrolysis, fragmentation of the material, and finally a more extensive hydrolysis accompanied by phagocytosis, diffusion and metabolism (when ingested) (Anderson, 1997). By-products such as lactic acid are a common food ingredient, and although it is expected to decrease pH, milk possesses buffering systems that would prevent pH changes. Therefore, the effective use of these polymers in dairy food systems is dependent on the amount of moisture in the food. PLA and PGA were declared as "Generally Recognized As Safe (GRAS)" for its intended uses as a polymer for fabricated containers that will hold and/or package food (Conn *et al*., 1996). Limited migration of food grade substances was not found to be of significant risk.

The objectives of this study were:

1. To measure release of antioxidants (α-tocopherol, and a combination of BHA and BHT) from poly(lactide-*co*-glycolide (PLGA) (50:50) into water and Miglyol 812® over eight weeks of storage at 4°C and 25°C.

2. To measure hexanal, pentanal, and heptanal content of whole milk- and buttermilk powder when stored at 25^{o}C for 4 weeks with antioxidant-loaded PLGA (50:50) films.

MATERIALS AND METHODS

Antioxidant-loaded PLGA films were prepared to meet two goals: *(i)* to evaluate release of antioxidants (α-tocopherol, BHA, BHT, and combinations thereof) into water, Miglyol 812®, dry whole milk, and dry buttermilk and *(ii)* to evaluate hexanal, pentanal, and heptanal content, as a measure of oxidation, in whole milk- and buttermilk powders when exposed to antioxidant-loaded films.

Materials

Poly(DL-lactide-*co*-glycolide) (50:50) was obtained from Polysciences (Warrington, PA). Antioxidants (BHA, BHT, and α-tocopherol) were purchased from Aldrich (Milwaukee, WI), while other chemicals and solvents used for identification standards, such as pentanal, hexanal, heptanal, methylene chloride and methanol were purchased from Sigma Chemical Company (St. Louis, MO). Whole milk powder (Naturetone 3968®) and buttermilk powder (San-a-creme 985 BM®) were obtained from Kerry Specialty Ingredients (Beloit, WI). Miglyol 812®, which was used as a food simulating liquid, was obtained from Condea Vista Co. (Cranford, NJ). It was chosen as a saturated oil substitute for milkfat, since it is comprised of saturated triglycerides, tricaprylin ($C_{8:0}$) and tricaprin ($C_{10:0}$). Milkfat comprises of approximately 20% short chain saturated fatty acids, 33% palmitic acid, 16% palmitoleic acid, and 30% unsaturated fatty acids.

Antioxidant-loaded PLGA Films

Antioxidant-loaded PLGA (50:50) films were prepared by homogeneously dispersing (i) 2% α-tocopherol, and (ii) a combination of 1% BHA and 1% BHT, and polymer in a solution with methylene chloride. Subsequently, films were cast by pouring the dispersion into polytetrafluoroethylene (PTFE or Teflon) Petri dishes (Supelco, Bellefonte, PA), and allowing them to dry at room temperature in the dark for 48 h. The dried films were cut into rectangles (1

cm x 4 cm) for subsequent immersion in water and Miglyol 812®, or half-circles (r = 2.9 cm) for contact in dry whole milk and dry buttermilk. Film thickness was constant at approximately 100 μm ± 9.4 μm (n = 10), as measured with a micrometer gauge (Gümüsderelioglu and Deniz, 2000; Andreopoulos *et al.*, 1999).

Characterization of Polymer Films

Polymer films were characterized by nuclear magnetic resonance spectroscopy (NMR) and gel permeation chromatography (GPC), also known as size exclusion chromatography (SEC). NMR analysis determined true antioxidant content of films while GPC analysis determined molecular weight (M_n) and molecular weight distribution (M_w/M_n) information. GPC analysis was done at 40°C in tetrahydrofuran (THF) using polystyrene standards on a Waters size exclusion chromatographer (Waters Inc., Burnsville, MN) equipped with 3 in-line PLgel 5 mm MIXED-C columns, an autosampler, and 410 refractive-index detector. ^{1}H NMR spectra were collected at 399,944 MHz on a Varian Inova 400 spectrometer (Varian Inc., Palo Alto, CA) at ambient temperature. Spectra were recorded in $CHCL_3$ containing tetramethylsilane (TMS) as internal standard.

Characterization of Dry Whole Milk and Dry Buttermilk

Dry whole milk and dry buttermilk were evaluated for moisture content (AOAC, 1997), total solid content (AOAC, 1997), fat content, and phospholipids content. Fat content was determined by the Bligh and Dyer fat extraction (Bligh and Dyer, 1959), as well as the Folch method (Folch *et al*., 1957). Phospholipid content was determined as described in Rouser *et al*. (1966). The values obtained in the phosphorous analysis were multiplied by a factor of 25 to convert from phosphorous content to phospholipids content as described by Anderson *et al.* (1977).

Release of Antioxidants and Degradation of PLGA Films in Water and Miglyol 812®

Preweighed PLGA (50:50) films (1 cm x 4 cm x 100 μm) containing (i) 2% α-tocopherol, and (ii) a combination of 1% BHA and 1% BHT were immersed in 5-ml aliquots of water and Miglyol 812®. Samples were stored in 40-ml clear glass containers, capped with white Teflon caps (Supelco, Bellefonte, PA), and placed in an automated shaker (Lab-line Orbit Environ, Lab-line Instruments, Inc., Melrose Park, ILL). Gentle rocking of samples (140 rpm) commenced while stored in the dark at room temperature (25 ± 3°C) and in a refrigeration unit (Tonka, Hopkins, Minnesota) (4 ± 1°C) for eight weeks (Wessling *et al*., 1999). At d 0, wk 1, 2, 3, 4, 5, 6, 7, and 8 of storage, three individual water and oil samples of each treatment were assayed for antioxidant concentration by measuring absorbance at 296 nm (α-tocopherol), 292 nm (BHA), and 288 nm (BHT) with a double beam UV-spectrophotometer (Hitachi model 150-20) (Hitachi High-Technologies, Schaumburg, IL). The degradation characteristics of the polymer films were evaluated by measuring polymer molecular weight by GPC, as described above.

Release of Antioxidants and Degradation of PLGA films in Whole Milk Powder and Buttermilk Powder

Preweighed PLGA (50:50) films (half circle with r = 2.9 cm) containing (i) 2% α-tocopherol, and (ii) a combination of 1% BHA and 1% BHT were completely immersed in 12-g dry whole milk and dry buttermilk. Film dimensions were chosen to compare with the surface area to milk powder ratio of commercial 272-g milk powder packaging (Our study, 2.20 cm^2 per g powder; Commercial, 2.76 cm^2 per g powder). Samples were contained in 40-ml clear glass bottles fitted with Teflon septa (Supelco, Bellefonte, PA). Control whole and buttermilk powders (without antioxidant-loaded PLGA films) were exposed to two levels (light-protected, light-exposed) of light. Light-protected samples were covered with aluminum foil to prevent any light exposure, whereas light-exposed samples were not covered with foil. All samples were placed horizontally under a row of fluorescent lights (1100-1300 lx) for light exposure of 12 h per day, every day, for four weeks at 25 ± 3°C (Van Aardt *et al.*, 2001). Three individual samples of each treatment were randomly chosen for volatile analysis on d 0, wk 1, 2, 3, and 4 of storage. Possible PLGA

degradation was measured by GPC. After four weeks of storage, NMR analysis of PLGA films was used to evaluate antioxidant content.

Volatile Analysis

Volatiles were extracted and concentrated from whole milk- and buttermilk powders using solid-phase microextraction (SPME), and separated and quantified by gas chromatography (GC) as described in Chapter IV and V (Van Aardt *et al.*, 2001). One exception was the absence of magnetic stirring in the powder samples as opposed to fluid milk samples in Chapter III. Pentanal, hexanal, and heptanal concentrations were determined in triplicate by using calibration curves as described in Chapter IV (McNair and Miller, 1997).

Lipid Extraction and HPLC Analysis of Antioxidants in Powders

After 12-g whole milk- and buttermilk powder samples were analyzed for volatile compounds, the same powders were reconstituted with 20 ml water, heated for 15 min in a 40^{o}C waterbath, and ultra-centrifuged (Beckman L2-65B, Beckman Instruments, Inc. Palo Alto, CA) for 30 min at 15,000 rpm under refrigeration (10^{o}C to 15^{o}C). The top fat layer was extracted three times with 2 ml, 1 ml, and 1 ml aliquots of methanol to dissolve all antioxidants (AOAC, 1997). Approximately 1.5 ml of each methanol extract was placed in a 2-ml clear glass crimp vial for subsequent HPLC analysis of antioxidants (Chapter IV).

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to test H_0 (H_0: $\mu_{control\text{-}no\ light} = \mu_{control\text{-}light} = \mu_{tocopherol} = \mu_{BHA/BHT}$), while Tukey's least significant difference (LSD) was used to compare means.) Hexanal, pentanal, and heptanal were quantified by calculating means (n = 3) and standard errors. Degrees of freedom (n-1) were 95 and treatments included (i) control – light, (ii) control-no light, (iii) 2% tocopherol, and (iv) 1% BHA and 1% BHT for seven time levels (d 0, wk 1, 2, 3, 4, 5, and 6). Compositional analysis of powders and antioxidant content of powders

also were determined by calculating means (n = 3) and standard errors. For all analysis, significant differences were defined at $P < 0.05$ (SAS, 1998).

RESULTS AND DISCUSSION

Antioxidant release, polymer degradation, and volatile compound concentration were evaluated when antioxidant-loaded PLGA (50:50) films were immersed in water, Miglyol 812®, whole milk powder, and buttermilk powder. Table 1 shows characterization results of polymer films. Number average molecular weight (M_n) is reported as an indication of polymer chain lengths before and after degradation. A smaller M_n indicates shorter polymer chains, while a larger M_n indicates long polymer chains. Although PLGA with 2% a-tocopherol shows a slightly lower M_n (34,500 $g.mol^{-1}$) than PLGA with 1% BHA and 1% BHT added (51,250 $g.mol^{-1}$), both these values fall outside the specification for molecular weight of the purchased polymer (M_n; 16,500 $g.mol^{-1}$ to 22,000 $g.mol^{-1}$). They are however similar enough to draw the conclusion that the addition of antioxidants at these levels does not greatly influence molecular weight. Molecular weight distribution, also called polydispersity index (M_w/M_n), is used as an indication of the breadth of molecular weight range in a polymer sample. A system having a range of molecular weight, such as partially degraded PLGA, is said to be polydisperse (Stevens, 1999). Polydispersity values for PLGA with 2% a-tocopherol (2.14) and PLGA with 1% BHA and 1% BHT (2.25) were similar. Preliminary studies also showed that the addition of antioxidants did not influence polydispersity values. NMR data shows the actual concentration of comonomer (lactide, glycolide) and antioxidant in antioxidant-loaded PLGA films. Both a-tocopherol- and BHA/BHT-loaded films consisted of approximately 58% lactide and 41% glycolide monomers. Films loaded with 2% a-tocopherol (wt %) actually contained 1.28% a-tocopherol (wt %), yielding a recovery of 66%. PLGA films loaded with 1% BHA and 1% BHT (wt %) actually contained 0.77% and 0.78% of the antioxidants, respectively, yielding a combined recovery after processing of 77%. It is not uncommon for antioxidants to be lost or degraded during processing. Wessling *et al.* (2000) found that two-thirds of a-tocopherol added to LDPE films was lost during processing.

Release of Antioxidants and Degradation of PLGA Films in Water and Miglyol 812®

Degradation of PLGA (50:50) films prepared in this study was measured by comparing decrease in molecular weight (M_n) and the subsequent increase in molecular weight distribution, while the

diffusion and release of α-tocopherol, BHA, and BHT in water and oil were measured with UV-spectrophotometry. Studies done at refrigerated storage showed no decrease in M_n (d 0 = 33,500 g.mol^{-1}, wk 8 = 33,890 g.mol^{-1}), no increase in molecular weight distribution of polymer films (M_w/M_n; d 0 = 2.09, wk 8 = 1.50), and no release of antioxidants into water or oil medium over eight weeks of storage. Much research has been done on the degradation of PLGA in buffered solutions at body temperature (37^{o}C) (Gümüsderelioglu and Deniz, 2000). However, little information exists regarding hydrolysis of PLGA at refrigeration temperature (4^{o}C). Conn *et al.* (1995) determined the rate of hydrolysis for L-lactide in 8% ethanol at 21^{o}C (k = 0.23 per h). It is well understood that temperature and pH plays a very important role on the rate of migration and hydrolysis.

At room temperature, antioxidant-loaded PLGA films stored in oil medium did not show a decrease in molecular weight or an increase in molecular weight distribution. However, PLGA loaded with BHA and BHT showed a decrease in molecular weight (51,250 g.mol^{-1} to 764 g.mol^{-1}) and increase in molecular weight distribution (2.09 to 4.29) due to the presence of low molecular weight fractions over eight weeks of storage (Table 2). The same trend was seen when PLGA films loaded with α-tocopherol were stored in water medium. Molecular weight decreased from 34,500 g.mol^{-1} to 4760 g.mol^{-1}. This emphasizes the role that moisture plays in the hydrolytic degradation of PLGA. It is interesting to see that PLGA films loaded with a-tocopherol and stored in water did not show an increase in polydispersity as did BHA/BHT-loaded PLGA films stored in water. Preliminary studies done on PLA and PLGA (85:15) films also did not show mass loss during eight weeks of storage at 4^{o}C, indicating that both temperature and glycolide content plays an important role in degradation rate. Gümüsderelioglu and Deniz (2000) measured mass loss of PLGA (70:30) and PLGA (90:10) at 37^{o}C in phosphate buffered saline, and found that PLGA (70:30) degraded faster than PLGA (90:10). Molecular weight of PLGA (70:30) decreased by 40% after 70 days, while the same decrease was observed in PLGA (90:10) after 110 days. In our study, refrigeration temperature was originally chosen to simulate milk storage conditions. Since no release of antioxidants were achieved at this temperature from antioxidant-loaded PLA, -PLGA (85:15) and -PLGA (50:50), storage temperature was increased to 25^{o}C. At this storage temperature, the release of antioxidants from polymer films could help stabilize milkfat of ultra high temperature processed dairy products.

UV-spectrophotometry showed an increase in BHT content in water when stored at room temperature in the presence of BHA/BHT-loaded PLGA (50:50) films (Figure 1). After eight weeks of storage BHT increased to 192.0 $mg.kg^{-1}$ when stored in water. Since BHT is insoluble in water, it is more likely to be contained in the hydrophobic PLGA. Therefore, slow release of BHT was expected. Due to the degradation process of PLGA through hydrolysis of ester groups, polymer molecular weight decreased (Table 2) and molecular weight distribution increased. This resulted in permeability of BHT within the polymer matrix and subsequent release of BHT into the water matrix over time. However, no BHA or tocopherol was observed in water or oil media over eight weeks of storage at 4°C or 25°C. Preliminary studies showed that no BHA, BHT, or α-tocopherol was observed in water or oil stored with antioxidant-loaded PLA or PLGA (90:10) films at refrigeration temperature. Gümüsderelioglu and Deniz (2000) studied the effects of homopolymer (PLA) molecular weight, comonomer ratio (glycolide), film thickness, and drug loading on mitomycin-C release in phosphate buffered saline at 37°C. They found slow release of hydrophilic mitomycin-C due to diffusion until significant PLA mass loss was observed after 90 days of storage, after which release rate increased due to polymer degradation.

Scanning electron microscopy could potentially be used to study the surface characteristics of the antioxidant-loaded PLGA films, as well as the mechanism of diffusion (bulk vs. surface) of antioxidants. Unfortunately, SEM analysis of PLA films showed extensive electron beam damage (Figure 2). SEM uses electron beams to view topography and to chemically analyze samples. Because electrons have much shorter wavelengths than visible light, they produce images of samples with much higher resolution than optical microscopes. Unlike light microscopes, however, electron microscopes can damage sample surfaces in the process of analyzing them. Further evaluation of this technique on PLGA films is necessary.

Release of Antioxidants and Degradation of PLGA Films in Dry Whole Milk and Dry Buttermilk

The effect of antioxidant-loaded PLGA (50:50) films on the stability of dry whole milk and dry buttermilk, when exposed to light, was evaluated. Serving as control, dry whole milk and dry buttermilk samples were stored without antioxidant-loaded PLGA films in the presence and in the absence of light. Table 3 provides a compositional characterization of each powder, documenting the high lipid content of whole milk powder and the high phospholipids content of the buttermilk powders.

Hexanal, pentanal, and heptanal content was measured as an indicator of lipid oxidation. Since lipid autoxidation increase with increasing water activity of a food, it is expected that dry milk products is prone to oxidation with a water activity between 0.6 and 0.8. Figure 3, 4, and 5 shows the increase in hexanal -, pentanal -, and heptanal content in dry whole milk and dry buttermilk, respectively. When comparing volatile content in dry whole milk and dry buttermilk, the former showed substantially higher levels, which can be attributed to the fact that whole milk powder has a substantially higher fat content (24.5%) than buttermilk powder (2.4%) (Table 3). Two-way ANOVA showed significant interactions between time (wk) and treatment (antioxidant-loaded PLGA films), which can be seen visually by observing slope changes in Figures 3 to 5. Although hexanal content in light-exposed control and light-exposed antioxidant-treated samples increased over four weeks of storage, one-way ANOVA showed no significant differences between treated and control powders for each week of storage. Pentanal content also increased in light-exposed control and light-exposed treated powders, but was significantly reduced in buttermilk powder in contact with BHA/BHT-loaded PLGA throughout the first three weeks of storage. In whole milk powder, a-tocopherol-loaded PLGA reduced pentanal content for week two and three of storage. The same trend was observed when heptanal content was determined. PLGA films loaded with a-tocopherol decreased heptanal content in whole milk powder for week one and week four of storage, while BHA/BHT-loaded PLGA films decreased heptanal content in buttermilk powders at week four.

These limited effects that was observed suggest that no significant amount of antioxidant diffused from the antioxidant-loaded PLGA films, or that the bound moisture of the powders was too low to sufficiently degrade PLGA films to the point where antioxidants are released (Table 3). Since antioxidants used in this study had large phenolic groups, it might be that they were trapped in the polymer matrix. However, NMR analysis showed a decrease in the antioxidant content of PLGA films over four weeks of storage (Table 4), and a subsequent increase in antioxidant content of whole milk powder (Figure 6). α-Tocopherol, BHA, and BHT migrated into whole mik powder to a maximum of 21.9 $mg.kg^{-1}$, 60.0 $mg.kg^{-1}$, and 191.9 $mg.kg^{-1}$ milkfat over 4 weeks of storage, respectively. In buttermilk powder, much lower migration maxima was observed. The migration of antioxidants into powders was most likely through surface contact with milkfat and less likely through volatilization as is described for stability of oat cereal by Wessling et al. (2000).

α-Tocopherol content of PLGA films in contact with whole milk and buttermilk powder decreased by 90% and 60%, respectively, while BHA and BHT content of PLGA films decreased by 92% and 35%, when in contact with whole milk, and buttermilk powders. Therefore, although limited decrease was observed in hexanal, pentanal, and heptanal content of powders, the potential stability of milkfat achieved from the migration of antioxidants from polymeric food packaging cannot be excluded. Figure 7 and 8 shows examples of NMR spectra of α-tocopherol-loaded PLGA and BHA/BHT-loaded PLGA films.

Some studies have evaluated the migration of BHA and BHT from food packaging into dry foods such as cereal and oatmeal. Wessling *et al.* (2000) stored oatmeal in antioxidant-loaded LDPE and PP pouches and observed that BHT was rapidly lost from pouches within days of storage, while pouches loaded with 0.4% α-tocopherol lost its antioxidant content completely after six weeks at 20^{o}C. Antioxidant content of oat cereal samples was not determined therefore no conclusion relating the loss of antioxidants from the pouches to increased antioxidant levels in the food was made.

In our study GPC analysis showed no increase in molecular weight distribution of a-tocopherol-loaded PLGA films, whether stored in contact with dry whole milk or dry buttermilk (M_w/M_n; d

0 = 2.14; wk 4 = 2.24), and BHA/BHT-loaded films stored in contact with dry buttermilk (M_w/M_n; d 0 = 2.25; wk 4 = 1.14). These results indicate no hydrolytic degradation of the polymer during that time. Since dry whole milk and dry buttermilk contain only 3.01% and 4.60% bound moisture, respectively, one might conclude that PLGA (50:50) needs higher moisture content for degradation to occur. However, it is interesting to note that BHA/BHT-loaded PLGA films stored in contact with dry whole milk showed a significant increase in molecular weight distribution from 2.25 (d 0) to 4.24 (w 4).

Since BHA and BHT have a higher volatility (lower vapor pressure) when compared to a-tocopherol, it was expected that the former two antioxidants would be more suitable for dry food applications. However, in this study a-tocopherol showed limited stability of whole milk powder. Wessling *et al.* (2000) found that BHT was rapidly lost from LDPE films stored in contact with oatmeal at 20^{o}C, 30^{o}C, and 40^{o}C as compared to α-tocopherol that was retained in the polymer for longer periods.

CONCLUSION

The implementation of antioxidant-loaded PLGA (50:50), a biodegradable polymer, in stability of milkfat was evaluated in this study. Migration of antioxidants from antioxidant-loaded films into dry milk products with water content of < 4.60% was not driven by hydrolytic degradation of PLGA (50:50), but instead, by diffusion. However, in water medium, PLGA (50:50) did show hydrolytic degradation and release of BHT into water. Potential use of biodegradable polymers as unique active packaging options for sustained delivery of antioxidants could be a benefit to the dairy industry by limiting oxidation of some dairy products.

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TABLES

Table 1. Gel permeation chromatographic (GPC) and nuclear magnetic resonance spectroscopic (NMR) characterization of antioxidant-loaded PLGA (50:50) films.

Analysis	PLGA with 2% Tocopherol	PLGA with 1% BHA, 1% BHT
GPC		
M_n[1] (g.mol^{-1})	34500	51250
M_w/M_n[2]	2.14	2.25
NMR		
Antioxidant content[3] (wt %)	1.28	0.77; 0.78
Lactide content (wt %)	57.99	57.79
Glycolide content (wt%)	40.73	40.66

[1] M_n: Number average molecular weight

[2] M_w/M_n: Molecular weight distribution or polydispersity index

Table 2. Mass loss of antioxidant-loaded PLGA (50:50) in water and oil media stored at 25^{o}C for eight weeks.

PLGA film ; Medium	M_n[1] (g.mol^{-1}) d 0	M_n[1] (g.mol^{-1}) wk 8	M_w/M_n[2] d 0	M_w/M_n[2] wk 8
2% Tocopherol ; Water	34500	4760	3.15	1.07
2% Tocopherol ; Miglyol 812®	34500	33890	3.15	1.47
1% BHA, 1% BHT ; Water	51250	764	2.09	4.29
1% BHA, 1% BHT ; Miglyol 812®	51250	39560	2.09	1.49

[1] M_n: Number average molecular weight measured by GPC

[2] M_w/M_n: Molecular weight distribution or polydispersity index measured by GPC

[3] Antioxidant content (spiked by weight) and recovered (by weight) measured by NMR

Table 3. Compositional characterization of dry whole milk and dry buttermilk.

Component	Whole milk powder	Buttermilk powder
Moisture (%)	3.01 ± 0.04	4.60 ± 0.01
Total solids (%)	97.0 ± 0.04	95.4 ± 0.01
Fat (%)		
Bligh and Dyer method	20.0 ± 1.22	2.35 ± 0.30
Folch method	24.5 ± 1.21	
Phospholipid (% of fat)		
Bligh and Dyer method	1.97 ± 0.25	22.1 ± 2.80
Folch method	2.07 ± 0.20	

Table 4. Antioxidant content of PLGA films before and after light-exposed (1100-1300 lx) storage in whole milk and buttermilk powders, as determined by NMR analysis.

Treatment	α-Tocopherol content (%)	BHA content (%)	BHA content (%)
WM[1], Tocopherol-loaded PLGA film			
d 0	1.28		
wk 4	0.12		
WM[1], BHA/BHT-loaded PLGA film			
d 0		0.77	0.78
wk 4		0.05	0.07
BM[2], Tocopherol-loaded PLGA film			
d 0	1.28		
wk 4	0.52		
BM[2], BHA/BHT-loaded PLGA film			
d 0		0.77	0.78
wk 4		0.47	054

[1] Whole milk powder

[2] Buttermilk powder

FIGURES

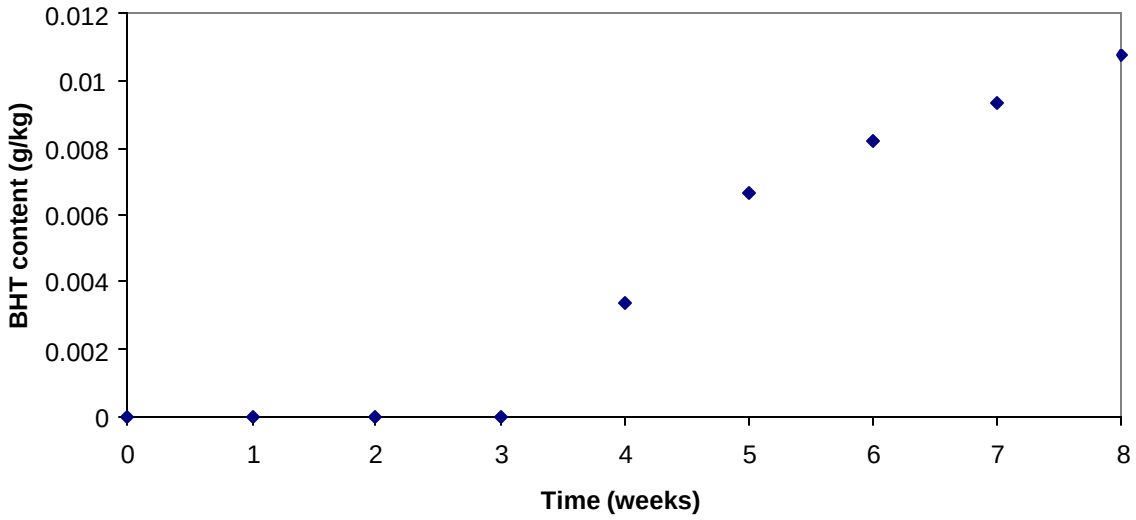


Figure 1. BHT concentration in water, when stored in contact with BHA/BHT-loaded PLGA (50:50) film at 25^{o}C for eight weeks.

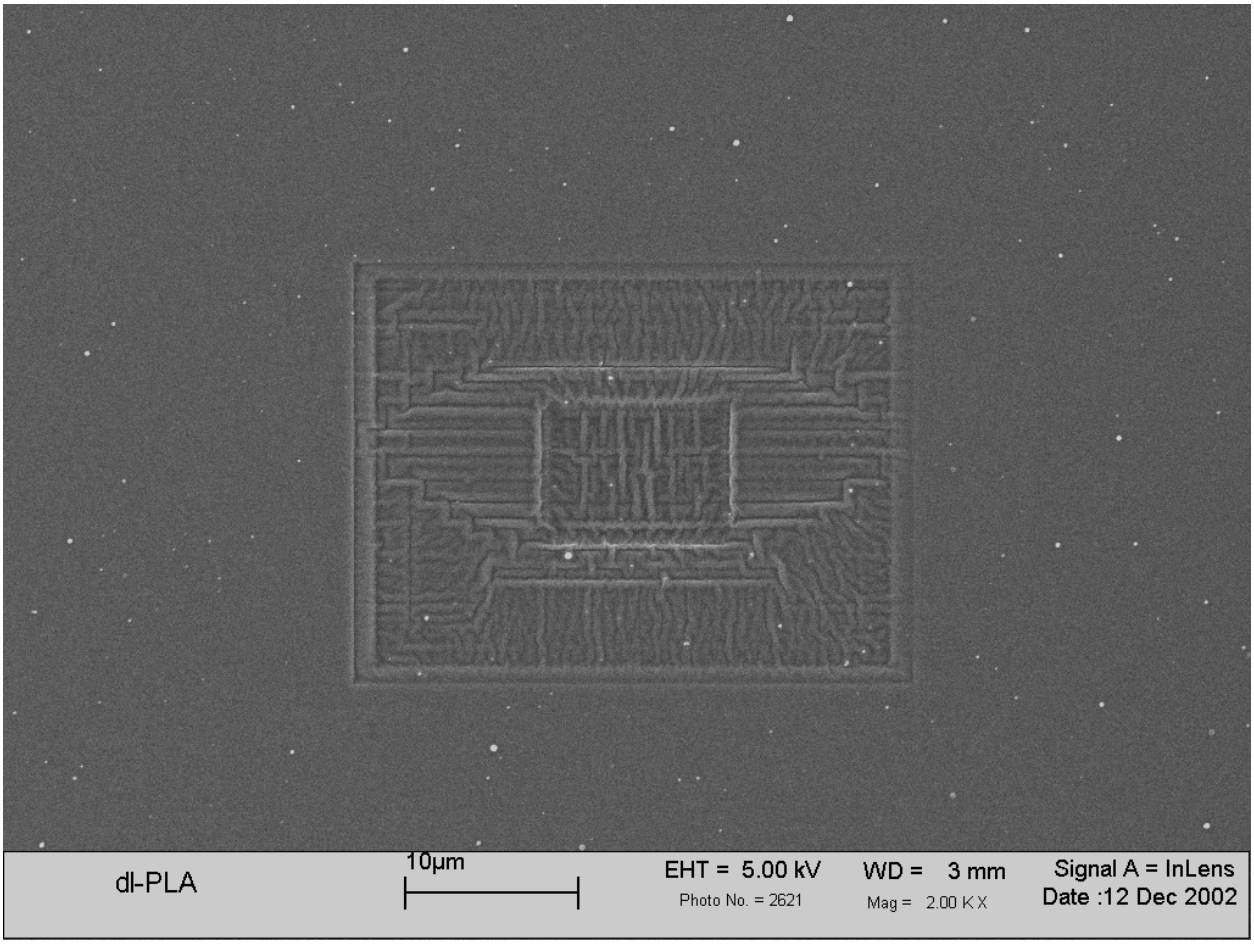
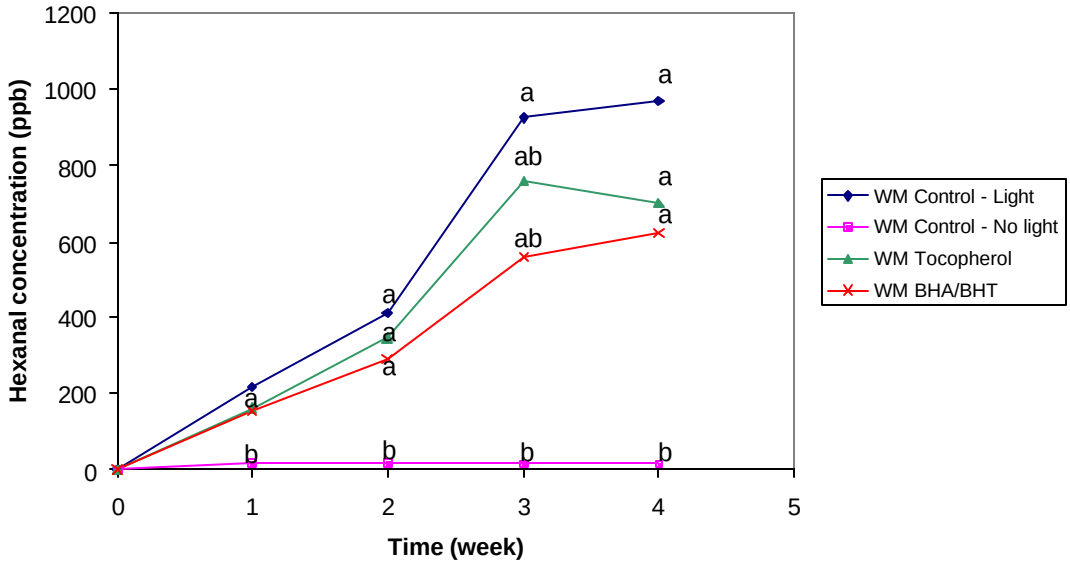
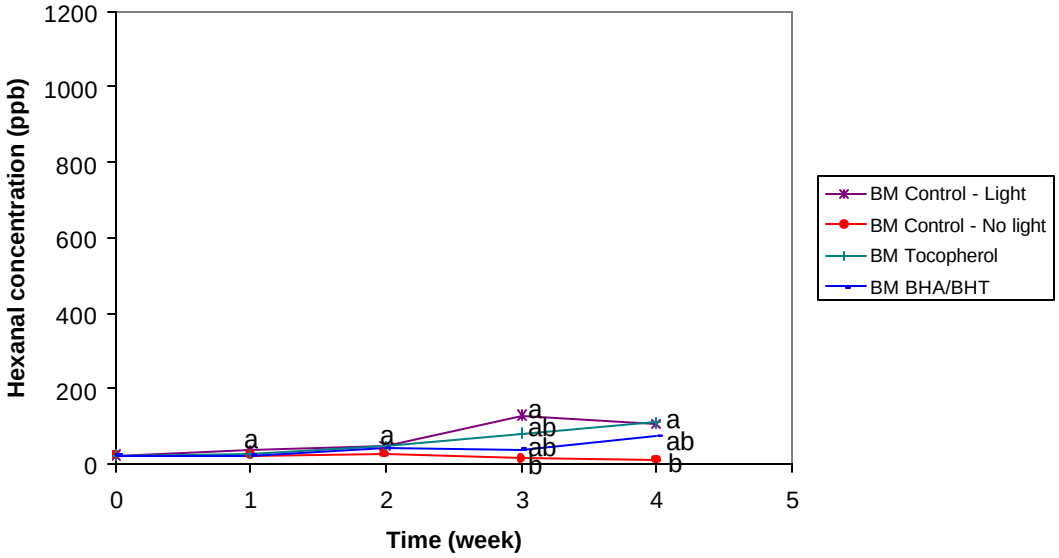


Figure 2. Scanning electron microscopy of dl-PLA film showed electron beam damage.

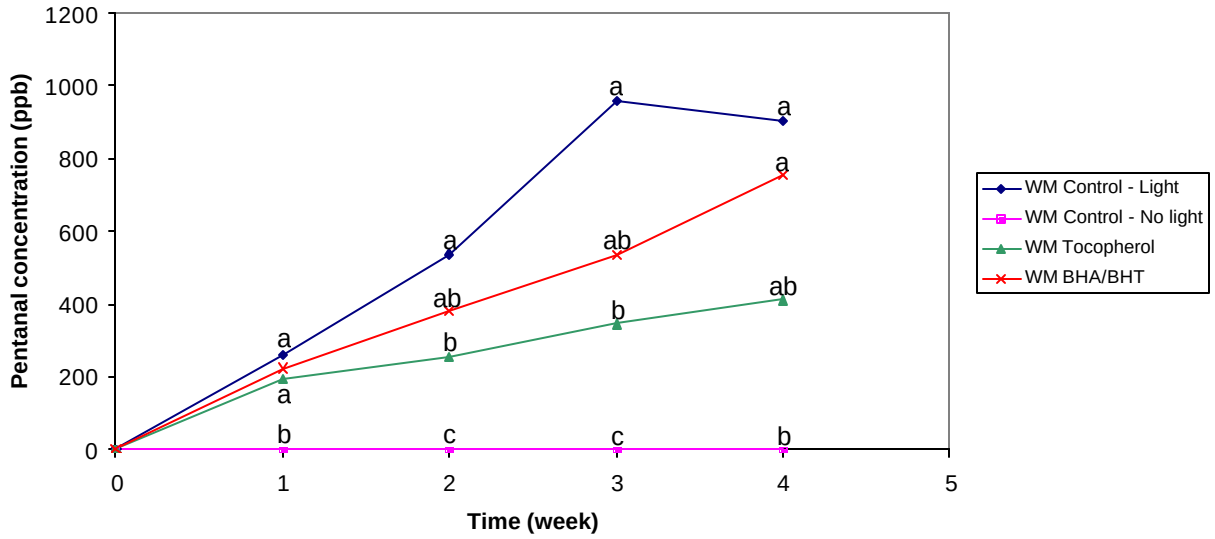


(a)

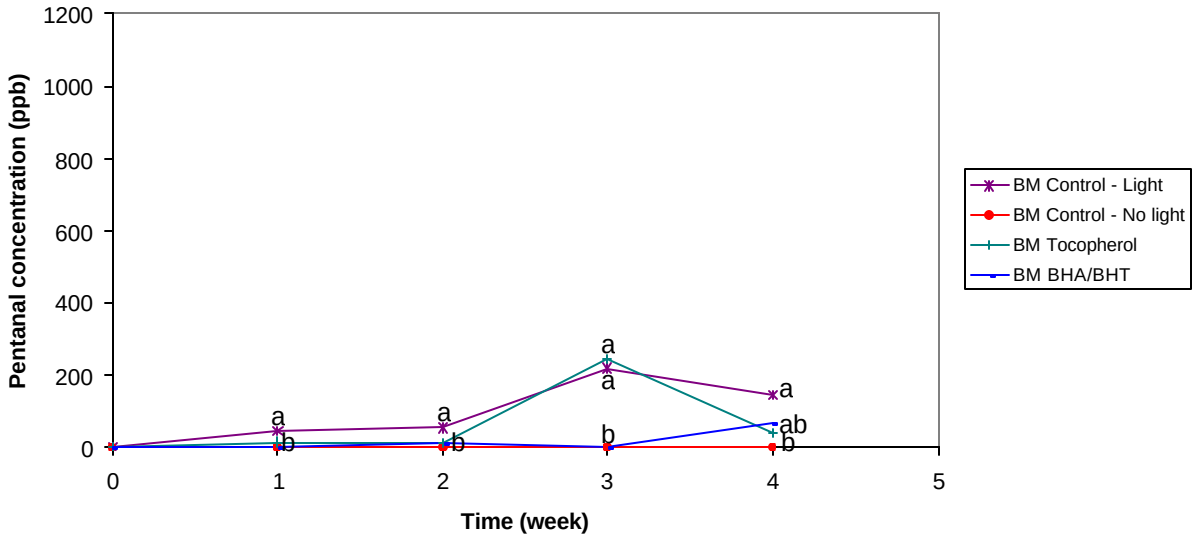


(b)

Figure 3. Hexanal concentration (ppb = mg.kg^{-1}) of (a) dry whole milk and (b) dry buttermilk after eight weeks of light-exposed (1100-1300 lx) storage at 25°C with antioxidant-loaded PLGA (50:50) films. $^{a-c}$ Means (n=3) in the same week with different superscripts are significantly different ($P < 0.05$).

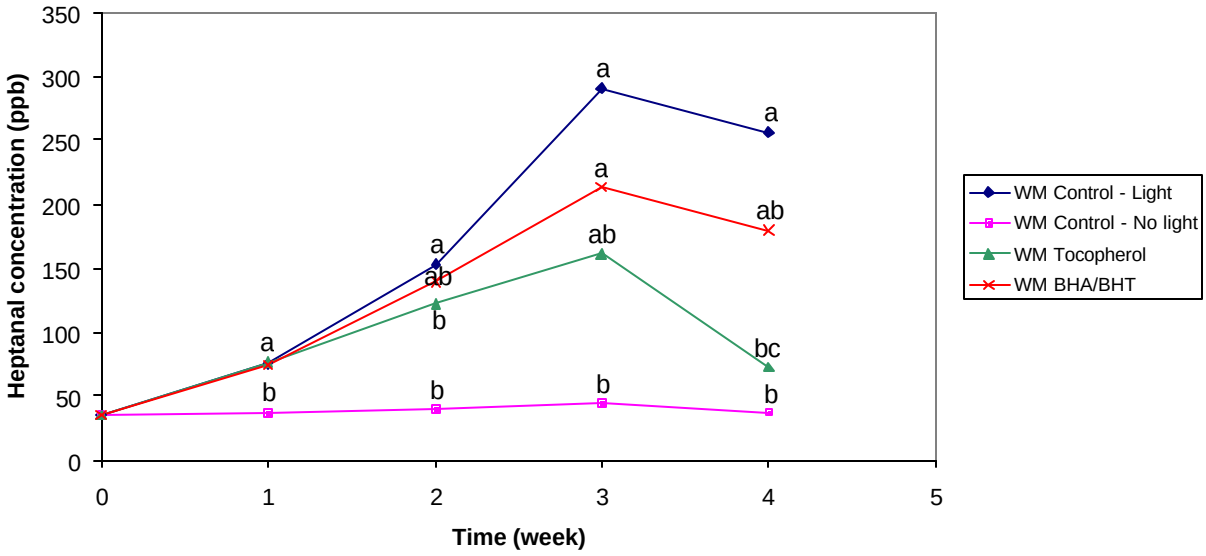


(a)

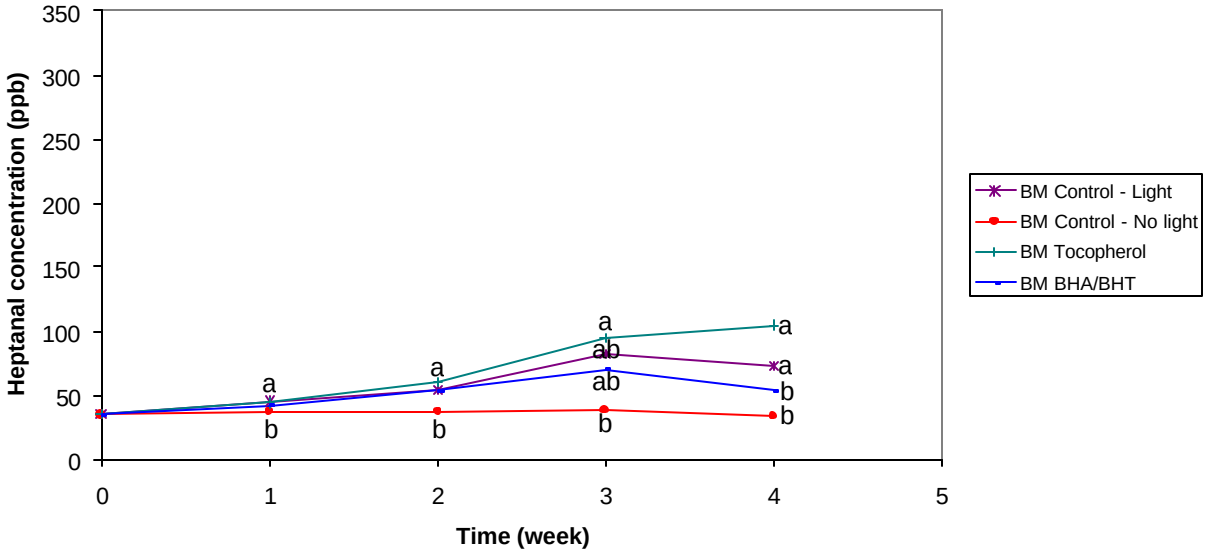


(b)

Figure 4. Pentanal concentration (ppb = mg.kg^{-1}) of (a) dry whole milk and (b) dry buttermilk after eight weeks of light-exposed (1100-1300 lx) storage at 25°C with antioxidant-loaded PLGA (50:50) films. $^{a-c}$ Means (n=3) in the same week with different superscripts are significantly different ($P < 0.05$).

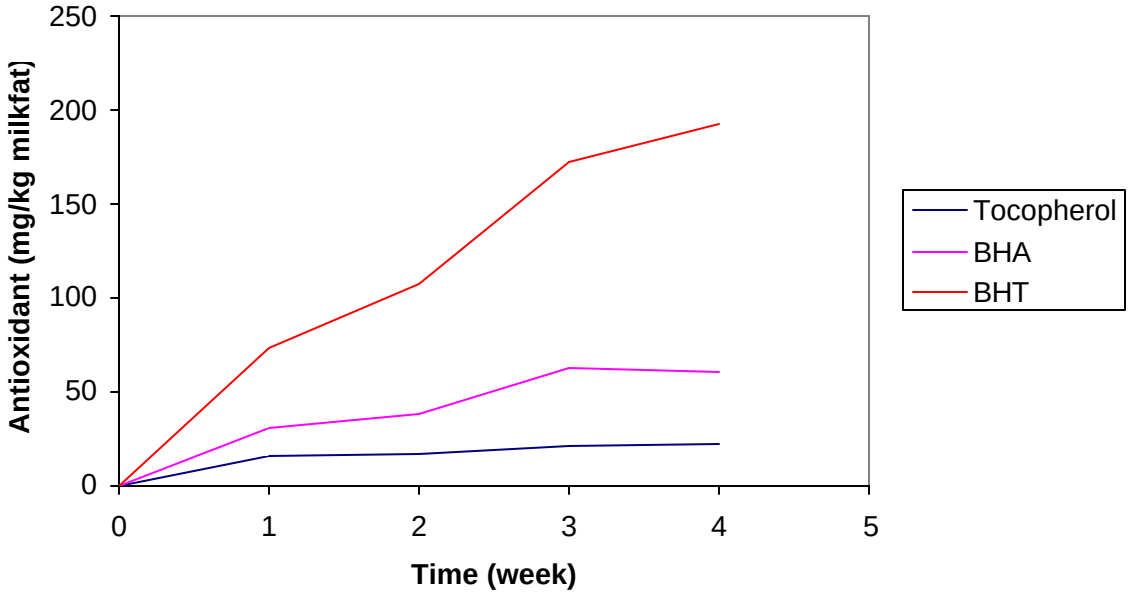


(a)

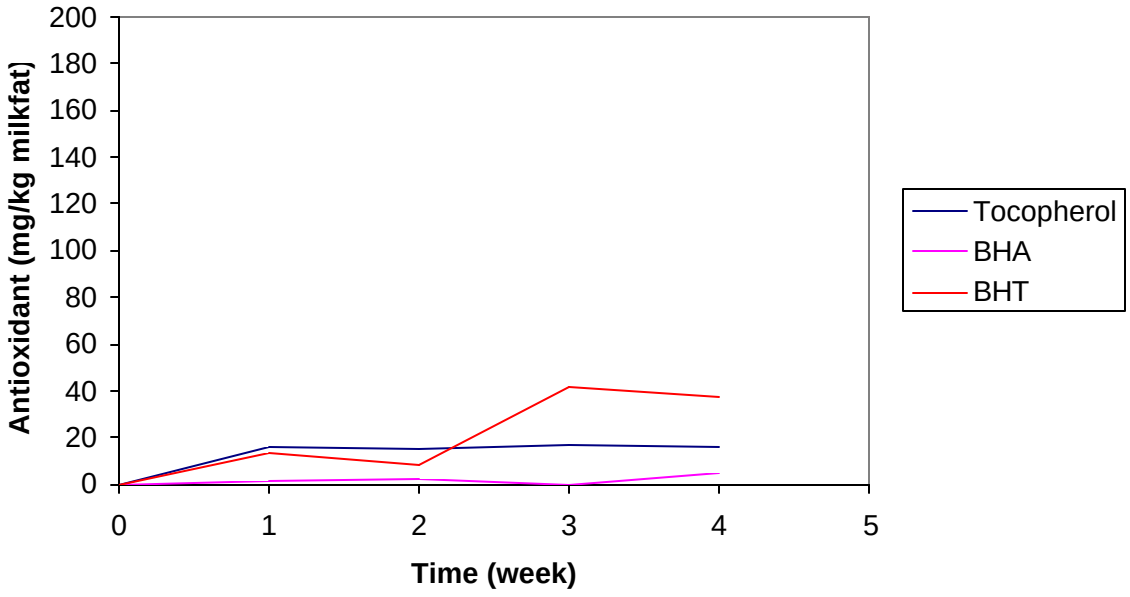


(b)

Figure 5. Heptanal concentration (ppb = mg.kg^{-1}) of (a) dry whole milk and (b) dry buttermilk after eight weeks of light-exposed (1100-1300 lx) storage at 25°C with antioxidant-loaded PLGA (50:50) films. $^{a-c}$ Means (n=3) in the same week with different superscripts are significantly different ($P < 0.05$).



(a)



(b)

Figure 6. Antioxidant content of (a) dry whole milk and (b) dry buttermilk after four weeks of light-exposed (1100-1300 lx) storage with antioxidant-loaded PLGA (50:50) films.

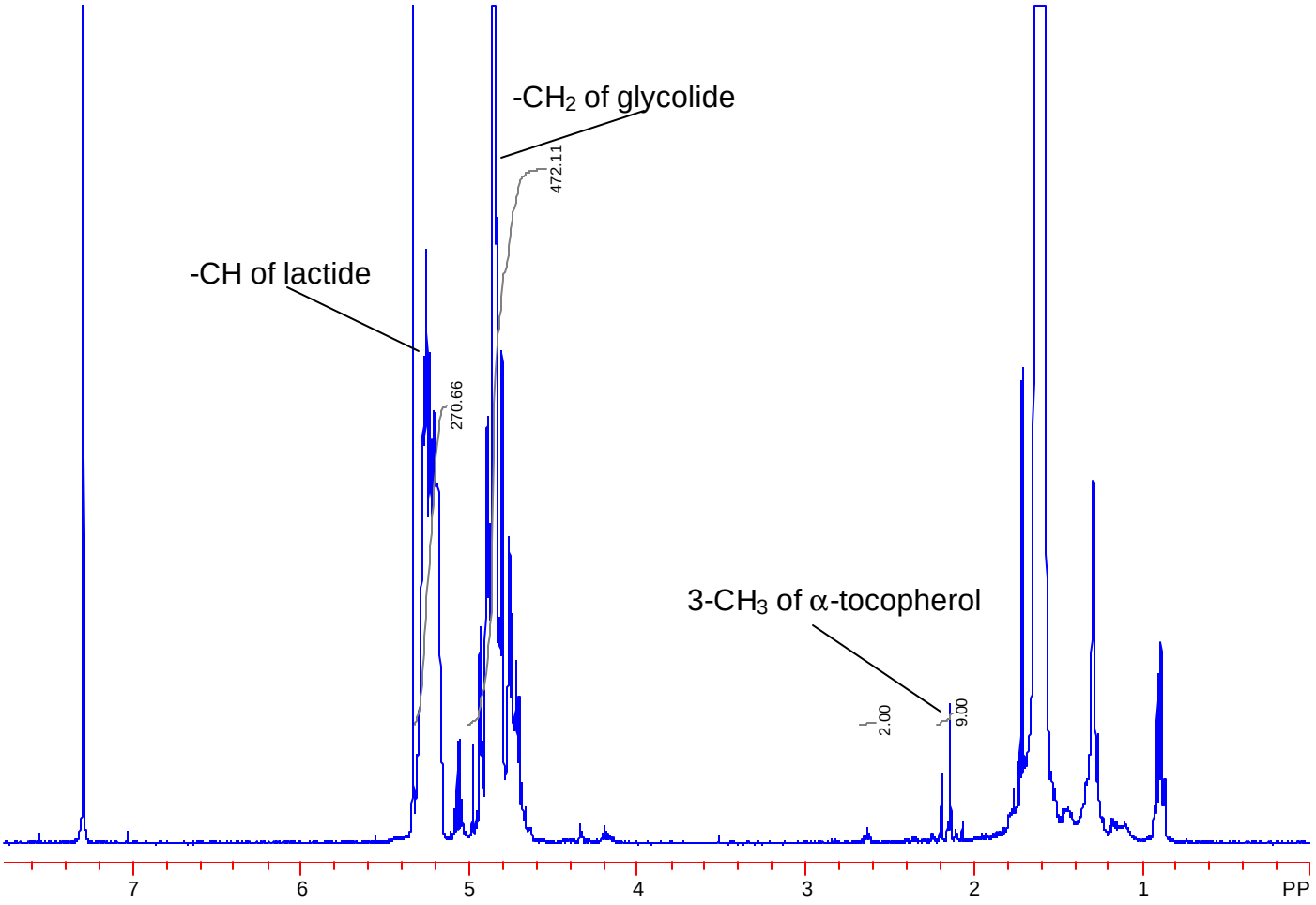


Figure 7. Nuclear magnetic resonance spectroscopy (NMR) of PLGA (50:50) films loaded with 2% a **-tocopherol.**

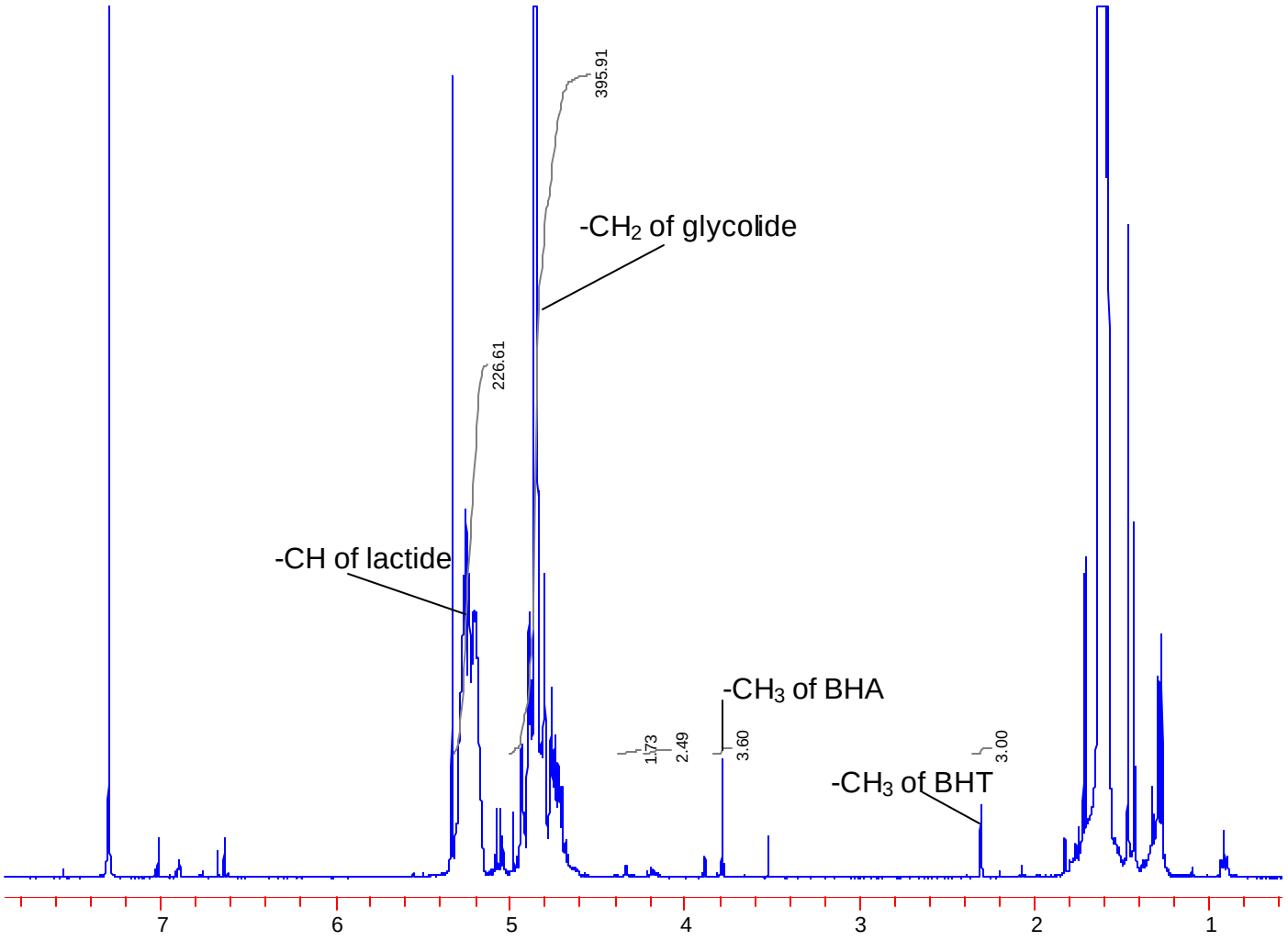


Figure 8. Nuclear magnetic resonance spectroscopy (NMR) of PLGA (50:50) films loaded with 1% BHA and 1% BHT.

APPENDICES

APPENDIX A

Human Subjects Forms for Sensory Evaluation

Protocol for Projects of Sensory Evaluation

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

Check all that apply:

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

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

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

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

5. Describe the storage protocols for the product that are necessary to maintain the product in safe condition.
   Milk is stored at 4^{o}C until sensory analysis. Milk is analyzed within 48 hours of processing.

6. If microbiological cultures are a part of the food processing or preparation procedure, describe what cultures will be used, if they will be active on consumption, and give evidence that these cultures are known to be safe for human consumption.
   No microbiological cultures used.

7. Allergies
   - __NO__ Are any ingredients to be used potentially allergenic as consumed or by topical application? If yes, describe. Have panelists been made aware of these ingredients?

When you have completed this form, indicate the risk level to the panelists of this project. Complete the appropriate form; for "not at risk", the Certificate of Exemption form; for "at minimal risk", the Request for Approval form.

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

Title of Project: Effect of Light Exposure on Milk Flavor when Fortified with α-Tocopherol and Ascorbic Acid

Principal Investigator: Marleen van Aardt

I. THE PURPOSE OF THIS PROJECT

You are invited to participate on a sensory evaluation panel about milk. Our objective is to determine if natural antioxidants will control oxidation flavor in light exposed milk.

II. PROCEDURES

There will be one session involving about 10 minutes. You will be presented with 9 samples. As a panelist, it is critical to the project that you complete your session. Should you find a sample unpalatable or offensive, you may choose to spit it out and continue to other samples.

Certain individuals are sensitive to some foods such as milk, eggs, wheat gluten, strawberries, chocolate, artificial sweeteners, etc. If you are aware of any food or drug allergies, list them in the following space.

III. BENEFITS/RISKS OF THE PROJECT

Your participation in the project will provide the following information that may be helpful: Information about antioxidant effectivity in controlling oxidation flavor in milk. You may receive the results or summary of the panel when the project is completed. Some risk may be involved if you have an unknown food allergy.

IV. EXTENT OF AN0NYMITY AND CONFIDENTIALITY

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

V. COMPENSATION

No monetary compensation will be given, but a sweet treat will be offered at the end of the session.

VI. FREEDOM TO WITHDRAW

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

VII. APPROVAL OF RESEARCH

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

VIII. SUBJECT'S RESPONSIBILITIES

I know of no reason I cannot participate in this study, which will require tasting 9 milk samples in one session.

_____

Signature/Date

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

Address: _____ Phone: _____

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

IX. SUBJECT'S PERMISSION

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

I know of no reason I cannot participate in this study, which will require: (list sessions to be attended or other requirements.)

_____

Signature/Date

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

Marleen van Aardt (Investigator) Phone (540) 231-6806

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

Dr Tom Hurd (Chair, IRB) Phone (540) 231-6077

APPENDIX B

EFFECT OF LIGHT EXPOSURE ON MILK FLAVOR WHEN FORTIFIED WITH a -TOCOPHEROL AND ASCORBIC ACID

Judge # _____

Instructions

Please complete the human subjects consent form before starting sensory analysis. Rinse your mouth with water before beginning. Expectorate the water into the container provided.

You will be provided with 3 three-sample sets. Two of the three samples in a set are the same and one is different. Please taste the samples in the order presented, from left to right.

Circle the number of the sample in each set of three that tastes "different".

Rinse your mouth with water between samples and expectorate all samples and the water.

Set 1 _____ _____ _____ Description of taste _____

Set 2 _____ _____ _____ Description of taste _____

Set 3 _____ _____ _____ Description of taste _____

Thank you very much for your participation in this study.

APPENDIX C

Worksheet for Triangle Test 1
Similarity Testing for Antioxidant Flavor in Lowfat Milk

Date: April 4, 2000 **Test code:** Marleen

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

Samples: Control - Lowfat milk (2 % milkfat)
Treatment 1: 0.05 % α-tocopherol in lowfat milk
Treatment 2: 0.05 % ascorbic acid in lowfat milk
Treatment 3: 0.025 % α-tocopherol and 0.025 % ascorbic acid in lowfat milk

Type of test: Triangle test for similarity

Sample Identification		**Codes used for: Sets with 2 A's**		**Sets with 2 B's**	
Set 1	A: Control milk	381	294	138	
	B: Treated milk 1	516		206	649
Set 2	A: Control milk	413	894	246	
	B: Treated milk 2	587		365	751
Set 3	A: Control milk	326	149	802	
	B: Treated milk 3	211		477	945

Code serving containers as follows:

Subject #	**Set 1**			**Set 2**			**Set 3**		
1, 7, 13, 19,	381	516	294	365	246	751	149	326	211
2, 8, 14, 20,	294	381	516	365	751	246	945	477	802
3, 9, 15, 21,	516	381	294	246	751	365	326	211	149
4, 10, 16, 22,	206	138	649	413	587	894	802	477	945
5, 11, 17, 23,	649	206	138	894	413	587	477	802	945
6, 12, 18, 24,	138	206	649	587	413	894	211	326	149

Order of presentation is randomized in equal amounts between ABA, AAB, BAA, BAB, BBA, and ABB.

- Select samples with three-digit randomized codes for A and B, and place on tray from left to right; place correct scorecard with samples
- Write selected three-digit codes on panelist's scorecard before serving samples
- Receive filled-in scorecard and note whether reply was correct (C) or incorrect (I)

APPENDIX D: Bacterial Counts[1] (CFU.ml^{-1}) for extended shelf-life (ESL) milk treated with single- and weekly additions of antioxidants

	Standard Plate Count (log CFU.ml^{-1})						
Treatment[2]	Day 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Replication 1							
Control – light	0	0	0	0	0	0	5
Control – no light	0	0	0	5	0	0	0
Control (UHT) – light	0	0	0	0	0	0	0
Control (UHT) – no light	0	0	5	0	1	0	0
BHA/BHT – single addition	0	0	0	0	30	33	0
Toc/Asc – single addition	0	0	0	5	10	0	0
BHA/BHT – weekly addition	0	0	11	0	0	0	18
Toc/Asc – weekly addition	0	0	7	0	1	15	3
Replication 2							
Control – light	0	0	0	0	0	0	1
Control – no light	0	0	5	0	0	0	0
Control (UHT) – light	0	0	0	0	0	0	0
Control (UHT) – no light	0	0	0	0	0	0	0
BHA/BHT – single addition	0	0	0	0	0	30	0
Toc/Asc – single addition	0	0	0	0	0	0	0
BHA/BHT – weekly addition	0	0	6	0	10	0	8
Toc/Asc – weekly addition	0	0	2	0	1	0	4
Replication 3							
Control – light	0	0	0	0	0	0	0
Control – no light	0	0	0	0	0	0	2
Control (UHT) – light	0	0	0	0	0	0	0
Control (UHT) – no light	0	0	0	0	0	0	0
BHA/BHT – single addition	0	0	0	0	0	0	0
Toc/Asc – single addition	0	0	0	0	0	0	0
BHA/BHT – weekly addition	0	0	4	0	2	27	0
Toc/Asc – weekly addition	0	0	1	0	11	0	26

[1] BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; Toc, a-tocopherol; Asc, ascorbyl palmitate

[2] Coliform counts were 0 for all weeks of storage.

Appendix E: Odor-active compounds detected by GC-O of control and antioxidant[3]-treated milk when exposed to light (1100-1300 lx) for 12 h per day for 6 weeks.

CONTROL, LIGHT

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290	+	+				+	
3	0321	+	+				+	
4 (2-butanone)	0347		+	+			+	
5	0408						++	+
6 (pentanal)	0440				++		++	
6.4 (dimethyl disulfide)	0503	+					+	
7 (hexanal)	0567			++	++	++	+++	+
8 (1-hexanol)	0639	++			++		+	+
9 (2-heptanone)	0687							
10 (n-heptanal)	0702	++	++	+	++	++	+++	+++
11 (1-heptanol)	0792						++	
11.5 (1-octene-3-ol)	0805		++		++	+		+
12	0836			+			+	
12.5 (1-octanol)	0907							+
13 (nonanal)	0962				+		+	+

CONTROL, NO LIGHT

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290					++		
3	0321						++	
4 (2-butanone)	0347			+	+			
5	0408					+		+
6 (pentanal)	0440						+	+
6.4 (dimethyl disulfide)	0503				+			
6.5 (pentanol)	0529							+
7 (hexanal)	0567				+			
9 (2-heptanone)	0687							+
10 (n-heptanal)	0702		+				+	

CONTROL UHT, LIGHT

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290			+		++	+	
3	0321		++		+		++	+
4 (2-butanone)	0347			+	+		++	
5	0408		+		+		+	
6 (pentanal)	0440		+	+	+	+	+	+
6.4 (dimethyl disulfide)	0503			+				++
7 (hexanal)	0567		++	++	++		+++	+++
8 (1-hexanol)	0639		+					
9 (2-heptanone)	0687				+			
10 (n-heptanal)	0702		+	+++	+++		+++	+
11.5 (1-octene-3-ol)	0805		++	++	++		+++	++
12.5	0907						+	
13 (nonanal)	0962		+		+	++		

CONTROL UHT, NO LIGHT

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290		+				+	
3	0321			+			++	
4 (2-butanone)	0347			+		+		
5	0408				+		+	
6 (pentanal)	0440						+	
6.4 (dimethyl disulfide)	0503				+		+	
7 (hexanal)	0567					+	++	+
9 (2-heptanone)	0687		++		+		+	
10 (n-heptanal)	0702		++		+++	++	+++	
11.5 (1-octene-3-ol)	0805		++	+				+
12	0836		+				++	
12.5	0907		++				+	
13 (nonanal)	0962					+	++	+

TOCOPHEROL AND ASCORBYL PALMITATE, SINGLE ADDITION

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290						++	
3	0321		+	++	++		++	
4 (2-butanone)	0347		+	++		+		
5	0408		+	+				
6 (pentanal)	0440						++	+
6.4 (dimethyl disulfide)	0503						++	
7 (hexanal)	0567		+	++	+		+++	+++
8 (1-hexanol)	0639			+			++	+
9 (2-heptanone)	0687						++	+
10 (n-heptanal)	0702	+	++	++	++	+	+++	++
11 (1-heptanol)	0792							
11.5 (1-octene-3-ol)	0805		++	++	+		+++	
12	0836						++	
12.5	0907						+++	
13 (nonanal)	0962		+		+		+++	

TOCOPHEROL AND ASCORBYL PALMITATE, BEFORE WEEKLY ADDITION

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
3	0321		+	+			+	
4 (2-butanone)	0347		+	+			+	
5	0408		+					++
6.4 (dimethyl disulfide)	0503			+		+		++
7 (hexanal)	0567		+		+	+	+++	++
9 (2-heptanone)	0687						+	
10 (n-heptanal)	0702		++	++	++		+++	+++
11.5 (1-octene-3-ol)	0805		++		++		++	+
12	0836				+			
12.5	0907					+		
13 (nonanal)	0962		+	+		+		

TOCOPHEROL AND ASCORBYL PALMITATE, AFTER WEEKLY ADDITION

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290						+	
3	0321		+	+			++	
4 (2-butanone)	0347			+		+	++	
5	0408		+					
6.4 (dimethyl disulfide)	0503				+	+	+	
7 (hexanal)	0567		++		+	+	+++	
8 (1-hexanol)	0639		+++		+	+	+	
9 (2-heptanone)	0687						++	
10 (n-heptanal)	0702		+++	++			+++	
11 (1-heptanol)	0792		+				+++	
11.5 (1-octene-3-ol)	0805		++	++			+++	
12	0836		+	+			++	
12.5	0907				+		+	
13 (nonanal)	0962				+			

BHA AND BHT, SINGLE ADDITION

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290		+				++	
3	0321				+		++	
4 (2-butanone)	0347		+	+			+	
5	0408	++						++
6 (pentanal)	0440	+	+					+
6.4 (dimethyl disulfide)	0503		+					+
7 (hexanal)	0567		++	++	+	+	+	+
8 (1-hexanol)	0639							+++
9 (2-heptanone)	0687	++		++			++	
10 (n-heptanal)	0702	++	+	++	++		+++	+++
11 (1-heptanol)	0792						++	
11.5 (1-octene-3-ol)	0907		+	++	+			+
12	0836		+					
12.5	0907		+				++	

BHA AND BHT, BEFORE WEEKLY ADDITION

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290		+	+				
3	0321			++				
4 (2-butanone)	0347		+	+				
5	0408					+	+	
6 (pentanal)	0440		+			+		
6.4 (dimethyl disulfide)	0503		+					++
7 (hexanal)	0567		++	+	+	+	++	++
8 (1-hexanol)	0639			+				
10 (n-heptanal)	0702		+	++	++		+++	++
11.5 (1-octene-3-ol)	0805		+	+++	++		++	++
12	0836		+		+			
12.5	0907		+				++	

BHA AND BHT, AFTER WEEKLY ADDITION

Compound[1]	Retention Index[2]	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
2 (2-propanone)	0290					+	++	
3	0321		++					
4 (2-butanone)	0347		++					
5	0408		+		+	+		
6 (pentanal)	0440				+			
7 (hexanal)	0567				+		+	
8 (1-hexanol)	0639			+				
10 (n-heptanal)	0702		+++				+++	
11 (1-heptanol)	0792			++				
11.5 (1-octene-3-ol)	0805		+++	++		+	++	
12.5	0907			+				
13 (nonanal)	0962			+		+		

[1] Compound identified by comparison of its mass spectrum

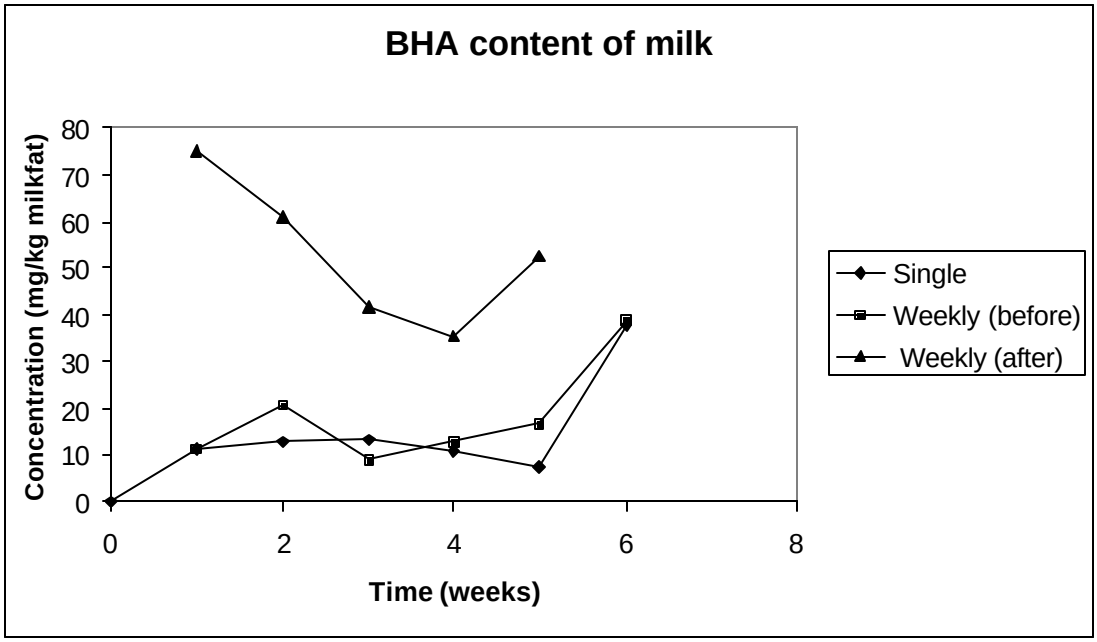
[2] Retention indices from GC-O data

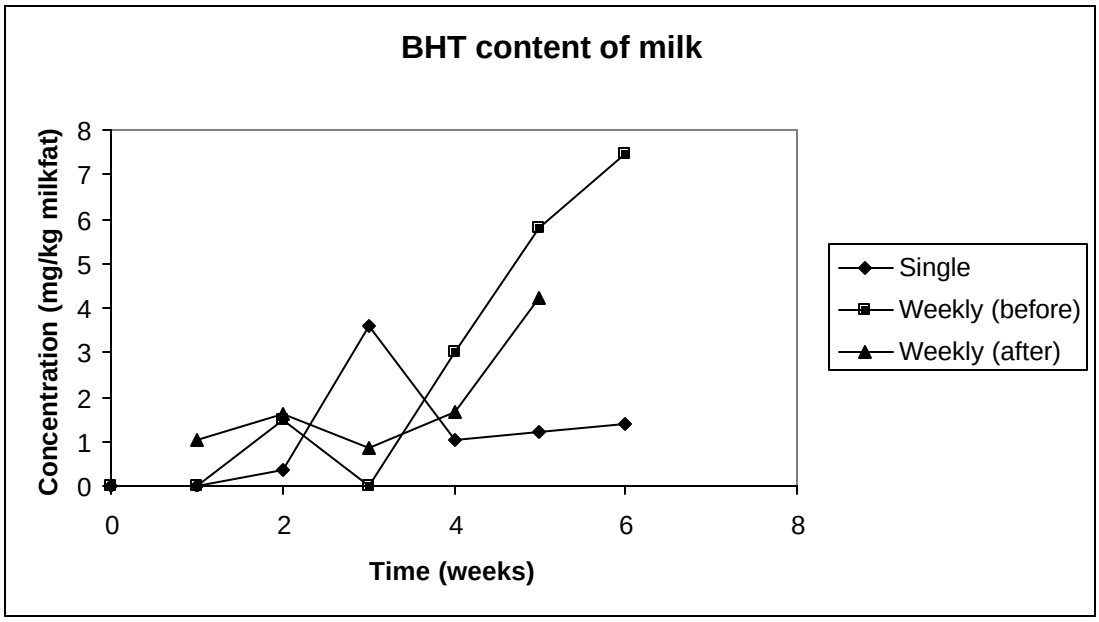
[3] Treatments included: Control milk (unspiked); Control milk with UHT milk added at the same amounts as antioxidant samples spiked weekly; Single addition of 100 mg.kg^{-1} milkfat of BHT and 100 mg.kg^{-1} milkfat of BHA; Single addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate; GCO analysis before and after weekly addition of 100 mg.kg^{-1} milkfat of BHT and 100 mg.kg^{-1} milkfat of BHA dissolved in a small quantity of

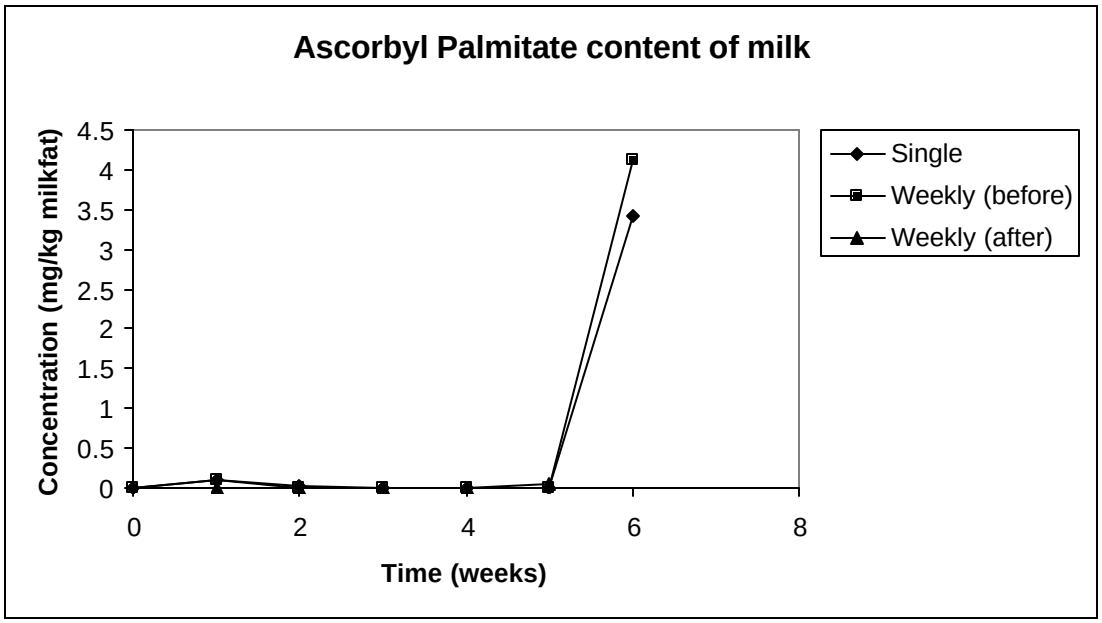
UHT milk; GCO analysis before and after weekly addition of 100 mg.kg^{-1} milkfat of tocopherol and 100 mg.kg^{-1} milkfat of ascorbyl palmitate dissolved in a small quantity of UHT milk.

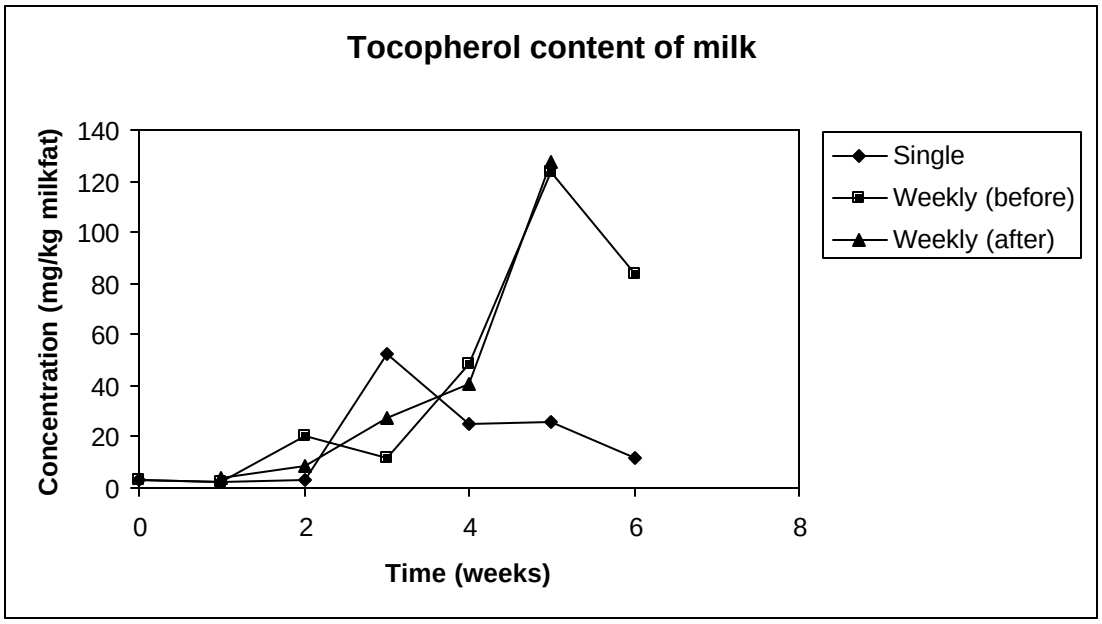
Odor intensities: + (0-1), ++ (2-3), +++ (4-5)

APPENDIX F: Antioxidant content of antioxidant-treated milk during 6 weeks of storage









Single addition: Antioxidants were added in one single dose at the beginning of the study at 100 $mg.kg^{-1}$ milkfat for each antioxidant.

Weekly addition: Antioxidants were added weekly at 100 $g.kg^{-1}$ milkfat for each antioxidant. HPLC analysis was done on samples before and after weekly addition of antioxidants.

CURRICULUM VITA
Marleen van Aardt

ADDRESS

Dept. of Food Science and Technology
Virginia Polytechnic Institute and State University
Blacksburg, VA
24061-0418

Phone (W): (01) (540) 231-6806
Phone (H): (01) (540) 961-6799
Fax: (01) (540) 231-9293
E-mail: mvanaard@vt.edu

MISSION STATEMENT

My goal is to enter academia to establish a research program spanning the areas of food packaging and its interaction with foods. There is a need for scientists familiar with the biomolecule-macromolecule interface, to bridge the gap between the food processor and polymer chemist. Emphasis will be put on sensory evaluation of foods and its relationship to flavor chemistry. I also wish to develop my interaction with students by teaching advanced courses in food packaging, food chemistry, and sensory evaluation of foods.

EDUCATION / QUALIFICATIONS

Doctor of Philosophy in Food Science and Technology, Dec 2003. Virginia Polytechnic Institute and State University, Blacksburg, VA. Advisor: Dr. Susan E. Duncan
Dissertation: "Controlled release of antioxidants by polymeric films into milk and dried milk products"

Masters of Science in Food Science and Technology, May 2000. Virginia Polytechnic Institute and State University, Blacksburg, VA. (GPA: 3.7/4.0) Advisor: Dr. Susan E. Duncan
Thesis: "Effect of shelf-life and light exposure on acetaldehyde concentration in milk packaged in HDPE and PETE bottles"

Bachelors of Science in Food Science and Technology, December 1997. Major: Chemistry. University of Stellenbosch, Stellenbosch, South Africa.

EMPLOYMENT

Graduate Research Assistant, Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA. (January 1999 – current)
Skills developed: sensory analysis of food; analysis of volatile flavor compounds using solid phase micro-extraction gas chromatography, and thermogravimetric analysis; development and characterization of antioxidant-loaded biodegradable polymeric films.

Dairy Research Technician, Agricultural Research Council of South Africa, Elsenburg, South Africa. (December 1997 – July 1998)
Skills developed: Analyzed various dairy products for nutritional information, somatic cell counts, and chemical composition; Prepared milk calibration samples for countrywide distribution.

Product Development Technician (Internship), South African Dried Fruit Company, Wellington, South Africa. (December 1995 – February 1996)
Skills developed: Developed citrus flavored dried prunes; Performed routine analysis of moisture content, sulfur content, and sugar content on various dried fruits and dried fruit products.

EXPERIENCE

Teaching:

Graduate Teaching Assistant for the undergraduate Food Chemistry Course (FST 4984) at the Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA. (August 2002 - December 2002)

Graduate Teaching Assistant for the undergraduate Dairy Products Sensory Evaluation Course (FST 2014) at the Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA (August 2000 – December 2000)

Assistant Coach to the Dairy Products Evaluation Team of Virginia Polytechnic Institute and State University and the University of the Orange Free State, South Africa (exchange program). These teams competed in the Collegiate Dairy Products Evaluation contest in San Francisco, CA. (August 2000 – November 2000). Students were trained to identify and evaluate the off-flavors associated with defects in various dairy products.
(Competed in the same competition as a graduate student member in Chicago, IL, November 1999)

Graduate Teaching Assistant for the graduate Sensory Evaluation of Foods Course (FST 5014) at the Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA. (January 2000 – May 2000)

Research:

Grants awarded on national level: (had direct involvement in writing and experimental design)

Dairy Management Inc. Research Grant: "Polymeric inhibition of photosensitive reactions of milk components" ($75,000) (2001-2003)

Dairy Management Inc. Research Grant: "Controlled release of antioxidants by polymeric films into milk" ($78,908) (2002-2004)

Grants awarded through Virginia Polytechnic Institute and State University: (had direct involvement in writing and experimental design)

Graduate Research Development Project Grant: Dissertation: "Controlled release of antioxidants by polymeric films into milk" (August 2000)

Graduate Research Development Project grant: Thesis: "Effect of shelf-life and light exposure on acetaldehyde concentration in milk packaged in HDPE and PETE bottles" (June 1999)

Peer review of scientific publications and proposals:

Vassila, E., Moyssiadi, T., Kondyli, E., Badeka, A., Savaidis, I., Kontominas, M.G. (in review) Chemical and microbiological changes in fluid milk as affected by packaging conditions. Int. Dairy J.

Public Service:

Secretary, Graduate Student Assembly, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, (June 2002 – June 2003)

Member, University Library Committee, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, (June 2002 – June 2003)

Chair, Graduate Research and Development Program, Division of the Graduate Student Assembly, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, (August 2001 – June 2002)

Delegate, representing the Department of Food Science and Technology at the Graduate Student Assembly, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, (June 2001 – June 2002)

President, Dairy Products Evaluation Teams, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, (November 1999 – June 2001)

HONORS / SPECIAL AWARDS

Package Education Forum Scholarship for outstanding achievement and contribution to Food Packaging (April 2002)

Dairy Evaluation Scholarship, Virginia Polytechnic Institute and State University (July 2001)

Invited to compete in the Graduate Student Paper Competition at the annual American Dairy Science Association meeting in Baltimore, MD. Abstract # 565 (July, 2000)

Gamma Sigma Delta (Honor Society of Agriculture, Virginia Polytechnic Institute and State University Chapter), Membership for high scholarship, outstanding achievement or service (March, 2000)

Virginia Dairy Technology Society: Dairy Products Evaluation Scholarship for outstanding achievement in Food Science and Technology (November 1999)

ADM Cocoa Award for outstanding achievement in Dairy Products Evaluation (November 1999)

Phi Tau Sigma Honorary member (March 1999)

PROFESSIONAL SPEAKING ENGAGEMENTS AND POSTER PRESENTATIONS

Invited Posters and Presentations:

Presentation: Effect of antioxidants on stability of edible fats and oils: Thermogravimetric analysis. M. van Aardt, S.E. Duncan, T.E. Long, S.F. O'Keefe, J.E. Marcy, S.R. Nielsen, Institute of Food Technologists annual meeting, Anaheim, CA (June 15 – 19, 2002)

Poster: Controlled release of antioxidants from polymeric films to control lipid oxidation in milk. M. van Aardt, S.E. Duncan, T.E. Long, S.F. O'Keefe, J.E. Marcy, S.R. Nielsen, American Dairy Science Association annual meeting in Indianapolis, IN. (July 24 – 28, 2001)

Presentation: Effect of shelf life and light exposure on acetaldehyde concentration in milk packaged in HDPE and PETE bottles. M. van Aardt, S.E. Duncan, D. Bourne, J.E. Marcy, T.E. Long, C.R. Hackney, C. Heisey. Virginia Dairy Quality Control Conference at Virginia Polytechnic Institute and State University, Blacksburg, VA. (September 2000)

Presentation: Effect of shelf life and light exposure on acetaldehyde concentration in milk packaged in HDPE and PETE bottles. M van Aardt, S.E. Duncan, D. Bourne, J.E. Marcy, T.E. Long, C.R. Hackney, C. Heisey. Compete in Graduate Student Paper Competition, American Dairy Science Association annual meeting in Baltimore, MD. (July 2000)

Presentation: Flavor threshold for acetaldehyde in milk, chocolate milk, and spring water using solid phase micro-extraction gas chromatography (SPME-GC) for quantification. M. van Aardt, S.E. Duncan, D. Bourne, J.E. Marcy, T.E. Long, C.R. Hackney, C. Heisey. Virginia Dairy Quality Control Conference, Virginia Polytechnic Institute and State University, Blacksburg, VA. (September 1999)

Volunteered Posters and Presentations:

Poster: Controlled release of antioxidants from polymeric films to control lipid oxidation in milk. M. van Aardt, S.E. Duncan, T.E. Long, S.F. O'Keefe, J.E. Marcy, S.R. Nielsen, Joint University Day, Eastman Chemical Co., Kingsport, TN. (August 2001)

Poster: Controlled release of antioxidants from polymeric films to control lipid oxidation in milk. M. van Aardt, S.E. Duncan, T.E. Long, S.F. O'Keefe, J.E. Marcy, S.R. Nielsen, American Dairy Science Association annual meeting in Baltimore, MD. (July 2000)

Presentation: Effect of shelf life and light exposure on acetaldehyde concentration in milk packaged in HDPE and PETE bottles. M**.** van Aardt, Project Sponsors (Eastman Chemical Co.) and Graduate Committee at Virginia Polytechnic Institute and State University, Blacksburg, VA. (February 2000)

MEMBERSHIPS

Institute of Food Technologists - Student Member, Packaging and Sensory Evaluation Divisions, (January 2000 – present)

Food Science Club of Virginia Polytechnic Institute and State University, member (January 1999 – present)

American Dairy Science Association, Student Member (June 2001 – present)

PUBLICATIONS

Van Aardt, M., Duncan, S.E., Marcy, J.E., Long, T.E., Hackney, C.R. 2001. Effectiveness of poly(ethylene terephthalate) and high-density polyethylene in protection of milk flavor. J. Dairy Sci. 84:1341-1347.

Van Aardt, M., Duncan, S.E., Bourne, D., Marcy, J.E., Long, T.E., Hackney, C.R., Heisey, C. 2001. Flavor threshold for acetaldehyde in milk, chocolate milk, and spring water using solid phase micro-extraction gas chromatography (SPME-GC) for quantification. J. Agric. Food Chem. 49(3): 1377-1381.

Van Aardt, M., Duncan, S.E., Bourne, D., Marcy, J.E., Long, T.E., Hackney, C.R., Heisey, C. 2000. Human flavor threshold for acetaldehyde in milk of various fat content, chocolate milk, and spring water. J. Dairy Sci. 83:(Suppl. 1) Abstract # 565 p. 132

Van Aardt, M., Duncan, S.E., Bourne, D., Marcy, J.E., Long, T.E., Hackney, C.R., Heisey, C. 2000. Effect of shelf-life and light exposure on acetaldehyde concentration in milk packaged in HDPE and PETE bottles. J. Dairy Sci. 83:(Suppl. 1) Abstract # 347 p. 82

REFERENCES

1. Susan E. Duncan, Ph.D.
   Associate Professor
   Food Science and Technology, Virginia Polytechnic Institute and State University
   Blacksburg, VA, 24061-0418
   Phone: (540) 231-8675
   E-mail: duncans@vt.edu

2. Joseph E. Marcy, Ph.D.
   Associate Professor
   Food Science and Technology, Virginia Polytechnic Institute and State University
   Blacksburg, VA, 24061-0418
   Phone: (540) 231-7850
   E-mail: jmarcy@vt.edu

3. Timothy E. Long, Ph.D.
   Associate Professor
   Chemistry, Virginia Polytechnic Institute and State University
   Blacksburg, VA, 24061-0418
   Phone: (540) 231-2480
   E-mail: telong@vt.edu