Antioxidant Protection of an Omega-3 Fatty Acid Fortified Dairy-Based Beverage

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ABSTRACT

Skim, butter-derived aqueous phase, anhydrous milk fat, and fish oil were used to formulate ultra high temperature (UHT) processed extended shelf-life omega-3 fatty acid fortified dairy-based beverages with fat contents mimicking whole milk (3.25%). Oxidation of the lipids in the formulated beverages was investigated during storage for 35 days at 4°C using GC/MS analysis, conjugated diene analysis, and headspace solid phase micro-extraction GC/MS (SPME-GC/MS) analysis of headspace. Omega-3 fatty acid fortified dairy-based beverages were produced that mimicked the physical properties of 3.25% fat whole milk. Oxidation resulted in only small changes in omega-3 lipid content and sensory analysis by an untrained panel indicated that the overall aroma was no different than that of commercially available UHT processed milk. An omega-3 fatty acid fortified dairy-based beverage was produced that delivered 440mg of omega-3 fatty acid per 8oz serving. When consumed daily, the beverage could provide the equivalent amount of omega-3 fatty acids recommended by the American Heart Association, and the equivalent amount of omega-3 fatty acids found in two fatty fish meals over the period of one week.

Antioxidants were added to the lipid phase, immediately prior to processing, of additionally produced formulations to determine if a reduction in omega-3 lipid oxidation was observed. No overall reduction in oxidation was observed, as indicated by GC/MS and SPME-GC/MS analysis. Sensory analysis indicated that oxidative aromas increased during storage for the antioxidant and omega-3 fatty acid fortified dairy-based beverage. Ascorbyl palmitate was determined to have a pro-oxidative effect on the formulated omega-3 fortified dairy-based beverages. Antioxidants present in the commercial grade fish oil used for fortification were effective in controlling oxidation in the formulated omega-3 fatty acid fortified dairy-based beverages.

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CHAPTER I
INTRODUCTION

Omega-3 polyunsaturated fatty acids (FA) include α-linolenic acid (ALA, 18:3ω3), eicosapentaenoic acid (EPA, 20:5ω3), and docosahexaenoic acid (DHA, 22:6ω3). The potential health benefits of regularly consuming omega-3 FA have been extensively studied. EPA and DHA are recognized as the principle omega-3 FA associated with increased health benefits. Omega-3 FA have been associated with reduced risk and alleviation of many health conditions, such as coronary heart disease and arthritis, and are important for the development and functions of the brain and nervous systems [1-5].

The primary dietary sources of omega-3 FA are marine sources, such as algae and cold water fatty fish, but they are also found in terrestrial sources such as seeds and nuts.
Modern Western diets include low amounts of natural sources of omega-3 FA. Food products fortified with omega-3 FA provide a means of dietary supplementation that could increase the overall intake of omega-3 FA by individuals looking to supplement their dietary intake or by individuals who do not prefer natural sources.

Due to the low current intake levels of omega-3 FA through the diet, there is a need for fortified products that can deliver substantial amounts of omega-3 FA. The World Health Organization (WHO) and American Heart Association (AHA) both recommend two fatty fish meals per week to help supplement omega-3 FA intake. A product that could deliver the equivalent amount of omega-3 FA recommended by the WHO and AHA, by consuming a serving every day, would be advantageous in the problem of achieving suggested omega-3 levels in the diet.

However, developing omega-3 fortified foods is challenging because of sensory issues from two sources: 1) characteristic flavor and aroma of omega-3 FA sources; and 2) volatile by-products of oxidative degradation of these highly susceptible unsaturated fatty acids. EPA and DHA are sourced mainly from marine origins, so fortification into food products may result in unacceptable sensory characteristics. Due to the high level of unsaturation in the molecular structures of EPA and DHA, they are highly susceptible to oxidation. Oxidation results in

reduction of the nutritional impact of the omega-3 FA, and in the formation of small molecular weight volatile compounds. McFarlane and others [6] reported that compounds produced from omega-3 FA oxidation have very low aroma and flavor threshold levels and can negatively impact the aromas and flavors of omega-3 FA fortified products.

The addition of antioxidants to food matrices fortified with omega-3 FA is one approach used to inhibit or reduce oxidation. Preventing and reducing oxidation of omega-3 FA in both bulk lipids and food matrices by the addition of antioxidants have been extensively studied [7-15]. Processing and formulation, storage conditions (temperature, light exposure), and storage time play important roles in omega-3 lipid oxidation. Light, heat, and metals all can serve as initiators in oxidation reactions. Foods that are good candidates for delivering (fortification with) omega-3 fatty acids include those that are consumed frequently, are not subjected to intense heat treatments, light or oxygen, and are not stored for long time periods [16].

In this project, we targeted formulation of a dairy-based beverage that contained, in one serving (8 fluid oz), $1/7^{th}$ of the amount of EPA and DHA found in two fatty fish meals. The desired characteristics of this dairy-based beverage included a long shelf-life at refrigerated temperatures, positive sensory attributes, minimal changes in unsaturated fat composition throughout the shelf-life, and delivery of a sufficient omega-3 FA level per serving.

The objectives of this project included:

1. evaluating oxidative and physical stability and aroma impact of a fish oil-fortified ultrahigh temperature (UHT) processed omega-3 fatty acid dairy-based beverage, formulated to deliver 500mg of EPA+DHA per 8oz. serving, during 35 days of storage at 4°C;
2. identifying natural antioxidants that provide efficacy in reducing or inhibiting oxidation in an omega-3 fortified UHT processed dairy-based beverage fortified with fish oil;
3. assessing oxidative and physical stability in an antioxidant-enriched omega-3 fatty acid fortified UHT processed dairy beverage over 35 days at 4°C.

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CHAPTER II
REVIEW OF LITERATURE

Omega-3 Fatty Acids in Consumer and New Food Product Trends

There is a growing number of consumers who purchase foods because of the health benefits associated with consumption. These foods, referred to as functional foods, are beneficial because they could deliver levels of bioactive nutrients that are not delivered through the standard diet. Functional foods have increased in sales over the past few years, with an estimated growth rate of 10% annually, compared to an overall food and beverage market growth of about 2% annually [1]. Omega-3 polyunsaturated fatty acids (PUFA) provide positive health effects, but are consumed at low levels in the traditional American diet. Omega-3 fortified products are one of the growing segments in the functional foods area, with 120 omega-3 enriched food and beverage products in 2005 and that number doubling by 2006, covering the bakery, dairy, and sports products sectors [2].

Health Benefits of Omega-3 Fatty Acids

Consumption of dietary omega-3 fatty acids (FA) for health benefits has been extensively studied by the medical, scientific, and nonscientific communities; in recent years a vast amount of literature has been published on their findings. Omega-3 FA have significant positive effects on health and general wellbeing [3-7]. The Food and Drug Administration (FDA) announced a qualified health claim stating a connection between omega-3 FA and a reduced risk of coronary heart disease based on sufficient scientific studies documenting a positive correlation to coronary and cardiovascular health [8]. Omega-3 FA also may contribute to alleviation of inflammatory diseases such as arthritis and cystic fibrosis, mood and behavior disorders, cardiovascular disease (CVD), and aiding in human brain development [9-13].

One of the primary health effects of omega-3 FA is the positive role they play in reducing the risk of CVD. This was first observed in an epidemiological study of Greenland Inuits who rarely suffer from coronary disease and consume high levels of fish, which contain omega-3 FA [14]. Similar observations have been made of other populations, such as the Greek population [15], where the diet is rich in omega-3 FA. Although the mechanisms by which omega-3 FA

contribute to coronary health are not fully understood, it is hypothesized that they help prevent arrhythmias, have anti-inflammatory properties, are prostaglandin and leukotriene precursors, and inhibit synthesis of cytokines [16, 17].

Omega-3 lipids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), play important roles in the body and in its development. EPA and DHA are major constituents of cell membranes where they aid in the fluidity of plasma within cells. EPA is recognized as less physiologically important than DHA for brain and visual functioning. DHA is an essential nutrient for the proper functions of the brain and retinas of the eyes where high concentrations are required for providing optimal mental and visual acuity. DHA plays a very important role in maintaining the fluid microenvironment of phospholipid components in the grey matter in the brains and cell membranes of the nervous system of mammals. DHA alters the basic properties of cell membranes, such as their permeability, elastic compressibility, and interactions with regulatory proteins. DHA is thought to support the electrical signaling of the nervous system and support brain functions such as memory and learning ability [9]. Infants, born to mothers who supplemented their diets during pregnancy with DHA, were found to have better visual, cognitive, and motor development at six and eleven months of age than infants who had lower levels of DHA supplemented to them during gestation [18].
Omega-3 FA play a role in mental health as well. Studies have shown that omega-3 FA, specifically EPA, could be used as an alternative treatment approach to schizophrenia in levels that are free of harmful side-effects [10]. Other conditions, such as Alzheimer's disease and depression, have been shown to be responsive to omega-3 treatments, and links between reduced suicide attempts and omega-3 FA consumption have been studied [19-21].

Omega-3 Fatty Acids in the Diet

Omega-3 FA are a group of PUFA that have in common the location of the first carbon-carbon double bond, relative to the end of the FA molecule. This carbon-carbon bond occurs as the third bond from the methyl, or "omega", end of the molecule, also known as the n-3 position. The structures of the main omega-3 and omega-6 PUFAs can be seen in Figure 2-1. Omega-3

PUFAs, as well as omega-6 PUFAs, are classified as essential FA because the human body cannot synthesize them. The human body is capable of producing unsaturated FA but it cannot produce FA having double bonds below the ninth carbon atom, which includes omega-3 and omega-6 FA. Since the body cannot synthesize these essential FA, they must be supplied via dietary intake or nutritional supplements.

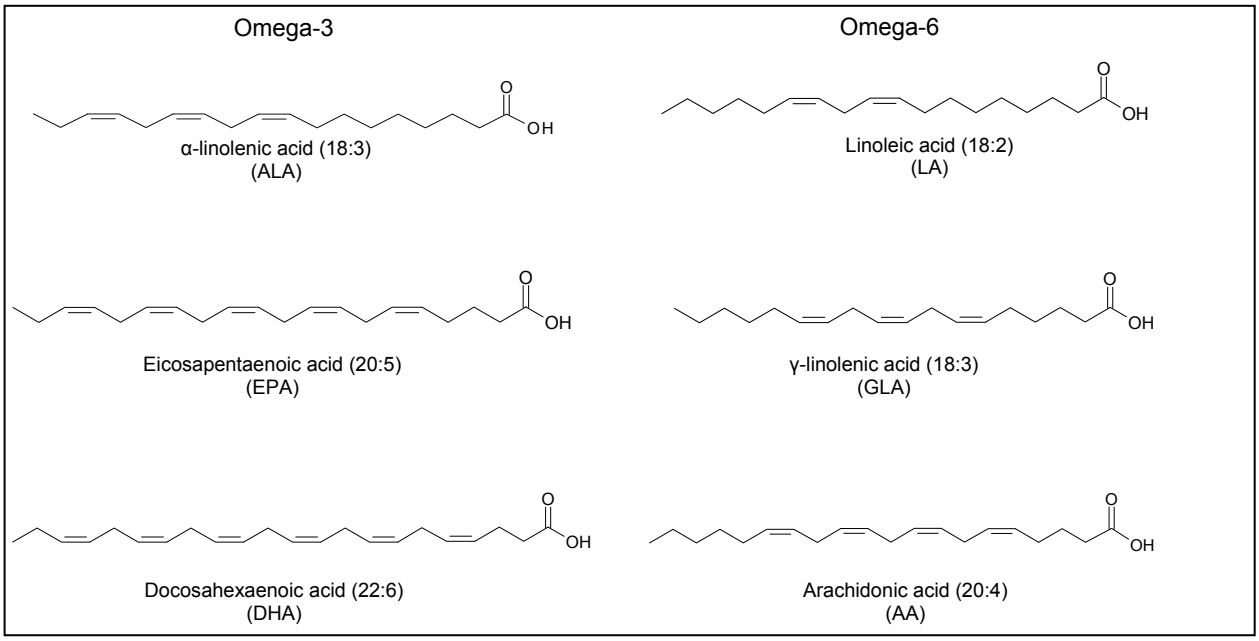


Figure 2-1. Structures of major omega-3 and omega-6 fatty acids

Dietary sources of the essential PUFAs differ by type. Omega-6 FA are found in rich supply in vegetable oils, such as corn and canola oils, and meat from animals fed on diets of cereal grains such as corn and rice. Omega-3 FA can be found in both plant and marine sources. Alpha-linoleic acid (ALA) is found in plant sources and also in animals that eat the plant sources. ALA is most abundant in seeds, oils, and nuts, such as flax seeds/oil, hemp seeds/oil, pumpkin seeds/oil, olive oil, rapeseed oil, and walnuts. Flax has been found to contain the highest levels of ALA of the plant sources, while walnuts have been found to contain the highest concentration of ALA of any tree nut. Purslane, a common succulent wild plant, considered a weed in the U.S. but used as a food source in Europe and Asia [22], has been found to contain high levels of ALA [23]. EPA and DHA are found in marine sources such as algae and cold water fatty fish, like salmon, tuna, anchovies, and menhaden, but also can be found in crustaceans and mollusks. It should be noted though that most fish get their omega-3 FA from eating smaller organisms that

eat the algae that produce the lipids. The omega-3 FA content of selected foods can be seen in Table 2-1.

Table 2-1. Omega-3 concentration (ALA, EPA+DHA)[1] in selected plant, nut, oil, and fish sources [24-26].

Source of omega-3 FA	ALA (mg/g)	EPA+DHA (mg/serving[2])
Flaxseeds	82.8	
Flaxseed oil	533.0	
Pumpkin seeds	1.8	
Walnuts, black	5.5	
Walnuts, English	90.8	
Olive oil	7.6	
Rapeseed oil	95.7	
Hemp seeds	150-200	
Purslane	3-4	
Salmon		1825
Mackerel		1571
Tuna, canned		733
Swordfish		696
Shrimp		460

[1] ALA= α-linoleic acid; EPA= eicosapentaenoic acid; DHA= docosahexaenoic acid

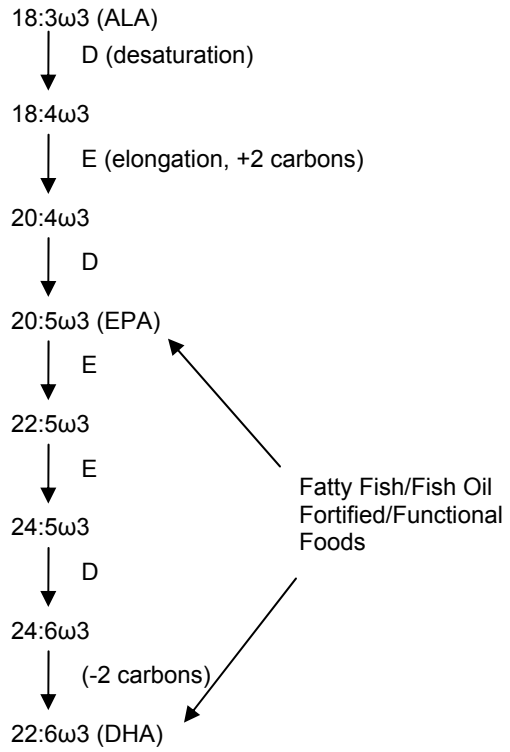
[2] 1 serving = 85g (3 oz) cooked portions

Since omega-3 FA must be provided through the diet or supplementation, humans must get them by consuming the seeds, nuts, oils, and meats in which they are found. Common vegetable oil sources of ALA include soybean and rapeseed oils, which contain 4-11% and 6-14% ALA, respectively. Flax seeds contain high levels of ALA at ~50% while walnuts contain around 13%

[27]. Salmon and tuna contain ~1.8% and ~1.5%, [24] respectively, of EPA+DHA combined[26].

Recent studies have shown that in the U.S. population the average current intake of omega-3 FA is ~1.6 g/day, of which 1.4 g are ALA and 100 to 200 mg are EPA+DHA [28]. In the Australian population, average intake is 1.4 g/day, of which ALA and EPA+DHA are 1.2 and 0.2 g, respectively [29], and in the U.K., the average omega-3 intake is 1.8 g/day, of which ALA and EPA+DHA are 1.5 and 0.3 g, respectively [30].
Recommended dietary intakes of omega-3 FA vary based upon the source of the recommendation. The World Health Organization (WHO) and the North Atlantic Treaty Organization (NATO) recommend 800 to 1100 mg/day of alpha-linolenic acid (ALA) and 300 to 500 mg/day of EPA+DHA [31]. The American Heart Association (AHA) recommends 1.5 to 3 gram/day of ALA plus 500 to 1800 mg/day of EPA+DHA for beneficial health effects, which include the reduced risk of cardiovascular disease [32]. While the recommended amounts of ALA are approximately met in the U.S. diet, the amount of EPA and DHA consumed through the diet is lacking on a daily basis, with the U.S. population receiving only about fifteen percent of the recommended daily amount [28].

When consumed in the diet, alpha-linolenic acid can be converted via desaturation and elongation reactions into EPA and DHA. The enzyme responsible for this conversion is the delta-6-desaturase enzyme (Figure 2-2).



Adapted from http://dhaomega3.org

Figure 2-2. Enzymatic conversion steps for elongation of omega-3 alpha-linoleic acid (ALA) to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

However, studies have shown that the conversion rate of the precursor ALA to the end products EPA and DHA is considerably low. Conversion from ALA to EPA varies from 0.2% to 21% and that of ALA to DHA varies from 0% to 9% [33, 34]. Multiple factors in the body contribute to these conversion rates, such as the presence of the omega-6 FA linoleic acid (LA) which competes for the same desaturase enzyme. This means that while some diets may approximately meet the dietary recommendations for ALA, the conversion to the more physiologically important EPA and DHA is not sufficient enough to compensate for the low levels of EPA and DHA obtained through the diet.

The modern Western diet generally includes low amounts of fish and other naturally rich sources of omega-3 FA in the form of EPA and DHA. The characteristic Western diet is heavily laden with vegetable oils, such as corn, soybean and canola oil, which can provide high amounts of ALA but also provide high amounts of omega-6 FA. The levels of omega-6 FA that are received through the modern diet are high compared to the levels of omega-3 FA. This high ratio of

omega-6:omega-3 FA is believed to be the cause of many modern ailments, such as heart and skin ailments, as our early ancestors consumed diets with an approximately equal ratio of 1-2:1 omega-6:omega-3 FA [35]. This approximately equivalent ratio was achieved by consuming foods such as nuts, seeds, berries, and wild animals. Wild animals also contained equivalent amounts of omega-6 and omega-3 FA as they ate the same food sources as our early ancestors.

The diet consumed today contains a different ratio of omega-6 and omega-3 FA, due in part to our industrialized agricultural methods. Modern agriculture has led to increased consumption of vegetable oils and a change in diet of our livestock, which are fed large amounts of cereal grains. This shift in agriculture and, ultimately, our diet, has led to consumption of omega-6 and omega-3 FA at a ratio of ~10:1 for omega-6 and omega-3 respectively [28, 36]. The end product of omega-6 FA desaturase is arachidonic acid (AA), which is a precursor for a number of pro-inflammatory and tumor-enhancing eicosanoids. Consuming omega-3 FA, whether through natural sources or through supplementation, helps to reduce the ratio of omega-6 and omega-3 FA in our diet, which in return may reduce the health effects associated with elevated omega-6 FA levels.

Increasing the intake of PUFA and omega-3 FA in the diet requires consideration of normal food consumption habits and knowledge of food sources rich in omega-3 FA. The World Health Organization (WHO) and American Heart Association (AHA) recommend two fatty fish meals per week to achieve the suggested dietary intake of omega-3 FA [31, 32]. Another strategy would be to alter dietary intake to include more foods that naturally contain omega-3 FA, such as nuts and seeds, while at the same time limiting the intake of foods that contain high levels of omega-6 FA, such as vegetable oils.

Fortified foods also provide a means to implement more omega-3 FA into the diet. There are many foods available that provide additional supplementation, some of which include baked goods such as breads, energy/nutrition bars, eggs, milks, cheeses, yogurts, and spreadable fats such as margarine. Table 2-2 provides a list of some of the fortified products in markets today and the levels of omega-3 FA (EPA and DHA) contained per serving.

Table 2-2. Examples of commercially available omega-3 fortified products and their levels of EPA+DHA per serving

Food	Serving size	EPA+DHA (mg)
Smart Balance Omega Plus Buttery Spread (Smart Balance, Inc., Paramus, NJ)	1 tbsp.	160
Land O' Lakes Omega-3 Eggs (Land O' Lakes, Inc., Saint Paul, MN)	1 egg	150
Breyers Smart DHA Omega-3 yogurt (Breyers Yogurt Company, North Lawrence, NV)	6 oz.	30
Horizon Organic DHA Omega-3 milk (Horizon, Boulder, CO)	8 oz	32
Silk Plus Omega-3 DHA Soy Milk (WhiteWave Foods, Broomfield, CO)	8 oz.	30

It is difficult to meet the AHA suggested daily intake levels of EPA+DHA using omega-3 enriched foods currently in the marketplace. The EPA+DHA levels per serving are low (Table 2-2) and would require consuming large volumes. For example, approximately 2 gallons of DHA omega-3 milk would be needed to meet the omega-3 FA (EPA+DHA) daily recommended amounts of 300-500mg/day and 500-1800mg/day by the WHO and AHA [31, 32], respectively. These products are best described as additional methods to augment omega-3 FA already in the diet because the concentration of omega-3 FA is too low to be physiologically beneficial as the principal dietary source of omega-3 FA. Because low amounts of omega-3 FA from natural sources are consumed in the average diet and because low amounts are found in most omega-3 FA fortified foods, there is a need for products fortified with omega-3 FA at levels that can be physiologically beneficial.

Potential Health Value of Omega-3 Fatty Acids in Milk and Dairy Foods

Many of the omega-3 fortified foods on the market are dairy-based products. Dairy products, such as milk, naturally contain low levels of omega-3 FA. Omega-3 FA comprise approximately 1% of the total fats that occur naturally in these products, although evidence has shown that there are differences in these levels depending on whether the cow was from an organic or

conventional farm [37, 38]. Investigators also have shown that omega-3 FA levels can be manipulated in the milk produced by cows by feeding them a diet rich in sources of omega-3 FA, such as diets containing flax and or fish oil [39].

Dairy products provide a good delivery system for omega-3 FA due to the nature in which they are stored and consumed. Investigators have concluded that the best products for omega-3 FA fortification are ones that are stored at low temperatures for short times, and in packages that prohibit the passage of air and light [40]. Dairy products are usually consumed within short periods from the time of purchase and meet the aforementioned storage parameters, suggesting that they serve as good candidates for fortified delivery of omega-3 FA.

Milk, the basis of dairy products, is naturally designed as a nutrient dense food source that nourishes and provides immunological protection for mammalian offspring. Per 8 fl oz serving, milk contains 7.9 g protein, 276 mg calcium, 222 mg phosphorus, 349 mg potassium, 447 mg riboflavin, 883 mg vitamin B_5, 249 mg vitamin A, 98 mg vitamin D, as well as other vitamins and minerals [26]. With this assortment of biologically valuable nutrients and other bioactive molecules, milk is recognized as an important functional food.

Milk and dairy products provide calcium, which is biologically necessary for bone growth and bone remodeling. The absorbability, and ultimately bioavailability, of calcium from dairy products is high, due to bonding with peptides and proteins, which allows high calcium absorption under unfavorable physiological conditions [41]. The calcium in dairy products is easily utilized and provides a means of prevention and treatment of osteoporosis [42]. Osteoporosis, a disease that causes structural deterioration of bone tissue, affects an estimated 55% of persons age 50 and over. Currently in the US there are an estimated 10 million individuals who have the disease, and an estimated 34 million more that have low bone mass that places them at risk for developing osteoporosis [43]. According to the National Osteoporosis Foundation, one step to help prevent osteoporosis is to get the recommended daily amounts of calcium and vitamin D. Consumption of dairy products can contribute to meeting the recommended daily dietary intake of calcium.

The incorporation of calcium, by supplemental or dietary means, has been shown to have positive effects on weight loss by improving lipid metabolism and reducing energy storage [44], which may help fight obesity. Studies using supplemental and dietary calcium, where the dietary calcium originated from dairy, found a larger weight loss effect using dairy calcium. It has been suggested that other components in milk, such as whey proteins and peptides, may contribute to the weight loss effect of dairy calcium, or have individual effects. These dairy components may function synergistically with calcium to modulate lipid metabolism [45].

It is generally accepted that obesity can be linked to consuming more energy than the body needs, such as high intakes of dietary fat and calories. An increased energy intake, along with a decrease in physical activity may be related to the growing population of overweight or obese individuals. Recent findings suggest that the incorporation of omega-3 FA into the diet stimulate lipolysis in fat storing cells [46] and stimulate intestinal lipid metabolism[47]. The addition of exercise along with omega-3 consumption has been shown to significantly reduce body fat [48]. This suggests that supplementation of omega-3 FA in the diet in conjunction with exercise or increased physical activity may increase the rate of weight loss, which may help control or fight obesity. Dairy components in conjunction with omega-3 FA may positively alter lipid metabolism and storage, and may work synergistically to increase weight loss in obese or obese prone individuals.

Conjugated linoleic acid (CLA), a dairy lipid constituent derived from linoleic acid, also has been found to promote weight reduction. Research has found that CLA affects lipoprotein lipases and stearoyl-CoA desaturases, which causes a reduction in lipid uptake by adipose cells [49-52]. The carnitine palmitoyltransferase activity, which affects β-oxidation, also has been shown to be increased by CLA [49]. This means that CLA decreases the uptake of lipids in the body by adipocytes and stimulates the oxidation of fat in muscle cells. CLA helps regulate and control bone modeling and formation [53], which may contribute to the benefits of dairy consumption in controlling and or preventing osteoporosis.

Milk naturally contains low levels of omega-3 FA, at around 0.66% to 1.11% of the total FA that occur [37]. Due to the low levels of omega-3 FA that occur naturally in milk, much research has

been done to manipulate the FA composition of the milk that cows produce by altering their diet [54, 55]. While the amount of omega-3 FA, specifically ALA, and CLA in milk can be elevated by the cow's diet, there is low transfer efficiency of the omega-3 FA from the bovine diet into the milkfat [39]. Milk produced through dietary modifications also is more prone to oxidation due to the higher content of PUFA [56]. It is more economic and efficient to fortify the milk with omega-3 FA (EPA and DHA) through formulation and processing. Milk, or milk-based dairy products, that have been fortified with omega-3 FA may provide a means for supplementation of omega-3s into the diet. There are several aseptically packaged UHT processed milk products on the market in the U.K. that have been fortified with omega-3 FA. One product, produced by Dairy Crest, claims to provide 50% of the recommended daily intake of omega-3 FA with two glasses [57], although the source of the recommended daily intake and serving size are not stipulated.

Challenges Associated with Omega-3 Enrichment in Dairy Foods

Regulatory Challenges. One advantage of pre-harvest dietary modification for increasing omega-3 FA concentration in milk is that this natural approach is not restricted by federal regulations for the term "milk". Due to federal regulations pertaining to milk composition and processing, it is difficult to fortify milk with omega-3 FA and still maintain the descriptor of "milk". According to the Filled Milk Act of 1923 (21 U.S. Code, Section 61-64) it is unlawful to participate in the commerce of any "filled milk", which is defined as any milk, cream, or skimmed milk that has had any fat or oil, other than milk fat, blended or compounded within it to produce an imitation milk product or product that resembles milk. The standard of identity for milk described under 21 CFR 131.110 [58] also puts forth a hurdle in marketing omega-3 fortified milk, as omega-3 FA are not considered a viable addition to milk. Reformulation of a milk to contain foreign fat, i.e. fish oil containing omega-3 FA, would require labeling that specifies the product is a dairy or milk based beverage, not milk. Also, the use of synthetic antioxidants and many other ingredients are not permitted in many dairy systems, including milk, as regulated by the Pasteurized Milk Ordinance, although they are permitted for use in formulated food systems, which include formulated dairy-based food systems. Natural antioxidants, such as tocopherols, ascorbic acid, and ascorbyl palmitate are allowed for use in milk but they must be stated on the label [59].

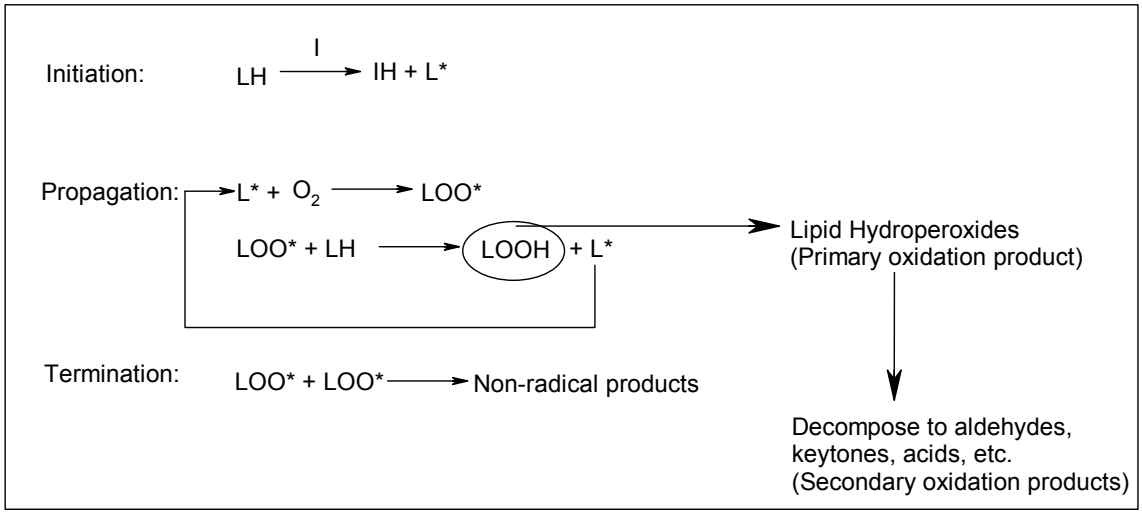
Sensory and Product Challenges. Dairy foods fortified with omega-3 FA, specifically EPA and DHA, may exhibit sensory characteristics of the source from which the omega-3 FA were derived. EPA and DHA, which can be added at low levels without any characteristic sensory attributes present in the product, can contribute characteristic "fishy" notes when added to foods at elevated levels [60].

High quality milk has bland sensory characteristics. Milk should exhibit no aftertaste other than the natural richness imparted by the milkfat and other milk solids [61]. The aroma should possess a mild, slightly cooked note that results from the pasteurization process. Good high quality milk should be almost neutral in flavor and leave a clean, pleasing sensation after swallowing or expectoration. Due to the expectedly bland sensory characteristics of milk, off-flavors and aromas are easily detectable [61]. Challenges of fortifying milk with omega-3 FA involve inhibiting any fishy flavor or odor that may be imparted in it as a result of fortification with fish oil.

Omega-3 FA are very susceptible to oxidation due to the double bonds present in the molecular structure. While omega-3 FA are flavorless, oxidation of these lipids, along with other milk lipids such as CLA, can produce volatile oxidation compounds that can contribute undesirable off-flavors and/or aromas. These volatile compounds have very low flavor threshold levels and research has shown that they are perceptible at parts per million and parts per billion concentrations [62].

Lipid oxidation is a free radical reaction that can be catalyzed by light, heat, metals, or by other free radicals, and it can occur while in the vapor phase or in non-polar solvents. Oxidation can be described in three processes: initiation, propagation, and termination. During the initiation process, a lipid loses a hydrogen radical, which results in a lipid free radical. The leads to the propagation process, where the lipid free radical reacts rapidly with molecular oxygen to form a lipid peroxyl radical. This peroxyl radical then is involved in a hydrogen transfer reaction with another unsaturated lipid, which forms new lipid radicals and lipid hydroperoxides. These steps are the first stages of oxidation, producing hydroperoxides as the primary oxidation products. In

the termination process, two peroxyl radicals react and form non-radical secondary products. Free radical reactions can be inhibited by antioxidants, which serve as radical acceptors. The primary oxidation products are very unstable and degrade easily to yield volatile secondary oxidation products. These volatiles impart off-flavors and can cause significant flavor deterioration of foods. Oxidation produces detrimental flavor and aroma compounds through the interaction and decomposition of lipid hydroperoxides, and it can reduce the nutritional quality and safety of foods. Hydroperoxides can react with proteins and other components in a food system, impacting flavor and oxidative stability as well as texture during cooking, processing, and storage. The products affect flavor and they may affect the safety of the lipid-containing food by damaging proteins and enzymes. The oxidation process is depicted in Figure 2-3.



LH – unsaturated lipid
I – initiator
L* - lipid free radical
LOO* - peroxyl radical

Figure 2-3. General reactions of lipid oxidation to yield primary (hydroperoxides) and secondary oxidation products.

Minimizing the sensory impacts of omega-3 lipids in dairy-based systems is needed. This includes consideration of the source and processing of the omega-3 oil product, as well as oxidation and degradation of the highly susceptible oils. Deodorization of fish oil, achieved through steam distillation, can reduce undesirable volatile compounds that contribute to the smell and taste of the oil. While this process can reduce the sensory characteristics of the oil, non-volatile degradation products, such as cyclic FA monomers and geometrical isomers of EPA and DHA, can be formed. The formation of these products may reduce the valuable nutritional components in the fish oil. Another method is to control oxidation of the oils used for fortification. The oxidation process can be initiated by several factors such as light, heat, enzymes, and metals, such as iron. Incorporation of antioxidants into foods fortified with omega-3 FA serves as one method to approach the issue of flavors imparted by lipid oxidation. Milk contains metals in small amounts, such as Fe^{2+} and Cu^{2+} at 0.07 and 0.027 mg/8oz [63], respectively, which may contribute to lipid oxidation by acting as catalysts. Antioxidants capable of chelating metal ions, especially in systems containing PUFA, may inhibit the metal catalysis of lipid oxidation by sequestering metal ions capable of participating in further oxidation reactions.

Some antioxidants that might be used to help control omega-3 lipid oxidation in dairy systems include tocopherols, ascorbyl palmitate (AP), ascorbic acid, and lipoic acid. Tocopherols, which are lipid soluble, exhibit low antioxidant properties when added to many food systems, and have been shown to exhibit prooxidant effects in high concentrations [64]. This may be a result of the natural presence of tocopherols at the maximum level of effectiveness against oxidation. AP and ascorbic acid both have shown to exhibit antioxidant properties in dairy products at low levels, but prooxidant properties at high concentrations. This prooxidant effect at high concentrations is thought to be a result of the ability of the AOX to promote the decomposition of peroxides. AP has shown to be an effective antioxidant when used at lower concentrations in milk and milk drinks containing 1.5-5% fat [65]. The formation of hexanal has been found to be inhibited by AP, which is amphiphilic, when added to the oil phase, and heptadienal formation has been found to be inhibited by ascorbic acid, which is water soluble, when added to the aqueous phase [65]. Hexanal and heptadienal are volatile oxidation products that can add unacceptable sensory

characteristics to milk. Lipoic acid, which is also an amphiphilic antioxidant, has been shown to work synergistically with ascorbic acid to reduce oxidation in vegetable oils [66]. The complex of the aforementioned antioxidants, added into the appropriate phases at the appropriate levels, may work synergistically together to inhibit or greatly reduce the oxidation of omega-3 FA so that no detectable levels of oxidation components can be determined.

An additional method for the incorporation of omega-3 FA in food systems is the use of microencapsulation for protecting the lipids from oxidation. Polyunsaturated lipids can be coated in a protective matrix that helps protect against oxidation and gives added thermostability against heat. These microencapsulated lipids then can be spray-dried to produce a potentially oxidatively stable powder that could be added to food products [67].

Dairy products, such as cheese and butter, have been shown to be good delivery systems for elevated levels of omega-3 FA. When fortified with long-chain omega-3 PUFAs from fish oil, solid high-fat dairy products such as butter, processed cheeses and fresh spreadable cheeses, had the highest ability to mask the sensory characteristics of fish oil, especially when flavorings were present in the products [60]. These authors also found that one portion (~30g) of the aforementioned products, when fortified at acceptable sensory levels, could provide 180-360 mg of omega-3 PUFAs. Other recent studies have shown that cheddar cheese could be produced with up to 75% of the milk fat replaced with omega-3 FA in the form of fish oil without altering the characteristics of the cheese [68], which also could provide substantial amounts of omega-3 FA per serving, as well as calcium.

Researchers also have found that it was possible to produce fish oil-enriched milk that contained 1% milk fat and 0.5% w/w of omega-3 FA rich oil. This milk formulation was found to have acceptable sensory characteristics if the fish oil was blended with rapeseed oil and the blended oil had an initial peroxide value below 0.5 meq/kg^{-1}. One serving of this enriched milk, 250 ml or ~8 fl. oz, would supply approximately 125 mg of EPA and DHA in total, and 60 mg of ALA [69, 70].

Previous studies have shown that omega-3 FA enriched products can be produced that exhibit little if any negative sensory characteristics as a result of fish oil addition. However, these fortified products deliver amounts of omega-3 FA that are inadequate to achieve potentially positive health benefits based upon their consumption alone. The goal of this study was to produce an extended shelf-life omega-3 FA fortified product, with acceptable sensory characteristics, that could deliver physiologically beneficial levels of healthy omega-3 FA in one serving, and deliver the omega-3 FA equivalent in two fatty fish meals over the period of seven days with daily consumption.

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CHAPTER III

DELIVERING OMEGA-3 FATTY ACIDS BY FORTIFICATION IN AN EXTENDED SHELF-LIFE DAIRY-BASED BEVERAGE SYSTEM

ABSTRACT

Skim milk, butter-derived aqueous phase (BAP), butter oil (BO), and fish oil (FO; 3 levels) were used to produce an ultrahigh temperature (UHT) pasteurized omega-3 fatty acid fortified beverage (3.1% fat) with targeted deliveries of 200, 500, and 800 mg eicosapentaenoic acid and docosahexaenoic acid (EPA+DHA/250 mL (8 fluid oz)). Microbial quality, emulsion stability, and oxidation of lipids over 35 days of storage at 4°C were evaluated. Overall difference in aroma, compared to commercially processed aseptically packaged milk, was conducted by triangle test using an untrained consumer panel. Omega-3 fortified dairy-based beverages were produced containing 3.1% total fat, 3.9% protein, and 11.5% total solids. Conjugated diene hydroperoxides were below 1% of beverage lipids throughout storage but were found at highest concentrations around day 21 of storage for all formulations. Volatile analysis indicated an increase in 1-penten-3-ol in the omega-3 fortified dairy-based beverage systems during storage. The beverage system with targeted delivery of 500 EPA+DHA mg/250 mL was not different in aroma compared to commercially available UHT processed milk. This formulation delivered 440 mg of omega-3 fatty acids per 250 mL (8 oz.) serving and was microbially and physically stable throughout the 35-day refrigerated storage period.

INTRODUCTION

Increasing medical evidence supports the health benefits such as improved cardiovascular health and aiding in brain development associated with regular consumption of omega-3 fatty acids (FA) [1-6] . Implied health benefits and low consumption of omega-3 FA, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), from their natural food sources [7-9], has caused an increased trend in fortification of foods with healthy omega-3 FA. Over 200 new omega-3-FA fortified products in 2006 and over 400 in 2007 have been developed [9-11]. Sales of omega-3 FA fortified foods are predicted to grow to $7 billion in 2011, up from $2 billion in 2006 [12]. Recent studies indicate that the current intake of EPA+DHA in the typical western diet is 100 to 200 mg/day [7]; however the World Health Organization and the North

Atlantic Treaty Organization recommend 300 to 500 mg EPA+DHA/day and The American Heart Association recommends 500 to 1800 mg EPA+DHA/day [9], which is approximately equivalent to two fatty fish meals per week.

Dairy products can serve as good food vehicles for delivering omega-3 FA and increasing consumption of these healthful lipids. Recommended criteria for foods that are well-suited for fortification with omega-3 FA include frequent consumption, and processing, storage and packaging conditions that protect omega-3 FA from oxidation [10]. Extended shelf-life dairy products, which are recognized for excellent nutritional value, are stored at refrigeration temperatures and should be protected from light in order to protect the inherent dairy nutrient quality. Let and others [13, 14] produced a high temperature short time pasteurized fish oil-enriched milk with 1% milk fat and 0.5% w/w of omega-3 FA rich oil. This milk formulation, stored at 5°C and evaluated using descriptive analysis by a panel trained in descriptive analysis of fishy off-flavors as well as an untrained panel for acceptability, had acceptable sensory characteristics if the fish oil was blended with rapeseed oil. One serving (250 mL; ~8 fl oz) of this enriched milk supplied approximately 125 mg of EPA+DHA.

Challenges associated with incorporating omega-3 rich oil sources into dairy-based systems include sensory issues of the source oil and oxidative deterioration of these polyunsaturated fatty acids. Primary sources of omega-3 FA are of marine origin, algae and fish oils (FO), which can contribute to the overall taste and aromas of oils used for product fortification and are more easily noticeable in low-flavor systems, such as milk. Food products fortified with FO are susceptible to aromas indicative of the oil origin as well as from omega-3 FA oxidation, which decreases the nutritional impact of the product and produces volatile oxidation end-compounds that can negatively impact the aromas and/or flavors of the product. Omega-3 FA are polyunsaturated compounds that are very susceptible to oxidation due to the large number of C-C double bonds and *bis*-allylic carbons in their molecular structure (Figure 3-1). Extended shelf-life dairy-based beverages have increased risk of quality and nutritive deterioration from oxidation than traditional high temperature short time pasteurized beverages stored at refrigeration temperature.

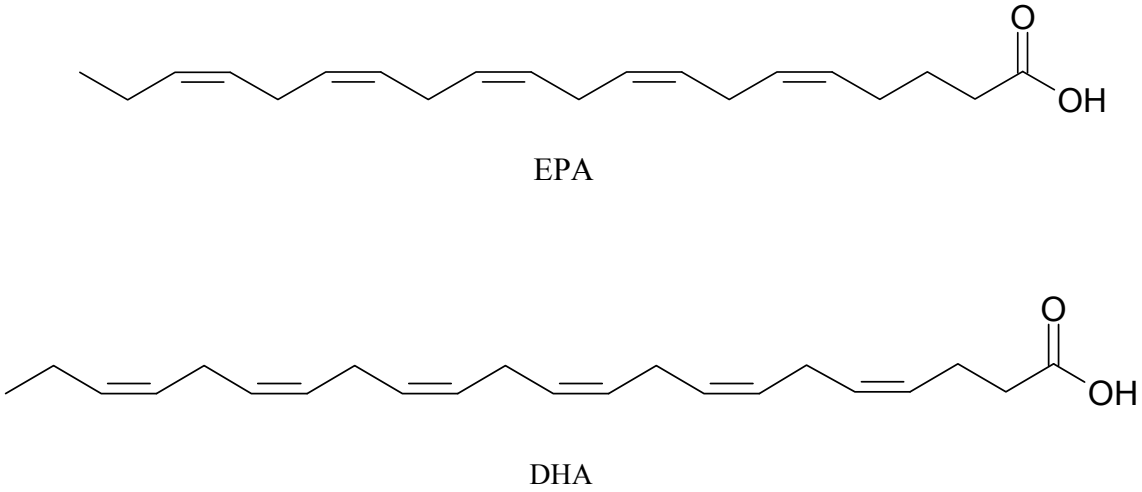

Figure 3-1. Molecular structures of eicosapentaenoic acid (20:5ω3) (EPA) and docosahexaenoic acid (22:6ω3) (DHA) omega-3 fatty acids.

The goal of this research was to develop an omega-3 FA fortified dairy-based fluid beverage system that could deliver at least 500 mg EPA+DHA per 250 mL (8 fl oz) serving. The objectives of this study were:

1. to produce an extended shelf-life omega-3 FA fortified dairy-based beverage system with physical and microbiological quality for 35 d shelf-life at 4°C. The low level of fish oil addition to the dairy-based system could contribute significantly to dietary EPA+DHA at 200 mg per serving. The higher levels of fish oil addition would provide, if one serving per day were consumed for a week, the equivalent EPA+DHA levels of 2 fatty fish meals per week;
2. to monitor oxidative degradation of omega-3 FA in the dairy-based system during storage;
3. to determine relationship of fish oil concentration to volatile chemistry from fish oil and oxidation of the system over 35 d shelf-life at 4°C;
4. to determine if aroma of omega-3 FA fortified dairy-based beverage was different than a commercially available UHT processed milk, using an untrained consumer panel.

MATERIALS AND METHODS

Dairy-Based Ingredients and Fish Oil for Formulation of Beverage Systems

Fresh raw milk was obtained from the Virginia Tech dairy farm, heated to 55°C then separated into cream (30 to 35% milk fat) and skim milk (skim) using a pilot plant separator (Elecrem, model 1G, 6400 rpm, Bonanza Industries, Inc., Calgary, Alberta, Canada). Skim was pasteurized at 63°C for 30 minutes then immediately stored at 4°C until subsequent use. Cream was pasteurized at 68°C for 30 minutes and immediately cooled via ice bath to 13°C. Pasteurized cream was tempered overnight in an incubator at 13°C, then churned using a mechanical churn (Gem Dandy Standard Electric Churn, Bonanza Industries, Inc., Calgary, Canada) to produce butter and buttermilk. Butter granules were separated from buttermilk by filtering through cheesecloth and rinsed (16 times) with cool water to remove any remaining buttermilk. Butter was liquefied by heating to 60°C with occasional stirring. Melted butter was transferred into separatory funnels for separation of oil and aqueous phase. Butter-derived aqueous phase (BAP) was collected in glass containers, purged with N_2 gas and frozen until time of use. Butter oil (BO) was transferred into centrifuge bottles and centrifuged (Sorvall RC-5B Refrigerated Superspeed Centrifuge) at 8000 RPM (10415 X g) for 15 minutes at 25°C. Centrifuged BO was decanted from residual BAP into a glass container, purged with N_2 gas, and then stored at -70°C in a commercial freezer (Forma Scientific, Inc, model 5479) until time of use.

Additional BAP was obtained commercially from Grassland Dairy Products, Inc. (Greenwood, WI) to supplement the BAP obtained from pilot plant sources. BAP was packaged with ice packs and shipped in insulated coolers. Upon receiving, BAP was immediately aliquoted into 400 ml volumes and stored at -18°C until use.

Food grade fish oil (Omega-3 TG grade fish oil) was obtained from a commercial supplier (Ocean Nutrition Canada LTD, Dartmouth, Nova Scotia, Canada). Fish oil consisted of anchovy and sardine oils with the addition of canola oil, natural flavor, tocopherols, sunflower oil, and citric acid, as stated on the packaging label. The ratio of the different types of fish and vegetable oils, and the levels of added antioxidants, all considered proprietary, were not disclosed. Fish oil

was aliquoted into 40 ml amber glass bottles, purged with N_2 gas and stored at 4°C until time of use.

Fatty Acid Profile and Conjugated Diene Value of Fish Oil and Butter Oil

Fatty acid (FA) profiles of FO and BO were determined prior to formulation of the dairy-based beverage systems. FA profiles were determined using gas chromatography after conversion to FA methyl esters (FAME) following AOCS official method Ce 1b-89 [15], without the use of a correction factor since a flame ionization detector was not used. A Shimadzu QP 5050 GC/MS (Shimadzu, Kyoto, Japan) equipped with a SP-2560 (cross-linked and bonded *bis*-cyanopropyl polysiloxane, Supelco Corp., Sigma-Aldrich, St. Louis, MO) capillary column (100m x 0.25mm) was used for fatty acid analysis. Helium carrier gas flow was 26.9 cm/sec and column flow was 1.7 mL/min. A program using a split ratio of 1:20, injector temperature of 270°C, and detector temperature of 230°C was used. Initial run temperature was 130°C ramped to 240°C at a rate of 2°C/min with a hold time of 10 min. Total run time was 65 min. Fatty acids were identified using a quadrupole mass analyzer. Solvent (1µL) was directly injected into the GC/MS for analysis. A 37 component FAME mixture (Supelco, Bellefonte, PA) was used as an external reference standard to identify retention times of FAMEs. EPA and DHA FAME in the FO, as well as other major FAME in the FO and BO, were quantified following AOCS methods [15]. Methyl tricosanoate (C23:0) methyl ester (Nu-Chek-Prep Inc., Elysian, MN) was used an internal standard. Internal standard (25mg/ml) was added (1ml) to lipid samples prior to quantification following AOCS methods [15].

Conjugated dienes were determined for BO and FO following AOCS official method Ti-1a-64[16]. Each lipid (25 mg) was dissolved into 25ml of isooctane to achieve stock solutions of 1 g/L. Stock solutions were diluted using isooctane to prepare test solutions of ~0.4 g/L. Absorbance at 233nm was measured in triplicate for each test solution using a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). Results were reported as % conjugated dienoic acid.

Production of Omega-3 Fortified Dairy-Based Beverages

Four dairy-based beverages were prepared with different concentrations of fish oil. Formulations targeting a total fat content of 3.25% fat, but differing in fatty acid concentration based on FO content, were prepared using skim milk, BAP, BO, and FO. Table 3-1 shows each formulation and ratio of BO:FO per beverage formulation.

Dairy-based beverage formulations were homogenized and ultra-pasteurized. Homogenization occurred in two stages (13.6MPa (2000psi), 3.4Mpa (500psi)) using a laboratory homogenizer (Microthermics, Laboratory In-Line Homogenizer, Type NS2006H , Microthermics, Inc., Raleigh, NC) and ultra-pasteurized using a tubular laboratory pasteurization system (Microthermics, UHT/HTST Laboratory 25-HV, Microthermics, Inc., Raleigh, NC) at 148°C for 2 seconds. Immediately after processing, formulations were transferred into sterilized 40ml glass sampling containers (for physical properties analysis) and stored in a walk-in cooler (Tonka, Hopkins, MN) at 4°C for 35 days.

Evaluation of Physical and Chemical Quality Parameters

Sampling schedule for all analyses (microbiological, composition, and chemical quality parameters) were completed for all formulations as indicated in Table 3-2.

Table 3-1. Omega-3 fortified dairy-based beverage formulation composition based upon lipid content with calculated estimate of omega-3 fatty acid per serving (250 mL)

BO:FO[1] Treatments	100:0 (control)	90:10	78:22*	62:38
Proportion of lipids (3.25%)				
BO (%)[2]	3.25	2.90	2.55	2.00
FO (%)[2]	0.00	0.35	0.70	1.25
Proportion of aqueous components (96.75%)				
Skim milk (%)	90	90	90	90
BAP[1] (%)	10	10	10	10
Fat (g) per 8 oz. serving				
Milk fat (BO)	7.93	7.08	6.22	4.88
FO	0.00	0.85	1.71	3.05
Omega-3 FA	0.00	0.20	0.50	0.80

*Indicates formulation designed for targeted delivery amount of omega-3 fatty acids

[1]BO: butter oil; FO: fish oil; BAP: butter oil-derived aqueous phase

[2]wt/wt %

Microbial Analysis. Standard plate counts of aerobic, coliform, and yeast and mold organisms were performed based on standard methods [17] to ensure each dairy-based beverage was properly pasteurized and maintained satisfactory microbial quality. Dairy formulations were plated at 10^{-1}, 10^{-2}, and 10^{-3} concentrations using Petrifilm™ Aerobic Count Plates, Petrifilm Coliform Count Plates, and Petrifilm Yeast and Mold Count Plates (3M, St. Paul, MN). Dairy blanks used for dilutions in the microbial analyses were produced using solutions of $MgPO_4$, KCl, and deionized water following standard methods[17]. Aerobic count plates and coliform count plates were incubated at 32°C for 48 h. Yeast and mold count plates were incubated at 25°C for 72 h.

Table 3-2. Sampling schedule and analytical analyses for omega-3 fortified dairy-based beverage systems stored at 4°C.

Test↓ Day→	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	21	28	35
Microbiological	*							*							*	*	*	*
Fatty acid analysis	*	*						*							*			*
Protein content			*															
Total lipid					*													
Total solids							*											
Emulsion stability	*		*		*			*		*		*			*	*	*	*
Conjugated dienes	*	*		*		*		*				*			*	*	*	*
Headspace GC/MS	*	*		*		*		*				*			*	*	*	*

Fat, Protein, and Total Solids Determination. Total fat content was determined by analyzing the formulations, in duplicate, using the Babcock procedure [18]. Protein content was determined spectrophotometrically using a dye-binding assay (RC DC BioRad assay, BioRad laboratories, Hercules, CA). Bovine serum albumin (BSA, Sigma) was used to prepare protein standards (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL BSA) used for protein analysis. Total solids (TS) were determined by analyzing the formulations, in duplicate, following standard methods [19]. Dairy formulations were weighed (2-3 g) into pre-dried and pre-weighed aluminum weigh pans. Samples were place in a forced air oven (Blue M, model OY-490A-2, Blue Island, Il.), heated/dried for 6 hrs at 103°C, placed into desiccator for 30 min, then weighed.

Fatty Acid Analysis. Lipids were extracted using a modified Folch method [20]. Each formulation (15 mL) was combined with 110 mL of solvent (2:1 methanol: chloroform) and shaken for 5 min. Solutions were filtered and filtrates transferred into separatory funnels along with 40 mL of 0.9% NaCl solution. Separatory funnels were shaken and allowed to sit overnight for phase separation. The bottom layer, containing solvent and extracted lipids, was collected in round bottom flasks. Solvent was removed from lipids using a rotary evaporator (Evapotec, Haake Buchler, Germany). Extracted lipids were analyzed for fatty acid profiles using a Shimadzu CG-17A gas chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD) following protocols previously described.

Emulsion Stability. Emulsion stability of dairy-based beverages was determined over 35 days of storage following the procedures of Elling and Duncan, 1996 [21]. Dairy-based beverages were

placed into 100 mL graduated cylinders and stored at 4°C on the day of processing. Emulsion stability was tested frequently over the first 14 days of storage then on a weekly basis (Table 3.2). For each test day, two cylinders of each dairy-based beverage formulation were tested (duplicates) using the Babcock procedure [18]. The change in fat content in top and bottom layers within each cylinder was determined by:

Change in fat percentage = [((fat content of top or bottom layer) / (initial fat content) x 100) – 100]

Determination of Oxidative Stability. Oxidation of lipids in the dairy-based beverages was tracked as a function of time during the 35 day storage period. Lipids were extracted from the dairy-based beverages using the modified Folch method [20] previously described. Extracted lipids were transferred into glass storage containers, purged with nitrogen, and then stored at -80 °C until analysis. Oxidation was assessed via determination of conjugated dienes and fatty acid analysis as previously described. Conjugated diene analysis [16] was used to monitor hydroperoxide development originating from oxidation of fatty acids, and fatty acid analysis [15] to determine changes in omega-3 lipid concentration over time. Conjugated diene (in duplicate) and fatty acid analysis was performed on each formulation on days indicated in Table 3.2.

Determining Volatile Chemistry by Solid Phase Micro-Extraction GC/MS. Volatile oxidation compounds formed during storage of the dairy-based beverages were monitored using a solid phase micro-extraction method coupled with GC/MS. On the day of formulation, 1 g of each formulation was transferred into 20 ml amber glass head space vials, sealed with a headspace aluminum cap equipped with a PTFE septum, and stored at 4°C until time of analysis.

Volatile oxidation compounds were extracted and concentrated using a 75 μm carboxen-polydimethyl siloxane (CAR-PDMS) solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA). The CAR-PDMS fiber was conditioned at temperatures recommended by the manufacturer before use. A blank analysis was performed using an empty sealed vial to ensure that extraneous compounds adsorbed from the atmosphere were desorbed from the fiber before sample analysis. Sample vials were heated to 50 ºC while being agitated at 250rpm. The SPME

fiber was exposed 22mm into the vial headspace for 15 min during heating and agitation. Volatile oxidation products adsorbed onto the SPME fiber were desorbed and analyzed using an HP5890A (Hewlett Packard, Palo Alto, CA) coupled with a HP5972 series mass selective detector (Hewlett Packard, Palo Alto, CA). A DB-5 capillary column (30m x 0.25 mm I.D. x 0.25μm film thickness, J&W Scientific, Folsom, CA) was used for volatiles separation. Injector temperature was 250 ºC, detector temperature was 265 ºC, and program was run in splitless mode with helium carrier gas at a constant flow rate of 1 mL/min. Initial oven temperature was 35 ºC, held for 7 min. Temperature then was ramped to 120 ºC at a rate of 5 ºC/min, then to 220 ºC at a rate of 10 ºC/min and held for 10 min [22]. Total GC run time was 44 min. Oxidation volatiles were identified by MS internal library searches and comparison of retention times of external standards. SPME-GC/MS was performed on each formulation on days indicated in Table 3.2. External standards of acetaldehyde, propanal, pentanal, 1-penten-3-one, 1-penten-3-ol, hexanal, and 2,4-heptadienal were used to identify and track oxidation products. Fresh and oxidized reference samples of FO, BO, and commercially available UHT milk (2% fat) were analyzed for headspace volatiles to help identify compounds that may be present during storage of the dairy-based beverages.

Sensory Evaluation of Dairy-Based Beverage Systems

Sensory Influence of Fish Oil. One dairy-based formulation (78% BO:22% FO) was evaluated, within one week of processing, for the aroma influence of FO addition. Effect of time (shelf-life) was not studied during sensory evaluation as the purpose was to determine if addition of FO influenced the aroma of the dairy-based beverage. Sensory studies were pre-approved by the Institutional Review Board (IRB) of Virginia Tech (Appendix A). Twenty-five untrained panelists participated in each sensory session. Panelists were recruited from the Virginia Tech Department of Food Science and Technology and consisted of faculty, staff, graduate and undergraduate students. A triangle test for difference [23] was used to evaluate if aroma characteristics of FO and the dairy-based beverage system were different from a commercial UHT pasteurized aseptically packaged 2% fat milk (Parmalat, Parma, Italy) containing no FO. Approximately 15 ml of each dairy product were poured into 1 oz plastic soufflé cups, capped, and stored at 4°C. Samples were assigned three digit codes and randomized for a balanced order of presentation to sensory panelists. Panelists were presented with two sets of three samples with

the task of smelling each sample and identifying the odd sample in each set. Sensory tests occurred in the sensory laboratory of Virginia Tech's Department of Food Science and Technology under white fluorescent lighting. SIMS 2000 (Sensory Information Management System, Sensory Computer Systems, LLC, Morristown, NJ) was used for sensory test design and data analysis.

Panelists completed a demographics survey (Appendix B) after completion of the sensory samples. The survey asked demographic questions and also inquired about panelists' attitudes toward dairy and omega-3 FA consumption. All panelists completed human consent forms for each day of sensory analysis (Appendix C).

Statistical Analysis

This study was replicated two times. Analytical analyses were completed in duplicate within each replication with the exception of fatty acid analyses. The main factors in the experiment were the ratio of lipids (milkfat:omega 3), time, and replication. However, since time was not independent, a two-way analysis of variance with repeated measures was used to assess changes in oxidation and physical stability due to ratio of lipids. Statistical analyses were completed using JMP (SAS Institute, Cary, NC). Alpha level of 0.05 was preset for determining significant differences. Least Significant Differences test was used for mean separation when significant differences were found.

Sensory data from triangle tests was analyzed based upon statistical parameters of $\alpha = 0.05$, $\beta = 0.4$, $P_d = 30\%$, and equations outlined in Meilgaard and others (2007) by replication as well as for commingled data.

RESULTS AND DISCUSSION

Dairy formulations were not different in total fat (3.08% ±0.08), protein (3.89% ±0.83%), total solids (11.45% ±0.17), and total solids not fat (8.37% ±0.21) compared to the composition of whole milk (Table 3-3). Total solids not fat were above the 8.25% required by the CFR [24]. UHT processing of the dairy-based beverages and refrigerated storage conditions effectively controlled microbial growth during the 35 day storage period (Appendix D).

Emulsion stability of the dairy-based emulsions was tracked during storage. As the emulsion stability decreases during storage, increasing amounts of fat will migrate to the top of the emulsion. This layer of fat is referred to as a cream plug and is characteristically observed in unhomogenized milk and cream. Emulsion stability of the dairy-based systems indicated that beverages were properly homogenized. Emulsion stability data indicated no significant difference ($p>0.05$) in change in percent fat of the upper and bottom layers of the emulsions during storage, though differences were significant between replications ($p<0.05$) (Appendix E). The formulated dairy-based beverage systems were comparable in gross composition and physical stability.

Table 3-3. Gross composition ($\bar{x}$ [1]± sd) of fish oil fortified UHT pasteurized dairy-based beverage systems

Formulation BO:FO (W/W %)	% fat $\bar{x}$ (± sd)	% protein $\bar{x}$ (± sd)	%TS $\bar{x}$ (± sd)	%TS not fat ($\bar{x}$ ± sd)
100 : 0	3.00(±0.02)	3.86(±0.56)	11.48(±0.02)	8.48(±0.02)
90 : 10	3.11(±0.04)	3.73(±0.54)	11.37(±0.19)	8.26(±0.25)
78 : 22	3.13(±0.06)	4.08(±0.49)	11.65(±0.04)	8.52(±0.02)
62 : 38	3.08(±0.03)	3.91(±0.72)	11.28(±0.14)	8.21(±0.16)

[1] Averages of 2 replications for each formulation
***** Means within columns are not significantly different (p>0.05)

Fish oil source contained a total of 34% (33.57) omega-3 fatty acids, including EPA at 21.4% and DHA at 12.1%. Major fatty acids found in BO included myristic, palmitic, stearic, oleic, and linoleic acids [25, 26]. FO and BO fatty acids were represented in the dairy-based beverages (Figure 3-2).

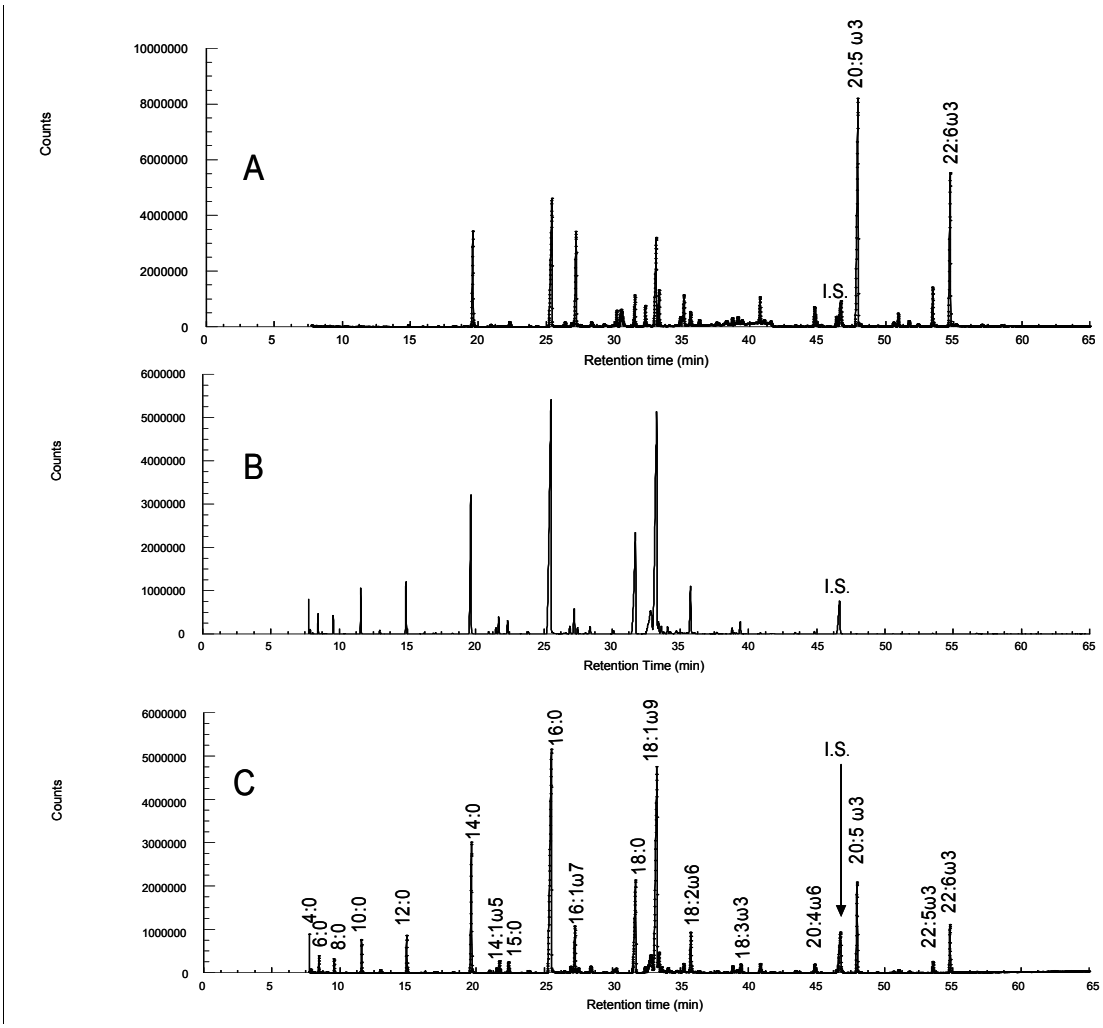

Figure 3-2. Chromatograms of transesterified (A) fish oil, (B) butter oil, and (C) extracted lipids from 78:22 target formulation, showing peaks of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and $C_{23:0}$ internal standard (I.S.).

Using tricosanoic acid ($C_{23:0}$) methyl ester as the internal standard, the amount of omega-3 FA in the FO was determined to be 335.7 mg/g FO. The amount of EPA and DHA was determined to be 214.4 mg/g and 121.3 mg/g FO, respectively. Conjugated diene values for FO and BO were determined to be 0.99 (±0.08) and 0.70 (±0.02) respectively, indicating that the source oils were not oxidized.

Fatty acid profiles of lipids extracted from the omega-3 fortified dairy-based beverages were determined to monitor omega-3 lipid concentrations and track oxidation during storage. Figure 3-3 shows the amounts of EPA+DHA in the omega-3 fortified formulations throughout the 35-

day shelf-life. Table 3-4 shows the major fatty acids in the FO, BO, and 78:22 (BO:FO) dairy-based beverage extracted lipids during storage.

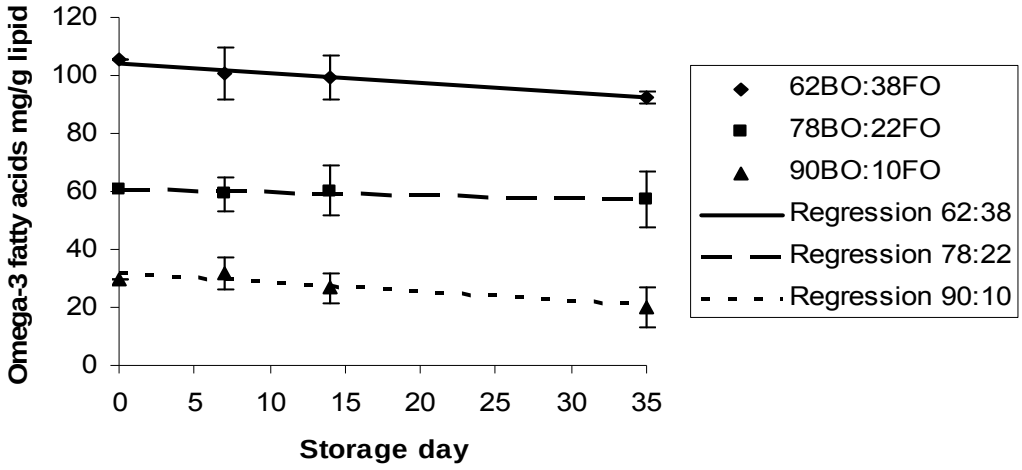

Figure 3-3. Total omega-3 fatty acids (EPA+DHA, mg/g extracted lipid) determined in omega-3 FA fortified dairy-based beverages during 35 days storage (4°C).

Table 3-4. Concentrations of major fatty acids identified in fish oil and butteroil source lipids and UHT processed omega-3 FA fortified dairy-based beverage (78:22 BO:FO) during 35 days of storage at 4°C.

			Concentration (mg/g) lipid ($\bar{x}$ ± sd)			
			Dairy-based beverage			
	Source Lipid		(100:0 %BO:%FO)		(78:22 %BO:%FO)	
Fatty acid	FO[A]	BO[A]	Day0[B]	Day 35[A]	Day0[B]	Day 35[A]
4:0	ND	1.1(± 0.3)	0.9	0.5(± 0.4)	0.5	0.5(± 0.1)
6:0	ND	3.3(± 1.2)	4.9	2.5(± 0.4)	3.1	2.4(± 0.3)
8:0	ND	3.5(± 1.1)	4.9	2.8(± 0.7)	3.1	2.9(± 0.2)
10:0	ND	11.0(± 3.5)	13.7	8.9(± 2.5)	9.3	9.6(± 0.9)
12:0	0.9(± 0.0)	15.5(± 4.3)	18.0	12.3(± 4.2)	12.6	14.1(± 2.2)
14:0	66.1(± 2.6)	61.7(±11.3)	69.6	46.7(±17.9)	59.5	63.0(± 9.1)
14:1ω5	1.1(± 0.4)	5.9(± 1.7)	7.0	4.3(± 1.2)	4.6	4.9(± 0.6)
15:0	3.6(± 0.2)	5.2(± 1.4)	5.8	3.8(± 1.3)	4.7	4.8(± 0.6)
16:0	147.5(±11.2)	214.4(±12.6)	220.7	144.6(±62.1)	181.7	185.3(±25.7)
16:1ω7	78.8(± 1.4)	11.1(± 2.6)	12.7	7.6(± 2.9)	23.6	22.7(± 1.2)
18:0	27.7(± 2.1)	84.1(± 1.3)	82.8	59.3(±30.7)	64.5	67.6(±11.9)
18:1ω9	91.2(± 4.0)	238.2(±21.4)	221.5	147.5(±73.3)	169.8	173.6(±31.1)
18:2ω6	10.4(± 0.5)	27.5(± 1.6)	28.2	19.7(±10.8)	21.9	21.5(± 2.3)
18:3ω3	5.2(± 0.2)	4.8(± 1.6)	2.5	2.0(± 1.2)	2.9	3.4(± 1.0)
20:4ω6	21.2(± 0.2)	1.1(± 0.1)	1.1	0.8(± 0.3)	4.4	4.5(± 0.5)
20:5ω3	213.7(± 3.9)	ND	ND	ND	40.7	38.0(± 5.5)
22:5ω3	27.4(± 3.6)	ND	ND	ND	5.0	4.7(± 0.6)
22:6ω3	114.2(±15.4)	ND	ND	ND	21.4	20.4(± 4.4)
Saturated	245.8(±11.7)	399.9(±17.9)	421.3	281.3(±71.7)	339.0	350.2(±29.9)
Mono+Poly unsaturated	563.2(±16.8)	288.7(±21.7)	273.1	181.9(±74.2)	294.4	293.7(±32.0)
EPA+DHA	327.9(±15.9)	ND	ND	ND	62.1	58.4(± 7.0)

[A]n=2 replications
[B] n=1 replications

Omega-3 FA are naturally present at very low levels in milk, approximately 0.25% of total fatty acids [27] and were not detected in the 100% butteroil control formulation in this study. Much work has been done to increase the levels of omega-3 FA and the nutritive quality of milk through pre-harvest technologies such as supplementing dairy cow diets with FO and flax seed or flax seed oil [27-32]. McDonald and Scott [33] reported that large increases in linoleic acid (18:2ω6) had been observed in cow's milk through dietary modifications, however the milk was more prone to autoxidation compared to conventional cow's milk. Petit and others [28] reported that alpha-linolenic acid was increased from 1.0 to 13.9% of milk fatty acids by using a duodenal infusion of linseed oil. In a study using different feed types used for fat sources, Petit [31] reported that feeding flaxseed to lactating Holstein cows resulted in the lowest omega-6-to-omega-3 FA ratio, which improved the nutritive quality of the milk from a human health aspect. In studies where diets of dairy cows were supplemented with FO, Shingfield and others [30] reported that EPA and DHA levels increased in milk; however, transfer efficiencies of the omega-3 FA from feed to milk was very low at 0.020 and 0.018% for EPA and DHA respectively. Donovan and others [27] reported that when dairy cows were fed 2 and 3% FO diets, total omega-3 FA in their milk increased 3.3 and 4.1%, respectively, from levels present when fed a 0% FO diet. While pre-harvest techniques have been shown to be effective in raising omega-3 FA levels in milk, the efficiencies of these methods are quite low. Investigators also have reported that FO supplemented diets resulted in decreased concentrations of total fat and protein in the milk [27, 28, 30, 32].

The FO fortified dairy-based beverages contained different amounts of omega-3 FA among formulations, reflecting levels of fortification. Omega-3 FA present in all fortified beverages decreased during storage, suggesting oxidation of lipids occurred. Table 3-5 shows the amounts of omega-3 FA (mg) in 8 oz of each fortified beverage, reflecting a serving, on days 0 and 35 of storage and also shows a decrease in omega-3 FA content over the storage period. However, due to no standard deviation for values on day 0 and a high standard deviation for values on day 35, there may be no significant difference in omega-3 FA levels in the dairy-based beverages at the beginning and end of the storage period. Total omega-3 FA decrease over 35 days of storage (mg omega-3 FA/8oz) in all omega-3 FA fortified dairy-based beverages ranged from 6.6 to 34.4%,

with the 78:22 formulation exhibiting the smallest decrease and the 90:10 formulation exhibiting the largest. AOX present in the FO may have contributed to inhibiting oxidation of lipids present in the dairy beverages.

Table 3-5. Omega-3 FA levels (mg / 8oz) in omega-3 FA fortified dairy-based beverages (3.1% fat) during 35 days storage at 4°C

Formulation (%BO:%FO)	Fatty acid	Day 0 (mg/8oz) [1] $\bar{x}$ ± sd	Day 35 (mg/8oz)[1] $\bar{x}$ ± sd	% decrease[2]
90:10	EPA	156.6	101.5(±32.9)	35.1
	DHA	75.3	50.6(±16.3)	32.8
	Total ω -3	231.8	152.2(±49.3)	34.3
*78:22	EPA	312.3	288.5(±46.6)	7.6
	DHA	160.5	151.8(±34.9)	5.4
	Total ω -3	472.8	440.3(±58.2)	6.8
62:38	EPA	546.2	458.4(±25.8)	16.1
	DHA	271.1	244.8(±11.4)	9.7
	Total ω -3	817.3	703.3(±28.2)	14.0

[1] 1 replication for day 0; 2 replications for day 35
[2] Based on change in means between day 0 and day 35
*Indicates formulation designed for targeted delivery amount of omega-3 fatty acids
EPA= Eicosapentaenoic acid, DHA=Docosahexaenoic acid

EPA, DHA, and total ω3 FA decreased during storage (Table 3-5). The targeted delivery formulation (78:22, BO:FO) on storage day 35 provided 440.3 mg of ω3 FA per 8oz, close to the targeted value of 500 mg/8oz. Though a reduction of omega-3 lipid levels was observed between storage day 0 and day 35, the concentrations of omega-3 FA present in the target formulation were maintained at a level that would deliver a significant amount of the recommended daily intake of omega-3 FA. One 8 oz serving per day of this formulation, over the span of 1 week, would provide 3082mg of ω3 FA, which is nearly equivalent to the amount of ω3 FA found in two servings of fatty fish, such as salmon (~1825mg EPA+DHA/serving(3oz)) [34], and the ~500mg daily intake recommendation of the American Heart Association [35]. Let and others

[13] , in a study on sensory stability, produced a HTST FO-enriched milk product that was calculated to contain approximately 125 mg EPA+DHA per 8oz. Other omega-3 fortified milk products on the current market provide much smaller amounts of EPA+DHA. Horizon Organic™ DHA Omega-3 milk (Horizon, Boulder, CO) was calculated to provide 32mg of EPA+DHA/8oz, or approximately 224mg per week if one serving is consumed per day.

Determination of Oxidative Stability of Omega-3 FA Fortified Dairy Based-Beverages

Susceptibility to oxidization of the added polyunsaturated fatty acids (PUFA) is of concern due to reduced nutritional value and the production of oxidative products that contribute to negative sensory qualities. Conjugated diene analysis was a convenient and rapid method for monitoring oxidation because this method required minimal reagents and no reaction waiting time.

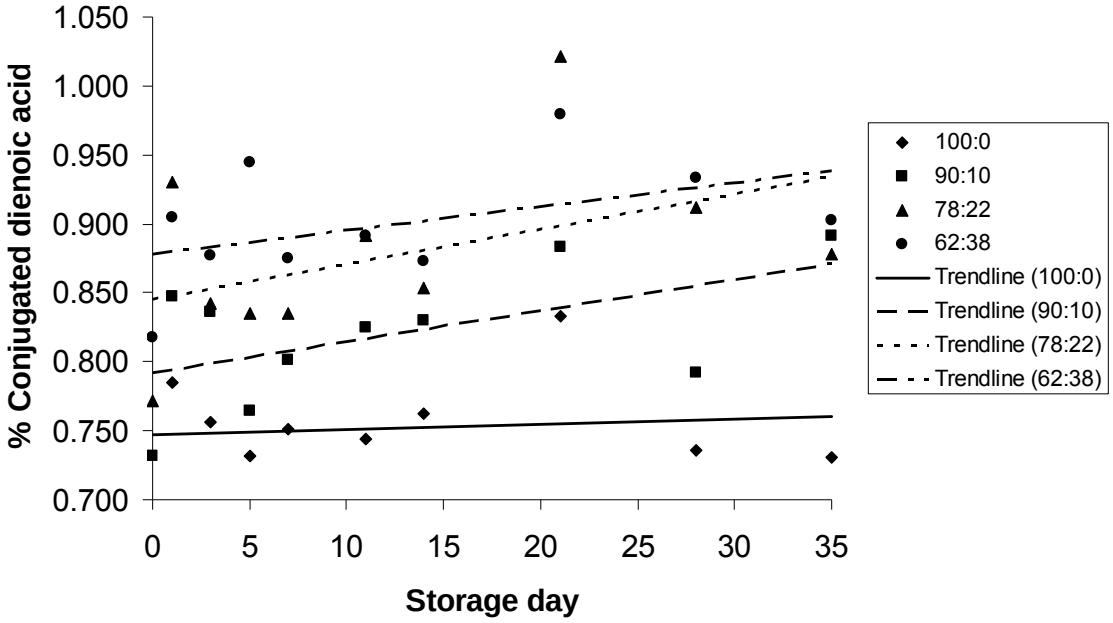

Figure 3-4. Percent conjugated dienoic acid for all UHT processed omega-3 FA fortified (%butter oil:% fish oil) dairy-based beverages (3.08% fat) during 35 days of storage at 4°C.

All formulations exhibited a spike in %CD around day 21 of storage (Figure 3-4). Conjugated diene hydroperoxides are primary oxidation products in which formation would follow oxidation of the lipids present in the sample. This increase in %CD around day 21 suggests that the highest level of lipid oxidation occurred around that time. Results from CD analysis were modeled via linear regression trendlines to indicate an overall trend in CD formation. Trendlines indicate that

an overall increase in CD formation occurred for each formulation during the storage period. The increase in CD values suggests that as storage time increased, oxidation of lipids occurred. Initial CD values of each formulation corresponded to the ratio of FO in the formulation and reflected the differences in CD of the source oils. All omega-3 FA fortified beverages exhibited an overall, but not significant ($p>.05$),increase in CD values during storage, with steeper slopes of trendlines for omega-3 FA fortified beverages than for the 100% butteroil formulation (Figure 3-4), though this increase was not significant ($p>0.05$) either.

Mean values of conjugated diene values were typically below 1.0 for all formulations throughout storage (Table 3-6). There were no significant differences among CD values for all formulations for each day of analysis. Replication was found to be significantly different ($p<0.05$). The lack of significant differences and low CD values throughout storage may indicate that AOX present in the bulk FO contributed to inhibiting oxidation in the fortified dairy-based beverage systems. Results from conjugated diene hydroperoxide analysis have been shown to correlate well with results from peroxide value analysis [36-39]. Frankel [40] reported that conjugated diene formation parallels the formation of hydroperoxides, which occurs in the early stages of lipid oxidation, and subsequent decomposition into secondary oxidation products. Marmesat and others [38] reported that in high linoleic and high oleic sunflower oils, conjugated diene values of 2.6 and 1.7, respectively, corresponded to peroxide values of 2meq O_2/kg oil, which generally indicates a slight degree of oxidation. It may be concluded that CD values less than one indicate a low degree of oxidation.

Table 3-6. Percent conjugated dienoic acid ($\bar{x}$ ± sd) of omega-3 FA fortified dairy-based beverages (3.1% fat) over 35 days of storage (4°C)

	% Conjugated Dienoic Acid By Formulation (Butter oil:Fish oil)			
Storage day	100:0 $\bar{x}$ ± sd	90:10 $\bar{x}$ ± sd	78:22 $\bar{x}$ ± sd	62:38 $\bar{x}$ ± sd
0	0.69(±0.06)	0.73(±0.03)	0.77(±0.07)	0.88(±0.01)
1	0.78(±0.00)	0.85(±0.09)	0.93(±0.03)	0.87(±0.07)
3	0.76(±0.11)	0.84(±0.13)	0.84(±0.15)	0.94(±0.08)
5	0.73(±0.00)	0.76(±0.00)	0.84(±0.06)	0.91(±0.10)
7	0.75(±0.02)	0.80(±0.06)	0.84(±0.00)	0.93(±0.03)
11	0.74(±0.05)	0.83(±0.03)	0.89(±0.04)	0.85(±0.02)
14	0.76(±0.08)	0.83(±0.10)	0.85(±0.10)	0.95(±0.03)
21	0.83(±0.05)	0.88(±0.03)	1.02(±0.04)	1.00(±0.00)
28	0.74(±0.08)	0.79(±0.11)	0.91(±0.10)	0.95(±0.11)
35	0.73(±0.06)	0.89(0.06)	0.88(±0.11)	0.96(±0.02)

[1]n=2 replications
*Results are expressed mean ± standard error of mean.

Volatile Analyses for Aroma and Oxidative Stability Indices

Analysis of volatile compounds via SPME/GC/MS is an effective technique for isolating and identifying flavor compounds in food. The technique is simple, quick, requires no solvent, and can be used to effectively analyze volatiles of solid, liquid, and gaseous samples [41]. In this study, SPME/GC/MS was used to monitor the formation of volatile oxidation products that could negatively impact the aroma qualities of the omega-3 fortified dairy-based beverages. PUFAs present in fish oil, mainly omega-3s, oxidize and the result is the formation of lipid hydroperoxides. Frankel [42] reported that lipid hydroperoxides are odorless and tasteless, and

that further oxidation of these compounds into secondary oxidation products are accountable for the off-aromas and flavors found in oxidized foods. Acetaldehyde, propanal, 2-butanone, 2-pentanone, 1-penten-3-ol, hexanal, and 2-heptanone were identified in the omega-3 FA fortified dairy-based beverages and in fish oil, butteroil, and commercial shelf-stable UHT milk. These compounds are in agreement with those previously reported in FO and omega-3 FA enriched dairy products [22, 43-47]. Jimenez-Alvarez and others [22] and Iglesias and others [45] reported the formation of acetaldehyde, 1-penten-3-ol and propanal as oxidative volatiles produced in the headspace of FO and FO emulsions. Contarini and others [48] analyzed UHT pasteurized milk using headspace GC and concluded that ketones, aldehydes, terpenes, and sulfur and aromatic compounds were all present, with ketones representing the most abundant class of volatiles identified including 2-butanone, 2-pentanone, and 2-heptanone.

Retention times of the external standards used during headspace volatiles analysis can be seen in Appendix F and full-scan chromatograms of the FO, BO, and commercial UHT milk external references can be seen in Appendix G. From the full scan external reference chromatograms, it can be observed that most of the volatile compounds adsorbed onto the SMPE fiber eluted from the GC column within 20 minutes of the GC run. Adsorbed compounds eluting from the GC column after 20 minutes were identified as being present in the polymer septums of the headspace vials. Levels of septum compounds increased with storage time as noted in the full scan chromatograms. Figure 3-5 shows partial chromatograms (up to 5 min) for fresh and oxidized FO.

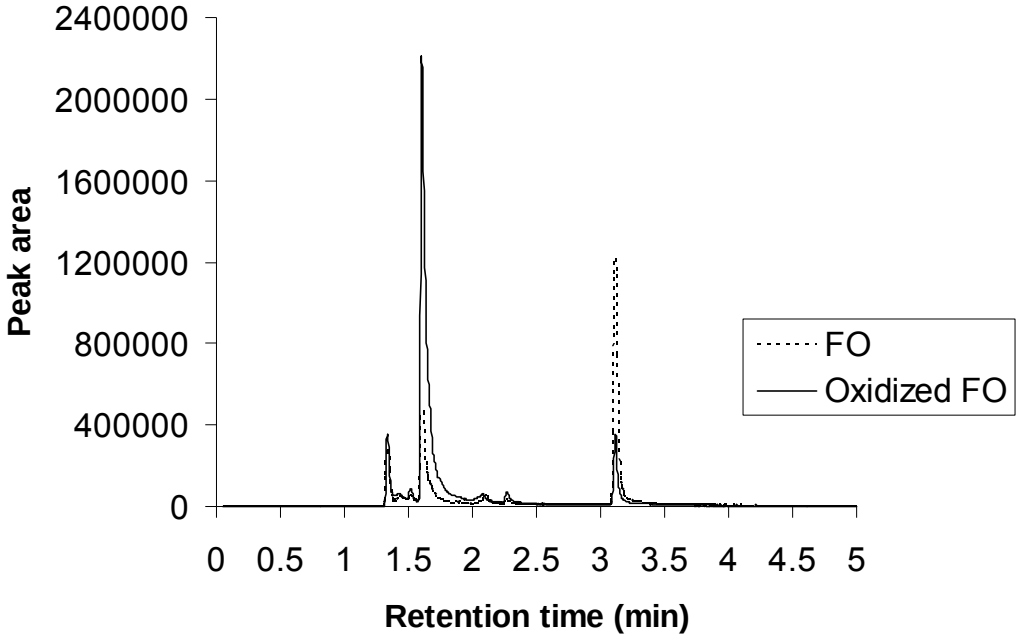

Figure 3-5. Partial headspace analysis chromatograms of volatiles collected from fresh and oxidized fish oil.

Chromatogram peaks are of similar intensity for both fresh and oxidized FO samples with the exception of peaks at 1.6 min and 3.2 min, identified as propanal and 1-penten-3-ol, respectively. Oxidized FO had a larger peak at 1.6 min while fresh FO had a larger peak at 3.2 min. Figure 3-6 shows partial headspace chromatograms of fresh and opened commercial UHT milk.

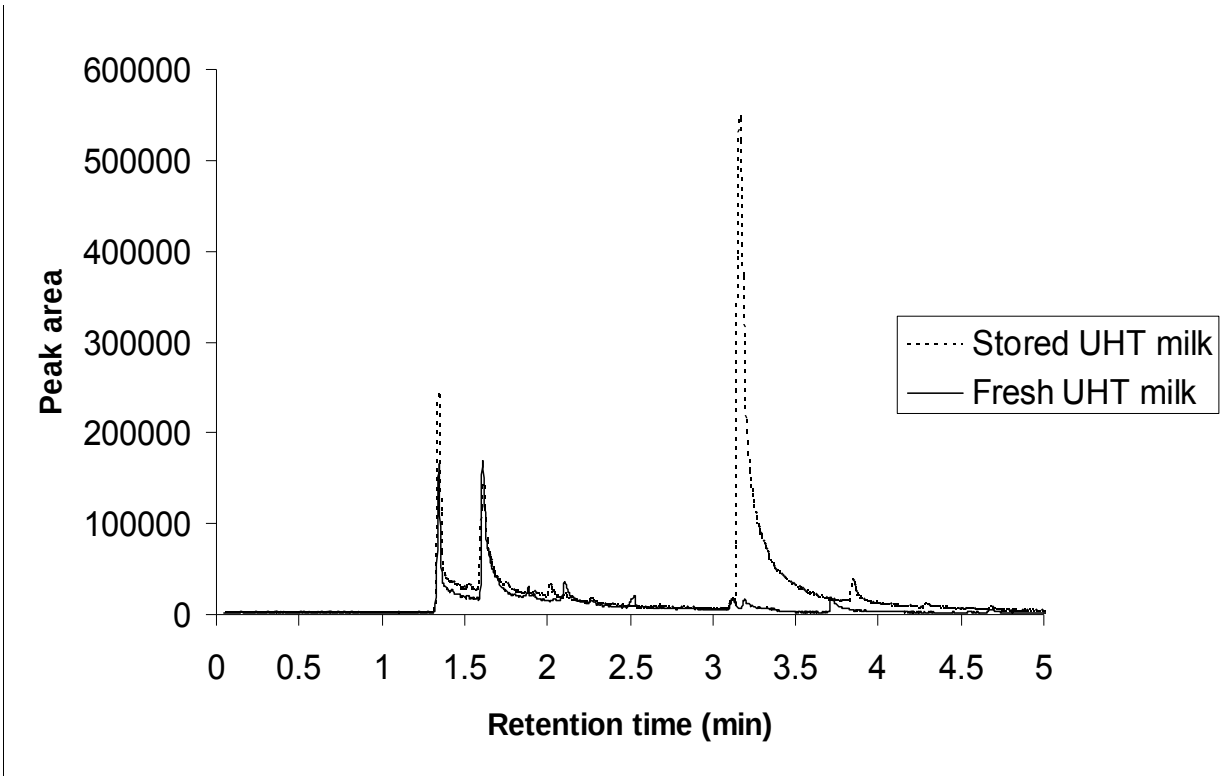

Figure 3-6. Partial headspace analysis chromatograms of volatiles collected from fresh and opened commercial UHT milk.

Chromatogram peaks are similar for both stored and fresh commercial UHT with the exception of peaks at 1.4 and 3.2 min, identified as acetaldehyde and 2-pentanone, respectively. Stored commercial UHT milk had larger peaks at 1.4 and 3.2 min. It also was observed that a peak eluting at 11.4 min, identified as 2-heptanone, was noticeably larger in the stored sample than in the fresh sample. Contarini and others [48] concluded that keeping UHT processed milk at 4°C decreased processes that contribute to the development of flavor defects during the extended shelf-life of the product. These findings suggest that the compounds increased as the UHT milk aged after opening, possibly due to oxidation or enzymatic reactions. It should be noted that 1-penten-3-ol and 2-pentanone were both observed to elute from the GC column at approximately 3.2 minutes and both have been shown to be present as a result of degradation in FO and UHT processed milk, respectively, so it may be concluded that both compounds would contribute to the total peak area of the peak at 3.2 min observed in the chromatograms for the omega-3 FA fortified dairy-based beverages.

Omega-3 FA-fortified formulations were analyzed for changes in volatile compound content. Figure 3-7 reports peak area vs storage day for propanal, 1-penten-3-ol and hexanal in all dairy-based beverages during storage.

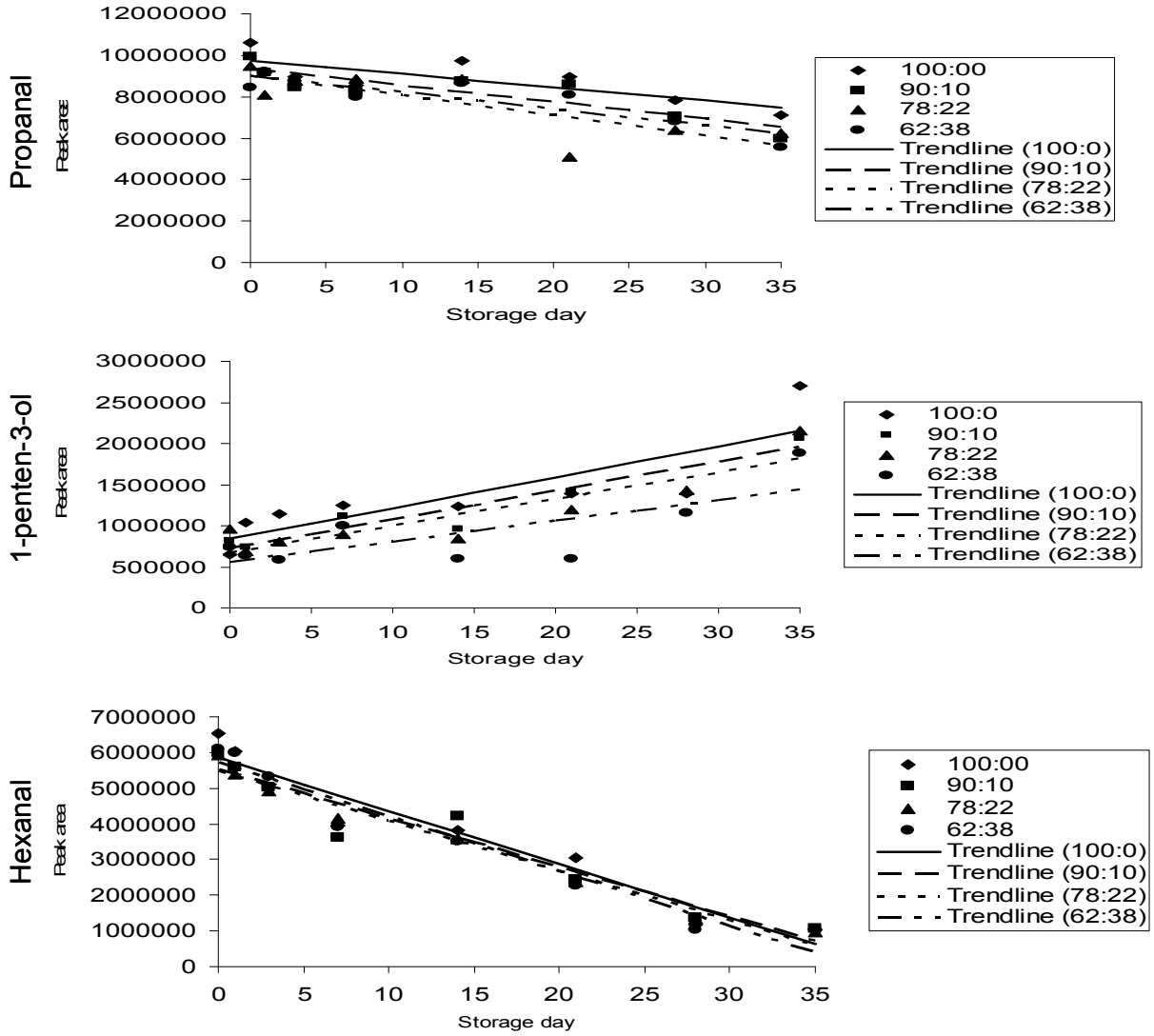

Figure 3-7. Peak areas of propanal, 1-penten-3-ol, and hexanal determined in headspace of all UHT processed dairy-based formulations (% butter oil:% fish oil) during 35 days of storage at 4°C.

Propanal and hexanal decreased during storage while 1-penten-3-ol increased. The decrease in propanal and hexanal levels during storage suggests oxidation of lipids occurred over time.

These findings contradict previous studies [22, 45, 49], which showed increases in hexanal levels as storage time and oxidation increases. Frankel [50] reported that propanal, formed from the decomposition of linolenate hydroperoxides, can further decompose via removal of two hydrogen radicals into propenal, which may explain the reduction in propanal observed. Jimenez-Alvarez and others [22] studied the volatile compounds produced in milk containing FO (5%/vol cod oil) and reported hexanal and 1-penten-3-ol were some of the most abundant volatile compounds formed and levels increased during storage (37 °C). Frankel [51] reported the formation of 1-penten-3-ol in oxidized cod oil and Jafar and others [52] reported the formation of it in FO-enriched mayonnaise was a result of enzymatic reactions. Hexanal has been shown to be derived from linoleate hydroperoxide decomposition [53, 54], which may originate from the vegetable oils added to the commercial FO. The decrease in hexanal levels may suggest that its formation from primary oxidation products was inhibited during storage, and it decomposed into shorter chain volatile compounds that were not measured, such as lower aldehydes, hydrocarbons, alcohols and acids reported by Frankel [53]. Jimenez-Alvarez and others [22] and Hartvigsen and others [46] reported the formation and increase in levels of 1-penten-3-ol during storage of FO-enriched milk and mayonnaise products. Figure 3-11 shows a comparison of the major volatile compound peaks between days 0 and 35 of the 78%BO:22%FO dairy-based beverage.

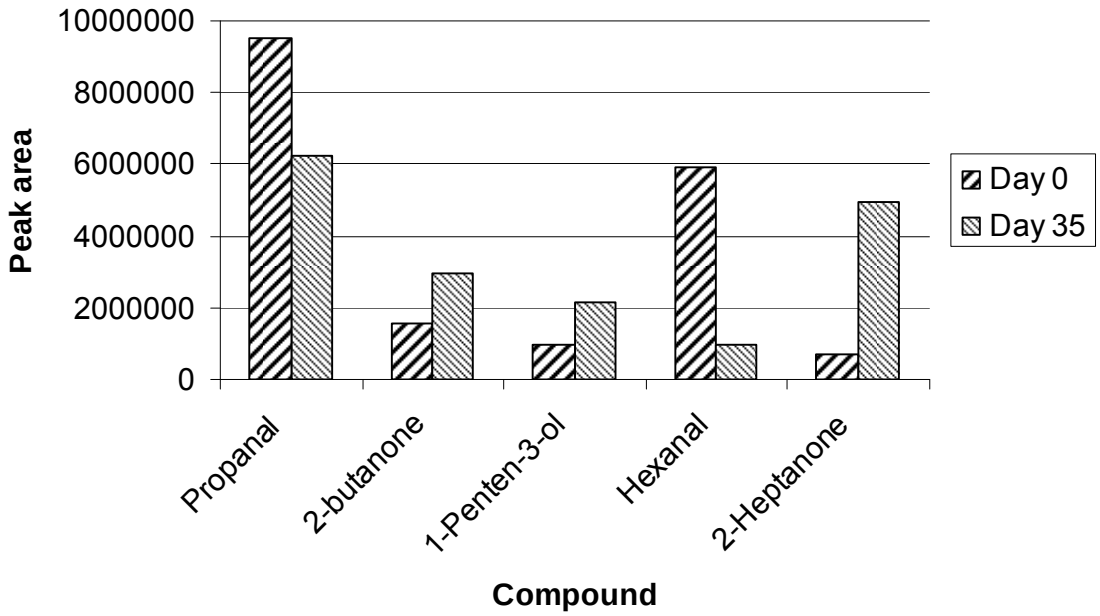

Figure 3-8. Comparison of major volatile compounds identified in headspace analysis chromatograms of omega-3 FA fortified dairy-based beverage (78%BO:22%FO formulation) on days 0 and 35 of storage (4°C).

Peak areas of compounds present in the 78:22 formulations varied during storage. Peak areas for 2-butanone, 1-penten-3-ol, and 2-heptanone increased during storage, suggesting oxidation with storage time, while areas for propanal and hexanal decreased. 2-Heptanone exhibited the largest increase in volatile concentration during storage.

Instrument sensitivity may have contributed to the inability to successfully monitor all volatile compounds in the headspace samples. Gorecki and Pawliszyn [55] reported that the volume of sample present in the headspace vials, and thus the amount of headspace volume in the vial, is a critical factor in SPME analysis. Headspace volume should be minimized so that extraction efficiency of volatiles from the headspace can be maximized. In this study 20 ml headspace vials were used incorporating 1g of sample into each vial. This low volume of sample may have negatively affected the ability of the SPME fiber to efficiently extract volatiles in amounts necessary for appropriate identification and quantification.

Sensory Evaluation

The aroma impact of fish oil addition to dairy products has been extensively studied [13, 22, 44, 56-58]. Venkateshwarlu and others [43] concluded that the volatile compounds that contribute to the odor profile of fish oil enriched milk were vinyl ketones, alkenals, and alkadienals, which are secondary oxidation products. In a study on FO fortified mayonnaise and milk drinks, Timm-Heinrich and others [44] reported that the decrease in overall sensory quality of fortified products, by the development of fishy off-flavors, was a result of oxidative deterioration. Let and others [57] reported that milk could be enriched with fish oil at high levels (15g FO/ kg milk) without significant development of fishy off-flavors during storage (2°C), provided the initial level of oxidation of the FO was low before emulsion formulation.

UHT processed milk has a "cooked" aroma due to the high pasteurization temperatures used. This aroma is different than the aroma of high temperature short time (HTST) pasteurized milk. The cooked aroma of the UHT processed dairy-based beverage, including the aroma contributions of free sulfhydryl groups resulting from UHT pasteurization [59, 60], may overpower and mask the aromas due to the presence of fish oil in the early stages of storage. Two dairy-based beverage systems, both formulated at 78:22 (BO:FO), with different total fat loads (2.5% and 3.1%) were compared to commercial UHT milk.

The dairy-based beverage (2.5% fat) used in sensory session 1 was determined to be significantly different ($p<0.05$) from the control (commercially available UHT milk) used in the test. Of the 25 panelists who participated in the sensory evaluation, 48% (12 panelists) were able to identify the odd sample in both tests. The dairy-based beverage (3.1% fat) from the second test session was determined to be not significantly different ($p>0.05$) from the control. Of the 25 panelists, only16% (4 panelists) were able to correctly identify the odd sample in both tests in the second session (Table 3-7).

Table 3-7. Differences in aroma between commercial UHT milk (2.0% fat) and 78% BO:22% FO-fortified UHT processed dairy-based beverage systems (2.5% or 3.1% total fat) by triangle sensory tests[1].

	Sensory session 1		Sensory session 2	
	2.5% fat 78:22 vs 2.0% fat comm.. UHT		3.1% fat 78:22 vs 2.0% fat comm. UHT	
Triangle test # →	Δ1	Δ2	Δ1	Δ2
# Correct	13[A]	19[B]	7[C]	12[C]
Correct both tests[2]	12		4	

[A] Significant ($p<0.05$)
[B] Significant ($p<0.001$
[C] Not significant ($p>0.05$)
[1] $\alpha = .05$; ß=.30; $p_d=0.30$; n=25; critical number =13
[2]Number of panelists who identified the difference correctly in both test sets within the session.

Fatty acid profiles of the dairy-based beverages used for sensory analysis were determined and compared. The major fatty acids identified in the (78%BO:22%FO) dairy-based beverages used for sensory analysis and the relative amounts in each beverage can be seen in Appendix H.

The reason for the significant difference in aroma found in the first sensory test is not clear. Storage parameters for sensory samples were kept constant for both sensory tests; all samples were stored away from light at 4°C until immediately before presentation to panelists. The fatty acid composition of samples was comparable in fatty acid content. The omega-3 FA-fortified dairy-based beverage (2.5% total fat, session 1) contained higher levels of EPA and DHA, by 2.2% and 1.3% respectively, than did the beverage system from the second sensory test. The second test session product contained higher percentages of palmitic and oleic acids, by 2.9% and 1.8% respectively. Some panelists had previous training in dairy sensory evaluation and oxidative aroma detection/evaluation, though none were specifically sought out or rejected for the sensory sessions. Sensory session 1 had a higher percentage of participants on the panel that had previous experience in detecting fishy and oxidative off-aromas. Panelists that participated in sensory session 1 were asked not to participate in session 2 in an attempt to increase the

diversity of panelists. The differences in FA content noted between the two sensory samples, specifically EPA and DHA, and the presence of experienced panelists that participated in session 1 may have contributed to the statistical difference between the test and control samples.

The majority of sensory panelists reported to be knowledgeable about the potential health benefits of consuming fatty fish and omega-3 fatty acids (Appendix I). Based upon the overall aroma of the dairy-based beverage and the panelists' overall knowledge of potential omega-3 FA health benefits, 84% said they would consume an omega-3 fortified dairy product and 34% reported they would consume it 1-3 days per week.

E. Conclusion

A dairy-based beverage system was successfully produced that delivered approximately 440 mg of omega-3 fatty acids per 250 mL (8 oz.) serving and maintained physical and microbiological quality characteristics throughout the 35 day refrigerated storage period. Oxidative and volatile changes in the product over 35 days were minimal but consideration of antioxidants in the system is needed. Consumer interest in omega-3 FA fortified dairy-based beverages to meet suggested dietary intake levels of omega-3 FA indicate that there may be a market for such products.

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CHAPTER IV

SENSORY AND CHEMICAL CHANGES IN AN EXTENDED SHELF-LIFE OMEGA-3 FATTY ACID AND ANTIOXIDANT FORTIFIED DAIRY-BASED SYSTEM

ABSTRACT

Two exploratory pilot studies were carried out to evaluate the effects of added antioxidants (AOX) on the oxidative stability and sensory profiles of an ultra-high temperature (UHT) processed omega-3 fatty acid (FA) fortified dairy-based beverage (3% fat) with a targeted delivery of 500mg eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)/250 mL (8oz). Skim milk, butter-derived aqueous phase (BAP), butter oil (BO), and fish oil (FO) were used to produce the dairy-based beverages. In exploratory study 1, ascorbyl palmitate (AP), kudzu root extract (KR), boldine (BD), and lipoic acid (LA) were tested for their AOX effect on protecting against oxidation and maintaining quality through a 35 day shelf-life (4°C). Conjugated diene values for all products were generally below 1.0, even with light exposure to initiate oxidation. Antioxidants ascorbyl palmitate, kudzu root extract, boldine, and lipoic acid showed no significant effects on oxidation as determined by conjugated diene analysis of the dairy-based beverages compared to a control dairy-based beverage with no additional antioxidants added. Major volatiles identified using solid-phase micro extraction GC/MS (SPME-GC/MS) included propanal, 2-butanone, 1-penten-3-ol, hexanal, and 2-heptanone. LA was observed to be most effective in preventing propanal formation. AP and KR were observed to be the most effective in controlling formation of hexanal.

In exploratory study 2, product composition was 3.0% fat, 3.7% protein, and 11.3% total solids. Conjugated diene values decreased during storage at 4°C for 35 days. Volatile analysis indicated an increase in formation of 1-penten-3-ol in the dairy-based beverage during storage. Sensory analysis, using a trained panel, indicated that oxidative aromas from FO oxidation increased during storage. The AOX and omega-3 FA fortified beverage delivered approximately 389 mg of omega-3 fatty acids per 250 mL serving and maintained microbiological and physical quality characteristics throughout the 35 day storage period.

INTRODUCTION

Milk and dairy products offer many advantages as dietary sources of omega-3-rich lipids including frequent consumption, inherent nutritional value, and storage and packaging conditions selected to protect against oxidation. Milk has many natural components that offer antioxidant (AOX) functionality, such as casein, whey, and lactoferrin proteins, carotenoids, tocopherols, ascorbic acid, and enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [1-5] . However, the AOX levels naturally found in milk may not be sufficient to protect additional PUFAs throughout shelf-life.

Food products fortified with fish oil (FO), which is rich in omega-3 fatty acids (FA), are more susceptible to oxidation due to the polyunsaturated nature of the FA, which decreases the nutritional impact of the product and produces volatile oxidation compounds that negatively impact aromas and/or flavors of the product. Controlling or reducing oxidation of FO-fortified food systems, such as milk and other dairy products, by addition of antioxidants has been studied by pre-harvest and post-harvest technologies [6-12]. Tocopherol (Vitamin E) and ascorbic acid (Vitamin C) derivatives have been studied most frequently [11-14]. Focant and others [12] found that 9616 IU of vitamin E orally supplemented to Holstein cows increased the α-tocopherol levels in milk by about 45% and was sufficient enough to prevent milk fat oxidation. Jung and others [13] found that ascorbic acid at 1000ppm was effective in lowering formation of dimethyl disulfide and off-flavors in skim milk.

Novel lipid soluble or amphiphilic AOX that function in the core lipid or at the fat globule surface, respectively, may provide additional protection against lipid oxidation over an extended refrigerated shelf-life [15-19]. Antioxidants that have shown to be efficient in bulk oils are usually efficient in emulsions, however the level of efficiency can be substantially different [20, 21]. Frankel and others [21] investigated polar and non-polar antioxidant effectiveness in bulk

oil and emulsions and concluded non-polar antioxidants were found to be more effective in emulsions than bulk oil and polar antioxidants were observed to behave in the opposite manner,. These differences were related to the antioxidant affinities to the air-oil or water-oil interfaces. In bulk oils, the polar antioxidants are situated at the air-oil interface where they protect the lipid from oxygen. In emulsions, non-polar antioxidants are located in and around the lipid droplets, protecting the lipids from water soluble reactive oxygen species. In oil-in-water emulsions, lipid hydroperoxides tend to migrate to the interfacial phase where they could react with metals catalysts in the aqueous phase. Oxidation reactions are more prevalent at the oil-water interface and amphiphilic AOX could orient there where they could inhibit the oxidation reactions. Chaiyasit and others concluded that γ-tocopherol was more surface active and thus a more effective antioxidant compare to α-tocopherol in corn oil-in-water emulsions [22].

Ascorbyl palmitate (AP), kudzu root extract (KR), boldine (BD), and lipoic acid (LA) have previously been shown to exhibit AOX properties in lipids and lipid-rich foods [17, 19, 23-25]. AP AOX activity is attributed to its ability to scavenge free radicals, chelate metals, and work synergistically with other AOX, such as α-tocopherol, where it donates hydrogen atoms to AOX radicals [26, 27]. Let and others [7] reported that when AP was added to a FO-enriched milk product (300 mg AP/ kg FO) it almost completely inhibited oxidation in the system. LA is found in both eukaryote and prokaryote organisms [28, 29] in essential enzyme complexes involved in metabolic processes. LA was reported by Bingham and others [30] to occur naturally in milk where is tightly bound to protein components in the fat globule membrane. LA has been reported to exhibit peroxyl radical, hydroxyl radical, and singlet oxygen scavenging AOX abilities [31-33]. Drinda and Warner [25] reported that LA was effective in prolonging the oxidation induction period in vegetable oils and lard, and was reported to have a synergist effect when coupled with AP, however, AOX effectiveness was higher in oils containing lower levels of polyunsaturated FA. AP and LA are both amphiphilic molecules and may exhibit AOX activity in the core lipid and oil-water interfacial area.

KR and BD are lipophilic molecules that would exhibit AOX effectiveness in the core lipid of an emulsion system. KR comes from the kudzu plant (*Pueraria lobata*), a fast growing leguminous vine native to China and Japan. Kudzu was introduced into the United States in the late 19th

century at which time it was promoted as an ornamental plant and for erosion control due to its fast rate of growth. KR has been shown to exhibit AOX properties, which are associated with flavonoids isolated from KR extract [17, 18]. Jun and others [17] reported that the AOX activity of KR is attributed to five major isoflavone compounds: puerarin, daidzin, daidzein, biochanin A, and genistein. These compounds exhibit potent free-radical scavenging activities. BD, a natural alkaloid extracted from the boldo tree (*Peumus boldus*) native to Chile, has been reported to exhibit strong free radical scavenging AOX properties [34, 35]. Valenzuela and others [19] reported that BD was comparable to quercetin and more effective than α-tocopherol and other synthetic AOX against oxidation in bulk FO. Ganga and others [36] reported that a mixture of quercetin and boldine (2:1 wt/wt) significantly increased the oxidation induction period of sardine oil at 60 °C to 4.5 hours. Figure 4-1 shows the molecular structure of the AOX evaluated.

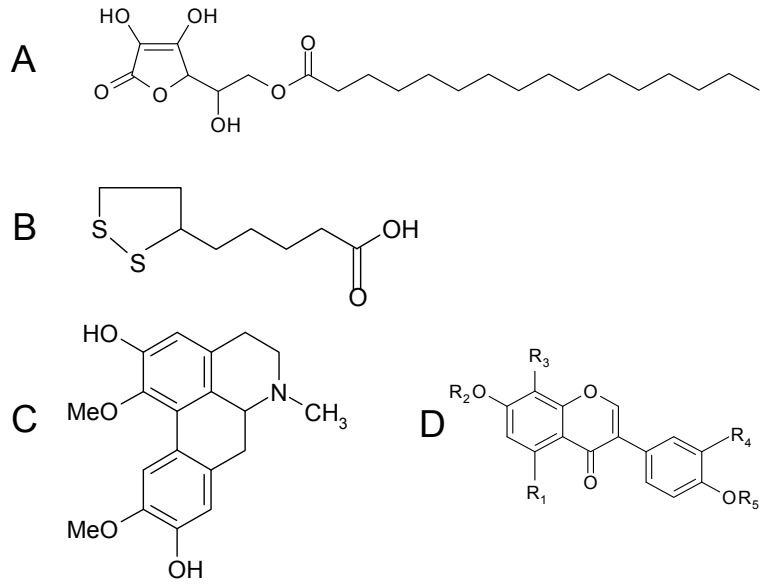

Figure 4-1. Chemical structures of lipid soluble and amphiphilic antioxidants from common and novel sources. A: amphiphilic Ascorbyl palmitate, B: amphiphilic lipoic acid, C: lipophilic Boldine, D: General structure of a lipophilic isoflavone contained in kudzu root extract.

Challenges of fortifying food products with omega-3 fatty acids involve inhibiting any fishy flavor or off-odors that may be imparted as a result of the omega-3 source, and preventing or

controlling oxidation of the omega-3 lipids. The increased susceptibility of the omega-3 FA to oxidize, due to the high number of C-C double bonds in their molecular structures compared to less unsaturated lipids, requires the addition of AOX to reduce the risk or extent of oxidation. While omega-3 fatty acids are flavorless, oxidation of these lipids can produce volatile oxidation compounds that can contribute undesirable off-flavors and/or aromas. These volatile compounds have very low flavor threshold levels and research has shown that they are perceptible at parts per million and parts per billion concentrations [37]. Volatile compounds formed as a result of oxidation of omega-3 lipids that can influence aroma and flavor profiles include aldehydes, alkenals, alcohols, alkadienals, alkatrienals, acids, epoxides, hydrocarbons, and ketones [38, 39].

Maintaining high quality aromas and flavors throughout the shelf-life of an omega-3 FA fortified product is important for consumer acceptability. Kolanowski and others [40, 41] reported that the flavors and aromas of omega-3 FA fortified food products are the most important and sensitive indicators of their quality and overall acceptability. Omega-3 FA, specifically EPA and DHA, can be added to food products at low levels without any characteristic sensory attributes present in the product, but can contribute characteristic "fishy" notes when added to foods at elevated levels.

High quality milk has bland sensory characteristics and should be almost neutral in flavor and exhibit no aftertaste other than the natural richness imparted by the milkfat and other milk solids. The aroma should possess a mild slightly cooked note that results from the pasteurization process [42]. UHT processed milk has a different aroma and flavor profile than HTST processed milk due to the different heat treatments used to destroy bacteria and inactivate enzymes. Due to the high heat treatment of UHT processed milk, aromas and flavors such as cooked, heated, oxidized, and stale develop [43]. Contarini and others [44] analyzed UHT pasteurized milk using headspace GC and concluded that ketones, aldehydes, terpenes, and sulfur and aromatic compounds were all present, with ketones representing the most abundant class of volatiles identified including 2-butanone, 2-pentanone, 2-heptanone. Due to the expectedly bland sensory characteristics of milk, off-flavors and aromas are easily detectable [42] and challenges of fortifying dairy-based beverages with omega-3 FA involve inhibiting any fishy flavor or odor that may be imparted in it as a result of fortification with the omega-3 FA source oil, most

commonly FO. The odors and flavors associated with UHT milk may contribute to masking any odors or flavors associated with omega-3 FA sources.

In a study on aromas developing from light induced oxidation of UHT processed extended shelf-life milk, van Aardt and others [45] concluded that the addition of AOX was effective in controlling the development of aroma active compounds, hexanal, pentanal, and 1-octen-3-ol, during 6 weeks of light exposure. Off-flavors and aromas reported in omega-3 fortified mayonnaise and milk drinks include fishy aroma/flavor, rancid aroma/flavor, metallic and bitter flavors [38, 46, 47]. Venkateshwarlu and others [38] reported that volatile compounds identified in fish oil-enriched milk emulsions were mostly carbonyl compounds including alkenals, alkadienals, alkatrienals, and vinyl ketones which can contribute to off-aromas and flavors of the product.

The objective of the two exploratory pilot studies was to evaluate the effectiveness of added AOX on oxidation and shelf-life of a UHT processed FO-fortified dairy-based beverage system stored at 4°C for 35 days. Effects of added AOX were evaluated by changes in conjugated diene (CD) hydroperoxide values, reported as % conjugated dienoic acid, and the formation of volatile headspace compounds as determined by SPME-GC/MS analysis.

The objectives of study 1 were to:

1. evaluate the effectiveness of added AOX on oxidation and shelf-life of a UHT processed extended shelf-life omega-3 FA fortified dairy-based beverage system stored at 4°C for 35 days;
2. evaluate the effectiveness of AOX present in the commercial FO on light-induced oxidation and shelf-life of a UHT processed extended shelf-life omega-3 FA fortified dairy-based beverage system stored at 4°C for 35 days;

The objectives of study 2 were to:

1. monitor changes in volatile compounds and sensory perception of oxidation as a function of time in a UHT processed extended shelf-life AOX and omega-3 FA fortified dairy-based beverage stored at 4°C for 35 days;

2. determine changes in oxidative degradation of omega-3 lipids during 35 days of storage at 4°C to evaluate if added AOX were effective in controlling oxidation and the formation of oxidative aromas in the dairy-based beverage.

The materials and methods for the two studies are similar and are described together. The results and discussion of each exploratory study follows sequentially. For simplicity, throughout the rest of the document the UHT processed extended shelf-life omega-3 FA fortified dairy-based beverage system will be referred to as the dairy-based beverage.

Materials and Methods

Milk Fractions, Fish Oil, and Antioxidant Sources

Milk fractions were obtained using the same processing methods and sources described in the Materials and Methods section of Chapter 3. FO, obtained commercially as reported previously (Ch. 3), was reported by the supplier to contain tocopherols and citric acid, though concentrations were not disclosed. This food grade source of fish oil also contained undisclosed levels of canola and sunflower oil perhaps as sources of natural AOX. Previous studies have indicated that canola oil significantly protected FO emulsions against oxidative deterioration, theorized as a result of tocopherols naturally present in canola oil [8, 48]. All milk fractions and oils were stored prior to processing into emulsions as described previously (Ch. 3).

Lipoic acid (Acros Organics, New Jersey, USA), boldine (Sigma, St. Louis, MO), ascorbyl palmitate (Spectrum, New Jersey, USA), and kudzu root extract (Sigma, St. Louis, MO) were obtained from commercial suppliers.

Processing of Dairy-Based Beverages

Dairy-based beverages were processed and stored as described in Chapter 3. A 100% BO: 0% FO formulation (Table 3-1) was produced to serve as a control. The control formulation was processed with a lipid phase (3.25%) composed of BO (100%) and a water phase (96.75%) composed of skim milk (90%) and butter-derived aqueous phase (BAP) (10%). No antioxidant was added. All beverage formulations produced contained the same proportion of skim and

BAP, but differed based upon BO and FO proportions in the lipid phase. The 62% BO: 38% FO formulation and the 78% BO: 22% FO formulation, as described in Chapter 3, were produced with and without AOX. For objective 1, formulations containing 78:22 and 62:38 BO:FO were prepared with no added AOX to assess the efficiency of AOX inherent in the FO and to serve as controls. For objective 2, formulations containing 62:38 BO:FO were used. Antioxidants (AP, KR, BD, LA) were added to the lipid phase, before processing, at 300, 500, 800, and 600 mg AOX/kg FO, respectively, prior to processing. For objectives 3 and 4, two dairy-based formulations were produced. A control (100%BO: 0%FO) with no added AOX and an experimental sample (78%BO: 22%FO) with AOX added into the oil phase before processing the emulsion. The experimental formulation targeted delivery of 500 mg of omega-3 fatty acids per 250 mL (8 oz) serving.

Evaluation of Physical and Chemical Quality Parameters

All analyses were completed following methods described in Chapter 3. Sampling for all analyses (microbiological, composition, and chemical quality parameters) was completed for all formulations as indicated in Table 4-1.

Table 4-1. Sampling schedule and analytical analyses for omega-3 fortified dairy-based beverage systems with and without antioxidants over 35 days (4°C).

Test↓ Day→	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	18	21	28	35
Microbiological	*							*							*		*	*	*
Fatty acid analysis	*	*						*							*				*
Protein content			*																
Total lipid					*														
Total solids							*												
Emulsion stability	*		*		*			*		*		*			*		*	*	*
Conjugated dienes	*	*		*		*		*				*			*		*	*	*
Headspace GC/MS	*	*		*		*		*				*			*		*	*	*
Sensory analysis				*											*	*			*

Light-Induced Oxidation of Omega-3 Fortified Dairy Based System

Two control omega-3 FA fortified dairy-based formulations (78:22 and 62:38 BO:FO) without added antioxidants were subjected to light to induce oxidation and determine the effect of AOX inherent in the milk and fish oil in controlling oxidation. Dairy-based samples, ~40 ml, were

placed into clear glass screw-top 40 mL containers and placed horizontally under fluorescent light banks (two 30-34 watt cool white fluorescent bulbs) for maximum surface area exposure and stored at 4°C for up to 35 days. Light at the bottle surface was ~1530 lux as measured by a light meter (Extech Instrument Corp., Walthaun, MA). Lipids were extracted and conjugated diene analysis was performed as described in Chapter 3 on storage days indicated in Table 4-2.

Sensory Evaluation for Oxidative Aromas

The dairy-based system was tested for oxidative aromas with a simple ranking test [49] utilizing panelists who were trained to recognize oxidative aromas in dairy systems. The study was approved by the Virginia Tech Institutional Review Board and all panelists provided informed consent prior to participation.

Potential panelists were screened for their ability to detect oxidative aromas using a triangle test for difference [50]. Samples consisted of freshly opened commercially available 2% fat UHT milk (Parmalat, Parma, Italy) and commercial 2% fat UHT milk homogenized with added oxidized vegetable oil. Approximately 15 ml of each sample were poured into 1 oz plastic soufflé cups, capped, and stored at 4°C. Samples were assigned three digit codes and randomized for presentation to sensory panelists. Panelists (n=21) were presented with three sets of three samples with the task of smelling each sample and identifying the odd sample in each set. Panelists who were correct in no less than two of the three screening tests (n=14) were asked to participate in the sensory training.

Panelists (n=9) were trained to recognize and evaluate the degree of oxidation during four training sessions. A beer taint kit (Brewing Research International), used to provide qualitative training of aromas indicative of oxidation, was used in combination with other known aromatic oxidative compounds to present panelists with oxidative aromas (Appendix J). Fish oil and butter oil samples were oxidized by 1) bubbling O_2 through the samples and 2) exposing the samples to direct sunlight. Conjugated diene analysis was used to assess the degree of oxidation of all dairy and lipid samples used for training and during testing. Panelists were validated for their ability to correctly rank oxidative aromas via two ranking tests using samples comprised of commercial 2% fat UHT milk and oils of known degrees of oxidation. Panelists who were unable to correctly

rank the samples were further trained until satisfactory results were achieved or were excused from participating in the panel.

Panelists received one set of four samples (4°C) in a balanced random order and were tasked to arrange them in rank order of oxidative aroma intensity, with 1 as the lowest intensity, 2 as the next lowest, and 4 as the most oxidized sample. The control, hypothesized as the lowest intensity, was a sample of the dairy-based system containing no FO. Reference dairy-based samples (n=2), prepared with oils oxidized to a known degree based on CD analysis, were used to track and compare the oxidation of the AOX and omega-3 FA fortified experimental sample during storage. Data collected from the oxidative aroma ranking tests were analyzed using Friedman analysis [49].

Sensory testing occurred in the sensory laboratory of Virginia Tech's Department of Food Science and Technology under white fluorescent lighting. SIMS 2000 (Sensory Information Management System, Sensory Computer Systems, LLC) was used for sensory test design and data analysis. Sensory analysis was performed on days 3, 14, 18, and 35 of storage, days previously determined by CD analysis to have the highest potential for the presence of oxidative aromas (Chapter 3).

Statistical Analysis

The exploratory pilot studies in objectives 1 and 2 were carried out in one replication, as the purpose was to estimate effects, if any, the added AOX contributed to oxidative stability of the dairy-based beverages. Objectives 3 and 4 were evaluated based on processing the formulation beverage (78:22 BO:FO) three times, representing three replications in the study. The control sample was only processed one time. Analytical analyses were completed in duplicate for each replication with the exception of fatty acid analyses. Replications were used to monitor physical, chemical, and microbiological stability of the emulsions. The main factors in the experiment were time and replication. However, since time is not independent, a one-way analysis of variance with repeated measures was used to assess changes in oxidation and physical stability. Statistical analyses were completed using JMP (SAS Institute, Cary, NC). Alpha level of 0.05

was preset for determining significant differences. Least Significant Differences test was used for mean separation when significant differences are found.
Ranking analyses was completed using Friedman analysis. Sensory data from ranking tests was determined by using $\chi^2 = 0.05$ and three degrees of freedom, and equations outlined in Meilgaard and others [49].

RESULTS AND DISCUSSION

UHT processing and refrigerated storage (4°C) of the dairy-based beverages effectively controlled microbial growth during the 35 days of storage. Emulsion stability data indicated no significant difference ($p>0.05$) in change in % fat of the upper and bottom layers of the emulsions on days 0 and 35 of storage, though a significant difference was indicated on day 11 of storage ($p<0.05$). Bolling and others [51] reported that pasteurization prior to homogenization resulted in more protein at the milk fat interface in 20% milk fat formulated creams, which contributed to emulsion stability. Skim and cream silk fractions used in this study were pasteurized separately before the beverage formulations were pasteurized, which may have contributed to increased stability of the dairy-based beverages.

Results from conjugated diene hydroperoxide analysis have been shown to correlate well with results from peroxide value analysis [52-55]. Frankel [56] reported that conjugated diene formation parallels the formation of hydroperoxides, which occurs in the early stages of lipid oxidation and then decomposes into secondary oxidation products.
Marmesat and others [54] reported that in high linoleic and high oleic sunflower oils, conjugated diene values of 2.6 and 1.7, respectively, corresponded to peroxide values of 2meq O_2/kg oil which generally indicates a slight degree of oxidation. The dairy-based beverages naturally contained levels of conjugated linolenic acid that also absorb at 233nm, which would influence the determined levels of oxidation based upon conjugated diene analysis. It is assumed that CLA isomers present in the dairy-based beverages influence conjugated diene values and the actual degree of oxidation determined for the beverages is slightly lower than calculated.

Exploratory Study 1. Novel Natural Antioxidants for Protecting Unsaturated Lipids in Dairy-Based Systems

Fat content was lower than the targeted 3.25% fat, and ranged from 1.55% to 2.15% fat, with a mean of 1.77% (±0.23) fat (Table 4-2). Mean fat content in two of the AOX formulations (AP, BD) was lower than the control ($p<0.05$) but mean fat content among AOX formulations was not significantly different. Protein (%) was significantly different ($p<0.05$), however, total solids (%) and total solids not fat (%) were not different among formulations. Protein (%), total solids (%), and total solids not fat (%) were within standard requirements, based on the Code of Federal Regulations, for fluid milk [57].

Table 4-2. Gross composition ($\bar{x}$ [1] ± sd) of omega-3 FA fortified UHT pasteurized dairy-based beverage systems (62% butter oil: 38% fish oil) with and without antioxidants. Data based on duplicate values from one replication.

AOX[2]	% fat $\bar{x}$ ± sd	% protein $\bar{x}$ ± sd	% TS[3] $\bar{x}$ ± sd	% TS not fat $\bar{x}$ ± sd
Control	2.15(±0.07) ^{A}	3.64(±0.00)^{D}	10.61(±0.03) ^{A}	8.45(±0.08) ^{A}
AP	1.55(±0.07) ^{B}	3.85(±0.00)^{B}	10.26(±0.04) ^{B}	8.71(±0.08) ^{A}
KR	1.70(±0.00) $^{A B}$	3.59(±0.00)^{E}	10.58(±0.02) ^{A}	8.88(±0.02) ^{A}
BD	1.65(±0.21) ^{B}	3.75(±0.00)^{C}	10.57(±0.04) ^{A}	8.92(±0.19) ^{A}
LA	1.80(±0.14) $^{A B}$	4.26(±0.00)^{A}	10.71(±0.01) ^{A}	8.91(±0.09) ^{A}

*Percentages within a column with the same letter are not significantly different ($p>0.05$)
[1]Mean and standard deviation based on duplicate measurements from one replication.
[2]Control has no antioxidants; AP: 300 mg ascorbyl palmitate/kg fish oil; KR: 500 mg kudzu root extract/kg fish oil; BD: 800 mg boldine/kg fish oil; LA: 600 mg lipoic acid/kg fish oil.
[3]TS: total solids

Conjugated diene values (%) for FO and BO sources were determined to be 0.89 and 0.40, respectively. Conjugated diene values (%) of the AOX fortified dairy-based beverages ranged from 0.56 to 1.43 (Appendix K) and were similar to those reported in Chapter 3. The conjugated diene values suggested that the dairy-based beverage systems oxidized during storage, though the degree of oxidation was low. The control formulation with no AOX showed the smallest

changes in CD content. KR and BD formulations exhibited the largest spikes in %CD, which occurred after the first week of storage (Figure 4-2).

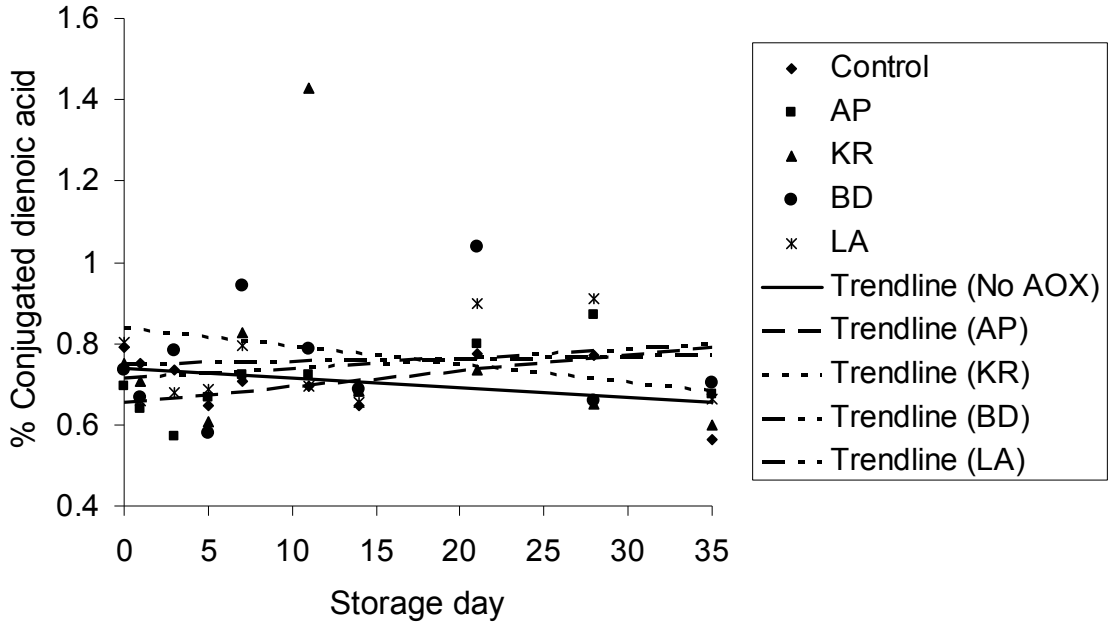

Figure 4-2. Percent conjugated dienoic acid for all UHT processed AOX and omega-3 FA fortified (62% butter oil: 38% fish oil) dairy-based beverage systems (1.7% fat) over 35 days of storage (4°C). Control: no added AOX, AP: ascorbyl palmitate (300 mg/kg fish oil), KR: kudzu root extract (500 mg/kg fish oil), BD: boldine (800 mg/kg fish oil), LA: lipoic acid (600 mg/kg fish oil). Data based on triplicate values from one replication.

Results from CD analysis were modeled via linear trendlines to indicate trends in CD formation. Trendlines indicate that an overall increase in %CD occurred for three of the five AOX formulations during the storage period. AP, BD, and LA AOX formulations all exhibited increases in %CD, while the control and KR formulations exhibited a decrease in CD values during storage. This may suggest that in the control and KR formulations AOX were inefficient in controlling the breakdown of primary oxidation products into volatile secondary oxidation products.

Valenzuela and others [19] reported that BD was an effective inhibitor against autoxidation and metal-induced oxidation, specifically Fe^{2+}-induced oxidation, where it was more effective than

α-tocopherol and BHT. Little information has been published about BD effectiveness as an AOX, especially in food matrices. In this study the BD fortified beverage was observed to increase %CD. The reported effectiveness of BD against metal-induced oxidation [19], and no decrease in CD values observed during this study may indicate that BD was effective as a secondary AOX via its metal chelating abilities. Further investigation may be warranted to conclude more information about its effectiveness against oxidation in food emulsion systems. Of the AOX tested, AP and LA maintained consistently low CD values through out the storage period (Appendix K), suggesting that they may be the most efficient AOX of those tested against the formation of primary oxidation products. It may be presumed the effect of AP is through its free radical scavenging and metal chelating abilities, and its ability to work synergistically with the other AOX present in the FO, and the effect of LA is through its ability to scavenge peroxyl and hydroxyl radicals and singlet oxygen.

Of the AOX added to the dairy-based beverages, none prevented the formation of CD better than the control formulation without any added AOX. The decrease in CD formation observed for the control and KR may suggest that the AOX already present in the FO and KR may be efficient in controlling the formation of primary oxidation products in the dairy-based beverages but not in preventing decomposition into volatile secondary products that may contribute to off-aromas.

SPME/GC/MS was used to monitor the formation of volatile oxidation products that could negatively impact the aroma qualities of the omega-3 fortified dairy-based system. PUFAs present in fish oil, mainly omega-3s, oxidize and the result is the formation of lipid hydroperoxides. Frankel [58] reported that lipid hydroperoxides are odorless and tasteless, and that the further oxidation of these compounds (into secondary oxidation products) are accountable for the off-aromas and flavors found in oxidized foods.
Major peaks in the AOX formulations were seen at 1.6, 2.1, 3.2, 6.8, and 11.6 minutes on the chromatograms. Peaks not identified by external standards were identified using the MS internal library. Peaks at 2.1, and 11.6 minutes were identified as 2-butanone and 2-heptanone, respectively, which are reported by Contarini and others [44] to originate from UHT processed milk. Peaks at 1.6, 3.2, and 6.8 minutes were identified as propanal, 1-penten-3-ol, and hexanal, respectively, previously identified to be present in FO emulsions [59, 60]. Figure 4-4 shows the

peak area vs storage day for propanal, 1-penten-3-ol, and hexanal in all AOX formulations. Propanal, reported by Boyd and others [61] as an indicator of oxidation of EPA and DHA FA, increased in all AOX formulations during storage with the exception of BD which exhibited no increase between day 0 and 35 of storage. Formulations with no added AOX (control) and KR were determined to contain the highest levels of propanal on day 35 of storage, while LA and BD were found to contain the lowest propanal levels. The formulation containing BD contained the lowest levels of propanal throughout storage, suggesting that BD was the most efficient against the formation of headspace propanal. A relatively large increase in 1-penten-3-ol was observed for the control formulation, while all other AOX formulations exhibited decreasing levels during storage. 1-Penten-3-ol is associated with marine oil oxidation and has shown to increase in FO-enriched milk as oxidation progresses [60]. The large increase in the control formulation may indicate that added AOX were effective in controlling 1-penten-3-ol during storage. Overall decreases in 1-penten-3-ol observed for formulations with added AOX may indicate that formation as a result of oxidation was predominant during processing, due to light, heat and oxygen exposure, and inhibited during storage. The LA-fortified beverage was observed to contain the lowest level of 1-penten-3-ol on day 0 compared to the other AOX tested. Higher levels on day 0 may indicate that the AOXs acted as pro-oxidants in the systems during processing. All formulations, with the exception of BD, showed decreasing levels of hexanal between days 0 and 35 of storage. All formulations exhibited a large hexanal level on day 0 of storage, which may suggest oxidation occurred during processing and was inhibited during storage. Hexanal levels were observed to decrease in all formulations during the first week of storage. The BD formulation was observed to contain similar levels of hexanal on day 0 and 35 of storage, indicating an increase in hexanal levels after the decrease during in week one of storage. The AP formulation was observed to contain the lowest levels of hexanal at the end of the 35 day storage period, suggesting it was most effective in controlling headspace hexanal formation during storage.

This supports findings by Jacobson and others [10] who reported that hexanal formation was inhibited by AP in milk drinks containing 1.5%-5% fat. LA and BD trendlines indicated an increase in hexanal levels during storage. Figure 4-3 shows the peak area for propanal, 1-penten-3-ol, and hexanal, respectively, in all AOX formulations determined during storage.

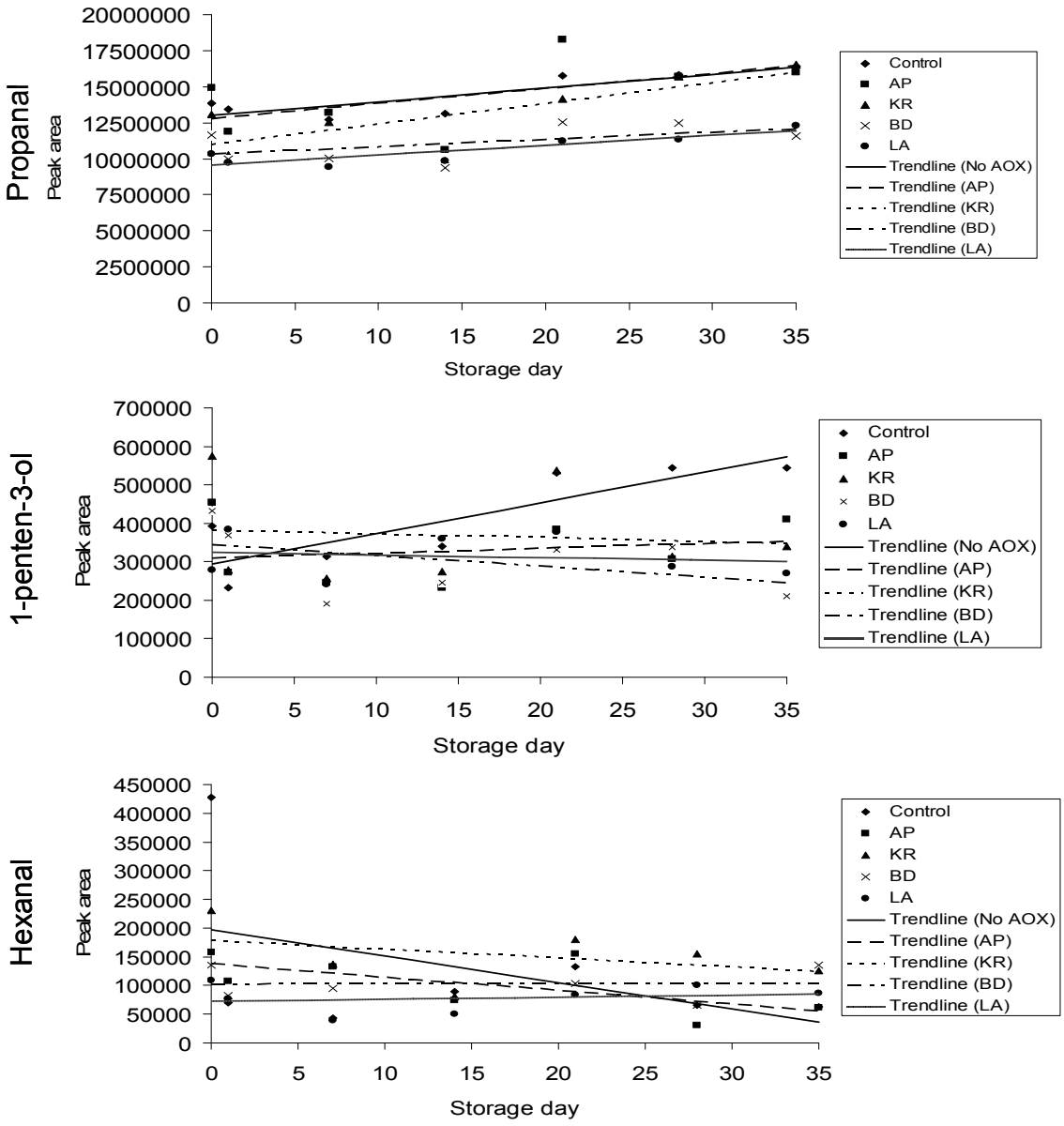

Figure 4-3. Peak area of propanal, 1-penten-3-ol, and hexanal over 35 days of storage (4°C) for all UHT processed AOX and omega-3 FA (62% butter oil: 38% fish oil) fortified dairy-based beverage systems (1.7% fat). Control: no added AOX, AP: ascorbyl palmitate (300 mg/kg fish oil), KR: kudzu root extract (500 mg/kg fish oil), BD: boldine (800 mg/kg fish oil), LA: lipoic acid (600 mg/kg fish oil). Data based on one replication.

The AOX fortified dairy-based beverage containing LA contained the lowest levels of total headspace oxidative volatiles on day 0 (Figure 4-4). In general, there were higher levels of volatile compounds, based on area counts of measured volatiles, after 35 days of storage for all formulations except for BD, which had approximately 2.5% less volatiles on day 35 as observed

on day 0. The headspace volatiles in the control, AP, KR, and LA formulations were found to increase by 14, 6, 18 and 16%, respectively, between day 0 and 35 of storage. This suggests that BD was effective in preventing the decomposition of primary oxidation products into volatile secondary oxidation products more effectively than the other AOX tested, which supports findings by CD analysis. LA and BD formulations contained the lowest levels of total headspace volatiles at the beginning and end of the storage period. These findings suggest that no specific AOX was efficient at preventing or controlling the formation of the three volatiles that were measured, but LA and BD were efficient at controlling the collective headspace volatiles that were monitored, suggesting the potential to reduce oxidation of omega-3 FA and its aroma impact.

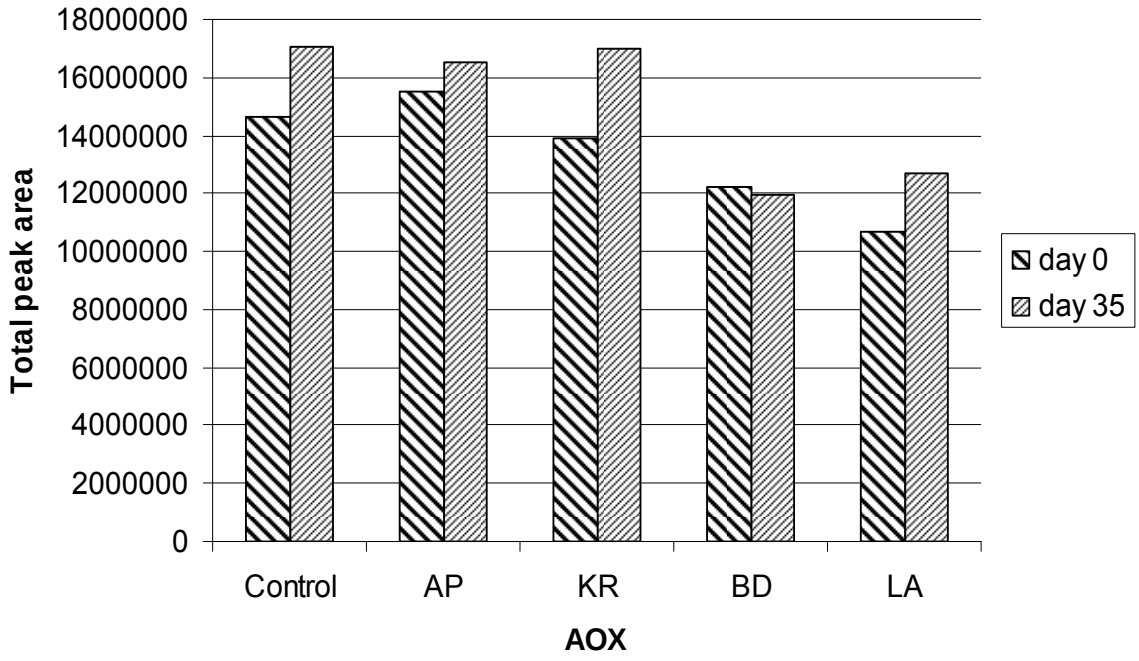

Figure 4-4. Comparison of total area counts of headspace volatiles identified in headspace of UHT processed antioxidant (AOX) and omega-3 FA fortified (62% butter oil: 38% fish oil) dairy-based beverage systems (1.7% fat) on days 0 and 35 of storage (4°C). Control: no added AOX, AP: ascorbyl palmitate (300 mg/kg fish oil), KR: kudzu root extract (500 mg/kg fish oil), BD: boldine (800 mg/kg fish oil), LA: lipoic acid (600 mg/kg fish oil). Data based on one replication. Data based on one replication.

Light-induced Oxidation Study

The relatively low degree of oxidation over 35 days of refrigerated storage for the dairy-based beverage containing omega-3 lipids and the control beverage suggested that there was some antioxidant activity occurring in the system prior to AOX addition. We decided to test this hypothesis by subjecting the system to light in order in induce oxidation (Objective 2). Two beverage formulations, with 22% and 38% fish oil in proportion with butteroil targeted to deliver 3.25% total fat, were evaluated. However, observed fat content was lower than expected with a mean of 2.35% (±0.04) fat (Table 4-3). Protein (%), total solids (%) and total solids not fat (%) were not different among formulations and were within standard requirements, based on the Code of Federal Regulations, for fluid milk [57].

Table 4-3. Gross composition ($\bar{x}$ [1]± sd) of omega-3 FA fortified UHT pasteurized dairy-based beverage systems used for light-induced oxidation study.

Formulation (%BO:%FO)	% fat $\bar{x}$ ± sd	% protein $\bar{x}$ ± sd	% TS[3] $\bar{x}$ ± sd	% TS not fat $\bar{x}$ ± sd
78:22	2.38(±0.11) [A]	3.41(±0.00) [A]	10.89(±0.00) [A]	8.51(±0.01) [A]
62:38	2.33(±0.04) [A]	3.67(±0.00) [A]	10.67(±0.01) [A]	8.35(±0.01) [A]

*Percentages within a column with the same letter are not significantly different (p>0.05)
[1]Mean and standard deviation based on duplicate measurements from one replication

Since there were no statistical differences in composition between the treatments, it was appropriate to compare the changes in volatile and oxidative chemistry. Conjugated diene values (%) for FO and BO sources were determined to be 1.07 and 0.85, respectively. Conjugated diene values (%) of the dairy-based beverages on day 0 were determined to be 0.87 for both formulations and were similar to those reported in Chapter 3. Conjugated diene values were similar for both formulations with and without light exposure (Appendix K) throughout the storage period (Figure 4-5).

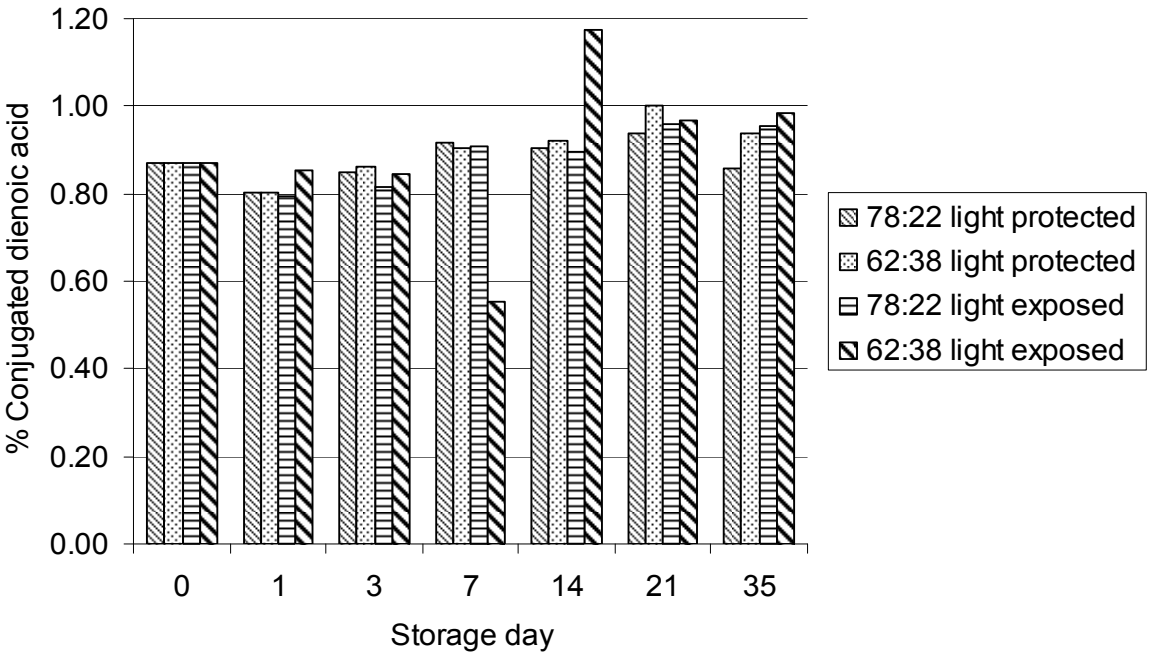

Figure 4-5. Comparison of conjugated diene values determined for UHT processed omega-3 FA fortified UHT pasteurized dairy-based beverage systems during 35 days of storage at 4°C with and without protection from UV light. Data based on one replication with triplicate values.

The light-exposed 62:38 formulation exhibited a spike in %C.D. on storage day 14 though no significant difference in CD values was detected between formulations, light-protected or light-exposed treatments ($p>0.05$) during storage. This data suggests that AOX present in the commercial FO, in combination with the natural AOX in milk, were efficient in controlling further oxidation induced by light exposure.

Conclusions

AOX already present in the commercial FO used in this pilot study, in combination with natural antioxidants in milk, were sufficient in controlling oxidation within the dairy-based system based on conjugated diene analysis and headspace analysis of volatiles, and even during light-exposure for 35 days based on conjugated diene analysis. Additional lipid soluble or amphiphilic AOX added to the dairy-based beverages were no more effective in controlling oxidation even though the non-polar antioxidants would be situated in the lipid phase, oriented at the oil-water interface, where oxidation would propagate. AP possibly acted synergistically with tocopherols present in

the FO. Let and others [7, 48] reported that AP (300mg/kg FO) was almost completely effective in controlling oxidation in FO-enriched milk emulsions (2:1 w/w% milkfat:FO) determined by measuring peroxide values, volatile oxidation products, and sensory evaluation. Coupland and McClements reported that α-tocopherol and ascorbyl palmitate were found to be more effective in oil-in-water emulsions, compared to bulk oils [62]. BD and LA were the most effective in reducing the formation of total volatiles monitored on days 0 and 35 of storage. Based upon these findings and literature support, AP was chosen to be used in future work on controlling oxidative aromas in omega-3 fortified dairy-based beverages. Future research should be done on the AOX efficiency of BD and KR in dairy-based FO emulsions to determine their efficiencies compared to more commonly utilized food lipid AOX.

Exploratory Study 2. **Oxidative Stability of an Omega-3 FA Fortified Dairy-Based Beverage with Ascorbyl Palmitate**

The previous study with novel AOX was not replicated and variation in volatiles and conjugated dienes may occur. AP was chosen to be fortified into a 78:22 (%BO: %FO) dairy-based beverage to further study the effects of volatiles on aroma related to oxidation in this dairy-based beverage. Compositions of the AP fortified dairy-based beverages were generally similar within this study although significant differences in total solids were noted ($p<0.05$). The 100% butteroil treatment was slightly higher in total solids and total solids not fat than the 78:22 formulations (Table 4-4). All formulations were within standard requirements, based on the Code of Federal Regulations, for fluid milk [57].

Table 4-4. Gross composition ($\bar{x}$ [1]± sd) of ascorbyl palmitate and omega-3 FA fortified UHT pasteurized dairy-based beverage systems.

Formulation BO:FO W/W %	% fat $\bar{x}$ ± sd	% protein $\bar{x}$ ± sd	%TS $\bar{x}$ ± sd	%TS not fat $\bar{x}$ ± sd
100 : 0	3.00(±0.00)^{A}	3.65(±0.00)^{A}	11.45(±0.00)^{A}	8.45(±0.00)^{A}
78 : 22	2.98(±0.07)^{A}	3.67(±0.26)^{A}	11.19(±0.02)^{B}	8.21(±0.06)^{B}

*Percentages within a column with the same letter are not significantly different ($p>0.05$).
[1]Mean and standard deviation based on duplicate measurements from one replication for the control and three replications for the experimental formulation.

The source FO used for formulation contained a total of 31% (30.54) omega-3 fatty acids, including EPA at 20.6% and DHA at 9.9%. CG/MS analysis indicated fatty acids present in the FO and BO were present in the dairy-based systems (Figure 4-6).

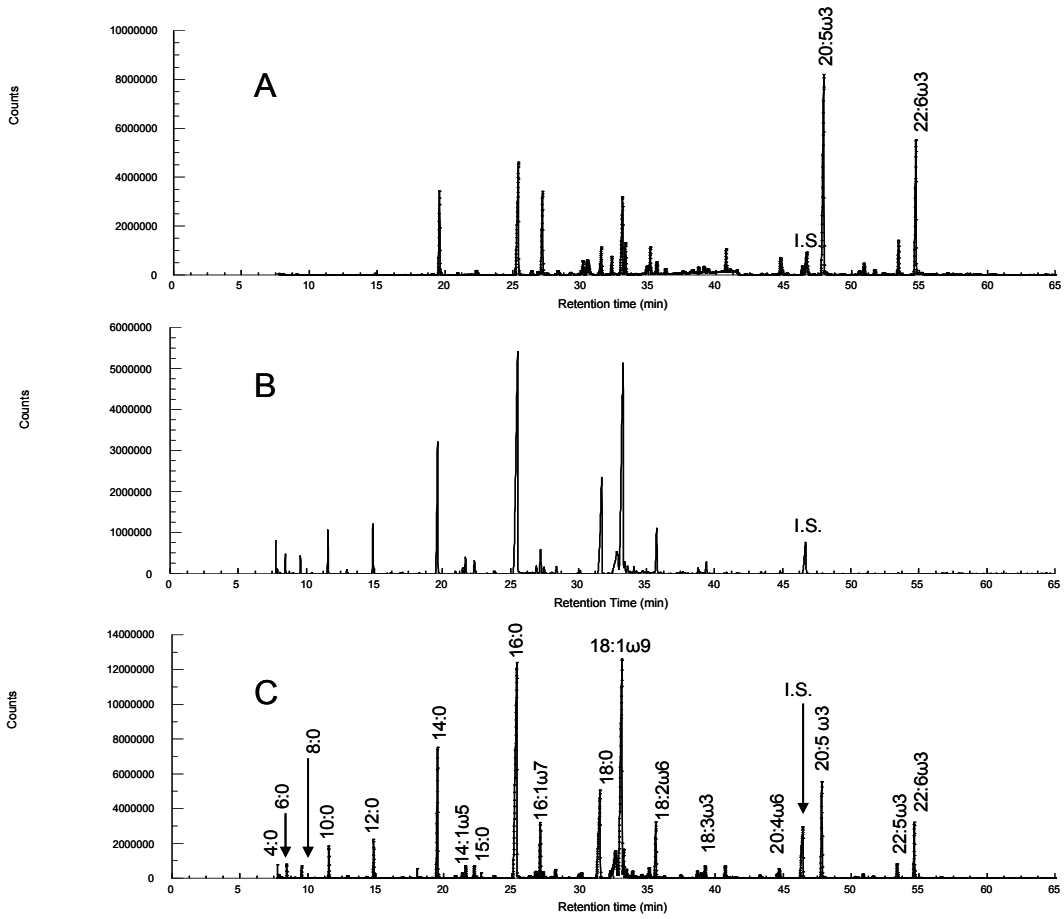

Figure 4-6. Chromatograms of transesterified fish oil (FO) (A), butteroil (BO) (B), and extracted lipids from 3% fat (78%BO:22%FO) UHT processed omega-3 fortified dairy-based beverage with added ascorbyl palmitate (300mg/kg FO) (C), showing peaks of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and C23:0 internal standard (I.S.).

Using tricosanoic acid ($C_{23:0}$) methyl ester as the internal standard, the amount of omega-3 FA in the FO was determined to be 309.2 mg/g FO. The amount of EPA and DHA was determined to be 206.4 mg/g and 99.0 mg/g FO, respectively. Conjugated diene values for the FO and BO, reported as % conjugated dienoic acid, were determined to be 1.13 and 0.82.

Fatty acid profiles of the omega-3 and AOX fortified dairy-based systems were determined to monitor omega-3 lipid concentrations during storage to track oxidation. Table 4-5 shows the major fatty acids in the FO, BO, and AP fortified dairy-based beverages during storage.

Table 4-5. Concentrations of major fatty acids identified in fish oil (FO), butteroil (BO), and ultrahigh temperature (UHT) processed omega-3 FA fortified dairy-based beverages during 35 days of storage at 4°C.

			Concentration (mg/g) lipid ($\bar{x}$ ± sd)			
			Dairy-based beverage			
	Source Lipid		(100:0 %BO:%FO)		(78:22 %BO:%FO)	
Fatty acid	FO[A]	BO[A]	Day0[A]	Day 35[A]	Day0[B]	Day 35[B]
4:0	ND	1.3	0.5	0.4	0.5(± 0.2)	0.4(± 0.1)
6:0	ND	2.5	3.1	2.8	2.5(± 0.1)	2.3(± 0.3)
8:0	ND	2.7	3.2	2.8	2.4(± 0.1)	2.4(± 0.3)
10:0	ND	8.5	9.9	8.3	7.3(± 0.5)	7.1(± 0.9)
12:0	0.9	12.5	13.7	11.7	10.5(± 0.6)	10.1(± 1.3)
14:0	64.3	53.7	52.9	45.9	50.2(± 3.5)	47.1(± 6.6)
14:1ω5	1.4	4.7	5.1	3.4	4.1(± 0.5)	3.6(± 0.6)
15:0	3.4	4.2	4.6	4.0	4.2(± 0.3)	3.9(± 0.6)
16:0	139.6	205.5	169.3	148.7	156.7(±12.5)	143.6(±21.5)
16:1ω7	77.8	9.3	10.1	8.9	20.7(± 1.5)	19.0(± 2.8)
18:0	26.2	83.1	73.9	66.3	63.5(± 4.6)	57.5(± 9.2)
18:1ω9	88.3	253.4	186.7	168.2	170.4(±13.7)	150.6(±27.4)
18:2ω6	10.8	26.3	25.4	22.6	22.2(± 1.4)	20.2(± 3.0)
18:3ω3	5.1	3.7	2.7	2.4	3.5(± 0.3)	3.2(± 0.6)
20:4ω6	21.1	1.2	1.4	1.3	4.4(± 1.3)	3.4(± 0.6)
20:5ω3	216.5	ND	ND	ND	37.5(± 4.6)	34.9(± 5.5)
22:5ω3	29.9	ND	ND	ND	5.4(± 0.5)	4.9(± 0.9)
22:6ω3	125.0	ND	ND	ND	20.6(± 2.4)	19.1(± 3.2)
Saturated	234.5	374.1	336.2	291.0	297.9(±13.8)	274.6(±24.4)
Mono+Poly unsaturated	575.9	298.6	231.4	206.7	288.9(±14.9)	258.9(±28.5)
EPA+DHA	341.6	ND	ND	ND	58.2(± 5.2)	54.0(± 6.4)

[A]n=1 replication
[B]n=3 replications

The concentration of most fatty acids present in the omega-3 and AP fortified dairy-based beverages were relatively similar because the addition of fish oil in proportion to butteroil was relatively small. Concentrations of palmitoleic acid (16:1) were increased substantially with the addition of fish oil and minor decreases in short chain fatty acids and the 18-carbon series of fatty acids were noted. The anticipated increases in EPA and DHA were observed. These changes resulted in an improved nutritional profile for the 78:22 BO:FO formulation as compared to the control formulation. The improved nutritional profile of the dairy-based beverage would allow for increased consumption of healthier PUFA, including EPA and DHA omega-3 FA..

Table 4-6 shows the amount ($\bar{x}$ ± sd) of omega-3 FA (mg/8oz.) in the 78:22 AP fortified formulations, compared to 78:22 with no added AOX and 100:0 control formulations, on days 0 and 35 of storage.

The total omega-3 FA determined in the AP fortified dairy-based beverages were similar compared to the beverages that contained no added AOX. Due to variations in formulation and processing, statistical differences were not determined between the beverages. The addition of AP did not provide additional protection against oxidation compared to the control (100%BO:0%FO) and 78:22 formulations, containing natural milk AOX and natural milk AOX plus AOX in the FO, respectively.

The AP fortified beverage on storage day 35 provided 388.6 mg of ω3 FA per 8oz. One serving per day of the 78:22 BO:FO formulation would deliver a significant amount of the recommended daily intake of omega-3 FA, and if one serving were consumed daily over the span of 1 week, could provide 2720mg of ω3 FA. This is about 77% of the ~500mg daily intake recommendation of the American Heart Association[63].

Table 4-6. Fatty acid levels in UHT processed dairy-based beverages during 35 days of storage at 4°C

	Saturated FA (mg/8oz)	Mono+poly-unsaturated FA (mg/8oz)	EPA (mg/8oz)	DHA (mg/8oz)	Total ω3 FA (mg/8oz)
Control[1]					
Day 0	327.8	220.0	ND	ND	ND
Day 35	288.0	197.7	ND	ND	ND
% decrease	12.1	10.1	ND	ND	ND
78:22[2]					
Day 0	335.6	274.2	312.3	160.5	472.8
Day 35	346.8(±29.6)	273.0(±31.6)	288.5(±46.6)	151.8(±34.9)	440.3(±58.2)
% decrease	ND	0.4	7.6	5.4	6.8
78:22 +AP[3]					
Day 0	295.7(±12.4)	269.1(±13.7)	272.2(±33.1)	146.5(±17.0)	418.7(±50.0)
Day 35	272.8(±24.8)	241.6(±28.8)	252.9(±39.9)	135.6(±22.8)	388.6(±62.7)
% decrease	7.7	10.2	7.1	7.4	7.2

[2]Control = 100:0 (%BO:FO). n= 1 replication
[2]78%BO:22%FO beverage with no added antioxidants. Day 0 based on 1 replication. Day 35 based upon 2 replications.
[3]78%BO:22%FO beverage with added ascorbyl palmitate (300mg/kg FO). n= 3 replications

Determination of Oxidative Stability of AP and Omega-3 FA Fortified Dairy Based-Systems

The susceptibility of the added omega-3 polyunsaturated fatty acids to oxidization is of concern due to reduced nutritional value and the production of oxidative products that contribute to negative sensory qualities. Conjugated diene analysis was a convenient and rapid method to monitor oxidation, requiring minimal reagents and no reaction waiting time.

Conjugated diene values (%) of the dairy-based beverages ranged from 0.71 to 1.07 (Appendix K) and were similar to those reported in Chapter 3 and reported in the previous exploratory study even though the fat content was higher in this study (1.55% vs 2.98%, respectively). The CD values indicate that the dairy-based beverage systems oxidized slightly during storage, though the degree of oxidation was low. The control formulation and the AP and omega-3 FA fortified formulations were observed to deviate in CD values around days 3 and 28 of storage. This may suggest oxidation as a result of processing and as a result of nearing the end of the shelf-life of the products.

Interpreting the relationship of CD values and oxidation can be challenging. The dairy-based beverages exhibited an overall decrease in CD values during storage (Appendix K). CD values of the source FO and BO, previously reported, used in the beverage formulations were similar to the initial CD values determined for all formulations on day 0. Results from CD analysis, modeled via linear regression trendlines, indicated an overall reduction of conjugated diene hydroperoxides (Figure 4-7). There was no significant difference between CD values between the control and the experimental formulation for each day of analysis. Conjugated diene hydroperoxides are primary oxidation products that are broken down into secondary oxidation products, which leads to a decrease in CD values via a decrease in absorbance at 233nm but this also may indicate an increase in volatile secondary oxidation products that influence aroma.

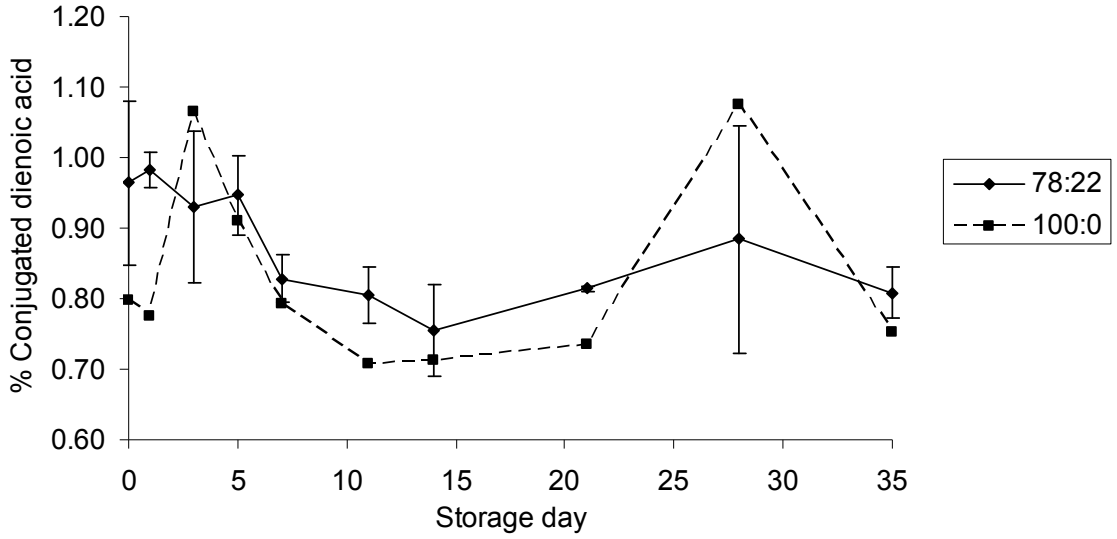

Figure 4-7. Percent conjugated dienoic acid for the UHT processed control (100% butter oil: 0% fish oil) and ascorbyl palmitate + omega-3 FA fortified (78% butter oil: 22% fish oil) dairy-based beverages (3.08% fat) during 35 days of storage at 4°C.

In this study, SPME/GC/MS was used to monitor the formation of volatile oxidation products that could negatively impact the aroma qualities of the dairy-based beverages. SPME headspace chromatograms for the control and AP and omega-3 FA fortified (78:22) formulations on days 1 and 35 of storage can be seen in Appendix L. Based upon volatile chemistry reported in Chapter III, the development of propanal, 1-penten-3-ol, and hexanal were the volatile compounds monitored. Figure 4-8 reports the peak areas determined during storage for propanal, 1-penten-3-ol, and hexanal, in the control and AP fortified dairy-based beverages during storage.

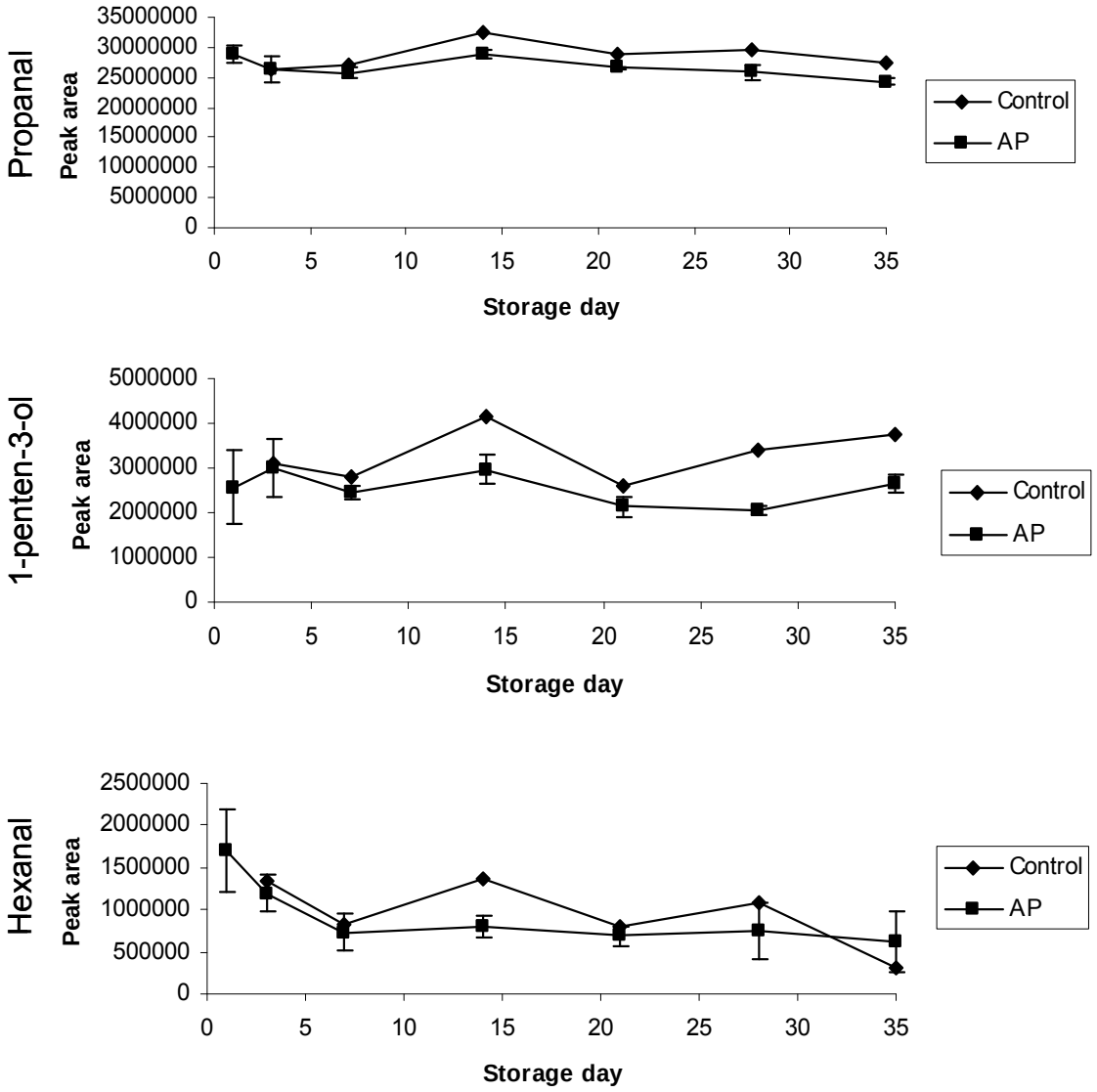

Figure 4-8. Peak areas of propanal, 1-penten-3-ol, and hexanal determined in headspace of control (100:0 BO:FO, n=1 replication) and AP (ascorbyl palmitate (300mg/kg FO)) and omega-3 FA fortified (78:22 BO:FO, n=3 replications) dairy-based beverages during 35 days of storage at 4°C.

The control beverage was observed to contain higher levels of volatiles throughout the storage period compared to the AP and FO fortified beverage. Since the control beverage contained no FO, this may suggest that AOX present in the 78:22 beverages were effective in reducing the formation of headspace volatiles originating from the dairy fractions in the beverage.

Trends in formation of headspace volatiles monitored in the AP and omega-3 FA fortified dairy-based beverages during storage followed the trends in formation of headspace volatiles in the omega-3 FA fortified dairy-based beverages with no added AOX from Chapter III (Figure 4-9). The omega-3 FA fortified beverages were generally lower in headspace volatile levels compared to the control beverage, with the exception of hexanal which was highest in the dairy-based beverage with no added AOX. The peak areas of propanal and 1 penten-3-ol formed in the AP fortified beverage were larger, by more than a factor of 3, compared to beverage with no added AOX (Appendix M). This may suggest that the addition of AP did not inhibit or reduce the formation of headspace volatiles, but may have enhanced the formation. Let and others reported that the addition of AP to FO enriched milk emulsions provided a high degree of oxidative stability and almost completely blocked oxidation of the product stored at 2°C for 12 days [48]. AP may have inhibited the decomposition of linoleate hydroperoxides which may account for the reduction in headspace hexanal observed in the AOX fortified systems.

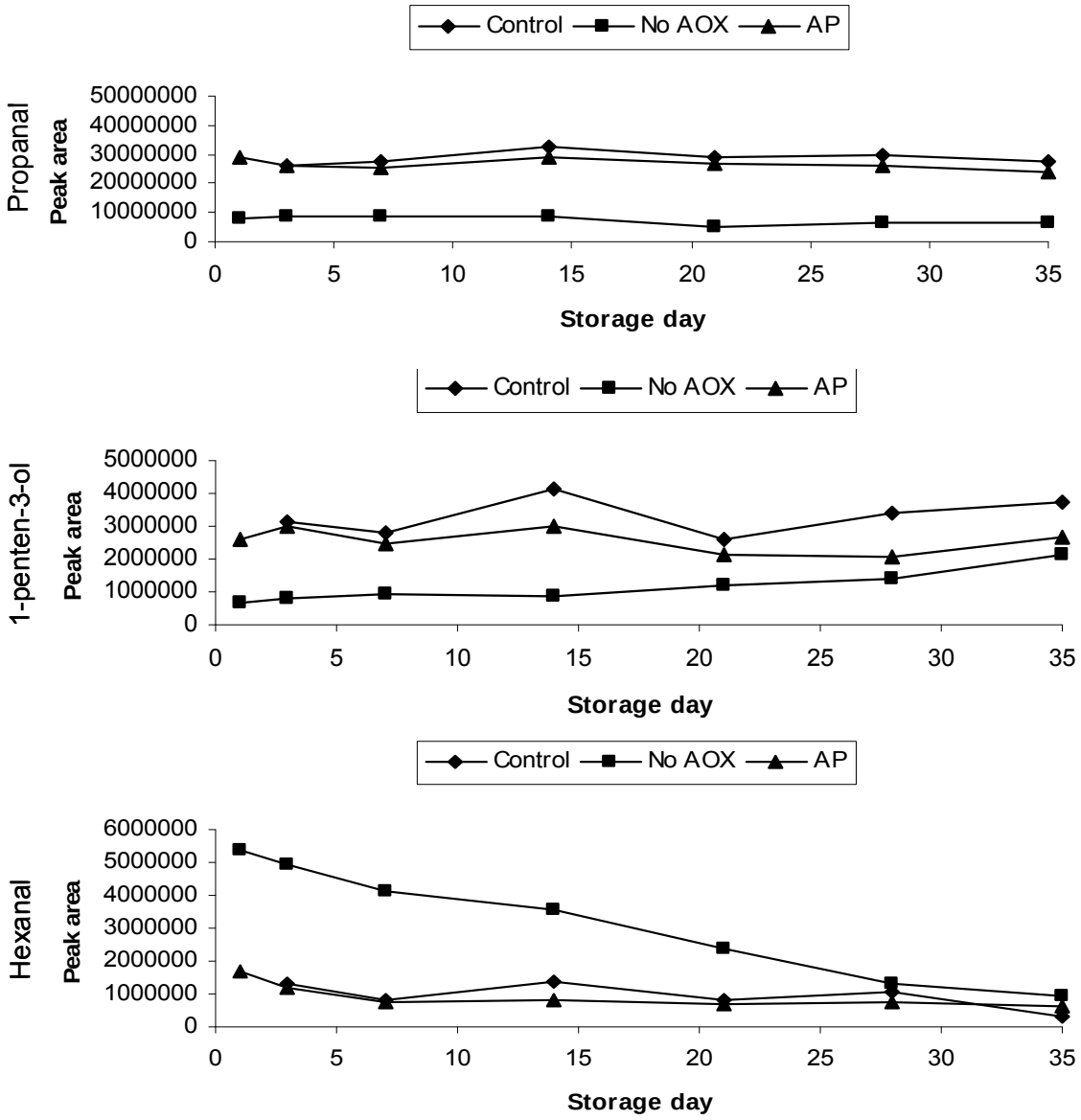

Figure 4-9. Formation of (A) propanal, (B) 1-penten-3-ol, and (C) hexanal during 35 days of storage at 4°C in UHT processed omega-3 FA and AP + omega-3 FA fortified dairy-based beverages. Control = 100%BO:0%FO. No AOX = 78%BO:22%FO. AP = 78%BO:22%FO + ascorbyl palmitate (300mg/kg FO).

Sensory Analysis of Odor in an AP and Omega-3 Fortified Dairy Based Beverage

A ranking system was utilized in this research, rather than a rating system, to evaluate oxidative aromas originating from omega-3 FA oxidation. The ranking test provided a relative order of oxidative aroma intensity, which allowed for a measurement of the AOX efficacy against oxidative aromas. The ranking test, however, gave less information about the oxidative aromas, compared to a rating test, because the intervals of aroma intensity could not be determined [64]. Because a ranking test was used, the intensity of oxidative aromas in the omega-3 fortified systems, compared to the control samples, was unable to be determined.

Conjugated diene analysis was used to determine degrees of oxidation of samples used during sensory analysis. Reference samples were consistent in CD values during sensory analysis while the control and test samples were observed to decrease during storage (Appendix K). There was no significant difference in oxidation odor intensity among the four samples on storage days 3, 14, and 35. However, a significant difference in oxidation odor was found among samples on day 18.The reference samples were ranked to have higher oxidative aromas throughout storage compared to the control and omega-3 fortified beverages, with the exception of day 3, where the omega-3 fortified beverage was ranked higher in oxidative aroma intensity compared to the mildly oxidized reference sample (Appendix N). These sensory findings may suggest that added antioxidants helped to inhibit oxidation and suppress aromas indicative of fish oil (omega-3) oxidation during storage, though no differences were detected at the beginning and end of the storage period. Jacobsen and others reported that in a 2.5% fat milk drink supplemented with fish oil, AP acted as a prooxidant and promoted oxidative odors [10].

Reference samples prepared with oxidized lipids showed consistency in CD values, indicating the samples were consistent in degree of oxidation throughout the sensory study. The repeatability of these samples, in regards to oxidation and CD values, allowed the tracking of increased oxidative aromas of the control and test samples throughout the storage and testing period. CD values determined for all sensory samples were relatively similar during storage. However, differences in aromas of the samples were found suggesting that CD analysis was an inadequate method to correctly determine the degree of oxidation of the dairy-based beverages. The evolution of free sulfhydryl groups in freshly processed UHT milk products, reported by Hutton and Patton[65], may have contributed to volatile aromas in the sensory formulations,

resulting in the findings of no difference among samples on day 3. Oxidation of the lipids in the control and test samples may have contributed to the oxidative aromas of the samples, which may suggest the lack of significant difference found between the sensory samples on day 35 of storage. Sensory analysis for oxidative aromas, via ranking tests using a trained panel, indicated that the AP and omega-3 FA fortified dairy-based beverage increased in oxidative aromas during storage. These findings suggest that AP, added at 300mg/kg FO, acted as a pro-oxidant in the omega-3 FA fortified dairy based-beverage. This supports the increase in volatiles associated with ascorbyl palmitate observed in the first exploratory study.

Overall Conclusions

An AOX and omega-3 FA fortified dairy-based beverage was successfully produced that delivered approximately 389mg of omega-3 fatty acids per 8 oz. serving and was microbially and physically stable throughout a 35 day storage period at 4°C. The fatty acid profile of the omega-3 FA-fortified beverage, compared to the control beverage with no omega-3 FA fortification, was nutritionally enhanced via a reduction of saturated FA and in increase in mono and polyunsaturated FA. A reduction of saturated fats in the diet is encouraged due to their negative effects on health such as cardiovascular disease and high LDL cholesterol levels [66, 67]. These changes allow the dairy-based beverage to deliver a significant amount of omega-3 FA and provide a means for better inclusion of omega-3 FA in the diet through routine consumption. The addition of FO with commercially added AOX seemed to provide protection against oxidation in the beverage without further addition of AOX. This protection was also observed under conditions of light exposure suggesting that the dairy-based beverage could maintain an extended shelf-life under retail and vending operation conditions without a significant loss of nutritional value. AP did not provide additional protection against oxidation under the conditions of this study. LA and BD may be effective in reducing the development of volatile secondary oxidation products that could negatively affect aromas in the dairy-based beverages, though, further investigation is needed.

References

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V. Appendices

Appendix A: Institutional Review Board Approval Letter

VirginiaTech

Office of Research Compliance
Institutional Review Board
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24061
540/231-4991 Fax 540/231-0959
e-mail moored@vt.edu
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FWA00000572(expires 1/20/2010)
IRB # is IRB00000667

DATE: September 18, 2008

MEMORANDUM

TO: Susan E. Duncan
Robert Moore
Kim M. Waterman

Approval date: 9/16/2008
Continuing Review Due Date:9/1/2009
Expiration Date: 9/15/2009

FROM: David M. Moore

SUBJECT: **IRB Expedited Approval:** "Formulation of an Ultra High Temperature Processed Omega-3 Fatty Acid and Antioxidant Fortified Extended Shelf-Life Dairy Based Beverage" , IRB # 08-526

This memo is regarding the above-mentioned protocol. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. As Chair of the Virginia Tech Institutional Review Board, I have granted approval to the study for a period of 12 months, effective September 16, 2008.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study's closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher's responsibility to obtain re-approval from the IRB before the study's expiration date.
4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

Important:
If you are conducting **federally funded non-exempt research**, please send the applicable OSP/grant proposal to the IRB office, once available. OSP funds may not be released until the IRB has compared and found consistent the proposal and related IRB applicaton.

cc: File

Appendix B: Demographics Questionnaire

Panelist # _____ (match with sensory scorecard)

Demographic Questionnaire

1. Indicate your age group:
   - o 18-25
   - o 26-35
   - o 36-45
   - o 46-55
   - o 56-65
   - o over 65
     a. **You must be at least 18 years old to participate in this study. If you are under 18, please do not continue. Come to the sensory kitchen to receive a token of appreciation for your interest in the study.**

2. Indicate your gender:
   - o Male
   - o Female

3. How often (# times per week) do you consume fatty fish (such as salmon, tuna, mackerel) with a meal?
   - o Never or up to 3x per month
   - o 1-2 meals per week
   - o 3-4 meals per week
   - o 4-5 meals per week
   - o 6-7 meals per week
   - o more than 7 meals per week
4. How often (# times per week) do you consume dairy products?
   - o Never or up to several times per month
   - o 1-3 servings per week
   - o 4-6 servings per week
   - o 7-9 servings per week
   - o 10-12 servings per week
   - o more than 12 servings per week
5. Identify the statement that best fits.
   - o I am not aware of any potential health benefits associated with regular consumption of fatty fish.
   - o I am vaguely aware of potential health benefits associated with regular consumption of fatty fish.
   - o I am generally aware of potential health benefits associated with regular consumption of fatty fish.
   - o I am very aware of potential health benefits associated with regular consumption of fatty fish.

6. Identify the statement that best fits.
   - o I am not aware of any potential health benefits associated with regular consumption of omega-3 fatty acids.
   - o I am vaguely aware of potential health benefits associated with regular consumption of omega-3 fatty acids.
   - o I am generally aware of potential health benefits associated with regular consumption of omega-3 fatty acids.
   - o I am very aware of potential health benefits associated with regular consumption of omega-3 fatty acids.

7. Do you make any attempt to supplement your diet or increase your dietary intake of omega-3 fatty acids?
    - o No (continue with Question 8)
    - o Yes (continue with the second part of this question; mark all that apply)
        - o By eating more fish
        - o By consuming omega-3 fortified foods
        - o By taking omega-3 fatty acid supplements
            - o Fish oil supplements
            - o Flax oil supplements
        - o By consuming other foods naturally rich in omega-3 fatty acids

            List foods:

            _____

            _____

8. Omega-3 fatty acids have potential health benefits which include improved cardiovascular health and brain functions. If you could consume one serving daily of an omega-3 fortified dairy product to achieve the level of omega-3 fatty acids for potential health benefits (choose one statement that best matches your attitude).
    - o I would not choose omega-3 enriched dairy products or I might choose an omega-3 enriched dairy product occasionally over a few weeks.
    - o I might choose to consume an omega-3 enriched dairy product a few (1-3) days each week.
    - o I might choose to consume an omega-3 enriched dairy product most (4-5) days each week.
    - o I might choose to consume an omega-3 enriched dairy product every day.
    - o I would definitely choose to consume omega-3 enriched most (5-7) days of the week.

Appendix C: Human Subjects Consent Form

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

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

Title of Project: Formulation Of An Ultra High Temperature Processed Omega-3 Fatty Acid and Antioxidant Fortified Extended Shelf-Life Dairy Based Beverage

Investigators: Robert Moore, MS, Susan E. Duncan, PhD, RD

I. Purpose of this Research/Project

You are invited to participate in a sensory test to determine the acceptability level of a dairy based product supplemented with omega-3 fatty acids (FA) and antioxidants. Omega-3 FA have been shown to be beneficial to one's health in areas such as the brain and heart. Studies have shown that most people don't consume adequate levels of omega-3 FA via their daily diet to achieve these health benefits. Inclusion of appropriate levels of omega-3 FA in a commonly consumed healthy beverage, such as a dairy beverage, may have the potential to deliver the health benefits of omega-3 FA to more consumers. The purpose of this study is to determine how acceptable the proposed level of omega-3 FA supplemented dairy based products is to consumers.

II. Procedures

There will be one sensory testing session lasting approximately 15 minutes. You will first complete a consumer survey. After that, you will be presented with two sets of three dairy product samples. For both sets of samples, you will be asked to identify the one sample that is different from the other two, based on the aroma of the samples. Do not taste or drink the samples.

Some individuals are sensitive to certain foods such as dairy products. If you are aware of any food or drug allergies you may let the investigator know and indicate those allergies in the survey.

III. Risks

There are no more than minimal risks for participating in this study. If you are aware of any allergy reactions to dairy products or fish, please inform the investigator.

IV. Benefits

Your participation on this study will provide valuable information about the acceptability level of omega-3 FA supplemented dairy based products. Results from this sensory evaluation will be used to determine if proposed levels of supplemented omega-3 FA are distinguishable and/or acceptable to consumers. If you would like a summary of the research results, please contact the researcher at a later time.

V. Extent of Anonymity and Confidentiality

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

VI. Compensation

You will not be compensated for participating in this study. You will receive a candy treat for participating.

VII. Freedom to Withdraw

If you agree to participate in this study, you are free to withdraw from the study at any time without penalty. There may be reasons under which the investigator may determine you should not participate in this study. If you have allergies to dairy products or fish, or are under the age of 18, you are asked to refrain from participating.

VIII. Subject's Responsibilities

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

1) Complete a consumer survey
2) Smell the dairy based products and identify the one sample that is different from the other two based on aroma.

IX. Subject's Permission

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

_____ Date_____
Subject signature

---For human subject to keep------

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

Robert Moore, Graduate Research Assistant, Investigator (540) 231-6806;
moore78@vt.edu

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

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

Appendix D: Microbial counts (colony forming units) on aerobic, coliform, and yeast and mold Petrifilm™ count plates for chapter 3 dairy-based formulations during 35 days storage at 4°C.

Day 0

Plate→	Aerobic			Coliform			Yeast/mold		
Dilution→	10^{-1}*	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Formulation %BO:%FO									
100:0	0,0[1]	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
90:10	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
78:22	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
62:38	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0

Day 7

	Aerobic			Coliform			Yeast/mold		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
100:0	0,0	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0
90:10	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
78:22	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
62:38	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0

Day 14

	Aerobic			Coliform			Yeast/mold		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
100:0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
90:10	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0
78:22	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0
62:38	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0

Appendix D cont.

	Day 21								
	Aerobic			Coliform			Yeast/mold		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
100:0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
90:10	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,1
78:22	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
62:38	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
	Day 28								
	Aerobic			Coliform			Yeast/mold		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
100:0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
90:10	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
78:22	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
62:38	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
	Day 35								
	Aerobic			Coliform			Yeast/mold		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
100:0	0,0	0,0	0,1	0,0	0,0	0,0	0,0	0,0	0,0
90:10	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
78:22	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
62:38	0,104	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0

* 10^{-1}= 1:10 dilution, 10^{-2}= 1:100 dilution, 10^{-3}= 1:1000 dilution

[1] colony forming units in replication 1, colony forming units in replication 2

Appendix E: Emulsion stability data showing % change in fat of top and bottom layers for omega-3 FA fortified dairy-based beverages during 35 days of storage at 4°C.

		Percent fat					
		Replication 1			Replication 2		
Formulation (%BO:%FO)	Storage day	Upper	Lower	% change	Upper	Lower	% change
100:0	0	3.0	3.1	3	2.9	3.2	9
100:0	2	2.9	2.9	0	2.3	3.7	38
100:0	4	3.0	2.8	-7	2.3	4.1	44
100:0	7	3.0	2.6	-15	2.0	3.8	47
100:0	11	3.2	2.5	-28	1.9	3.9	51
100:0	14	3.0	2.5	-20	2.1	3.6	42
100:0	21	3.1	2.5	-24	2.3	3.9	41
100:0	28	2.9	2.1	-38	2.2	3.9	44
100:0	35	3.3	2.0	-65	2.1	3.4	38
90:10	0	3.1	3.0	-3	3.0	3.2	6
90:10	2	3.2	3.0	-7	2.4	3.6	33
90:10	4	3.2	2.9	-10	2.4	4.3	44
90:10	7	3.2	2.7	-19	2.1	4.2	50
90:10	11	3.2	2.6	-23	2.1	4.2	50
90:10	14	3.3	2.4	-38	2.3	4.0	43
90:10	21	3.3	2.4	-38	2.1	4.4	52
90:10	28	3.1	1.9	-63	2.2	3.9	44
90:10	35	3.2	1.9	-68	2.1	4.2	50
78:22	0	3.1	3.1	0	3.2	3.3	3
78:22	2	3.1	2.9	-7	2.4	3.7	35
78:22	4	3.1	2.8	-11	2.2	4.3	49
78:22	11	3.3	2.5	-32	1.9	4.2	55
78:22	14	3.1	2.4	-29	2.0	4.5	56

78:22	21	3.2	2.3	-39	2.0	4.2	52
78:22	28	3.1	1.8	-72	2.1	4.0	48
78:22	35	3.2	1.7	-88	1.9	3.6	47
62:38	0	3.0	3.1	3	3.2	3.2	0
62:38	2	3.0	3.1	3	2.3	3.5	34
62:38	4	2.9	3.1	6	2.4	4.4	45
62:38	7	2.7	3.2	16	2.1	4.1	49
62:38	11	3.1	2.4	-29	2.2	4.1	46
62:38	14	2.4	3.1	23	2.3	4.0	43
62:38	21	2.4	3.2	25	2.2	4.1	46
62:38	28	1.9	3.1	39	2.2	4.0	45
62:38	35	1.9	3.2	41	2.0	4.1	51

Appendix F: Retention Times of HS-SPME-GC/MS External Reference Compounds.

Compound	Retention time (min)
Acetaldehyde	1.4
Propanal	1.6
1-penten-3-ol	3.2
1-penten-3-one	3.5
Hexanal	6.7
2,4-heptadienal	17.6

Appendix G

Figure G-1. SPME-GC/MS Chromatogram of Fish Oil Used for Dairy-Based Beverage Formulation. (A) Fresh Fish Oil, (B) Oxidized Fish Oil.

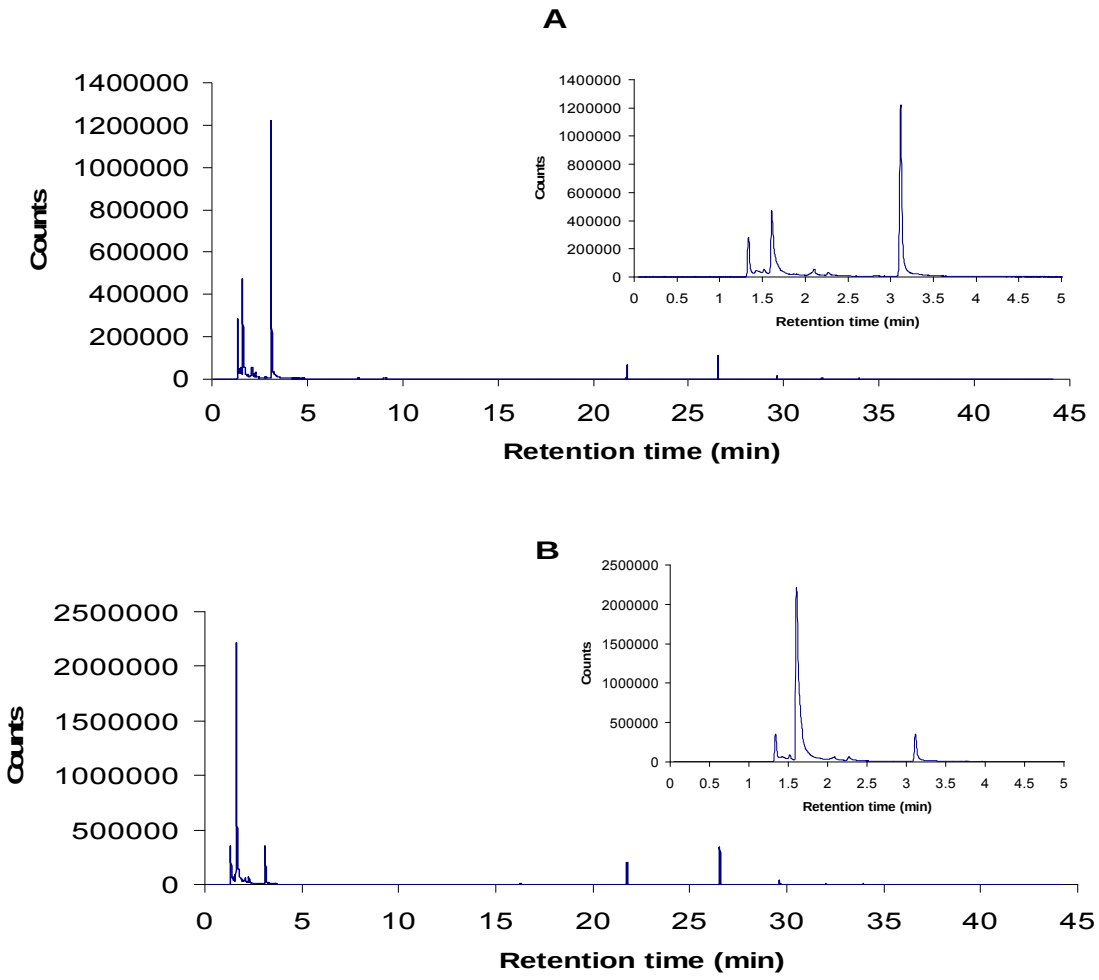


Figure G-2. SPME-GC/MS Chromatogram of Butter Oil Used for Dairy-Based Beverage Formulation. (A) Fresh Butter Oil, (B) Oxidized Butter Oil.

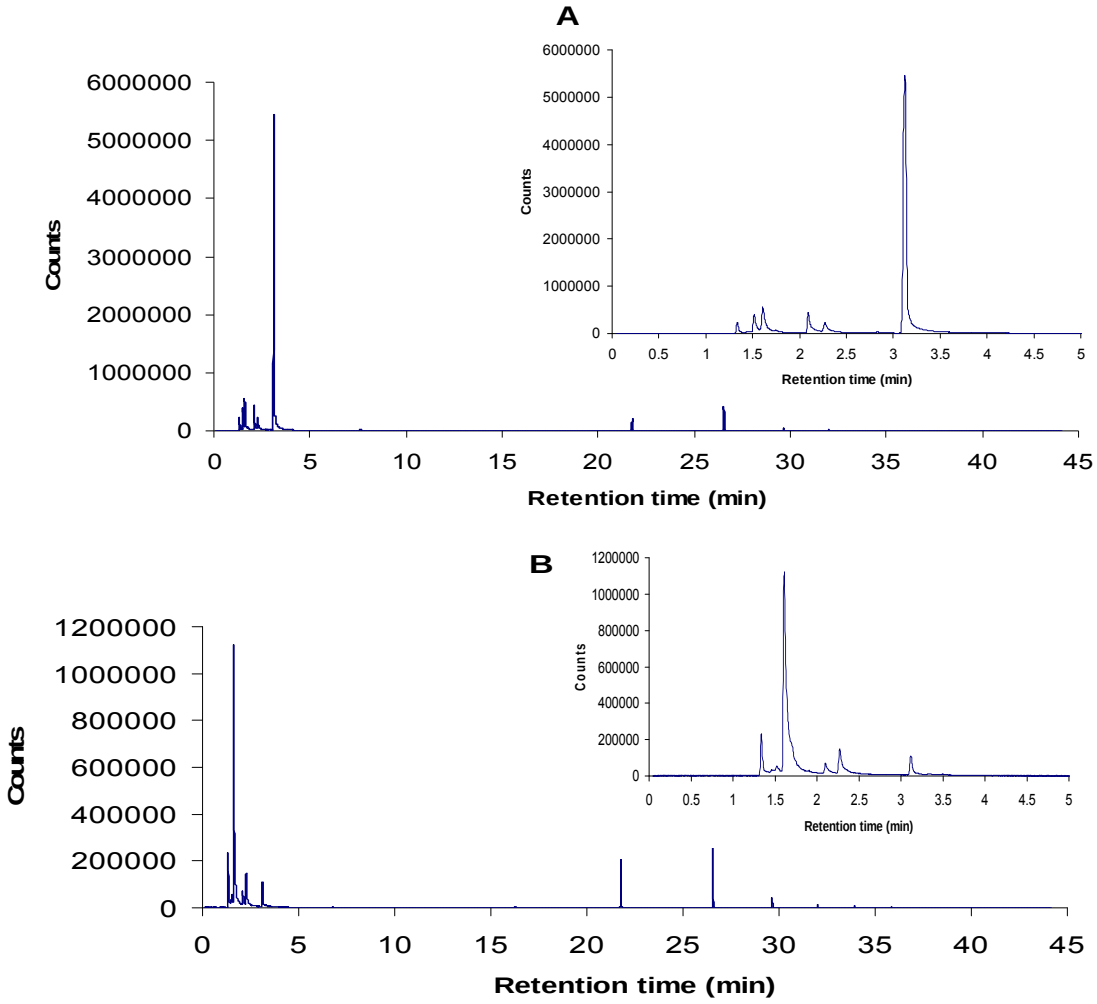
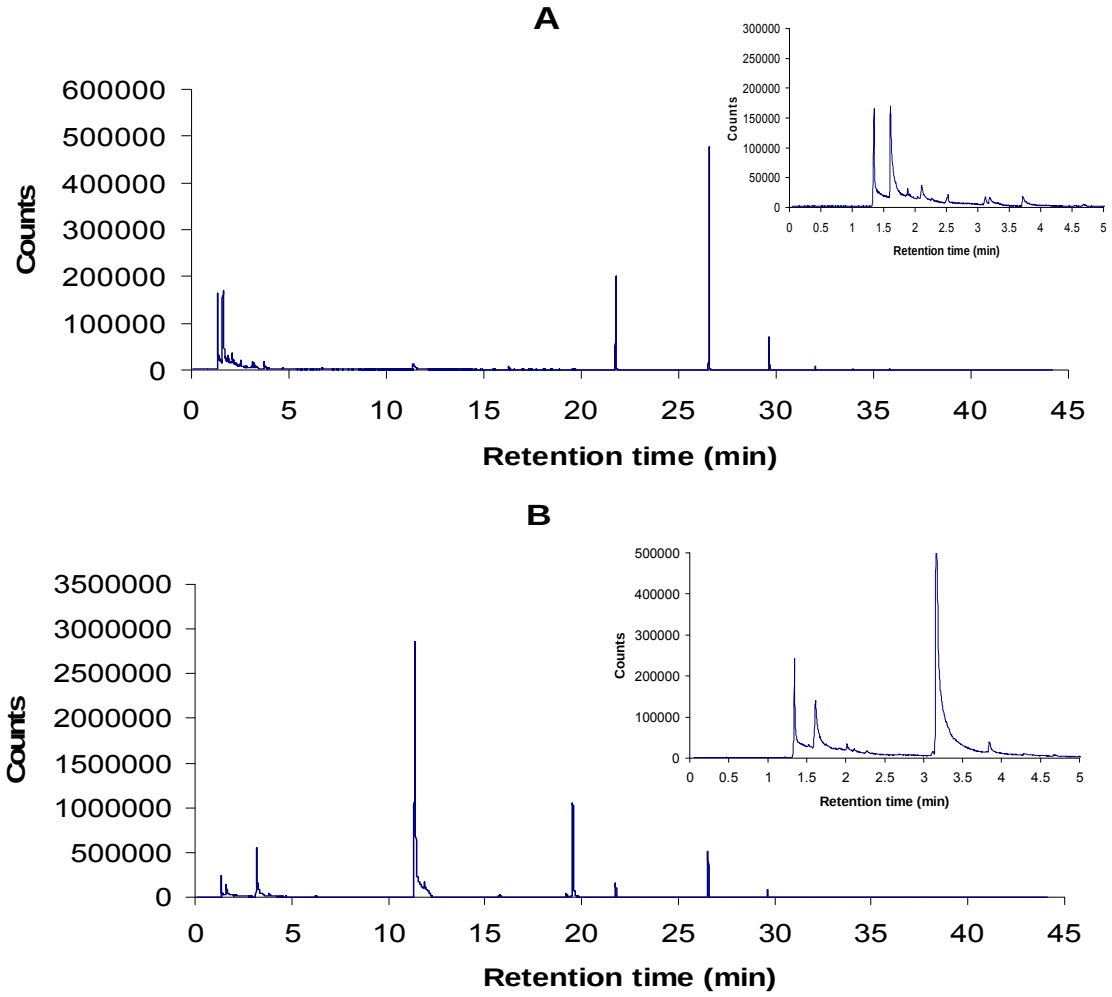


Figure G-3. SPME-GC/MS Chromatogram of Commercial 2.0% Fat UHT Milk. (A) Fresh Opened, (B) Stored Opened.

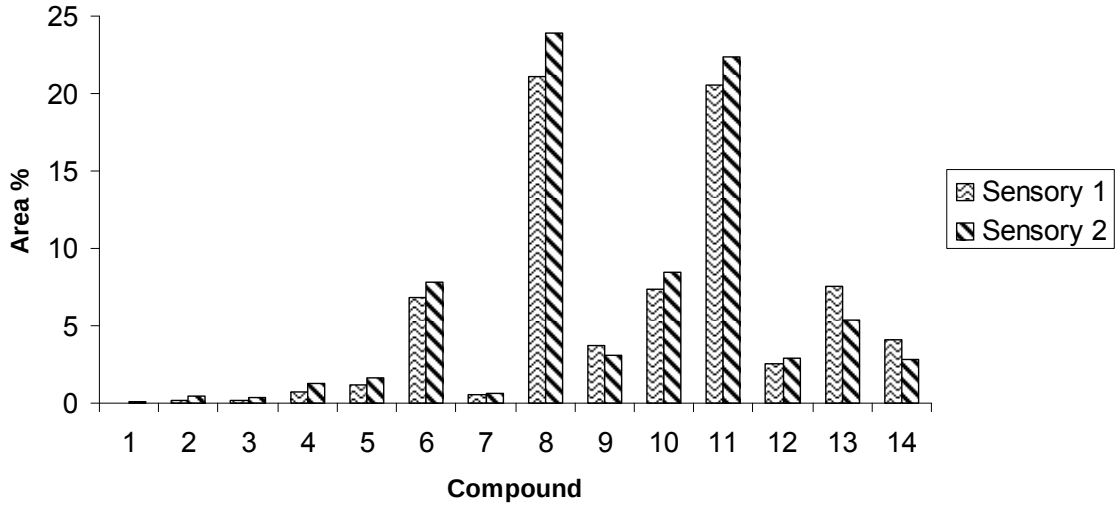


Appendix H

Table H-1: Fatty acids identified via GC/MS in sensory samples (78%BO:22%FO) used for overall aroma analysis during sensory sessions 1 and 2.

Compound Number	Systematic Name	Common Name
1	Butanoic Acid	Butyric acid
2	Hexanoic Acid	Caprioc acid
3	Octanoic Acid	Caprylic acid
4	Decanoic Acid	Capric acid
5	Dodecanoic Acid	Lauric acid
6	Tetradecanoic Acid	Myristic acid
7	Pentadecanoic Acid	Isopalmitic acid
8	Hexadecanoic Acid	Palmitic acid
9	9-Hexadecenoic Acid	Palmitoleic acid
10	Octadecanoic Acid	Stearic acid
11	9-Octadecenoic Acid	Oleic acid
12	9,12-Octadecadienoic Acid	Linoleic acid
13	Eicosapentaenoic Acid	Eicosapentaenoic Acid
14	Docosahexaenoic Acid	Docosahexaenoic Acid

Figure H-1: Relative amounts of fatty acids identified via GC/MS in sensory samples (78%BO:22%FO) used for overall aroma analysis during sensory sessions 1 and 2.



Appendix I: Demographics Questionnaire Results

		Overall sample (n=50)	
		number of responses	Percentage
Gender	Female	36	72
	Male	14	28
Age range			
Age Group	18-25 y	28	56
	26-35 y	13	26
	36-45 y	3	6
	46-55 y	3	6
	56-65 y	1	2
	Over 65 y	2	4
Fish consumption			
Meals per week	never or < 3/month	28	56
	1-2	21	42
	3-4	1	2
	4-5	0	0
	6-7	0	0
	>7	0	0
Dairy consumption			
Times per week	never or < 3/month	0	0
	1-3	5	10
	4-6	18	36
	7-9	0	0
	10-12	23	46
	>12	4	8
Knowledge of potential health benefits of consuming fatty fish			
Awareness level	Not aware of benefits	0	0

	Vaguely aware	7	14
	Generally aware	21	42
	Very aware	22	44
Knowledge of potential health benefits of consuming omega-3 fatty acids			
Awareness level	Not aware of benefits	0	0
	Vaguely aware	1	2
	Generally aware	22	44
	Very aware	27	54
Attempt to supplement or increase intake of omega-3 fatty acids?			
Response	No	20	40
	Yes	30	60
If yes, how?	Eat more fish	12	40
	Omega-3 fortified foods	8	26
	Omega-3 supplements	17	56
	Foods naturally rich in omega-3 fatty acids	13	43
Do you take omega-3 fatty acid supplements?			
Response	No	34	68
	Yes	16	32
If yes, what type?	Fish oil	11	69
	Flax oil	6	37
Willingness to consume omega-3 fortified dairy products			
Response	Would not consume	8	16
	Occasionally (<4/month)	16	32
	Might consume 1-3 days/wk	17	34
	Might consume 4-5 days/wk	3	6

	Might consume every day	3	6
	Definitely would consume 5-7 days/wk	3	6

Appendix J: Compound names and aroma descriptors used for oxidative aroma panel training.

Chemical name	Aroma/odor/scent[1]
Trans-2-nonenal	Cardboard, woody, waxy
p-Menthane-8-thiol-3-one	Catty, body odor, acidic
Isovaleric acid	Cheesy, sweaty
Dimethyl disulfide	Cooked vegetable
5-methyl furfural	Papery, leather, cigar
Butyric acid	Rancid, spoiled
Hexanal[A]	Green, grassy, walnuts
2,4-heptadienal[A]	Nutty, spicy, fatty, cucumber
1-penten-3-one[A]	Pungent, green, painty
	Musty[B], decaying leaves
	Medicinal[B], chemical, rubbery
	Light struck[B], skunky

[1] Aromas/odors given by the kit and described by the aroma panel.
[A] Not included in beer taint aroma kit.
[B] Included in beer taint aroma kit with no compound name.

Appendix K

Table K-1: Percent Conjugated dienoic acid values of omega-3 FA fortified UHT pasteurized dairy-based beverage systems (62% butter oil: 38% fish oil) with and without antioxidants during 35 days of storage at 4°C.

	% Conjugated Dienoic Acid by AOX Formulation[1]				
Storage day	Control[2]	AP[3]	KR[3]	BD[3]	LA[3]
0	0.79	0.69	0.75	0.73	0.80
1	0.75	0.64	0.71	0.67	0.66
5	0.65	0.67	0.61	0.58	0.68
7	0.71	0.72	0.83	0.94	0.79
11	0.69	0.72	1.43	0.79	0.70
21	0.78	0.80	0.74	1.04	0.90
28	0.77	0.87	0.65	0.66	0.91
35	0.56	0.67	0.60	0.70	0.66

[1] n=1 replication
[2] Contains no AOX
[3] AP: ascorbyl palmitate, KR: kudzu root, BD: boldine, LA: lipoic acid

Table K-2: Conjugated diene values determined for UHT processed FO fortified formulations during 35 days of storage at 4°C with and without protection from UV light.

% Conjugated dienoic acid by formulation (BO:FO)				
	Light protected samples		Light exposed samples	
Storage day	78:22	62:38	78:22	62:38
0	0.87	0.87	0.87	0.87
1	0.80	0.80	0.79	0.85
3	0.85	0.86	0.82	0.85
7	0.92	0.90	0.91	0.56
14	0.90	0.92	0.90	1.18
21	0.94	1.00	0.96	0.97
35	0.86	0.94	0.96	0.98

*n=1 replication

Table K-3: Conjugated diene values (% conjugated dienoic acid ($\bar{x}$ ± sd)) of AP and omega-3 FA fortified dairy-based beverages by storage day (4°C).

Storage day	% Conjugated Dienoic Acid By Formulation	
	100:0[1](control) $\bar{x}$ ± sd	78:22[2] $\bar{x}$ ± sd
0	0.80(±0.00)	0.96(±0.12)
1	0.77(±0.00)	0.98(±0.03)
3	1.07(±0.00)	0.93(±0.12)
5	0.91(±0.00)	0.95(±0.06)
7	0.79(±0.00)	0.83(±0.03)
11	0.71(±0.00)	0.81(±0.04)
14	0.71(±0.00)	0.75(±0.07)
21	0.74(±0.00)	0.81(±0.00)
28	1.07(±0.00)	0.88(±0.16)
35	0.75(±0.00)	0.81(±0.04)

*Results are expressed as mean ± standard error of mean. Values within each row and column are not significantly different.

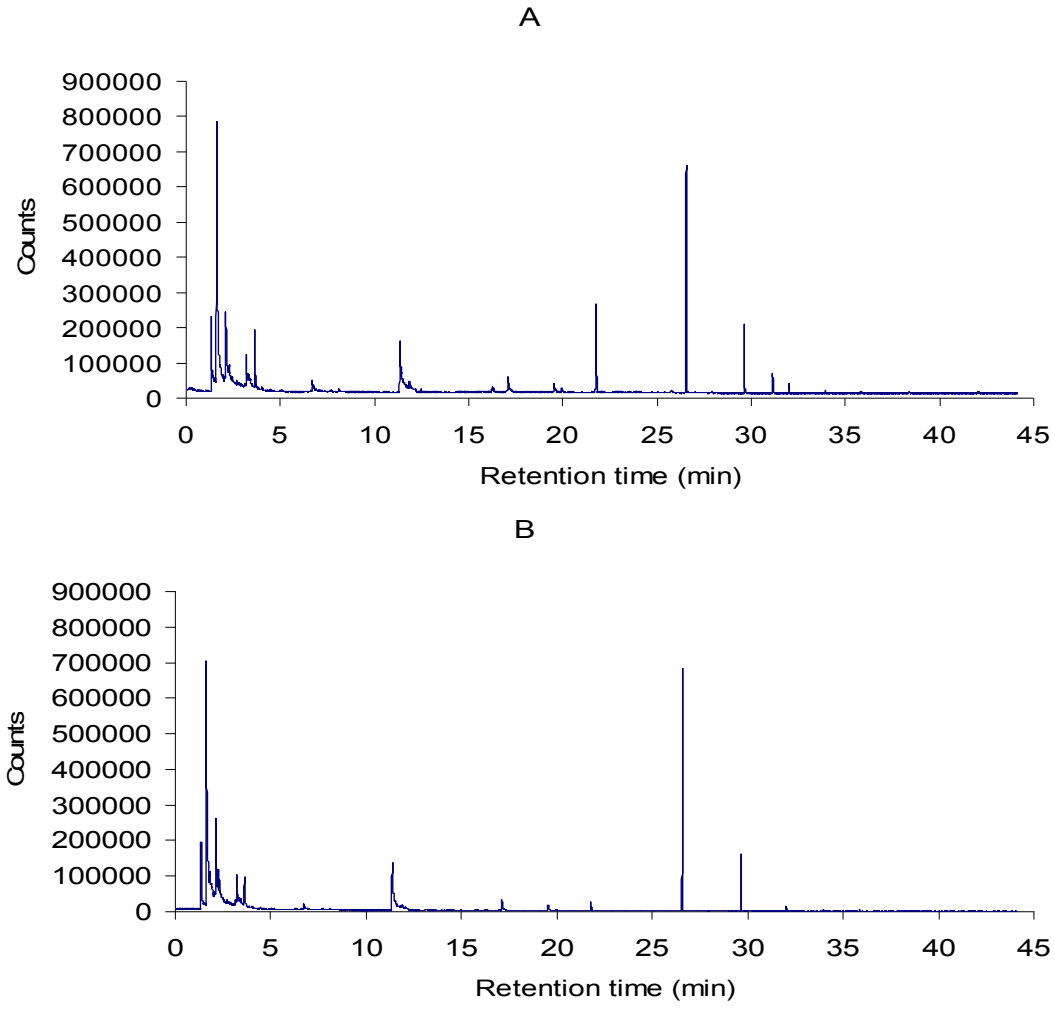
[1]n=1 replication analyzed in triplicate. sd < 0.00

[2]n=3 replications analyzed in triplicate

Table K-4: Conjugated diene values (% conjugated dienoic acid) determined in sensory sample used for oxidative aroma analysis via ranking tests.

	% conjugated dienoic acid by formulation			
	Sensory Sample			
Storage day	Control (100:0)	Test (78:22)	Reference 1	Reference 2
3	1.065	0.930	0.766	0.803
14	0.712	0.754	0.775	0.801
18	0.735	0.815	0.784	0.805
35	0.752	0.808	0.776	0.807

Appendix L: SPME-GC/MS chromatograms of ascorbyl palmitate (300mg/kg fish oil) and omega-3 FA fortified (78:22) formulations on (A) day 1 and (B) day 35 of storage.



Appendix M. Peak areas of major headspace volatiles formed during storage at 4°C in UHT processed omega-3 FA fortified dairy-based beverages.

	Control (100:0)		No AOX (78:22)		AOX (78:22)	
Compound	Day 3	Day 35	Day 3	Day 35	Day 3	Day 35
Propanal	26262245	27306737	8741276	6246285	26359784	24272652
2-butanone	9394148	12019003	1200000	2931306	10886431	9224752
1-penten-3-ol	3111869	3728405	801468	2153019	3018593	2647951
Hexanal	1342169	299673	4922453	965762	1193814	621132
2-heptanone	6798173	7270813	872582	4919997	7857366	7713346
Total peak area→	46908604	50624631	16537779	17216369	49315988	44479832

Control= 100%BO:0%FO. No AOX= 78%FO:22%BO. AOX= 78%FO:22%BO + ascorbyl palmitate (300mg/kg FO)

Appendix N: Ranking of dairy-based beverage samples for oxidative aromas from most oxidized to least oxidized using the rank sum average.

	Storage day			
Overall rank	**3**	**14**	**18**	**35**
Most oxidized	H.O (3.1) [1]	L.O (3.4)	H.O (3.4)	H.O (3.0)
↕	AOX ω3 (2.8)	H.O (2.6)	L.O (3.1)	L.O (3.0)
	L.O (2.3)	AOX ω3 (2.3)	Control (1.9)	AOX ω3 (2.0)
Least oxidized	Control (1.8)	Control (1.8)	AOX ω3 (1.6)	Control (2.0)
	T=5.6	T=6.8	T=11.1	T=4.8

[1]Sample ID (rank sum average).
χ^2=7.8 at 3 degrees of freedom. Samples are significantly different if T> χ^2
Control=100:0 %BO:%FO, AOX ω3 = 78:22 %BO:FO ascorbyl palmitate and omega-3 FA fortified beverage, L.O= mildly oxidized reference sample, H.O= highly oxidized reference sample