Fate of Omega-3 Fatty Acids from Algae in Mozzarella Cheese

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ABSTRACT

Increased consumer interest in omega-3 fatty acids (FA) has led to novel foods with added omega-3 FA. Additional information regarding omega-3 FA fate within foods is needed for improving quality and stability. This research modeled DHA, an omega-3 FA, fate and explored means of preventing degradation and oxidation of FA in algal oil and mozzarella cheese.

In algal oil, TBHQ (synthetic antioxidant) at 0.0175g/g algal oil prevented DHA degradation for at least 6 weeks, and mixed tocopherols (natural antioxidant) at 400ppm prevented DHA degradation and oxidation for about 4 weeks. DHA degradation in algal oil was modeled by an autocatalytic equation.

The fate of DHA from algal oil in mozzarella cheese was also modeled by an autocatalytic equation. In an effort to prevent DHA degradation and oxidation, mixed tocopherols were added. The optimum combination of those tested was found, using a response surface design, to be 3% algal oil with 110ppm mixed tocopherols for maximum DHA and minimum oxidation over 2 weeks. This algal oil/antioxidant combination in mozzarella cheese successfully prevented oxidation and DHA degradation over 3 weeks of storage. Approximately 0.1g DHA may be consumed from a 28g serving of this cheese. Approximately 0.5-18 servings of this cheese are equivalent to DHA consumed from a 3oz serving of fish, depending on fish type. Sensory evaluation tests found consumers could distinguish between mozzarella cheese with/without algal oil. Results from this study improve understanding of omega-3 FA behavior in mozzarella cheese and provide a means for preserving quality and nutrition.

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CHAPTER 1
INTRODUCTION

1.1 RATIONALE AND SIGNIFICANCE

Omega-3 fatty acids, which are essential fatty acids, are necessary for human health. Despite recent increased awareness regarding the importance of omega-3 fatty acids (IFIC 2008), many American diets do not consume adequate amounts of these fatty acids to realize the health benefits (Kris-Etherton and others 1998; Newton 1998; Raper and others 1998). Common sources of these nutrients are fish, oils, nuts, and supplements (Hunter 1990). However, many sources of omega-3 fatty acids do not include docosahexaenoic acid (DHA), a specific type of omega-3 fatty acid especially important for cardiovascular, mental, and visual health (Wang and others 2004; Hodge and others 2005; Schachter and others 2005). Fish and omega-3 supplements are currently the major dietary sources of DHA (O'Flaherty 2004). Diversifying the dietary source of DHA, such as mozzarella cheese supplemented with algal oil DHA, may result in increased consumer consumption of this important nutrient.

Dairy foods, especially cheeses, are very popular in America. In particular, mozzarella cheese is a popular and commonly consumed product, whose consumption is increasing (Putnam and Allshouse 2003; FAPRI 2006; WMMB 2008). Additionally, dairy foods have health benefits, including improved bone health and weight management (Heaney and others 2000; Zemel and others 2004).

Algal oil is a good source of DHA and is currently used in products such as infant formula (Ward and Singh 2005). Algal oil is superior to fish oil as a source of DHA due to the vegetarian source that can be quickly and easily replenished, has no risk of heavy metal contamination, and yields a much higher DHA content (New and Wijkström 2002; Ward and Singh 2005).

Information regarding the fate and oxidation of DHA within food vehicles for omega-3 fatty acids is limited (Gulati and others 2002; Chee and others 2005; Allred and others 2006). By modeling the fate of DHA within food vehicles, the stability and shelf-life of such products can be better understood. Then, by addressing any fatty acid

degradation and oxidation during storage, the quality and shelf-life of these products may be improved.

1.2 HYPOTHESIS

The hypothesis of this research is that DHA from algal oil supplemented into mozzarella cheese can be preserved through antioxidants to increase the stability of the product.

1.3 OBJECTIVES

The objectives of this project are as follows:

1) determine the efficacy of synthetic and natural antioxidants in algal oil fatty acids;
2) study the fate of DHA in algal oil-fortified mozzarella cheese; and
3) optimize and determine the efficacy of mixed tocopherols in algal oil fortified mozzarella cheese.

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CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

Many Americans' diets do not contain sufficient omega-3 fatty acids, specifically docosahexaenoic acid (DHA, C22:6), to realize the health benefits this essential fatty acid can provide (Kris-Ehterton and others 1998; Newton 1998; Raper and others 1998). DHA has been shown to have effects ranging from decreased risk of cardiovascular disease to decreased risk of Alzheimer's disease (Wang and others 2004; MacLean and others 2005). In an effort to make this nutrient more available to consumers, various products have been developed which have been supplemented with omega-3 fatty acids, using sources such as fish oil, linseed oil, and algal oil (Cortinas and others 2003; Gallaher and others 2005). Algal oil is a good source of omega-3 fatty acids as it has a very high DHA content, and it is also a safe and "vegetarian" source (Abril and others 2003; Lloyd-Still and others 2006). Dairy products are an attractive option as a food vehicle to deliver this nutrient since dairy products also carry many other health nutrients and benefits, such as calcium for reduced risk of osteoporosis. Additionally, conjugated linoleic acids (CLA), a fat found in dairy, has potential health benefits such as reducing cancer risk (O'Shea and others 1998).

2.2 OMEGA-3 FATTY ACIDS

2.2.1 Health Benefits

Health benefits from omega-3 fatty acids include improvement and/or preventative effects regarding cardiovascular disease, cognitive aging, dementia, neurological disease, eye health, and mental health (Wang and others 2004; Hodge and others 2005; MacLean and others 2005; Schachter and others 2005). Additionally, several studies have shown a wide range of other benefits from dietary intake of omega-3 fatty acids. For example, one study explored the effects of DHA from algal oil in 20 cystic fibrosis patients (Lloyd-Still and others 2006). This randomized, controlled study looked at bioavailablility, tissue accretion, lung function, and safety of the DHA source

within the patients (Lloyd-Still and others 2006). Between 1g and 4.2g of DHA was given to each subject each day based on weight (50mg DHA/kg weight) or a placebo (Lloyd-Still and others 2006). DHA supplementation resulted in an increase in DHA levels and a decrease in arachidonic acid (ARA) as well as in the ARA:DHA ratio, while no adverse effects were seen (Lloyd-Still and others 2006).

Another examples is found in an in vitro study which found that eicosatrienoic acid, eicosapentaenoic acid (EPA), docosatrienoic acid, and docosapentaenoic acid (DPA) did not inhibit cancer cell growth for human melanoma A-375, breast carcinoma MCF-7, or prostate adenocarcinoma PC-3 cancer cells (Chiu and others 2005). DHA was the only PUFA that significantly retarded the cancer cell growth (Chiu and others 2005).

The amount of omega-3 fatty acids consumed is an important consideration for health benefits, safety, and product acceptability. Adequate levels of the fatty acid must be consumed to realize the health benefits without any possibility for adverse effects. Table 2-1 shows the amount of omega-3 fatty acids used or found effective in select studies.

Table 2-1. Concentration of omega-3 fatty acids used in select studies.

Intake Level	Source
1 g/d EPA/DHA	Williams (2000)
1 to 4.2 g/d DHA	Lloyd-Still and others (2006)
5.1 g/d EPA and DHA ethyl esters in 3 concentrations (62.5, 80, and 85% of TFA)	Bryhn and others (2006)
about 2 g/d DHA	Arterbum and others (2006)
g EPA/DHA per serving: 1.0 - margarine; 0.4 - salad cream; 0.4 - french dressing; 3.3 - frankfurters; 1.0 - salami	Barlow and others (1990)
up to 1% EPA/DHA w/o significant affect on sensory acceptability in spreadable fats	Kolanowski (2001)
more than 1% fish oil (0.03% EPA/DHA) strongly decreased palatability	Kolanowski (1999)
190 g vienna franks contained 200 mg long-chain n-3 PUFA (75% DHA, 25% EPA)	Volker and others (2005)
8.2 g EPA plus DHA per day	Wallingford (1991)
8 g/d EPA+DHA gave no increase in infections compared to control	Leaf (1992)
0.35g/d DHA+EPA	Kris-Etherton and others (1998)

The dietary ratio of omega-3 to omega-6 fatty acids is another important factor influencing the effectiveness of these fatty acids. One such study explored the effects of this ratio on ex vivo bone prostaglandin E_2 production and rate of bone formation in rats (Watkins and others 2000). For 42 days, these rats were fed 70g/kg additional fat consisting of safflower oil and menhaden oil (Watkins and others 2000). The ratio of omega-6:omega-3 fatty acids fed to the rats were 23.8, 9.8, 2.6, or 1.2, where the majority of the omega-6 fatty acids was AA and the majority of the omega-3 fatty acids was EPA (Watkins and others 2000). Results showed that a lower ratio of omega-6:omega-3 fatty acids fed to the rats correlated with lower production of ex vivo bone prostaglandin E_2, but a higher ratio of omega-6:omega-3 fatty acids correlated with a lower bone formation rate and a lower ratio of both omega-6:omega-3 fatty acids within the bone as well as ex vivo bone prostaglandin E_2 within the bone (Watkins and others 2000).

A similar study fed rats soybean oil (7.3:1 omega-6:omega-3 ratio) or menhaden oil and safflower oil (1.8:1 omega-6:omega-3 ratio) with 10g/kg CLA or no CLA in order to determine the effects of the omega-6:omega-3 fatty acid ratio on liver prostaglandin E_2, macrophage cytokine production, and spleen fatty acid composition in the rats (Turek and others 1998). Soybean oil and CLA consumption caused a decrease in 18:1 fatty acids in the liver of the rats and caused a slight but insignificant reduction in the liver prostaglandin E_2 (Turek and others 1998). The lower omega-6:omega-3 fatty acid ratio caused higher macrophage production and lower spleen prostaglandin E_2 production (Turek and others 1998).

2.2.2 Current Consumption

The omega-3 fatty acid intake in the United States of America has been estimated to be 1.6 g/day (Kris-Etherton and others 1998). Of this 1.6g, it is estimated that 1.4g is from linolenic acid (LNA) and 0.1-0.2g is from EPA and DHA (Kris-Etherton and others 1998). In 1985, the U.S. food supply contained 78mg of DHA and 46mg of EPA per person per day, which made up 4% of the total omega-3 fatty acids in the food supply (the remainder was LNA) (Raper and others 1998). Omega-3 fatty acids made up 0.7% of the food energy in 1985, while PUFA made up 8% and total fat made up 43% of the food energy (Raper and others 1998). Additionally, in 1985 the total fat, PUFA, and

omega-3 fatty acids available in the U.S. food supply was 169g, 33g, and 2.92g per person per day, respectively (Raper and others 1998). In 1985, the amounts of omega-3 fatty acids and DHA in the food supply was the highest it had been between the years of 1935 and 1985 (Raper and others 1998). However, the percent of DHA and EPA of the omega-3 fatty acids had a decreasing trend from 1935 to 1985 (Raper and others 1998).

2.2.3 Sources

Omega-3 fatty acids are a type of polyunsaturated fatty acids (PUFA). Omega-3 fatty acids include alpha-Linolenic acid (ALA, 18:3 n-3), Stearidonic acid (18:4 n-3), Eicosapentaenoic acid (EPA, 20:5 n-3), Docosapentaenoic acid (DPA, 22:5 n-3), and Docosahexaenoic acid (DHA, 22:6 n-3) (Finley and Shahidi 2001). ALA is typically found from nut and vegetable sources (such as soybeans, rapeseed, sunflower, corn, cottonseed, peanut, canola), while DPA and DHA are typically found from marine sources (Nettleton 1995). The human body converts ALA to DHA (see Figure 2-4), but the conversion is very inefficient (approximately less than 1%) (Brenna 2002; Goyens and others 2006).

Common sources of PUFA from fish oil are shark liver, salmon, cod liver, tuna, menhaden, trout, mackerel, herring, sardines, Atlantic bluefish, and anchovy (Nettleton 1995; Finley and Shahidi 2001). Potential alternatives include fungi and microalgae (Alonso and Maroto 2000). Due to concerns with fish such as methylmercury contamination, depletion of fish sources, and vegetarianism, alternative DHA sources are receiving more attention (New and Wijkström 2002; DHHS and EPA 2004).

Microbial sources of PUFA, such as *Schizochytrium* sp., are currently being used in infant formula, for pregnant and nursing women, adult dietary supplements, as a food additive, and for cardiovascular health (Ward and Singh 2005). Microbial species which have been used for omega-3 fatty acid production include *C. cohnii*, *T. aureum*, *T. roseum*, *S. limacinum*, *Thraustochytium* sp., *S. mangrovei*, and *Schizochytrium* sp (Ward and Singh 2005). Of these, *Schizochytrium* sp. has great potential due to its high productivity of biomass and DHA (Ward and Singh 2005).)

Previous research studying culture conditions for *Schizochytrium limacinum* has shown potential nitrogen (corn steep liquor) and carbon (glucose, glycerol) sources for

growing the microalga (Yokochi and others 1998). Recently, a process of producing DHA-containing *S. limacinum* has been developed by growing the algae in biodiesel-derived crude glycerol (Chi and others 2007). The resulting algal biomass has the same quality in terms of chemical composition and proximate analyses (Pyle and others 2008).

Algae has been used as a source of the omega-3 fatty acid DHA in feed for aquaculture, poultry (for meat and eggs), and milk (from cows); and in nutrient supplement capsules, nutritional bars, and soy milk drinks (Barclay and others 2005). Other potential applications are swine feed and other dairy products (i.e. yogurts, spreads, margarines, and cheese) (Barclay and others 2005). Other algal sources of DHA have also been proposed, such as *Ulkenia* sp. for which there was an application to Australia and New Zealand as a novel food (FSANZ 2005). *Ulkenia* sp. usually has around 45% DHA in its oil (FSANZ 2005). *Schizochytrium* sp. had already been approved as a rich source of DHA from the dried microalgae or oil from the microalgae (FSANZ 2005). This algae was proposed to be used in foods at the level of 200-300 mg dried algae per serving, which is about 30-40 mg DHA per serving (FSANZ 2002). Oil derived from this type of algae was also proposed to be used at up to 150 mg per serving, including in dairy products such as cheese products (FSANZ 2002).

2.2.4 Extraction

Several methods have been utilized for the extraction of oil from algae, such as the extraction of EPA and AA from *Porphyridium cruentum*, a microalga (Giménez Giménez and others 1998). This study used direct saponification of biomass for fatty acid extraction, urea inclusion complexing for concentration of PUFA, and finally HPLC for EPA isolation (Giménez Giménez and others 1998). The authors tried both ethanol and hexane/ethanol (1:2.5 v/v) for saponification and found 96% (v/v) ethanol to be the most efficient, extracting 75% of the fatty acids (Giménez Giménez and others 1998). The urea inclusion complexing used a ratio of 4:1 urea:fatty acid (w/w) (Giménez Giménez and others 1998). Crystallization temperatures of $4^{o}C$ and $28^{o}C$ were tested, and $28^{o}C$ was found to provide higher output of EPA and AA (Giménez Giménez and others 1998). The optimum HPLC conditions were found to be 4.7 i.d. x 30cm compression radial cartridge (Giménez Giménez and others 1998). By using all of these

optimized processes, 94.3% and 81.4% pure fractions of EPA and AA, respectively, were obtained (Giménez Giménez and others 1998). This study also compared *Phaeodactylum tricornutum*, and *Isochrysis galbana* as sources of EPA and found *Isochrysis galbana* to have the highest overall purity and *Phaeodactylum tricornutum* to have the overall highest yield (Giménez Giménez and others 1998).

The extraction of omega-3 fatty acids from fish typically involves grinding, pressing, hydrolysis/esterification, distillation, glycerolysis, bleaching/deodorization, stabilization, and encapsulation (Finley and Shahidi 2001). The production of omega-3 fatty acid rich algal oil usually involves drying, hexane addition for extraction, chilling/filtering, removing the solvent, centrifuging, bleaching, deodorizing, adding antioxidants, and packaging (Zeller and others 2001).

2.2.5 Incorporation of Omega-3 Fatty Acids into Foods

Various efforts have been made to incorporate omega-3 fatty acids (FA) into dairy products by adding omega-3 FA sources such as fish, algae, canola, soybean, linseed, cod liver, and rapeseed to foods such as milk, cheese (feta, cheddar), yogurt, cream, butter, and buttermilk (Ramaswamy and others 2001; Gulati and others 2002; Lacasse and others 2002; Papadopoulos and others 2002; Hauswirth and others 2004; Chee and others 2005; Let and others 2005; Allred and others 2006). This was achieved by either adding the omega-3 fatty acid source directly to the milk or by including the source in the cow's (or dairy ewe's) diet. However, limited information is available regarding the oxidation of the omega-3 fatty acids within the dairy food vehicle.

2.3 DAIRY FOODS AS A VEHICLE OF OMEGA-3 FATTY ACIDS

2.3.1 Nutritional Benefits

Dairy foods have been shown to have many health benefits including helping to prevent osteoporosis, hypertension, obesity, and heart disease (Griffith and others 1999; Appel and others 2000; Heaney and others 2000; Zemel and others 2004; NDC 2008). Recent studies have shown additional benefits. One study showed that increased dairy intake in addition to diet in obese adults increased weight and fat loss (Zemel and others

2004). Other studies have shown that CLA found in milk fat from cows may help prevent cancer (O'Shea and others 1998).

        Despite recent negative attitudes toward dairy fat due to heart disease, obesity, and cancer, dairy lipids have several nutritional benefits (Fox and McSweeney 2006). For example, the fat provides essential fatty acids and carries fat-soluble vitamins (A, D, E, K) (Fox and McSweeney 2006).

2.3.2 Consumption

        Cheese consumption, including mozzarella cheese (a type of Italian cheese), has had a continuous increasing trend (Figure 2-1) (ERS 2008). Other dairy products, however, have not had the same increasing trend, and cheese availability has surpassed that of all other dairy products except fluid milk and cream (Figure 2-2) (ERS 2008). Consumption is projected to continue to increase (Figure 2-3) (FAPRI 2006).
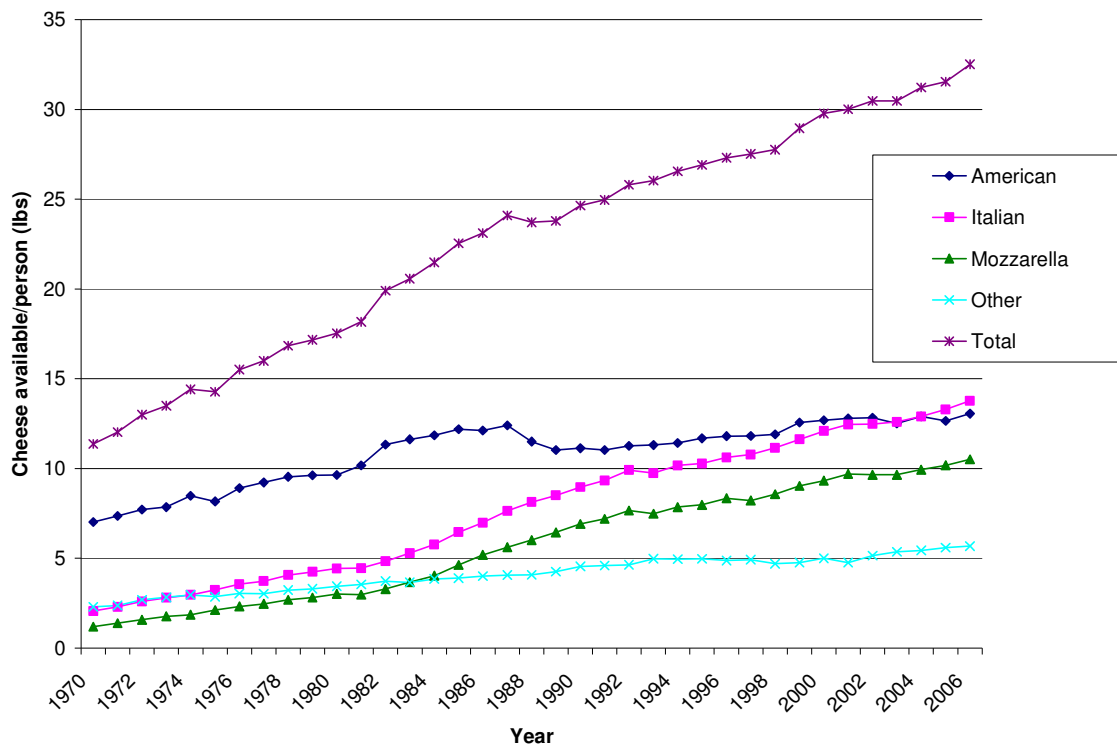
Figure 2-1. Cheese availability per capita in the U.S. from 1970-2006 (data from ERS 2008).
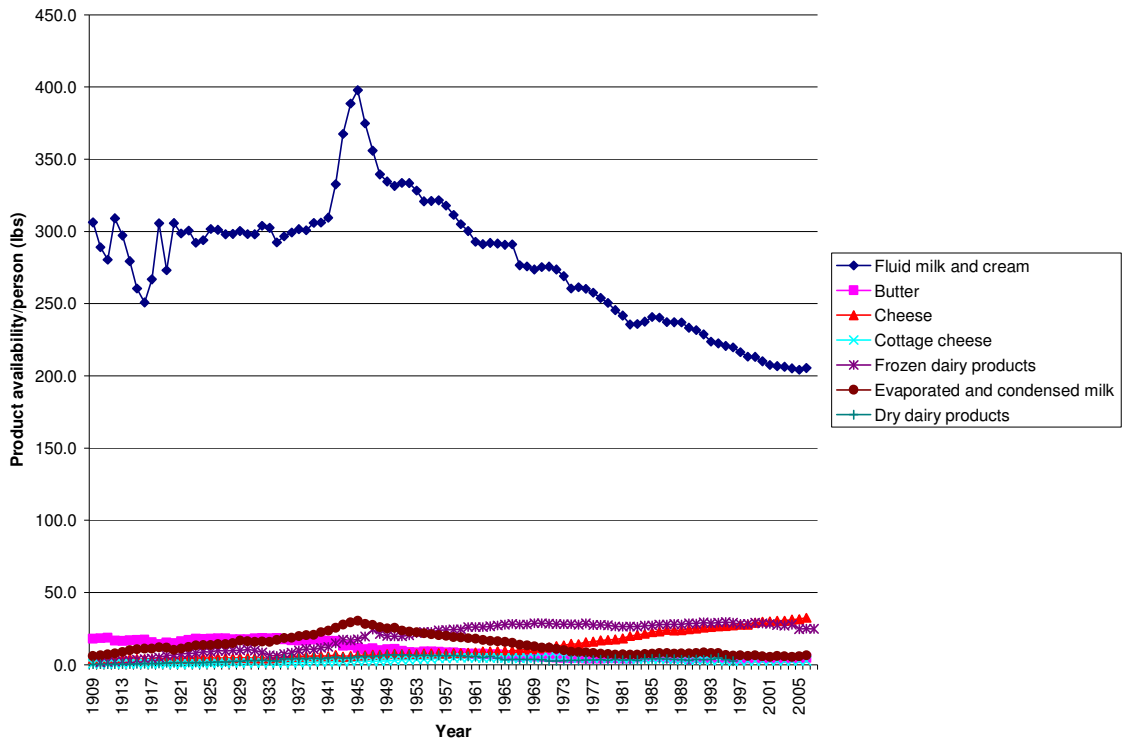
Figure 2-2. Dairy product availability per capita the U.S. from 1909-2006 (data from ERS 2008).
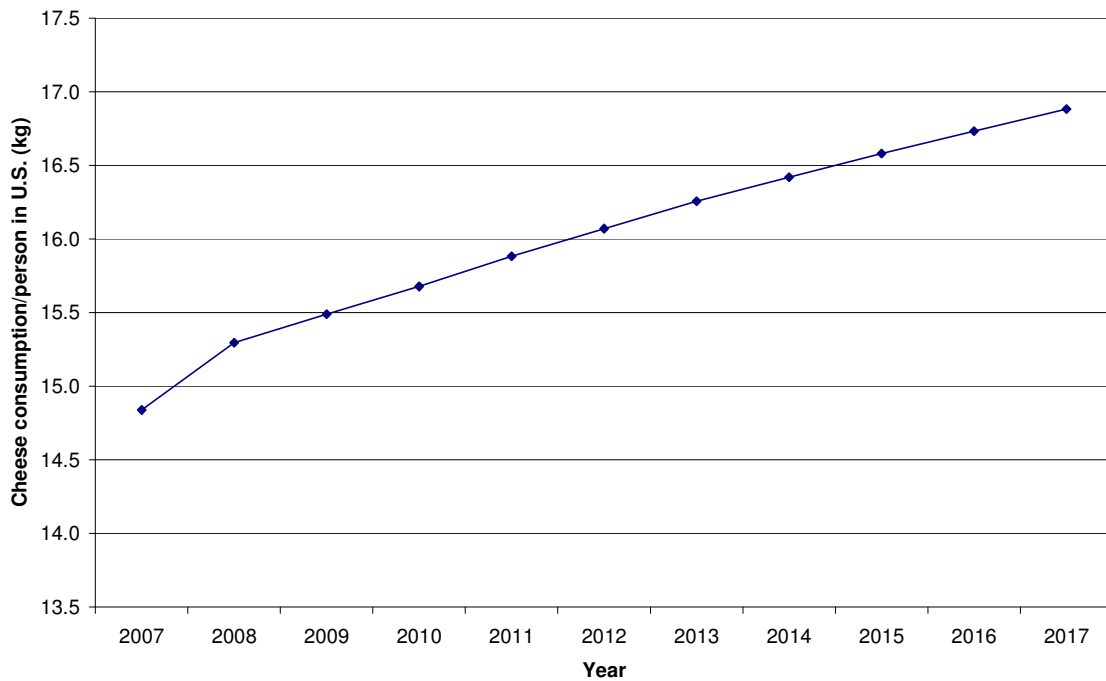
Figure 2-3. Projected per capita cheese consumption in the U.S. from 2007 to 2017 (data from FAPRI 2006).

2.3.3 Addition of Omega-3 Fatty Acids into Dairy Food

Several researchers have explored the benefits and problems of adding omega-3 fatty acids to foods, such as luncheon meats and eggs (Cortinas and others 2003; Volker and others 2005), as well as dairy products. For example, one study showed cows fed fish oil (protected or unprotected) produced milk that was more prone to oxidation, thus affecting sensory attributes (Lacasse and others 2002). Cows were fed fish (menhaden) oil, soybeans, or both in another study (randomized, complete block design) (Ramaswamy and others 2001). Raw milk from the cow, and cream, butter, and buttermilk made from the cow's milk were analyzed by fatty acid analysis of their butyl esters with a GLC (gas-liquid chromatographer) (Ramaswamy and others 2001). The results showed that fish oil and fish oil with extruded soybean added to the milk provided the most DHA (0.13-0.15g DHA/100g fatty acids) and CLA (2.17-2.30g CLA/100g fatty acids), while fish oil provided the most total omega-3 fatty acids (1.88g omega-3 fatty acids/100g fatty acids) (Ramaswamy and others 2001). In both of the previously mentioned studies, cows fed fish oil produced milk with a lower fat content (Ramaswamy and others 2001; Lacasse and others 2002).

Dairy ewes were fed algae supplements in order to determine the effects on their milk and milk products (Papadopoulos and others 2002). The algae added was *Schizochytrium* sp., and contained 385 g fat/kg dry matter (DM), with 147g omega-3 fatty acids ($C_{22:6}$)/kg DM (Papadopoulos and others 2002). Unlike the studies by Ramaswamy and others (2001) and Lacasse and others (2002), this study showed an increase in milk fat content for ewes fed more algae (Papadopoulos and others 2002). The milk from these ewes also increased in omega-3 fatty acid content by 4.3-12.4% (Papadopoulos and others 2002). The omega-3 fatty acids in the ewes milk were $C_{18:3}$, $C_{20:3}$, $C_{20:5}$, $C_{22:5}$, and $C_{22:6}$ (Papadopoulos and others 2002). $C_{20:5}$, $C_{22:5}$, and $C_{22:6}$ were not in the ewes' milk prior to addition of algae to their diet (Papadopoulos and others 2002). Omega-3 fatty acid concentrations in the milk, and yogurt and cheese made from the milk, increased except for $C_{18:3}$ and $C_{20:3}$, which remained about the same or decreased (Papadopoulos and others 2002). The PUFA (polyunsaturated fatty acid) content of the cheese made from the ewes' milk did not significantly change with time (Papadopoulos and others 2002).

Omega-3 fatty acids in the form of algal oil have been added to yogurt with a strawberry fruit base (Chee and others 2005). The algal oil supplement provided 500mg of omega-3 fatty acids per 272g of yogurt white mass (Chee and others 2005). The algae oil was added to the yogurt as an emulsion containing water, WPI (whey protein isolate), potassium sorbate, tocopherol, EDTA, and sodium citrate buffer (Chee and others 2005). The algal oil was added to the emulsion in a concentration of 250g algal oil/kg emulsion (Chee and others 2005). The algal oil emulsion was added to the yogurt before pasteurization and either before or after homogenization (Chee and others 2005). Chemical oxidation was measured by lipid hydroperoxide measurements, which showed that yogurt with the algal oil emulsion oxidized more than yogurt without the emulsion, oxidation increased with time, and oxidation was not affected by when the emulsion was added during processing (Chee and others 2005). After 22 days, a trained sensory panel detected a stronger fishy flavor (Chee and others 2005).

Omega-3 fatty acids have also been added to cow's milk through a natural means (Hauswirth and others 2004). Grass in certain alpine regions of Switzerland contain high levels of ALA (alpha-Linolenic acid), an omega-3 fatty acid (Hauswirth and others 2004). The cheese from cow's milk eating this grass was compared with cheese from cow's milk eating silage or linseed supplementation for omega-3 fatty acid concentration (Hauswirth and others 2004). Fatty acid methyl esters (FAME) of the cheeses were analyzed, and results showed that cheese from cows eating the alpine grass had the most total omega-3 fatty acids, ALA, EPA, and CLA, as well as fewer saturated fats (such as palmitic acid, $C_{16:0}$) and the lowest omega-6:omega-3 fatty acid ratio; this was followed by cows eating silage, and finally cows eating linseed (Hauswirth and others 2004).

A randomized block design for an experiment which fed cows calcium salts of palm and fish oil with or without soybean products (extruded full-fat soybeans or soybean oil) showed no adverse affects on consumer acceptability (Allred and others 2006). Unlike other studies, this experiment showed no change in nutrient intake, milk yield, or milk composition with these treatments (Allred and others 2006). The total omega-3 fatty acid concentration in each situation was as follows: control had 0.62g/100g fatty acids (FA), supplement of calcium salts of palm and fish oil had 0.69g/100g FA, supplement of calcium salts of palm and fish oil plus extruded soybeans

had 0.69g/100g FA, and supplement of calcium salts of palm and fish oil plus soybean oil had 0.67 g/100g FA (Allred and others 2006). The CLA content of the cheddar cheese produced from the cow's milk remained stable over a 24 week storage period, where a diet including 2.7% calcium salts of palm and fish oil plus 0.75% soybean oil gave the most CLA (about 1.7g/100g fat) (Allred and others 2006). Trained sensory panelists did not detect any off-flavors in any of the milk samples (Allred and others 2006). The panel found no difference in flavors between the cheese samples, except for a higher acid flavor in the sample from cow's milk fed calcium salts of palm and fish oil and extruded soybeans (Allred and others 2006). The sensory panel found no major changes in off-flavors of the control cheese during aging, a slight increase in flat and oxidized flavors in the cheese from milk with calcium salts of palm and fish oil as well as cheese from milk with calcium salts of palm and fish oil and extruded soybeans, and there was the most change in off-flavor during aging the with cheese from milk with calcium salts from palm and fish oil and soybean oil (Allred and others 2006). The off-flavor was mostly oxidized flavor, which increased from mild to medium cheddar, and decreased from medium cheddar to sharp cheddar during aging (Allred and others 2006). The cheese was stored for aging in vacuum-packaged air-tight plastic bags stored at 4^oC for 21 (mild cheddar), 30 (mild cheddar), 90 (medium cheddar), and 180 (sharp cheddar) days (Allred and others 2006). Aging did not significantly affect the fatty acids, including omega-3 fatty acids (Allred and others 2006).

Rumen-protected omega-3 fatty acids from canola oil and soybean oilseed (70:30 w/w), soybean oilseed and linseed oil (70:30, w/w), or soybean oilseed and tuna oil (70:30 w/w) were fed to dairy cows (Gulati and others 2002). Milk samples were stored at 4^oC for analysis and FAME of the milk were analyzed by gas chromatography (GC) (Gulati and others 2002). Only the cows fed soybean oilseed and tuna oil produced milk containing EPA and DHA (3.52% and 13.31% w/w, respectively) (Gulati and others 2002). All milk samples contained ALA, canola/soybean oil milk contained 7.03% (w/w), soybean/linseed oil milk contained 3.41% (w/w), and soybean/tuna oil contained 39.13% (w/w) (Gulati and others 2002). The melting profiles of all milkfat samples were similar, but there was less solid fat in milk with omega-3 fatty acid supplementation (Gulati and others 2002).

Cod liver oil or cod liver oil and rapeseed were added directly to milk in a study to determine the oxidative stability of milk based on fish oil quality and storage temperature (Let and others 2005). The milk contained 1.0% (w/w) milk fat and 0.5% (w/w) cod liver oil (no antioxidants added) or cod liver oil and rapeseed oil at a ratio of 1:1 (antioxidants added: 1840 ppm citric acid ester and 460 ppm propyl gallate) (Let and others 2005). Samples were evaluated by fatty acid composition, peroxide value, anisidine value, amount of free fatty acids, and tocopherol levels of oils (Let and others 2005). With a low initial peroxide value of the fish oil (less than 0.5), the oxidation of the milk fortified with fish oil could be controlled (Let and others 2005). In addition, the quality of the milk was better with a fortification of fish oil and rapeseed oil rather than just fish oil when stored at 2^oC (Let and others 2005). The oils were oxidized (using 40-50^oC temperature, air, no light, and stirring) from an initial peroxide value (PV) of 0.1 meq kg^{-1} and anisidine value (AV) of 2.5 for fish oil and 2.0 for fish oil/rapeseed oil to peroxide values of 0.1 (not oxidized), 0.5, 1.0, and 2.0 meq kg^{-1} (Let and others 2005). The PUFA were not significantly affected by the oxidation (Let and others 2005). Supplemented milk samples were stored at 2, 5, or 9^oC (with no light) (Let and others 2005). The Bligh and Dyer method was used to extract the lipids from the milk for analysis (Let and others 2005). Gas chromatography-mass spectroscopy (GC-MS) was used for dynamic headspace analysis of volatile secondary oxidation products (Let and others 2005). In addition, a trained sensory panel evaluated the milk samples, and samples with an added mixture of fish and rapeseed oils with less than 0.5 meq/kg peroxide value initially were acceptable by sensory evaluation (Let and others 2005).

2.4 OXIDATION OF OMEGA-3 FATTY ACIDS IN DAIRY FOODS

Oxidation causes undesirable off-flavors, shortened shelf-life, and decreased nutritional properties. The reaction of lipid oxidation involves the formation of free lipid radicals, primary reaction products (hydroperoxides), secondary oxidation products, and finally tertiary oxidation products (Kamal-Eldin and Pokorny 2005). In dairy products, oxidation is affected (positively or negatively) by oxygen, metals (i.e. copper, iron), light, temperature, water activity, proteins, enzymes, browning reaction products, constituents of the milk fat globule membrane, thiols, antioxidants, ascorbic acid, tocopherols, and

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carotenoids (Fox and McSweeney 2006). For minimum oxidation, it is necessary (when possible) to minimize oxygen, metals, light, and constituents of the milk fat globule membrane (Fox and McSweeney 2006). Proteins, browning reaction products, antioxidants, ascorbic acid, tocopherols, and carotenoids can help prevent oxidation (Fox and McSweeney 2006). However, the combination of these and other factors influence the anti- or pro- oxidative properties of each condition. Mixed results have been shown for the optimum temperature for dairy products (Fox and McSweeney 2006). Additionally, the oxidation effects of enzymes and thiols are unclear (Fox and McSweeney 2006). Water activity for the least oxidation has shown to be at about 0.4 (an intermediate level) (Fox and McSweeney 2006).

The oxidation of various dairy products have been extensively studied (Table 2-2). The oxidation of algal oil was measured in water and fluid milk in one study (Gallaher and others 2005). Samples were stored at $32+/-2^oC$ in the dark to measure oxidation due to temperature but not light. Lipid hydroperoxide (modified method of Shantha and Decker 2004) and propanal concentrations were evaluated to measure lipid oxidation. A trained sensory panel rated the fishy aroma of the samples. Sodium azide added to the samples at 200ppm (to prevent microbial growth) did not affect the oxidation. Additionally, iron added to the samples at 100ppm increased the rate of oxidation in the water samples, but not in the milk samples.

There are many considerations when supplementing a dairy product such as mozzarella cheese with an omega-3 fatty acid source such as algal oil to create a product with increased nutritional value. Due to the oxidative nature of PUFA such as omega-3 fatty acids, oxidation and fatty acid degradation must be understood and prevented.

Table 2-2. Methods used for measuring oxidation in various products.

Product	Oxidation test method	Source
Algal oil in water and milk	Lipid hydroperoxides (modified method of Shantha and Decker (1994)) Propanal concentration	Gallaher and others (2005)
Strawberry flavored yogurt with algal oil emulsion	Lipid hydroperoxides (modified method of Shantha and Decker (1994))	Chee and others (2005)
Milk (light-exposed)	Headspace gas chromatography	Webster (2006)
Milk with supplemental iron and copper	Thiobarbituric acid (TBA) test, modified method of Dunkley and Jennings (1951)	Hegenauer and others (1979)
Oil-in water emulsion including omega-3 fatty acids under heat processing with calcium ions	Lipid hydroperoxides (modified method of Shantha and Decker (1994)); Thiobarbituric acid-reactive substances (TBARS) test by method of McDonald and Hultin (1987))	Alamed and others (2006)
Butter and ice cream from milk from cows fed high oleic or high linoleic safflower oil	Peroxide value (AOAC 1997c); Free fatty acids (AOAC 1997b)	Gonzalez (2001) Gonzalez (2003)
Fish-oil-enriched mayonnaise	Lipid hydroperoxides by high performance liquid chromatography (HPLC); Secondary volatile oxidation products by (gas chromatography-mass spectroscopy (GC-MS)	Jacobsen and others (1999)
Oil-in-water emulsions containing fish and algal oil	Peroxide value by ferric thiocyanate method of Chapman and Mackay (1949); Propanal by static headspace gas chromatography of Frankel (1993)	Frankel and others (2002)
Milk enriched with fish oil	Peroxide value by colorimetric ferric-thiocyanate method (IDF 1991); volatile secondary oxidation products by GC-MS	Let and others (2003) Let and others (2005)
Edible oils stabilized with natural and synthetic antioxidants	Peroxide value and TBA value of David (1990)	Bera and others (2006)

2.5 SENSORY EVALUATION OF OMEGA-3 FATTY ACID FOODS

Studies have been done which have revealed sensory characteristics of certain dairy products supplemented with omega-3 fatty acids, but none have provided information regarding mozzarella cheese supplemented with omega-3 fatty acids (See Table 2-3).

In correlation with sensory acceptance, the oxidation of the product (which may occur due to temperature, light, oxygen exposure, etc.) and any resulting flavor changes are another concern. An illustration of this issue can be seen in one study by Let and others (2005) in which it was shown that milk with added fish and rapeseed oils (1.0% milkfat and 0.5% oils w/w) had acceptable sensory characteristics if the fish oil's initial peroxide value was less than 0.5 meq/kg (Let and others 2005). This study and another study by Let and others (2003) showed that a higher initial peroxide value caused faster oxidation of the oil (Let and others 2003; Let and others 2005). The second study (Let and others 2003) explored milk enriched with tuna or cod liver fish oil (1.5% w/w). Another experiment showed a significant difference between milk exposed to light with and without antioxidants (0.025% alpha-tocopherol and 0.025% ascorbic acid) through a sensory triangle test (van Aardt and others 2005). These results correlate with the thiobarbituric acid reactive substances test (TBARS), an oxidation test, which showed a lower value for the milk with antioxidants than the milk without antioxidants (van Aardt and others 2005).

A study regarding the effect of added iron on cheddar cheese quality found that a somewhat larger TBA value of the cheddar cheese did not negatively impact the sensory characteristics (Zhang and Mahoney 1989). The correlation coefficient of TBA to oxidized off-flavor was found to be 0.34, 0.19, 0.24 in three different experiments after seven days of aging (Zhang and Mahoney 1989). This experiment used a sensory panel consisting of dairy experts, nutritionists, and sensory experts to conduct a panel similar to a Quantitative Descriptive Analysis (Zhang and Mahoney 1989).

Table 2-3. Comparison of sensory tests of dairy products with omega-3 fatty acid supplements in select studies.

Dairy product	Omega-3 FA source	Sensory Test Used	Affects on sensory characteristics	Source
Strawberry flavored yogurt	Algal oil emulsion	Trained panel rating score (15 pt) of fishy perception; Untrained panel rating score (9 pt) of how well liked	Significant difference between control and algal oil added after 15 days storage for fishy perception; No difference between samples for overall liking	Chee and others (2005)
Milk Butter	Fish oil	Trained panel rating score (9 pt) of oxidized flavor for milk and butter, and for storage flavor for butter	Significant difference between oxidized flavor of samples with/without copper added, and after sample storage (except with both fish oil and copper added, and with fish oil in butter)	Baer and others (2001)
Milk	• None • Protected algae • Unprotected algae	Triangle test; Panelists familiar with dairy sensory evaluation	No difference; no off flavors reported	Franklin and others (1999)
Milk	Algal oil emulsion	Trained panel; rating score of fishy aroma (15 pt)	No significant difference	Gallaher and others (2005)
Dairy ewe milk, feta cheese, yogurt	• None • Low level algae (23.5g) • Medium level algae (47g) • High level algae (94g)	None		Papadopoulos and others (2002)
Milk Butter Cheddar cheese	• None • Fish meal	Rating score of quality (5 pt) for milk and butter Triangle test for milk, butter, and cheddar cheese	No significant difference for any product, but fish meal cheese had softer/smoother texture and stronger cheddar flavor after 6 months ripening	Avramis and others (2003)
Milk	Rumen-protected tuna oil	Single-blind test, rating score of flavor/smell (5 pt) Triangle test	Rating: no difference, no signs off flavor/fish oil flavor Triangle test: no significant difference	Kitessa and others (2004)

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CHAPTER 3
FATE OF ALGAL OIL FATTY ACIDS WITH SYNTHETIC OR NATURAL ANTIOXIDANTS

3.1 ABSTRACT

Omega-3 fatty acids are typically consumed in the form of fish, nuts, oils, and supplements. Due to adversities towards these sources such as vegetarianism, fear of heavy metals, environmental concern, and unpalatability, alternative sources of omega-3 fatty acids are desired. Algae, specifically *Schizochytrium* sp., has been shown to contain large amounts of DHA, an omega-3 fatty acid essential for health, in its oil.

Because of the high unsaturation of the PUFA in algal oil, oxidation is a major concern. Oxidation causes poor product quality and flavor as well as health risks due to free radicals formed. Antioxidants can help prevent or delay oxidation and degradation of fatty acids. Synthetic antioxidants are commonly used in the food industry, but increasingly health-conscious consumers may prefer natural antioxidants.

The main objective of this study was to determine the effects of synthetic and natural antioxidants on algal oil fatty acid degradation and oxidation, and to identify potential antioxidants for algal oil in supplemented mozzarella cheese. Algal oil was stored for up to 6 weeks with antioxidants BHT, TBHQ, or mixed tocopherols.

TBHQ was found to be an effective synthetic antioxidants for at least 6 weeks at the concentration of 0.0175 or 0.035g/g oil. Mixed tocopherols were found to be an effective natural antioxidant for about 4 weeks at the concentration of 400ppm.

3.2 INTRODUCTION

Omega-3 fatty acids have recently received increased attention due to its health benefits ranging from decreased risk of cardiovascular disease to decreased risk of Alzheimer's disease (Wang and others 2004; MacLean and others 2005). Even so, many people do not consume adequate amounts of omega-3 fatty acids (Kris-Ehterton and others 1998; Newton 1998; Raper and others 1998). Typical sources of this nutrient include fish, nuts, and supplements. Algal oil from *Schizochytrium* sp. is an alternative source of omega-3 fatty acids, which is especially high in DHA (Abril and others 2003;

Lloyd-Still and others 2006). Additionally, this source comes with no concerns that may be found with fish, regarding vegetarianism, heavy metal contamination, and depletion of fish.

Algal oil is prone to oxidation due to its long-chain PUFA composition. Ethylenediaminetetraacetic acid (EDTA) has been shown to be one effective antioxidant in an algal oil-in-water emulsion with low iron concentrations (Frankel and others 2002). Limited research regarding effective antioxidants in algal oil, specifically in the *Schizochytrium* sp., calls for further research in the prevention of oxidation of PUFA in the algal oil.

The primary focus of this study was to determine the effects of some natural and synthetic antioxidants on fatty acid degradation and oxidation in algal oil. The main objective of this study was to identify effective natural and synthetic antioxidants for potential use with algal oil in a food system.

3.3 MATERIALS AND METHODS

3.3.1 Algal Oil Preparation

The oil used in this study was extracted from the biomass of the alga *Schizochytrium limacinum* (ATCC MYA-1381). The alga was grown in a medium consisting of artificial seawater (18g/L NaCl, 2.44g/L $MgSO_4 \cdot 7H_2O$, 0.6g/L KCl, 1.0g/L $NaNO_3$, 0.3g/L $CaCl_2 \cdot 2H_2O$, 0.05g/L KH_2PO_4, 1.0g/L Tris buffer (Sigma Co.), 0.027g/L NH_4Cl, 15.0×10^{-8}g/L vitamin B_{12}, 3ml/L chelated iron solution, and 10ml/L trace element solution (boron, cobalt, manganese, zinc, molybdenum)) (Starr and Zeikus 1993), 1 g/L yeast, 1 g/L peptone, and 90 g/L glycerol from biodiesel waste or 50 g/L glucose. This is an ATCC 790 By+ medium, except the concentration of glucose/glycerol (typically 5g/L glucose), which was adjusted for optimal algae growth (Pyle 2008). Algae was grown in 250ml Erlenmeyer flasks at $20^{\circ}C$ on an orbital shaker at 170rpm until the algae reached the stationary growth phase (Pyle and others 2008).

The algae growing in the stationary phase were harvested and freeze-dried for oil extraction. Algae was freeze-dried using a Virtis Freezemobile 12SL (Gardiner, NY). The method of Bligh and Dyer (Bligh and Dyer 1959) with minor modifications was used

to extract the fat. Briefly, a 20g freeze-dried algae sample was used, and water was added to the sample to achieve 80% water composition. Chloroform was added to the rehydrated sample in the ratio of 1:1, and methanol then was added in the ratio of 2:1 methanol:sample. Solvents and sample were mixed in a Waring blender for 2 min, then chloroform was added to the mixture in the ratio of 1:1. The mixture was blended for another 30 sec, and then water was added in 1:1 ratio. Centrifugation at 7000rpm for 6 min was used to achieve separation and clarification of the chloroform/lipid phase in the mixture. After centrifugation, the chloroform/lipid phase (the lower phase) was separated using a pipette. Filtration was tried initially as a means of separation and clarification as called for in the Bligh and Dyer method, however, the homogenate particles were too large in size and volume to fit through the filter paper (even with a vacuum), causing clogging. After centrifugation, the chloroform/lipid phase (the lower phase) was separated from the methanol/algal solids phase using a pipette. The concentration of lipids in the solution was determined by mixing chloroform/lipid solution well to ensure homogeneity, then removing the chloroform from 1mL of extract of the solution using a nitrogen stream, and weighing the remaining lipid. Lipid concentration of the lipid/chloroform phase was calculated by the following equation:

$$LipidConcentration = \frac{MassDryLipid}{VolumeChloroform/LipidPhase}$$

Lipid concentration was determined to ensure consistent lipid weights among samples.

3.3.2 Comparison of 63°C and Ambient Storage

Based on the calculated lipid concentration, about 0.3ml of chloroform/lipid solution was placed into each test tube, so that each test tube contained approximately 20mg lipids after removing the chloroform from the lipids by a nitrogen stream. Eighteen of the test tubes containing the lipids were then stored in a 63°C oven for 6 days, and the other eighteen were stored at 20°C (room temperature) for 6 weeks. Samples were exposed to air, but not light (wrapped in foil).

3.3.3 Treatment of Algal Oil with Antioxidants

Antioxidants were added to the algal lipids in order to determine their effect on the DHA degradation. Antioxidants were added to the chloroform/lipid extract (rather than directly to the lipid after chloroform evaporation) because the antioxidants were more easily dissolved in the solution and this ensured the antioxidant was evenly distributed throughout the lipid. Lipid samples were weighed after removing the chloroform to verify equal masses of lipids, and therefore equal concentrations of antioxidant in the lipid samples. Antioxidants were added as follows:

The chloroform/lipid phase was stirred then separated equally into three 250ml Erlenmeyer flasks temporarily for antioxidant addition before dividing among test tubes. The antioxidants were not added directly to the test tubes because the amount of antioxidant was so small it would have been difficult to accurately weigh the correct amount in each test tube.

Effect of Synthetic Antioxidants on Algal Oil Fatty Acid Preservation

For the first experiment with BHT and TBHQ, antioxidants were added as follows to the chloroform/lipid solution from algae grown with glycerol (Bera and others 2006):

Solution 1: no antioxidants

Solution 2: 0.035 g butylated hydroxytoluene(BHT)/g lipid

Solution 3: 0.035 g tert-butylhydroquinone (TBHQ)/g lipid

A fourth container contained chloroform/lipid solution from algae grown with glucose and no antioxidants were added. Each solution was well mixed, and divided into 25ml test tubes so that the chloroform/lipid solution in each test tube contained an equal volume of the chloroform/lipid solution and approximately 20mg lipids.

The subsequent experiment used only a glycerol energy source to grow the algae and only TBHQ as the antioxidant. Antioxidants were added as follows to 20ml chloroform/lipid solution:

Solution 1: no TBHQ

Solution 2: 0.0175g TBHQ/g algal oil

Solution 3: 0.0350g TBHQ/g algal oil

The solutions were divided into 22 test tubes each to contain approximately 28.8mg lipids (0.8ml chloroform/lipid solution).

Effect of Natural Antioxidants (Mixed Tocopherols) on Algal Oil Fatty Acid Preservation

For the experiment using natural antioxidants (vitamin E mixed tocopherols, Covi-Ox T-30P Fortitech (company), Cognis Nutrition and Health (division), Schenectady, NY) and algae grown with glucose, antioxidants were added as follows to 15ml chloroform/lipid solution:

Solution 1: no vitamin E

Solution 2: 100ppm tocopherols

Solution 3: 200ppm tocopherols

Solution 4: 400ppm tocopherols

Each solution was well mixed and divided into test tubes so that the 0.3ml chloroform/lipid solution in each test tube contained approximately 20mg lipids. The tocopherol composition is typically 14% alpha-, 2% beta-, 60% gamma-, and 24% delta-tocopherols, but variation occurs in the formula.

For the preliminary experiment with the artificial antioxidants BHT and TBHQ and algae grown with glycerol or glucose, oil samples were stored at 63°C for up to 2 days (to simulate 2 weeks of storage at room temperature). For the subsequent experiments with TBHQ or mixed tocopherols, oil samples were stored at 63°C for up to 6 days. One day of storage at 63°C represents one week of storage at room temperature (Hoover and Nathan 1980). The chloroform was removed from stored samples by evaporation in the oven. The chloroform was removed from initial condition (day 0) samples and samples stored at ambient conditions using a nitrogen stream. The tops of the samples in the oven were loosely covered with foil to prevent any foreign material from entering the tubes. Each day of storage, samples were removed from the oven and stored in a -80°C freezer (Fisher Scientific Isotemp Freezer, Kendro Laboratory Products, Asheville, NC) until analysis. Samples were done in triplicate.

3.3.4 Analysis

Analysis of algal oil samples involved measurement of DHA content by GC. FAMEs of the oil samples were prepared using a chloroform, sulfuric acid, and methanol solution (1.7:0.3:2.0, respectively) according to the procedure of Chi and others (Chi and others 2007). A $C_{17:0}$ standard was added to the FAME for quantification of the fatty acids. To each algal oil sample, 8ml FAME solution was added to account for pure lipids rather than algal mass being analyzed as in the cited procedure. A Shimadzu 2010 GC (Shimadzu Scientific Instruments, Columbia, MD) with a polyethylene glycol coated SGE Sol Gel-Wax^{TM} capillary column (30mx0.25mx0.25um) was used and followed the apparatus requirements according to AOCS standards for fatty acid analysis (AOCS 1999). A split injection system and flame-ionization detector, with the detector temperature of $280^{o}C$, and helium carrier gas were used. Injector temperature was $250^{o}C$, and injection volume was 1ul. The following temperature program was used to separate the FAMEs: 0.5 minutes at $80^{o}C$, raised at $30^{o}C$ per minute to $175^{o}C$, raised at $5^{o}C$ per minute to $260^{o}C$, raised at $30^{o}C$ per minute to $280^{o}C$ and maintained for one minute (Chi and others 2007). The fatty acids in the GC samples were quantified by comparing the area of the curve for specific fatty acids with the area of the curve for the $C_{17:0}$ standard. The curves for the fatty acids were identified based on retention times of standards (Nu-Check Prep Inc, MN).

The thiobarbituric acid reactive substances (TBARS) method was used. TBA (2-thiobarbituric acid) working solution was added to the algal oil, mixed for 10-15 sec on a vortex mixer, heated for 45 min in a boiling water bath, cooled in tap water, and 5ml 0.28M trichloroacetic acid (TCA) solution added. This solution was mixed, centrifuged (Eppendorf Centrifuge 5804R, 15 amp version, Westbury, NY) for 6 min at 7000 rpm, and the absorbance of the top pink layer measured at 538nm (Ke and Woyewoda 1979) using a UV-Vis Spectrophotometer (Shimadzu UV mini 1240, Columbia, MD). Using a 0.01M 1,1,3,3-tetraethoxypropane (TEP) working standard, a calibration curve for absorbance and TBA value was made, and the TBA value of the oil samples were calculated.

Samples in the experiments were done in triplicate, and analysis was done using ANOVA (SAS 2007). Effects were determined based on change and significant

differences in DHA concentration and TBA values between samples for the different conditions tested.

3.4 RESULTS

3.4.1 Comparison of 63°C and Ambient Storage

An experiment was conducted in order to verify that storage at 63°C for one day corresponds to storage at ambient conditions for one week for algal oil as has been reported for peanuts (Ramos and others; Hoover and Nathan 1980). Algal oil was stored for 6 days at 63°C or 6 weeks at 20°C. The DHA in the algal oil corresponds as shown in Figure 3-1. This correspondence is likely due to the accelerated oxidation of the PUFA at higher temperatures. There is no significant difference between the DHA in algal oil stored at room temperature for one week and algal oil stored at 63°C for one day ($p > 0.05$). Results were analyzed using ANOVA (SAS 2007).
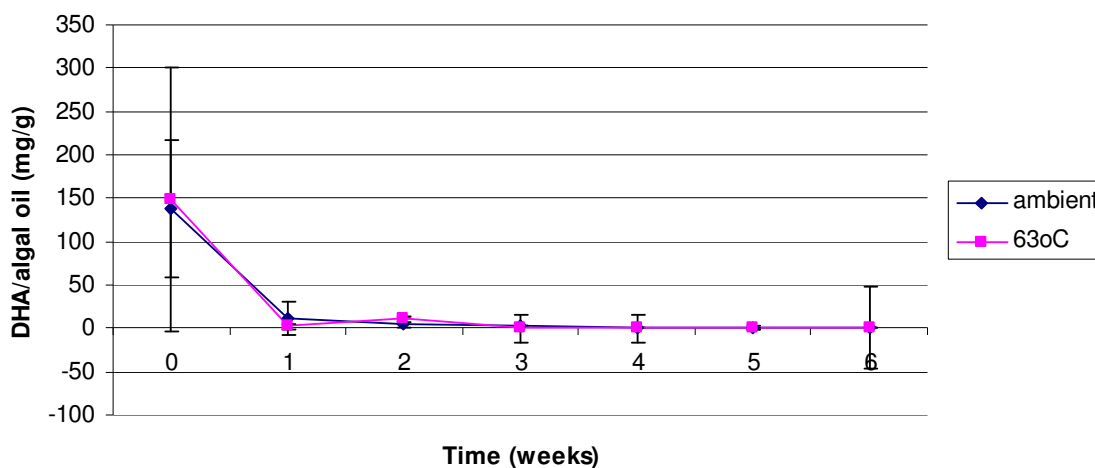
Figure 3-1. Comparison of DHA degradation (mean +/- st dev based on triplicate samples) of *Schizochytrium* sp. algal oil stored for 6 days at 63°C or 6 weeks at 20°C with no antioxidant.

The TBA values of the algal oil samples were compared as shown in Figure 3-2. Based on these results, it appears that the oxidation in algal oil stored at 6 days at 63°C and 6 weeks at ambient conditions correlate closely. However, the TBA values for weeks 5 and 6 are significantly different between algal oil from ambient and 63°C storage ($p < 0.05$).

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ANOVA was used to analyze the results (SAS 2007). The TBA values are below 19; TBA values less than 19 have been suggested to be acceptable in oils by a study with frozen mackerel (Ke and others 1975). Since it is known PUFA degradation is occurring based on the GC data, this may mean that the low TBA values are due to volatile TBARS (thiobarbituric acid reactive substances) which disappear during storage as suggested by Kaitaranta (Kaitaranta 1992) and the TBA value peaks and then decreases between weeks 0 and 1 and continues to slowly decrease from weeks 1 to 6. Future studies may study the TBA value of the oils stored between weeks 0 and 1 (at 20°C) and between days 0 and 1 (at 63°C).
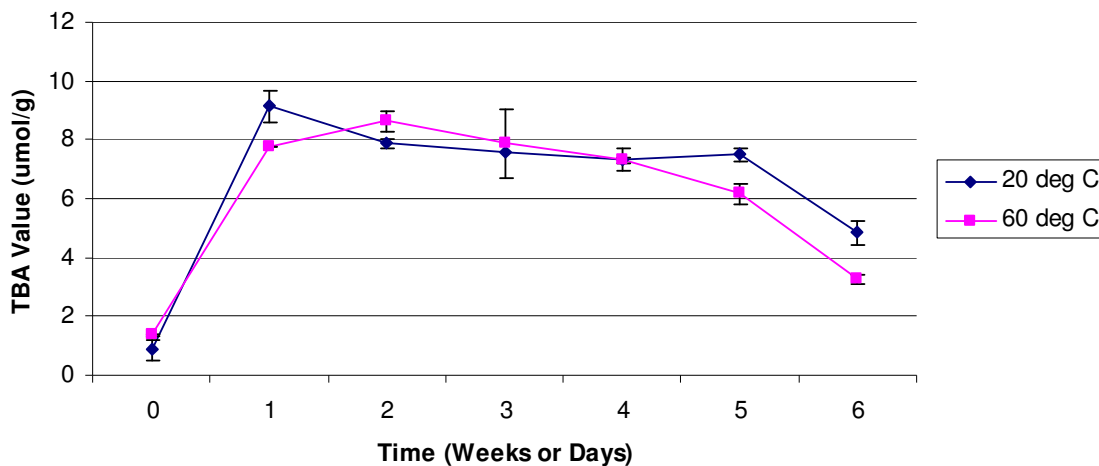
Figure 3-2. TBA values for algal oil stored for 6 days at 63°C or 6 weeks at room temperature. TBA values (mean +/- st dev based on triplicate samples) to measure oxidation for *Schizochytrium* sp. algal oil with no antioxidants. Oil was stored at 63°C for 6 days or for 6 weeks at 20°C.

3.4.2 Effect of Synthetic Antioxidants on Algal Oil Fatty Acid Preservation

A preliminary experiment compared the effects of BHT, TBHQ, or no antioxidant on DHA content in the algal oil during storage. Additionally, algal oil from algae grown with glucose or glycerol as the carbon source was compared to see if there was any significant difference between DHA degradation.

GC results showed that after 2 days of storage at 63°C (comparable to 2 weeks of storage at 20°C), there was no significant difference in total fatty acid (sum of the major fatty acids) or DHA degradation in algae grown with glucose and glycerol ($p > 0.05$).

Samples were only stored for two days as this was a preliminary experiment to understand the initial behavior of this samples. Time effects showed that there was no significant difference in total fatty acid or DHA degradation between glucose and glycerol carbon sources from day 0 to day 2 of storage ($p>0.05$). However, there was a significant difference in total fatty acid degradation, but not DHA degradation, for these samples from day 0 to day 1 of storage ($p<0.05$). Future studies may look into longer storage times to determine if a significant difference is found over longer storage time periods. GC results also showed that TBHQ is a slightly more effective antioxidant than BHT in the preservation of the fatty acids and DHA in the algal oil at the concentration of 0.035g antioxidant/g algal oil, but there is no significant difference between the two antioxidants ($p>0.05$) (SAS 2007) (Figures 3-3, 3-4). There was a significant difference between samples with and without antioxidants ($p<0.05$). A study with accelerated storage of fish oil also found TBHQ (0.01% concentration) to be a more effective antioxidant than BHT (0.02% concentration) based on TBA value and oil weight gain (Kaitaranta 1992). This is lower than the concentrations of TBHQ and BHT used in this experiment (3.5%). The study also tested the antioxidants anoxomer, ethoxyquin, and butylated hydroxyanisole (BHA) and found TBHQ to be the most effective of all tested . There is a significant difference between algal oil with and without antioxidants ($p<0.05$). Statistical analysis was done using ANOVA (SAS 2007).

TBHQ has been shown to be an effective antioxidant in other marine oils as well. In menhaden oil stored at 60°C for 7 days, TBHQ (200ug/g) was more effective than mixed tocopherols (500ug/g), alpha-tocopherol (500ug/g), mixed green tea catechins (200ug/g), epicatechin (200ug/g), epigallocatechin (200ug/g), epicatechin gallate (200ug/g), or epigallocatechin gallate (200ug/g) (Shahidi 1993; Shahidi and Kim 2002b). In seal blubber oil stored at 65°C for 125 hours under Schaal oven conditions (where 24 hours at these conditions is equivalent to one month of room temperature storage), TBHQ (200ug/g) was more effective than all of the previously mentioned antioxidants except for epigallocatechin (200ug/g) as measured by TBARS value and peroxide value (Shahidi 1993; Shahidi and Kim 2002a). The effectiveness of TBHQ in PUFA validates the common use of TBHQ in these fatty acids to improve oxidative stability (deMan 1999).
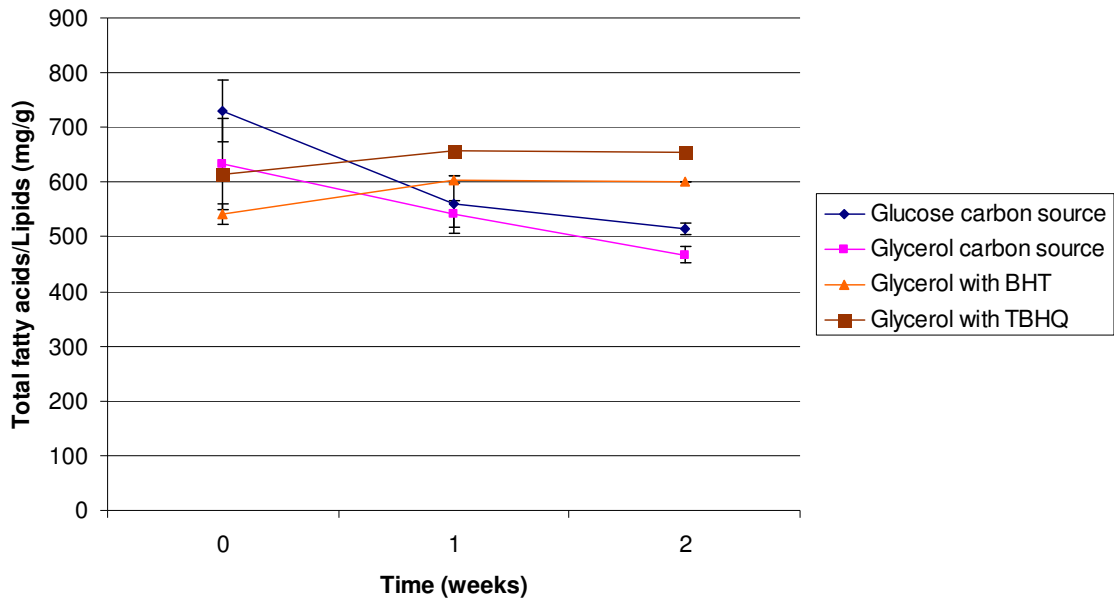
Figure 3-3. *Schizochytrium* sp. algal oil total fatty acid (mean +/- st dev based on triplicate samples) degradation with 0.035g BHT or TBHQ antioxidant/g oil. Algae was grown with a glucose or glycerol carbon source. Oil stored at 63°C for 2 days to simulate 2 weeks storage at 20°C.
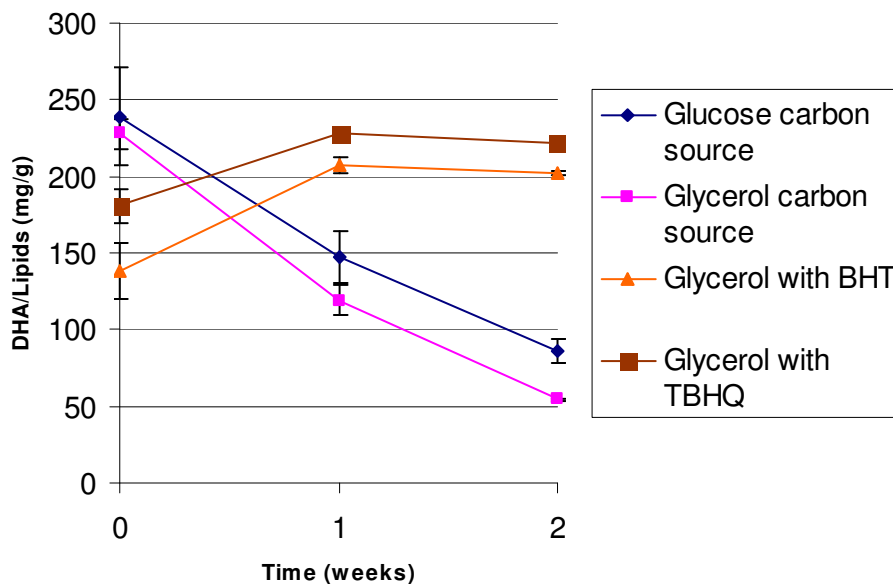
Figure 3-4. *Schizochytrium* sp. algal oil DHA content (mean +/- st dev based on triplicate samples) degradation with 0.035g BHT or TBHQ antioxidant/g oil. Algae was grown with a glucose or glycerol carbon source. Oil stored at 63°C for 2 days to simulate 2 weeks storage at 20°C.

In comparing different concentrations of TBHQ as an antioxidant in algal oil stored at 63°C for 6 days to mimic 20°C for 6 weeks, 0.0175 and 0.035g THBQ/g algal oil were shown to be equally effective in preventing fatty acid and DHA degradation ($p>0.05$), and both concentrations were shown to be significantly more effective than no TBHQ ($p<0.05$) (Figures 3-5 and 3-6). A significant difference between no antioxidant and 0.0175 or 0.035g TBHQ/g oil was first seen at day 2 based on time effects on DHA and total fatty acid content ($p<0.05$). Interestingly, no significant difference in change in total fatty acids from day 0 to 3 is found between no antioxidant and 0.035g TBHQ/g oil ($p>0.05$). ANOVA was used for the statistical analysis (SAS 2007).

Additionally, by looking at the percentage of total fatty acids for each fatty acid over the 6 weeks of storage, the percentages of DHA and DPA (PUFAs) decrease, while $C_{14:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$ increase for oil with no antioxidants. Oil with either 0.0175 or 0.035g TBHQ/g oil has nearly no change in fatty acid percentages over the 6 weeks of storage. This shows that the majority of the fatty acid degradation is due to degradation of PUFAs (DHA and DPA).
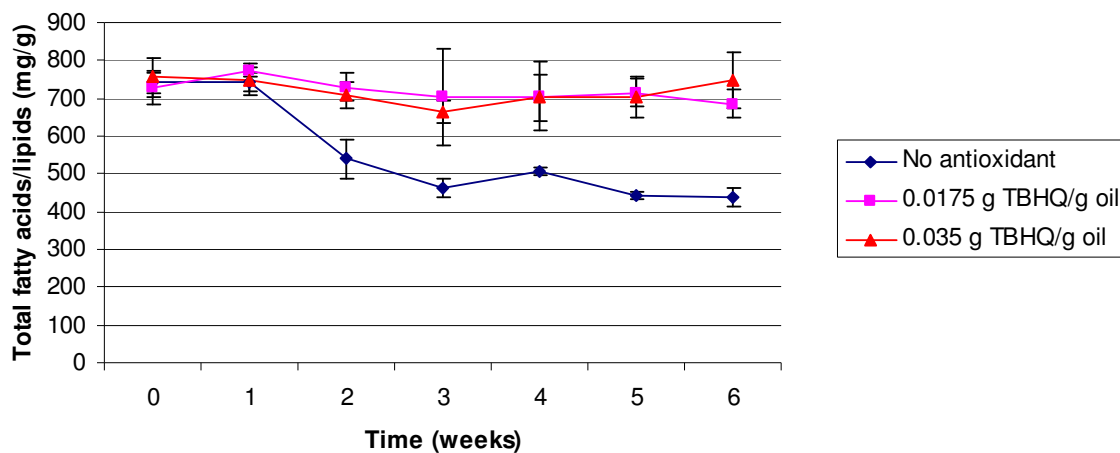
Figure 3-5. *Schizochytrium* sp. algal oil total fatty acid (mean +/- st dev based on triplicate samples) degradation with 0, 0.0175, or 0.035g TBHQ antioxidant/g algal oil. Oil was stored at 63°C for 6 days to simulate 6 weeks storage at 20°C.

Figure 3-6. *Schizochytrium* sp. algal oil DHA (mean +/- st dev based on triplicate samples) degradation with 0, 0.0175, or 0.035g TBHQ antioxidant/g algal oil. Oil was stored at 63°C for 6 days to simulate 6 weeks storage at 20°C.

Table 3-1. Percentage of total fatty acids for major fatty acids in *Schizochytrium* sp. algal oil stored at 63°C for 6 days (to mimic 6 weeks at 20°C) with no antioxidants.

Week	14:0	16:0	18:0	DPA	DHA
0	5.9	51.2	1.1	6.5	35.3
1	6.1	52.5	1.1	6.3	33.9
2	7.9	66.7	1.5	4.3	19.5
3	9.0	76.3	1.7	2.7	10.3
4	9.4	80.4	1.7	1.9	6.6
5	9.7	81.8	1.8	1.5	5.2
6	9.8	83.2	1.9	1.2	3.9

Table 3-2. Percentage of total fatty acids for major fatty acids in *Schizochytrium* sp. algal oil stored at 63°C for 6 days (to mimic 6 weeks at 20°C) with 0.0175g TBHQ/g oil.

Week	14:0	16:0	18:0	DPA	DHA
0	5.9	50.4	1.1	6.6	36.0
1	5.9	50.8	1.1	6.6	35.6
2	6.0	50.9	1.1	6.6	35.4
3	6.0	51.0	1.1	6.5	35.4
4	6.0	51.0	1.1	6.5	35.4
5	6.0	51.1	1.1	6.6	35.2
6	6.0	50.9	1.1	6.6	35.4

Table 3-3. Percentage of total fatty acids for major fatty acids in *Schizochytrium* sp. algal oil stored at 63°C for 6 days (to mimic 6 weeks at 20°C) with 0.035g TBHQ/g oil.

Week	14:0	16:0	18:0	DPA	DHA
0	5.9	50.1	1.1	6.6	36.3
1	5.9	50.4	1.1	6.6	36.1
2	5.9	51.0	1.1	6.5	35.5
3	6.0	50.4	1.1	6.6	35.9
4	6.0	51.3	1.1	6.4	35.2
5	6.0	51.3	1.1	6.5	35.1
6	6.0	51.1	1.1	6.5	35.3

The DHA in the algal oil can be modeled by the autocatalytic equation

$$\frac{dP}{dt} = kP(1-P)$$

where   P = DHA/original DHA (mg/mg), and

   k = reaction rate (Adachi and others 1995).

The reaction rate (units of 1/time) decreases as the TBHQ concentration increases, showing increased stability with higher concentrations of the antioxidant (Figure 3-7). However, antioxidant at too high of a concentration can cause pro-oxidative effects, so this decreasing reaction rate trend may not continue if higher TBHQ concentrations are used. The reaction rate is nearly equal for 0.0175 and 0.035g TBHQ/g oil, verifying these antioxidant concentrations are equally effective in preventing DHA degradation.

Figure 3-7. Reaction rate of autocatalytic equation describing DHA degradation in *Schizochytrium* sp. algal oil (stored at 63°C for 6 days to simulate storage at 20°C for 6 weeks) with 0, 0.0175, or 0.035g TBHQ/g algal oil.

Based on these results, it appears that 0.0175g TBHQ/g algal oil is the optimum antioxidant and concentration to be used among those compared for up to six weeks of storage at 20°C (since using less antioxidant is more economical). A smaller concentration of TBHQ than 0.0175g/g oil may be sufficient. Therefore, testing lower concentrations in future studies would be beneficial. Additionally, testing other antioxidants such as epigallocatechin, which has been shown to be more effective than TBHQ in seal blubber oil stored at 65°C for 125 hours under Schaal oven conditions (where 24 hours at these conditions is equivalent to one month of room temperature storage) as measured by TBARS value and peroxide value (Shahidi 1993; Shahidi and Kim 2002a), may provide relevant information. It is clear that the fatty acids, especially DHA, in the algal oil degrade rapidly after two days of accelerated storage (63°C) to simulate two weeks at 20°C without antioxidants, and the antioxidants aid in maintaining the fatty acids.

3.4.3 Effect of Natural Antioxidants (Mixed Tocopherols) on Algal Oil Fatty Acid Preservation

Analysis of algal oil samples supplemented with vitamin E in the form of alpha-, beta-, gamma-, and delta- tocopherols (14, 2, 60, and 24% composition, respectively) showed that 400ppm mixed tocopherols is the most effective concentration for the preservation of DHA in the algal oil, and appears to degrade after 4 days of storage at accelerated storage (63°C) to simulate 4 weeks of storage at 20°C (Figure 3-8). Algal oil with mixed tocopherols at 400ppm has significantly more DHA (mg DHA/g algal oil) than the other samples from weeks 2-5 ($p < 0.05$). ANOVA was used to analyze the results (SAS 2007). DHA in algal oil with 100 or 200ppm mixed tocopherols appears to degrade after one day of accelerated storage at 63°C (simulating one week of storage at 20°C), and DHA in algal oil with no tocopherols appears to be completely oxidized within one day of accelerated storage at 63°C (simulating one week of storage at 20°C). Looking at the time effects on the change in DHA content, there is a significant difference between the antioxidants ($p < 0.05$) until week four, when there is no longer a significant difference between the effectiveness of the antioxidant concentrations ($p > 0.05$).
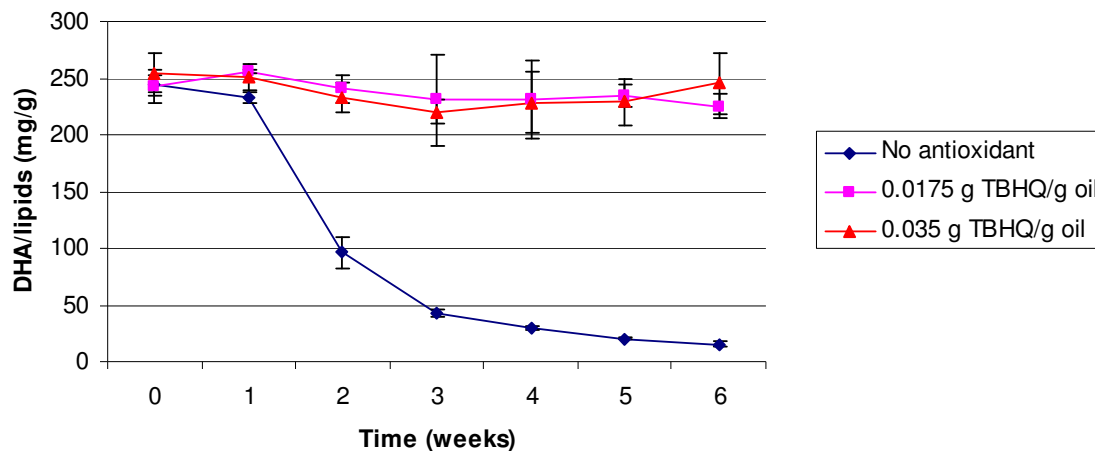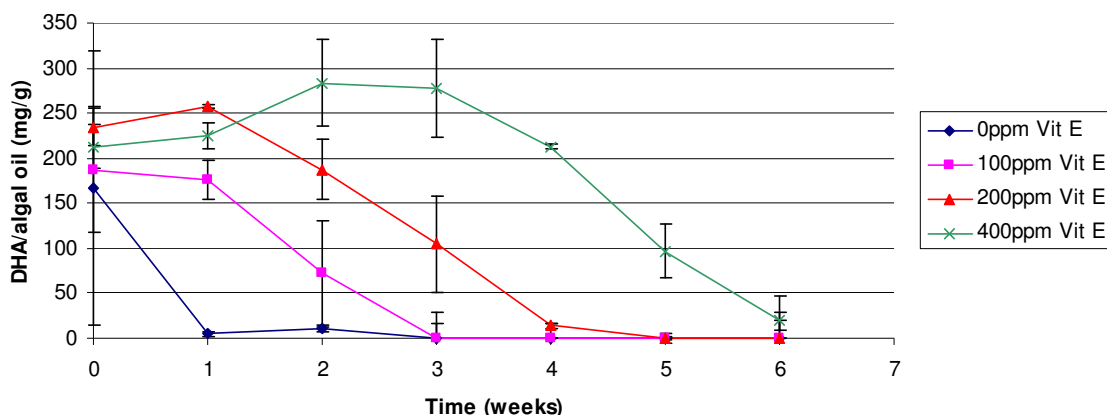
Figure 3-8. *Schizochytrium* sp. algal oil DHA (mean +/- st dev based on triplicate samples) degradation with 0, 100, 200, or 400ppm mixed tocopherols (alpha-, beta-, gamma-, and delta- tocopherols). Oil was stored at 63°C for 6 days to simulate 6 weeks storage at 20°C.

The DHA in the algal oil can be modeled by the autocatalytic equation $\dfrac{dP}{dt} = kP(1-P)$

where P = DHA/original DHA (mg/mg), and

       k = reaction rate (Adachi and others 1995).

The reaction rate changes decreases as the mixed tocopherols concentration increases,

showing increased stability with high concentrations of the antioxidant (Figure 3-9).
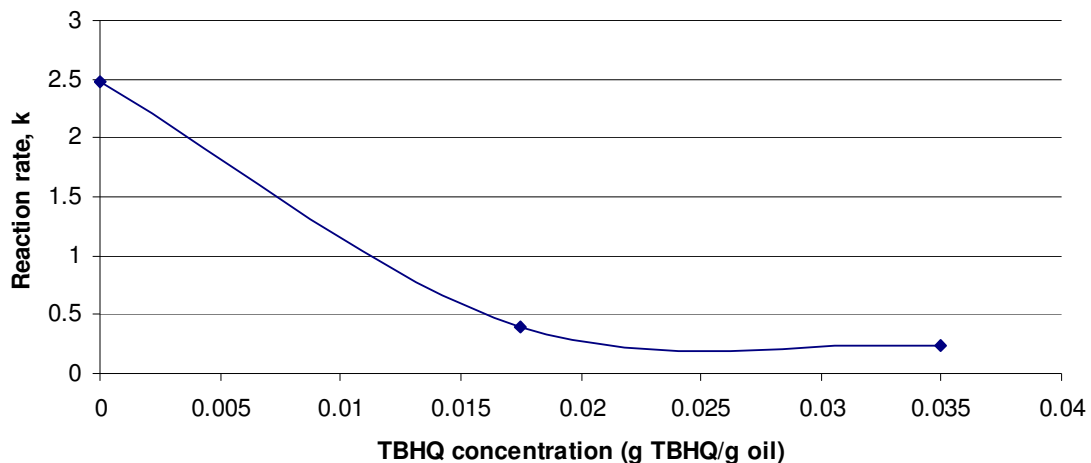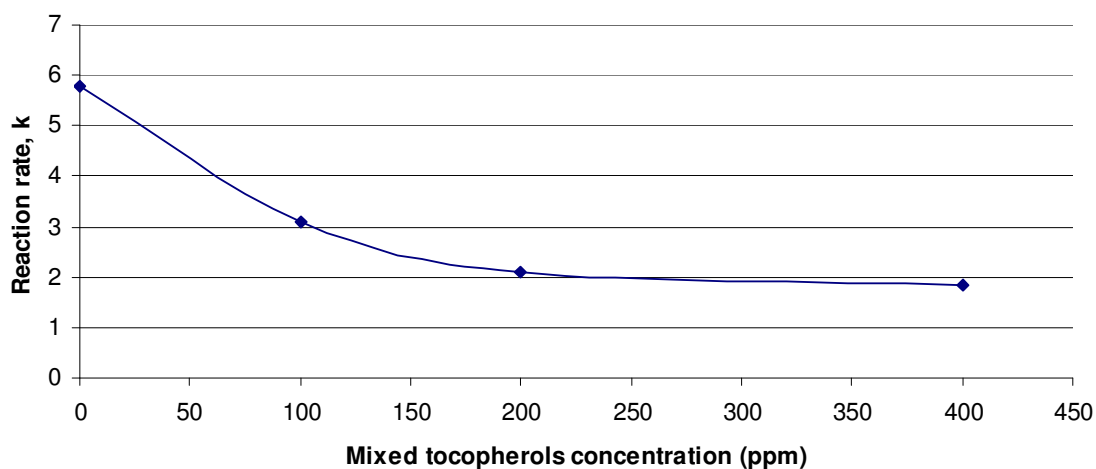

Figure 3-9. Reaction rate of autocatalytic equation describing DHA degradation in *Schizochytrium* sp. algal oil (stored at 63°C for 6 days to simulate storage at 20°C for 6 weeks) with 0, 100, 200, or 400ppm mixed tocopherols (alpha-, beta-, gamma-, and delta-tocopherols). Data has been normalized (offset by 1.5 days).

The variation in original DHA concentration (week 0) of the algal oil ranging from 166 to 235 mg DHA/g oil may be attributed to oxidation of the oil during extraction from the algae and/or while the chloroform was being removed from the oil samples. However, this does not explain the lack of variation in DHA at week 0 for the algal oil samples with TBHQ. The reaction rate for no antioxidant should be the same in the experiments with mixed tocopherols and TBHQ, yet this is not the case (Figures 3-7 and 3-9). This may be due to the higher original DHA concentration in the oil used in the experiment with mixed tocopherols resulting in more DHA degradation. Data for the mixed tocopherols samples was offset by 1.5 days for determining the reaction rate in order to normalize the data due to the variation in original DHA content.

Additionally, oxidation of the algal oil with these concentrations of tocopherols was measured by TBA value (Figure 3-10). For the concentrations tested, it appears that the higher the concentration of mixed tocopherols, the less oxidation results. This is based on triplicate samples. Mixed tocopherols at the concentration of 400ppm in the algal oil also appear to be the most effective in preventing oxidation. A study with frozen mackerel suggested TBA values of less than 19 to be acceptable in oils (Ke and others 1975). Other studies with marine oils have also used this acceptability range (Kaitaranta 1992). Using this measure of acceptability, all oil samples are acceptable regarding oxidation. The preservation of DHA and lower TBA values with increased concentrations of mixed tocopherols may be due to a delay in the stages of oxidation of the PUFA caused by the antioxidants scavenging free radicals. Alternatively, as suggested in the study by Kaitaranta with fish oil under accelerated storage conditions (60°C) with antioxidants (0.02% alpha-tocopherol acetate, 0.02% ascorbyl palmitate, 0.02% anoxomer, 0.02% ethoxyquin, 0.02% BHT, 0.02% butylated hydroxyanisole, or 0.01% TBHQ) (Kaitaranta 1992), the low TBA values may be due to volatile TBARS (thiobarbituric acid reactive substances) which disappear during storage.

A study with refined and deodorized fish oil (18% EPA, 12% DHA) stored at ambient conditions also found that combinations of natural antioxidants (specifically, 0.2-1.6% of tocopherols, rosemary extract, ascorbyol palmitate, and gallic acid) can inhibit oxidation (Robbins and Dooghe 2007). A separate study with flaxseed oil also found that a natural antioxidant (azoan extract) is equally, if not more, effective as a synthetic

antioxidant (TBHQ) for up to 2 hours for the temperature range 100-220°C, providing for almost no increase in PV and TBA values (Bera and others 2006). However, other studies have shown tocopherols to be less effective, as appears to be the case in these experiments (shown by lower reaction rates for TBHQ than mixed tocopherols, Figures 3-4 and 3-6). A study with menhaden oil stored for 7 days at 60°C found mixed tocopherols (500ug/g) to inhibit oxidation by only 32.5% compared with TBHQ (200ug/g) which inhibited oxidation by 54.7% based on oxidation measurement by TBARS values (Shahidi 1993; Shahidi and Kim 2002b). Additionally, a study with seal blubber oil stored at 65°C for 125 hours under Schaal oven conditions (where 24 hours at these conditions is equivalent to one month of room temperature storage) found alpha-tocopherol (200ug/g) to inhibit oxidation by 14.2% (Shahidi 1993; Shahidi and Kim 2002a). Oxidation was measured by peroxide value and TBARS value (Shahidi 1993; Shahidi and Kim 2002a) Another study found TBHQ (0.02%) to be more effective than alpha-tocopherol (0.1%), tempeh oil (5%), BHA (0.02%), and BHT (0.02%) in protecting mackerel skin lipids stored at 60°C for 8 days, measured by the weight gain method, peroxide value, and TBA molar value (Ke and others 1977). The present study inhibited DHA degradation as shown in Table 3-4.

Table 3-4. Percentage DHA degradation inhibition caused by TBHQ (0.0175, 0.035g TBHQ/g oil) or mixed tocopherols (100, 200, 400ppm) in *Schizochytrium* sp. algal oil stored at 63°C for 6 days by comparison of reaction rate (k) with reaction rate for no antioxidant addition.

Antioxidant	Reaction rate, k	Inhibition (%)
0g TBHQ/g oil	2.47363	0.00
0.0175g TBHQ/g oil	0.401601	83.76
0.035g TBHQ/g oil	0.242393	90.20
0ppm mixed tocopherols	5.78712	0.00
100ppm mixed tocopherols	3.09888	46.45
200ppm mixed tocopherols	2.10057	63.70
400ppm mixed tocopherols	1.82669	68.44

The synergistic effects of mixed tocopherols in combination with other natural antioxidants may also be tested in future studies. Additionally, the antioxidative capabilities of flavonoids in algal oil may be studied in future research as has been done

in fish oil (Nieto and others 1993). Other antioxidative methods to study in future research should include algal oil microencapsulation (Heinzelmann and Franke 1999).
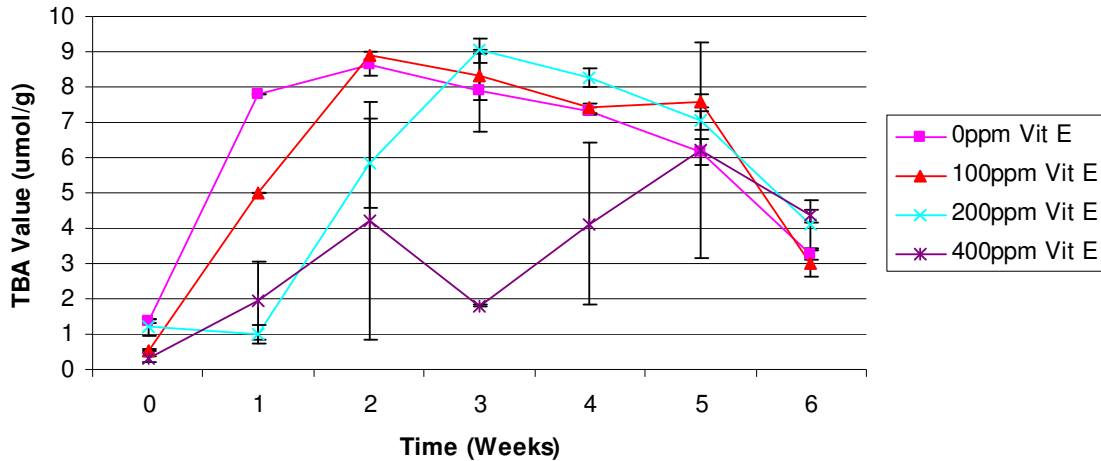
Figure 3-10. *Schizochytrium* sp. algal oil oxidation based on TBA value (mean +/- st dev based on triplicate samples) with 0, 100, 200, or 400ppm mixed tocopherols (alpha-, beta-, gamma-, and delta- tocopherols). Oil was stored at 63°C for 6 days to simulate 6 weeks storage at 20°C.

3.5 CONCLUSIONS

Algal oil storage at 63°C for one day mimics the DHA degradation and TBA value effects of algal oil stored at 20°C for one week.

The degradation of DHA in algal oil can be modeled by

$$\frac{dP}{dt} = kP(1-P)$$

where P = DHA/original DHA (mg/mg),

k = reaction rate.

Preventative measures are needed in order to inhibit this rapid degradation.

TBHQ is an effective synthetic antioxidant in prevention of DHA degradation for *Schizochytrium* sp. algal oil for at least 6 weeks at the concentrations of 0.0175g/g oil or 0.035g/g oil. Because using less antioxidant is more cost-effective, 0.0175g/g oil is suggested. Future studies may look into the effectiveness of concentrations lower than 0.0175g TBHQ/g algal oil.

Mixed tocopherols are an effective natural antioxidant in prevention of DHA degradation and oxidation for *Schizochytium* sp. algal oil for about 4 weeks at the concentration of 400ppm. Future studies may examine the effectiveness of higher concentrations of tocopherols. Higher concentrations may preserve the fatty acids for a longer period, but too much antioxidant may cause pro-oxidative effects.

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CHAPTER 4
FATE OF OMEGA-3 FATTY ACIDS IN MOZZARELLA CHEESE WITH OR WITHOUT ANTIOXIDANTS

4.1 ABSTRACT

Studies have shown omega-3 fatty acids, especially DHA, to have many health benefits, yet many consumers do not consume an adequate amount of these nutrients due to limited omega-3 fatty acid food sources that are generally not consumed at adequate levels. By incorporating omega-3 fatty acids into a variety of foods, consumers are given alternative sources that can be readily incorporated into their diets. Cheese is an attractive option as a food vehicle because of its high nutritional value, additional health benefits, and high level of consumption. Omega-3 fatty acids have been incorporated into various food systems (cite references), but there is limited information regarding the fate of omega-3 fatty acids in these foods, especially in cheese.

The main objectives of this study were to model the fate of DHA in mozzarella cheese, and to inhibit, through incorporation of antioxidants, any degradation and oxidation that occurs. Omega-3 supplemented cheese samples were stored at refrigerated conditions for up to 3 weeks.

The fate of DHA in mozzarella cheese can be modeled by the autocatalytic equation $\frac{dP}{dt} = kP(1 - P)$. DHA concentration decreased over 7 days. Using a response-surface design, 110ppm mixed tocopherols with 3% algal oil in mozzarella cheese is the optimum combination of antioxidant and algal oil for maximum DHA content and minimum oxidation. This combination preserves the DHA and prevents oxidation in the cheese for at least 3 weeks. Sensory evaluation of the cheese showed consumers can tell the difference between cheese with and without algal oil added.

4.2 INTRODUCTION

Cheese, including mozzarella cheese, is a popular food in the American diet and consumption is increasing (Putnam and Allshouse 2003). Additionally, dairy foods have several health benefits such as maintaining weight and bone health (Griffith and others

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1999; Appel and others 2000; Heaney and others 2000; Zemel and others 2004; NDC 2008). Therefore, mozzarella cheese is a good food vehicle for omega-3 fatty acids.

The effects of omega-3 enrichment into a variety of dairy foods has been studied (Table 4-1), but only a few studies (Kolanowski 1999; 2001; Let and others 2003; Let and others 2005) have reported degradation and oxidation of the omega-3 fatty acids in a cheese food vehicle. By understanding the fate and oxidation of the omega-3 fatty acids in the cheese, product quality can be improved and a more shelf-stable and nutrient-rich product can be provided to consumers.

Table 4-1. Previous research with omega-3 fatty acid enriched dairy products and issues addressed within the studies.

Dairy product	Issue addressed	Source
Yogurt	Processing effects	Dave and others (2002)
Fermented milk	Microbiological, rheological, sensory properties	Martin-Diana and others (2004)
Spray-dried milk	Shelf-life	Ramaprasad and others (2006)
Milk, Butter	Composition, flavor	Ramaswamy and others (2001)
Milk, Cheddar cheese	Flavor, composition	Allred and others (2006)
Feta cheese, yogurts	Composition	Papadopoulos and others (2002)
Milk	Sensory, oxidation, storage	Let and others (2005)
Flavored yogurt	Composition, sensory	Chee and others (2005)
Milk	Flavor, oxidation	Let and others (2003)
Milk, flavored yogurt, butter/oil, concentrated formula	Sensory, hydrolysis, oxidation	Kolanowski (1999)
Butter/oil	Quality, oxidation	Kolanowski (2001)
Milk	Milk yield, composition, sensory	Lacasse and others (2002)

The primary focus of this study was to better understand the behavior of DHA from algal oil in a mozzarella cheese food vehicle. The main objectives of this study were to model DHA degradation in mozzarella cheese; to attempt to prevent fatty acid degradation and oxidation through antioxidants; and to determine if consumers find mozzarella cheese with algal oil to be the same as mozzarella cheese without algal oil.

4.3 MATERIALS AND METHODS

Modifications in methodologies between experiments are due to different information desired (i.e. changes in antioxidants) or knowledge acquired based on preceding experiments.

4.3.1 Fate of Omega-3 Fatty Acids in Mozzarella Cheese without Antioxidants

Algal oil preparation. See section 3.3.1 Algal oil preparation. The following modifications apply here: Algal oil was extracted from *Schizochytrium* sp. by adding 40ml hexane:ethanol solvent (90:10 w/w) per gram of freeze-dried algae (Virtis Freezemobile 12SL, Gardiner, NY) which had been grown with a glucose energy source (Chen and Ju 2000). The mixture was nitrogen flushed in a flask and sealed, then stirred overnight (8-12 hours). Filtration with #1 then #50 Whatman filter paper with a vacuum was used to achieve separation. The solvent was removed from the lipids by using a rotavapor (Buchi 461 Water Bath, Brinkmann Rotavapor RE 121, Switzerland). Hexane solvent was used in order to remove any remaining oil in the rotavapor flask, and any residual solvent was removed by a nitrogen stream/evaporation under a fume hood. The oil was then frozen until use at -80°C.

Milk preparation. Raw milk was collected from the Virginia Tech dairy farm (Blacksburg, VA). Fat content of the raw milk was determined by Babcock method to ensure consistent milk fat content among samples (AOAC 989.04). Milk was split into three units (1 gallon each) and each unit was heated in a steam bath (stainless steel cylinder containers inside a steam bath) to 54.4^{o}C (130°F), Algal oil was added at 0, 1.5, or 3%, where percentages are amount of algal oil added per assumed 200g cheese yield from 1 gallon of milk. Milk was homogenized in two stages and pasteurized in stainless steel cylinder containers in a steam bath by heating to 65.6^{o}C (150°F) and holding for 30 min. The milk was cooled with ice for one hour.

Cheese preparation. Mozzarella cheese was prepared from the milk according to www.cheesemaking.com (Wallace 2007). Briefly, 1.5 teaspoons of citric acid was dissolved in ½ cup of water which was stirred into one gallon (3.79 L) of pasteurized milk. The milk was then stirred and heated to $32.2^{o}C$ ($90^{o}F$) in a water bath. Diluted rennet (¼ teaspoon of liquid rennet added to ¼ cup of water) was added, and the milk stirred gently, with up and down motions, for 30 sec and then allowed to rest for 5-10 min until curds formed. Curds were cut into 2.54cm (1 in) cubes and left undisturbed for another 2-3 min to allow for whey separation. Curds were then lifted using a slotted spoon from the whey into a colander for whey drainage from the curds. Curds were heated in hot water (approximately $79.4^{o}C$, $175^{o}F$), stretched by hand until smooth and pliable, and formed into balls.

Storage. Cheese pieces from mozzarella balls (approximately 5g) were stored in glass vials which were nitrogen flushed and sealed with parafilm. Cheese samples which were to be stored with exposure to no light were wrapped in foil. The samples were made in triplicate and stored for up to 14 days (samples taken at days 0, 7, 10, 12, 14), with or without light, with 0, 0.04, or 1.7% algal oil, and at 2 or $6^{o}C$. There were 36 cheese samples at each time point, except for day 0, when there were only 9 samples for initial conditions.

Analysis. The cheese samples were stored in a $-80^{o}C$ freezer (Fisher Scientific Isotemp Freezer, Kendro Laboratory Products, Asheville, NC) until analysis. Cheese fat was extracted using an alkaline extraction according to standard method AOAC 920.125 (AOAC 1997a). Analysis of cheese fat samples involved measurement of fatty acids, including DHA, content by GC (See section 3.3.3 Analysis for GC information). FAME of the oil samples were prepared using a chloroform, sulfuric acid, and methanol solution (1.7:0.3:2.0, respectively) according to the procedure of Chi and others (Chi and others 2007). A $C_{17:0}$ standard was added to the FAME for quantification of the fatty acids. The curves for the fatty acids were identified based on retention times of standards (Nu-Check Prep Inc, MN). For each 20mg fat sample, 8ml FAME solution and 1mg standard was

used. Fatty acids were quantified by the following equation:

$$FattyAcid / Lipid (mg / mg) = \frac{FattyAcidCurveArea}{C17:0CurveArea} * \frac{1mgC17:0}{20mgLipidSample}$$

4.3.2 Fate of Omega-3 Fatty Acids in Mozzarella Cheese with Antioxidants

The mozzarella cheese was prepared in the same manner (section 4.3.1) with the following modifications:

Algal oil extraction. *Schizochytrium limacinum* was grown with a glucose energy source and freeze-dried (Virtis Freezemobile 12SL, Gardiner, NY). The method of Bligh and Dyer (Bligh and Dyer 1959) was used to extract the fat with minor modifications. Briefly, a freeze-dried algae sample was used, and water was added to the sample to achieve 80% water composition. Chloroform was added to the sample in the ratio of 1:1 chloroform:sample (where the sample consisted of freeze-dried algae and 80% water), and methanol was added to the sample in the ratio of 2:1 methanol:sample. The mixture was blended in a Waring blender for 2 minutes, then chloroform was added to the mixture in the ratio of 1:1 chloroform:sample. The mixture was blended for another 30 seconds, and then water was added to mixture in ratio of 1:1 water:sample. Centrifugation at 7000rpm for 6 minutes was used to achieve separation and clarification of the chloroform/lipid phase in the mixture. Filtration was tried as called for in the Bligh and Dyer method, however, the homogenate particles were too large in size and volume to fit through the filter paper (even with a vacuum), causing clogging. After centrifugation, the chloroform/lipid phase (the lower phase) was separated using a pipette. The chloroform was removed using a rotavapor (Buchi 461 Water Bath, Brinkmann Rotavapor RE 121, Switzerland) and then removing any residual chloroform using a nitrogen stream.

Milk preparation. The batches of cheese were prepared with approximately 0, 1.5, or 3% algal oil added and 0, 200, or 400ppm mixed tocopherols (vitamin E mixed tocopherols, Covi-Ox T-30P (Cognis), Fortitech). Algal oil and mixed tocopherols were added based on milk fat.

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Storage. The cheese samples were individually packaged using a vacuum sealer (Koch Supplies Inc., Kansas City, MO, Model X200). Cheese samples were stored in refrigerated conditions at 2, 5, or $8^{\circ}C$. Samples were removed at 0 and 14 days for analysis for the experiment optimizing the algal oil and mixed tocopherols content; and removed at 0, 3, 7, 9, 11, 12, 14, 16, 18 and 21 days for the experiment measuring the fate of the omega-3 fatty acids in the cheese.

Analysis. Samples were analyzed by GC and by determining the TBA value as a measure of oxidation. GC analysis was achieved by direct transesterification of the freeze-dried cheese (Ulberth and Henninger 1992; Schreiner 2006). Briefly, individual cheese samples were freeze-dried in a Virtis Freezemobile 12SL (Gardiner, NY). One ml of $C_{17:0}$ standard solution (1mg $C_{17:0}$/ml toluene), 1ml toluene, and 3ml 5% methanolic HCl were added to approximately 30-40mg of accurately weighed freeze-dried cheese in a 25ml test tube. The 5% methanolic HCl for this procedure was prepared by adding acetyl chloride to methanol (Christie 2003). The headspace of the test tubes was flushed with N_2, tubes were capped, and the samples were placed in a $70^{\circ}C$ water bath for 2 hours. The tubes were cooled, 5ml 6% K_2CO_3 and 2ml toluene were added, tubes were vortexed, and the top layer was extracted to a centrifuge tube. Approximately 1mg Na_2SO_4 was added to the centrifuge tube, and the tube was centrifuged (Eppendorf Centrifuge 5804R, 15 amp version, Westbury, NY) at 1100 rpm for 10 minutes. About 0.5ml of the sample was removed to a GC vial, and the samples were analyzed by GC. The GC used was the same as described in Chapter 3.

The TBA value of the cheese fat was measured by first extracting the fat from freeze-dried cheese samples by the method of Bligh and Dyer with the following modifications: 80% water was added to freeze-dried cheese samples, homogenate was centrifuged (Eppendorf Centrifuge 5804R, 15 amp version, Westbury, NY) instead of filtered, and the chloroform solvent was removed from the fat samples using a nitrogen stream at room temperature (Bligh and Dyer 1959). Briefly, the TBA value was measured by adding 10ml of a TBA (2-thiobarbituric acid) working solution to the cheese fat, vortexing for 10-15 seconds, heating the samples for 45 minutes in a boiling water

bath, cooling in tap water, adding 5ml 0.28M TCA (trichloroacetic acid) solution, mixing, centrifuging (Eppendorf Centrifuge 5804R, 15 amp version, Westbury, NY) for 6 minutes at 7000 rpm, and measuring the absorbance of the top pink layer at 538nm (Ke and Woyewoda 1979) using a UV-Vis Spectrophotometer (Shimadzu UV mini 1240, Columbia, MD). Using a 0.01M TEP (1,1,3,3-tetraethoxypropane) working standard, a calibration curve for absorbance and TBA value was made, and the TBA value of the cheese samples could be calculated.

Samples in the experiments were done in triplicate, and analysis was done using ANOVA (SAS 2007). Effects were determined based on change and significant differences in DHA concentration and TBA values between samples for the different conditions tested.

4.3.3 Algal Oil Loss in Mozzarella Cheese during Processing

The mozzarella cheese was prepared in the same manner (section 4.3.1) with the following modifications:

Algal oil extraction. Algal oil was extracted by the same methods as for the "Fate of omega-3 fatty acids in mozzarella cheese with antioxidants" (Section 4.3.2).

Milk preparation. The milk was prepared by first reducing the fat content of the milk in an effort to allow more algal oil to bind to the milk proteins. This was achieved by separating the milk and milk fat, then adding milk fat back to the milk. A sample of the raw milk was taken for analysis. One gram of mixed tocopherols was added to 3.79L (1 gallon) of milk, and 1.287g algal oil (about 1% algal oil based on milkfat) was added to another gallon (3.79L) of milk. A sample of the homogenized and pasteurized milk with mixed tocopherols or algal oil was taken for analysis.

Cheese preparation. Samples of the whey from the cheese with mixed tocopherols and cheese with algal oil were taken for analysis. Samples of the final cheese product with mixed tocopherols or algal oil were also taken for analysis.

Analysis. The raw milk, homogenized/pasteurized milk, and whey samples were analyzed by freeze-drying (Virtis Freezemobile 12SL, Gardiner, NY) and oven-drying to determine water content. The fat from the freeze-dried milk and whey samples was extracted by the method of Bligh and Dyer (Bligh and Dyer 1959), and FAMEs prepared according to Chi and others were analyzed by GC (Chi and others 2007).

The cheese was analyzed by removing the fat according to AOAC 920.125 (AOAC 1997a), then FAMEs were prepared according to Chi and others were analyzed by GC (Chi and others 2007). The water content of the cheese was determined by oven-drying.

4.3.4 Sensory Evaluation of Mozzarella Cheese with Algal Oil

The mozzarella cheese was prepared in the same manner (section 4.3.1) with the following modifications:

Milk preparation. The batches of cheese were prepared with 1mg algal oil/g cheese. Algal oil was DHA-S oil from Martek (Baltimore, MD). The oil was of *Schizochytrium* sp., a marine alga, and contained sunflower lecithin, tocopherols, and ascorbyl palmitate. According to laboratory analysis of the oil sample sent by Martek, the oil contained 39.3% DHA.

Storage. Cheese samples were stored in a brine solution (3 Tablespoons salt dissolved in one quart of water) in plastic Ziploc bags in a dark 5°C refrigerator until sensory evaluation. Before evaluation, the cheese was dried in a cheese cloth to remove the brine. Stored cheese samples were stored for 7 days at 5°C in a brine solution (2 Tablespoons salt added to a quart of water) in a plastic bag before the sensory test. The cheese was dried on cheese cloth the day before the test so the cheese would not be soggy. Fresh cheese samples were prepared the day of the test. The cheese was divided into approximately 10g rectangular samples immediately before the sensory evaluation and was kept in covered plastic cups in refrigerated conditions (approximately 5°C) using ice packs and coolers during the sensory analysis before administration for accessibility purposes.

Sensory evaluation: Discrimination. A triangle test, using an untrained sensory panel (n=54), was used to determine if consumers could discriminate between fresh mozzarella cheese and mozzarella cheese fortified with algal oil. The number of panelists was based on the minimum number of panelists for an alpha value of 10%, a beta value of 5%, and a p_d value of 30%. In this test, it is desired to show if the mozzarella cheese with and without algal oil are perceived as the same, not to show if the cheese are different, because it is desired to be able to substitute the supplemented cheese for the plain cheese in the market. Therefore, the parameters (alpha, beta, and p_d values) were set for similarity testing, i.e. to determine if the cheese with algal oil added is perceived as the same as the cheese without algal oil added. Testing was conducted under white lighting in individual sensory booths, in the sensory evaluation laboratory of the Food Science and Technology Department at Virginia Tech. Each panelist was asked to evaluate three cheese samples (approximately 5^oC), identified with 3-digit codes selected from a table of randomized numbers, and identify the different sample. Equal numbers of six possible combinations were presented (ABB, BAA, AAB, BBA, ABA, and BAB where A was fresh mozzarella cheese and B was fresh mozzarella cheese with algal oil). Appendix A illustrates triangle test administration worksheets. Examples of the triangle test scorecard and consent form for instructions given to panelists are in Appendix B and C. Virginia Tech Institutional Review Board approval was received for all sensory tests conducted for this research (IRB # 08-157; Appendix D).

After completing the consent form, panelists were provided a cup for expectoration if desired, a cup of water for rinsing their mouth, a fork for transferring the cheese to their mouth, a napkin, and a scorecard/pencil along with their samples. The three cheese samples were presented simultaneously. A paper scorecard was used.

Results were evaluated by counting the number of correct responses and comparing this number to 23 (the critical number of correct responses in a triangle test for an alpha value of 10% and 54 panelists) to determine if the two samples were the same.

Sensory evaluation: Degree of oxidation. A sensory panel was used in a multisample difference test to determine degree of oxidation of cheese samples. Testing was completed in the sensory evaluation booths as previously described.

Panelists were screened to determine if they could perceive oxidized flavor using three samples of fluid milk (3.7% milkfat) with different degrees of light oxidation. Milk (about 2 gallons) was divided into three high-density polyethylene (HDPE) gallon containers. One container was not exposed to sunlight (control), one was exposed to sunlight for about 30 minutes, and one was exposed to sunlight for about an hour. The milk samples were tasted by an experienced dairy judge to verify the milk samples were oxidized to different intensity levels. The screening test occurred in the sensory laboratory. Using a paired comparison test, panelists were asked to evaluate three pairs (control: 30 minute oxidized sample; control: 60 minute oxidized sample; 30 minute: 60 minute samples) and identify which sample in each pair was more oxidized. Panelists correctly answering two of the three pairs correctly (n=8) were selected for participation in the experimental testing on mozzarella cheese. About one minute was allowed to elapse between each pair served so that the panelists did not experience sensory fatigue. See Appendix B and C for scorecards and consent form for instructions given to panelists, and Appendix A for test administration worksheets.

The multisample difference test, for mozzarella cheese was conducted by presenting all four samples, identified with 3-digit codes selected from a random numbers table, simultaneously and in random order. A 15-point universal scale, with 1 = imperceptible oxidation and 15 = extremely oxidized (Appendix C), was used to evaluate and rate four samples for oxidized flavor intensity: fresh mozzarella cheese, fresh mozzarella cheese with algal oil (28mg/28g cheese), stored mozzarella cheese, and stored mozzarella cheese with algal oil (28mg/28g cheese). Please see Appendix A for test administration worksheets. Since 8 of the 10 panelists completed the paired comparison milk test successfully, 8 samples (about 80g) of each type were needed. Please see attached scorecards and consent form for instructions given to panelists (Appendix B and C).

Results were evaluated using analysis of variance (ANOVA) using the statistical software SAS JMP (SAS 2007) to determine which samples were significantly different using an alpha value of 0.05.

4.4 RESULTS

4.4.1 Modeling DHA in Mozzarella Cheese without Antioxidants

Model. DHA in cheese supplemented with algal oil was measured by GC, and the results are as shown in Figure 4-1. Values shown are averages of cheese stored with or without exposure to light, and at 2^oC or 6^oC, as there was no significant difference found between these conditions.

Figure 4-1. DHA degradation in mozzarella cheese supplemented with *Schizochytrium* sp. algal oil and stored at 2 or 6^oC with or without exposure to light for 14 days (samples taken at days 0, 7, 10, 12, and 14).

Based on these results, an auto catalytic model was fitted to the DHA trend:

$$\frac{dP}{dt} = kP(1-P)$$

where P = DHA/original DHA (mg/mg), and

k = reaction rate (Adachi and others 1995).

For all conditions tested, there was no significant difference between the results, and therefore no significant difference between the reaction rate (k = 1.3). The various storage conditions tested included temperature (2 or 6°C) and light (with or without exposure). This model was solved using Berkeley Madonna, a dynamic simulation software (Macey and Oster 2006). A picture of this model is found in Figure 4-2. This is a typical kinetic curve of oxidation (Kamal-Eldin and Pokorny 2005).

Other studies have used a similar model to describe the autoxidation of DHA. Investigations into ethyl docosahexaenoate and ethyl eicosapentaenoate autoxidation used the equation

$$-\frac{dP}{dt} = kP(1-P)$$

where P = DHA/original DHA, and

$\quad$ k = apparent reaction rate constant (Yoshii and others 1999).

A study with ethyl docosahexaenoate, docosahexaenoic triglyceride (DHA oil), and DHA oil emulsion used the Langmuir-type autoxidation kinetic equation

$$-\frac{dP}{dt} = \frac{kOp}{K+Op}P(1-P)$$

where P = DHA/original DHA,

$\quad$ k = apparent reaction rate constant,

$\quad$ Op = oxygen pressure, and

$\quad$ K = Langmuir parameter for oxygen (Yoshii and others 2002).

Another study with ethyol docosaheaenoate and ethyl eicosapentaenoate also used the equation

$$-\frac{dP}{dt} = \frac{kOp}{K+Op}P(1-P)$$

where P = DHA/original DHA,

$\quad$ k = rate constant,

$\quad$ Op = unreacted oxygen concentration, and

$\quad$ K = saturation constant (Adachi and others 1995).

These studies found the model to be true for the situation where the fraction of unoxidized DHA was greater than or equal to 50%. Additionally, these studies found the model to be independent of temperature, as was found in this research.

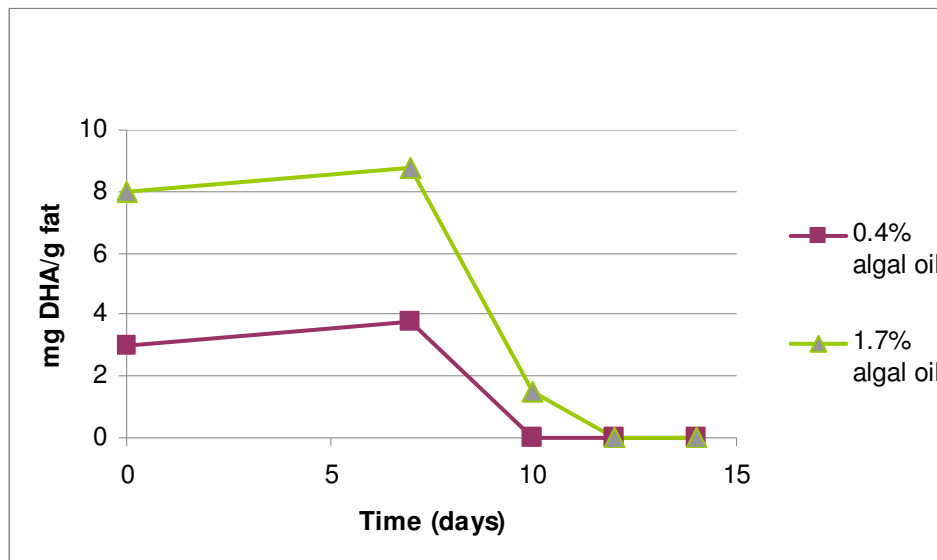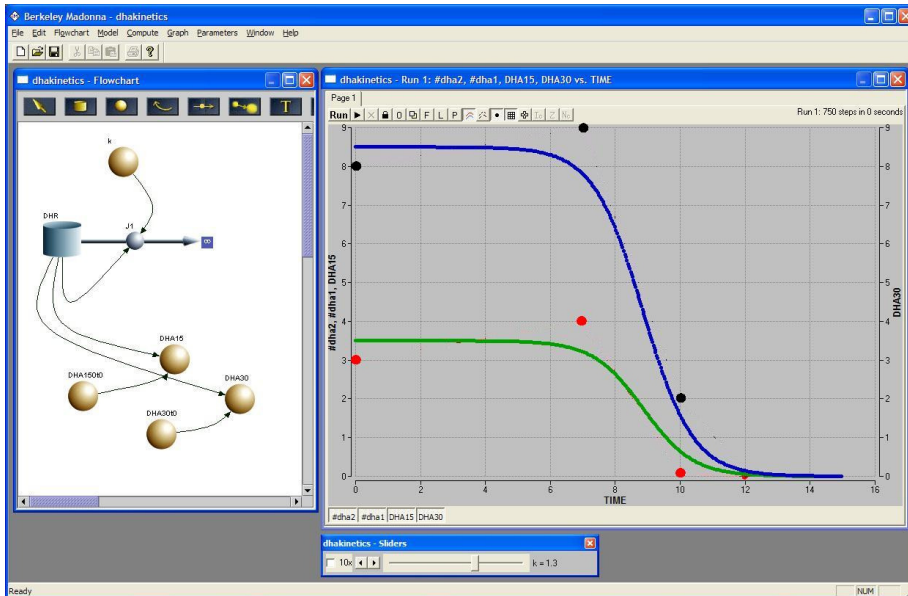**Figure 4-2**. Autocatalytic model of DHA content in mozzarella cheese supplemented with *Schizochytrium* sp. algal oil and stored at 2 or 6°C with or without exposure to light for 14 days (samples taken at days 0, 7, 10, 12, and 14).

There appears to be a clear distinction between the oxidation stages, with the induction period through day 7, followed by the accelerated oxidation stage. Future studies with mozzarella cheese supplemented with algal oil should explore effects of other oxidation factors, such as oxygen, metals, enzymes, pH, water activity, microbes, and fatty acid composition.

Oil loss during cheese processing. A mass balance of the components in cheese was done in order to understand the algal oil loss during the processing of the cheese. It appears that the majority of the algal oil (55mg DHA/g whey fat) is lost in the whey during the separation of the curds and whey and cheese stretching. This was determined by performing GC analysis of the fat from the whey. This whey could be used to make ricotta cheese supplemented with algal oil so that they algal oil is not wasted. More algal oil (about 50% more) must be added to the milk than is desired in the final cheese product in order to achieve the appropriate amount of algal oil and to account for algal oil

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lost during cheese processing. Methods of keeping the algal oil within the cheese product need to be explore in future work.

Future studies may also look into the effects of processing on product oxidation and ways to prevent processing oxidation, as significant oxidation has been shown to occur during pasteurization of milk for mozzarella cheese (Fedele and Bergamo 2001). Also, buffalo milk may be used in place of cow's milk as this may provide more protection against oxidation (Balestrieri and others 2002).

4.4.2 DHA and Oxidation in Mozzarella Cheese with Antioxidants

Optimization of algal oil and mixed tocopherols concentration. The optimum combination of algal oil and mixed tocopherols addition in mozzarella cheese for maximum DHA content and minimum oxidation appears to be 110ppm mixed tocopherols and 3% algal oil (per milk fat). These values were determined based on a response surface design using the program Stat-Ease Design Expert 7.0 (see Figure 4-3) (Stat-Ease 2005). Mozzarella cheese typically contains a much lower level of antioxidants naturally – 0.064-.116mmol/100g cheese (Halvorsen and others 2006).
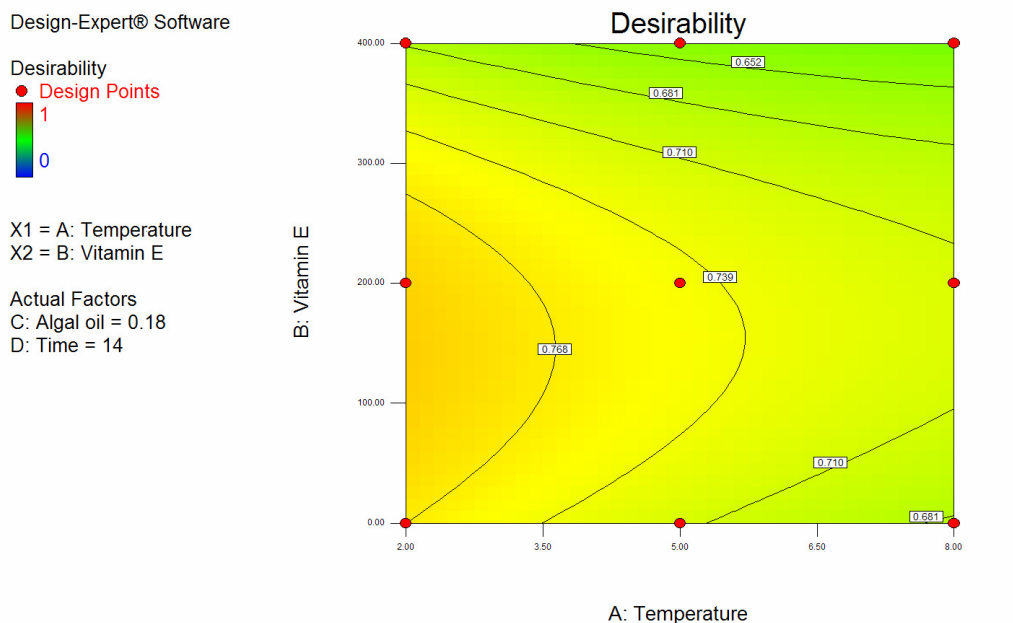
Figure 4-3. Graphical representation of mixed tocopherols (0, 100, 200, 400ppm) and algal oil (0, 1.5, 3%) optimization in mozzarella cheese stored at 2, 5, or 8°C for maximum DHA content (from GC data) and minimum oxidation (based on TBA value) from Design-Expert software (Stat-Ease 2005) response surface design.

Fate of DHA with optimum algal oil and mixed tocopherols concentration. The optimum algal oil (3%) and mixed tocopherols (110ppm) concentrations were incorporated into mozzarella cheese stored at 2, 5, or 8°C for 3 weeks, and the resulting DHA degradation and oxidation were measured. DHA degradation was minimal (Figure 4-4). Sample oxidation was also minimal (Figure 4-5). A study with frozen mackerel suggested TBA values of less than 19 to be acceptable in oils (Ke and others 1975). Other studies with marine oils have also used this acceptability range (Kaitaranta 1992).
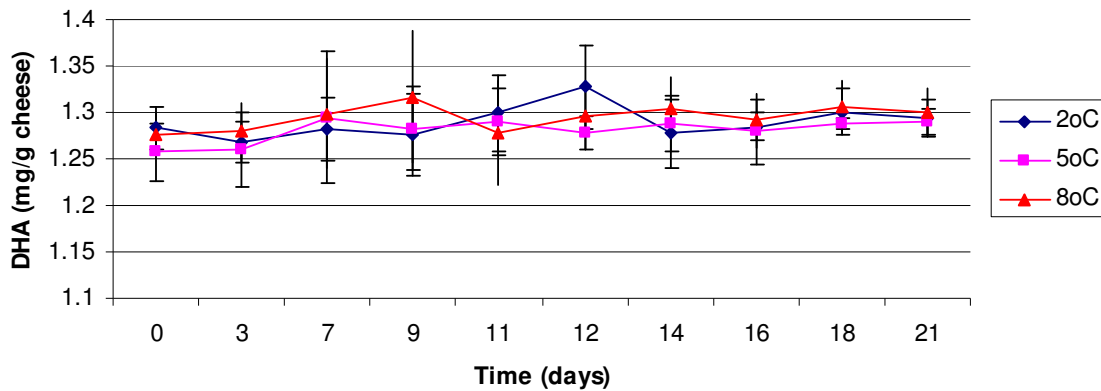
Figure 4-4. DHA content (mean +/- standard deviation) in mozzarella cheese with 3% *Schizochytrium* sp. algal oil and 110ppm mixed tocopherols stored for 21 days at 2, 5, or 8°C.
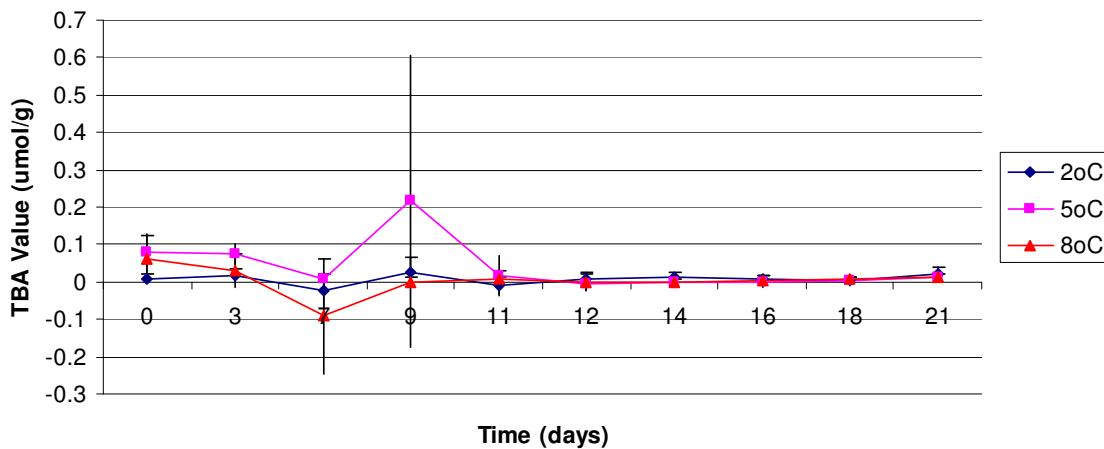
Figure 4-5. Oxidation (mean +/- standard deviation of TBA value) in mozzarella cheese with 3% *Schizochytrium* sp. algal oil and 110ppm mixed tocopherols stored for 21 days at 2, 5, or 8°C.

A study with milk enriched with fish oil found no significant difference between volatiles from samples stored at temperatures between 2 and 9°C (Let and others 2005), which is consistent with the present research with no significant difference between DHA content and TBA value between samples stored between 2 and 8°C (2, 5, and 8°C).

A study with fish oil, ground nut oil, or linseed oil incorporated into spray-dried milk found a significant increase ($p<0.05$) in the oxidation (peroxide value) of samples after one month of storage at 4, 27, or 37°C, and (as found here with tocopherols) a small decrease in the percentage DHA of the samples after one month of storage at the same temperatures (Ramaprasad and others 2006).

In another study, lipid hydroperoxides and propanal formation increased and was significantly greater for milk with algal oil than milk without algal oil over 25 hours at 32°C (Gallaher and others 2005). Ascorbic acid was found to be the most effective antioxidant compared with EDTA, sodium caseinate, whey protein isolate, and thermally denatured whey protein isolate over 20 days at 32°C (Gallaher and others 2005). This is another potential antioxidant to explore and compare in future research (Gallaher and others 2005).

Future studies may also explore the effects of microbial growth on oxidation, but a previous study with algal oil supplemented milk showed that inhibition of microbial growth by 200ppm sodium azide had no effect on oxidation (Gallaher and others 2005).

4.4.3 Sensory Evaluation of Mozzarella Cheese with Algal Oil

All sensory evaluation research was approved by the Virginia Tech Institutional Review Board (IRB# 08-157) (Appendix D).

Discrimination. Thirty-two out of 54 panelists correctly identified the odd sample. Since the critical number of responses for a triangle test with an alpha value of 0.10 and 54 panelists is 23, and since the number of correct responses is greater than the critical number of responses, the hypothesis that untrained panelists cannot discern the difference between mozzarella cheese and mozzarella cheese fortified with algal oil is rejected.

This result is not consistent with the results found by Franklin and others, who showed that trained dairy panelists could not detect the difference between milk from

cows fed or not fed algae over 42 days of storage (Franklin and others 1999). The milk contained about 0.46g DHA/g milk fat, whereas the cheese used in this experiment contained about 0.43mg DHA/g cheese fat (as measured by GC).

In the "comments" section of the scorecard, some panelists commented on the saltiness of the cheese. This may be due to certain parts of the cheese absorbing more salt from the brine solution, or this may be due to a salty flavor in the algal oil. Other panelists commented on other factors such as dryness, wetness, blandness, texture, appearance, sweetness, aftertaste, bitterness, smell, milkiness, hardness, and creaminess. One panelist commented on a flaxseed oil taste, while another commented on a fishy flavor. It appears that most panelists correctly identified the "odd" sample based on characteristics other than an algal oil/fishy/flaxseed flavor. However, these different characteristics may be caused by the algal oil addition.

Based on these results of the triangle test, it may be recommended to repeat the test with lower levels of algal oil added to determine the untrained panelists' threshold for detecting the algal oil. However, there should be adequate algal oil added in order to realize health benefits. In addition, since many panelists made comments about the saltiness of the cheese, the test may be repeated with no salt added to the cheese and/or no brine solution in order to remove this factor and prevent the saltiness from affecting the results. Additionally, factors such as saltiness of the algal oil alone may be evaluated to determine if the algal oil is the source of the salty flavor. Following this test it would be relevant to conduct a preference test. Although the panelists could tell the difference between the cheeses, it is unknown which they would prefer. Descriptive analysis may also be done on the cheese samples to determine what characteristics actually differ between the cheese samples.

Degree of oxidation. Panelist ratings (mean +/- standard deviation) were as follows (where 1 = imperceptible oxidation and 15 = extremely oxidized): 5.8 +/- 4.7 for fresh mozzarella cheese, 7.3 +/- 4.5 for stored mozzarella cheese, 7.3 +/- 4.5 for fresh mozzarella cheese with algal oil added, and 7.4 +/- 3.8 for stored mozzarella cheese with algal oil added. There was no significant difference between the oxidation ratings for the cheese samples based on ANOVA (SAS 2007). The mean oxidation ratings for all

samples correlate to a slightly to moderately perceptible oxidized flavor. The high standard deviation indicates the panel was not well trained and/or did not know how to use the scale.

The panelist rating results can also be converted into simple ranking data, based on the rating given, and analyzed by Friedman analysis. The T statistic was calculated by the equation

$$T = \left([12/bt(t+1)]\sum_{j=1}^{t} x_{.j}^{2} \right) - 3b(t+1)$$

where t = number of samples

b = number of panelists

$x_{.j}$ = rank sum of sample j

The T statistic (-4.35) was determined to be less than the Chi-squared value for 3 degrees of freedom at the 5% significance level (7.81), which shows the samples are not significantly different by ranking data either. This shows that the panelists were not rating the samples in the same order while using different parts of the scale.

The panelist rating results correlate with the results found by Chee and others, who found that there was no significant difference between fishy perception scores by a trained panel for yogurt with and without algal oil which was fresh or stored for 7 days (Chee and others 2005)

Additionally, TBA value analysis showed the order of oxidation to be as follows (from least to most oxidized): fresh mozzarella cheese, fresh mozzarella cheese with algal oil added, stored mozzarella cheese, stored mozzarella cheese with algal oil added. The TBA values of the fresh, stored, and fresh with algal oil mozzarella cheese samples were in the range of 0.004-0.00568, while the TBA value of the stored mozzarella cheese with algal oil was over twice this value. The TBA value of the stored mozzarella cheese with algal oil is probably much higher due to the unstable nature of the polyunsaturated fatty acids in the algal oil. However, it must be noted that these TBA values are only estimates, as only one replication was done for each sample. These estimates may be compared with the malonaldehyde value which has been reported for mozzarella cheese of 0.5 ug/g (Shamberger and others 1977). The TBA value results correlate with the lipid hydroperoxides found by Chee and others, which showed that there was a noticeable

increase in hydroperoxides after 7 days for yogurt with algal oil, as well as more lipid hydroperoxides in yogurt with algal oil that had not been stored than in yogurt without algal oil (Chee and others 2005).

Based on the results, the hypothesis that degree of oxidation of mozzarella cheese fortified with algal oil correlates to oxidized flavor described by a trained sensory panel as follows (from least to most oxidized): fresh mozzarella cheese, fresh mozzarella cheese with algal oil added, stored mozzarella cheese, and stored mozzarella cheese with algal oil added is rejected. This result may be due to the panelists associating a "fishy" taste with oxidation, or possibly finding it difficult to taste oxidized flavors because the algal oil masked any oxidized flavors causing panelists to guess.

Based on the oxidation ratings of the cheese and the TBA values, it appears that the mozzarella cheese does not experience significant oxidation with storage. However, the mozzarella cheese with algal oil does experience significant oxidation with storage based on the TBA value.

In future studies, the multisample difference test may be repeated using panelists with more extensive dairy oxidation training, as well as marine oil oxidation training, and a larger number of panelists. Panelists should also be screened for the ability to rate intensity and not just distinguish oxidized flavor from less or no oxidized flavor. The small number of panelists does not allow for a high level of confidence. Additionally, it may be interesting to use a panel trained only in seafood oxidation or a panel trained in both seafood and dairy oxidation. Methods of preventing oxidized flavor in the mozzarella cheese may also be explored, such as encapsulating the algal oil, addition of antioxidants, and improved storage methods.

4.5 CONCLUSIONS

The fate of DHA from algal oil in mozzarella cheese can be modeled by the equation $\dfrac{dP}{dt} = kP(1-P)$

where P = DHA/original DHA, and

k = reaction rate.

Future studies may explore modeling cheese under different conditions such as different concentrations of algal oil and cheese with various packaging.

The best combination of concentrations of algal oil and vitamin E is 3% algal oil and 110ppm mixed tocopherols in mozzarella cheese for maximum DHA content and minimum oxidation after 2 weeks of storage. This combination prevented DHA degradation and oxidation for 3 weeks of mozzarella cheese storage at 2, 5, and 8°C. Future studies may investigate the stability of higher concentrations of algal oil, and studies with deodorized or encapsulated algal oil.

Consuming a 28g serving of this fortified cheese (with 3% algal oil based on milkfat) would results in consuming about 0.1g DHA. Approximately 0.5-18 servings of the cheese would need to be consumed to intake the same amount of DHA as from a 3oz serving of fish, based on reported DHA concentration in fish and depending on the type of fish (i.e. catfish contains about 0.051g DHA/3oz, but mackerel contains about 1.8g DHA/3oz) (Hepburn and others 1986; Ackman 1988; Simopoulos and others 1999). Future research may investigate higher algal oil concentrations in the cheese with DHA concentrations closer to that of a serving of fish.

Consumers can distinguish between mozzarella cheese and mozzarella cheese with algal oil. Additionally, trained dairy judges find a more oxidized flavor in fresh mozzarella cheese with algal oil than in plain mozzarella cheese stored for a week. Further sensory studies need to be done regarding preference and acceptability of a mozzarella cheese product with algal oil.

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CHAPTER 5
SUMMARY AND CONCLUSIONS

An increased interest in omega-3 fatty acids has been brought on by the realization of the lack of this nutrient in many people's diets and the many benefits the nutrient can provide. DHA is a specific type of omega-3 fatty acids with various benefits shown through several studies. Algal oil is an abundant source of DHA which is also vegetarian, replenishable, and without concerns of heavy metal contamination. Functional foods are a good way to incorporate omega-3 fatty acids into diets by using popular foods that are already consumed. Mozzarella cheese is one such food whose consumption is increasing and has additional health benefits. The degradation and oxidation of PUFA within foods is not well understood. Modeling the degradation of DHA within mozzarella cheese will help with this understanding, and to provide further knowledge regarding the shelf-life and stability of such food products. Then, by identifying an appropriate method of preventing degradation and oxidation of the DHA, the quality and nutritional value of the food can be maintained.

This study first identified synthetic and natural antioxidants appropriate for preserving DHA in algal oil and preventing oxidation. TBHQ was found to be an effective synthetic antioxidant at a concentration of 0.0175g/g algal oil for at least 6 weeks. Mixed tocopherols were found to be an effective natural antioxidant at a concentration of 400ppm for up to 4 weeks. DHA degradation in algal oil was modeled using dynamic simulation software, Berkeley Madonna, by the auto catalytic equation

$$\frac{dP}{dt} = kP(1-P)$$

where   P = DHA/original DHA (mg/mg), and

   k = reaction rate (Adachi and others 1995).

The study then focused on algal oil (0.4% and 1.7%) within mozzarella cheese. DHA content in the mozzarella cheese stored over 2 weeks was modeled using dynamic simulation software to find the same auto catalytic model. This model has also been found to represent omega-3 fatty acid degradation in other studies. The DHA degradation and fatty acid oxidation within the cheese with 3% algal oil was then

controlled using a natural antioxidant of mixed tocopherols which was found to be optimally effective at a concentration of 110ppm. This antioxidant was effective for at least 3 weeks.

Future research should include looking into the use of other preventative measures for degradation and oxidation, such as encapsulation, which would also prevent any flavor and odor issues, and use of other antioxidants and synergists. Additionally, further modeling should be done for other food vehicles.

APPENDIX A
TEST ADMINISTRATION WORKSHEETS

TRIANGLE TEST WORKSHEET

Product Codes:

	Code 1	Code 2
A (0% DHA)	881	734
B (0.1% DHA)	959	698

Product Set Up: 10g mozzarella cheese for each treatment placed in small plastic cups. Each cup is marked with the corresponding code number.

Each panelist will get one sample set. Follow the coding sequence for each sample set.

CODE SEQUENCE:

Panelist Sequence	Sample Order	Code (left)	Code (middle)	Code (right)
1,7,13,19,25,31,37,43,49	AAB	881	734	959
2,8,14,20,26,32,38,44,50	ABA	881	959	734
3,9,15,21,27,33,39,45,51	BAA	698	881	734
4,10,16,22,28,34,40,46,52	BBA	959	698	881
5,11,17,23,29,35,41,47,53	BAB	959	881	698
6,12,18,24,30,36,42,48,54	ABB	881	959	698

PAIRED COMPARISON TEST WORKSHEET

Product codes:

Sample	Code 1	Code 2
A (no oxidation)	138	869
B (moderate oxidation)	496	375
C (extreme oxidation)	984	743

Product Set Up: 10ml of fluid milk for each treatment placed in small plastic cups. Each cup is marked with the corresponding code number.

Each panelist will get three sample sets. Follow the coding sequence for each sample set.

CODE SEQUENCE:

Panelist	Sample sequence (1)	Code (left)	Code (right)	Sample Sequence (2)	Code (left)	Code (right)	Sample sequence (3)	Code (left)	Code (right)
1	AB	138	496	BC	375	984	AC	869	743
2	BA	496	138	CB	984	375	CA	743	869
3	BC	496	984	AC	138	743	AB	869	375
4	CB	984	496	CA	743	138	BA	375	869
5	AC	138	984	AB	869	496	BC	375	743
6	CA	984	138	BA	496	869	CB	743	375
7	AB	138	496	BC	375	984	AC	869	743
8	BA	496	138	CB	984	375	CA	743	869
9	BC	496	984	AC	138	743	AB	869	375
10	CB	984	496	CA	743	138	BA	375	869
11	AC	138	984	AB	869	496	BC	375	743
12	CA	984	138	BA	496	869	CB	743	375
13	AB	138	496	BC	375	984	AC	869	743
14	BA	496	138	CB	984	375	CA	743	869
15	BC	496	984	AC	138	743	AB	869	375
16	CB	984	496	CA	743	138	BA	375	869
17	AC	138	984	AB	869	496	BC	375	743
18	CA	984	138	BA	496	869	CB	743	375
19	AB	138	496	BC	375	984	AC	869	743
20	BA	496	138	CB	984	375	CA	743	869
21	BC	496	984	AC	138	743	AB	869	375
22	CB	984	496	CA	743	138	BA	375	869
23	AC	138	984	AB	869	496	BC	375	743
24	CA	984	138	BA	496	869	CB	743	375

MULTISAMPLE DIFFERENCE TEST WORKSHEET

Product codes:

Sample	Code
A (fresh mozzarella)	337
B (fresh mozzarella with oil)	843
C (stored mozzarella)	562
D (stored mozzarella with oil)	924

Product Set Up: 10g mozzarella cheese for each treatment placed in small plastic cups. Each cup is marked with the corresponding code number.

Each panelist will get one sample set. Follow the coding sequence for each sample set.

CODE SEQUENCE:

Panelist	Sample Order	Code (left)	Code (middle left)	Code (middle right)	Code (right)
1	**ABCD**	337	843	562	924
2	**BACD**	843	337	562	924
3	**CABD**	562	337	843	924
4	**DABC**	924	337	843	562
5	**ADBC**	337	924	843	562
6	**BDAC**	843	924	337	562
7	**CBDA**	562	843	924	337
8	**DBCA**	924	843	562	337

APPENDIX B
CONSENT FORMS

TRIANGLE TEST

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

Title of Project: Evaluation of Mozzarella Cheese Supplemented with Algal Oil

Principal Investigator: Meg Orders

I.   THE PURPOSE OF THIS PROJECT

You are invited to participate on a sensory evaluation panel about mozzarella cheese. The purpose of this research is to test the flavor of mozzarella cheese when algal oil has been added as a dietary supplement.

II.   PROCEDURES

There will be 1 session involving about 10 minutes. You will be presented with 3 samples at the session. Should you find a sample unpalatable or offensive, you may choose to spit it out and continue to other samples.

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

ALLERGIES: _____

III.   BENEFITS/RISKS OF THE PROJECT

Your participation in the project will provide information that will help determine acceptability of a mozzarella cheese product supplemented with omega-3 fatty acids. You may receive the results or summary of the panel when the project is completed if you so wish. Some risk may be involved if you have an unknown food allergy. The cheese for this sensory test has been prepared in the Virginia Tech Food Science and Technology facilities, which have not been inspected by the Food and Drug Administration for cheese production.

IV.   EXTENT OF ANONYMITY AND CONFIDENTIALITY

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

V.   COMPENSATION

For participation in the project, you will receive some candy after the session.

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

VII.   APPROVAL OF RESEARCH

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

VIII.   SUBJECT'S RESPONSIBILITIES

I know of no reason I cannot participate in this study which will require tasting mozzarella cheese containing algal oil.

_____

Signature   Date

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

Address_____

Phone_____

---tear off--
IX.   SUBJECT'S PERMISSION (human subject may tear off and keep)

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

I know of no reason I cannot participate in this study which will require tasting mozzarella cheese.

_____

Signature   Date

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

Meg Orders, morders@vt.edu
Investigator/e-mail

(304) 389-0688
Faculty/Phone

Dr. David Moore   (540) 231-4991
Chair, IRB/Phone for Research Division

MULTISAMPLE DIFFERENCE/PAIRED COMPARISON TESTS

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

Title of Project: Evaluation of Mozzarella Cheese Supplemented with Algal Oil

Principal Investigator: Meg Orders

I.   THE PURPOSE OF THIS PROJECT

You are invited to participate on a sensory evaluation panel about milk and mozzarella cheese. The purpose of this research is to test the oxidation of mozzarella cheese when the cheese has been stored and algal oil has been added as a dietary supplement.

II.   PROCEDURES

There will be 2 sessions involving about 15 minutes each. You will be presented with 3 pairs of 2 samples of milk at the first session and 4 samples of mozzarella cheese at the second session. Should you find a sample unpalatable or offensive, you may choose to spit it out and continue to other samples.

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

ALLERGIES: _____

III.   BENEFITS/RISKS OF THE PROJECT

Your participation in the project will provide information that will help identify any oxidized flavor of mozzarella cheese supplemented with algal oil. You may receive the results or summary of the panel when the project is completed if you so wish. Some risk may be involved if you have an unknown food allergy. The cheese for this sensory test has been prepared in the Virginia Tech Food Science and Technology facilities, which have not been inspected by the Food and Drug Administration for cheese production.

IV.   EXTENT OF ANONYMITY AND CONFIDENTIALITY

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

V.   COMPENSATION

For participation in the project, you will receive some candy after the session.

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

VII.   APPROVAL OF RESEARCH

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

VIII.   SUBJECT'S RESPONSIBILITIES

I know of no reason I cannot participate in this study which will require tasting milk and mozzarella cheese containing algal oil.

_____
Signature   Date

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

Address_____

Phone_____

---tear off--
IX.   SUBJECT'S PERMISSION (human subject may tear off and keep)

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

I know of no reason I cannot participate in this study which will require tasting mozzarella cheese.

_____
Signature   Date

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

Meg Orders, morders@vt.edu_____
Investigator/e-mail

(304) 389-0688_____
Faculty/Phone

Dr. David Moore_____   (540) 231-4991
Chair, IRB/Phone for Research Division

SCORECARD
TRIANGLE TEST

Panelist Number_____

You will receive one set of samples for you to evaluate by tasting.

There are three samples in the sample set. Two of these samples are the same and one is different. Taste the samples from left to right and identify the odd sample. When you have identified the different sample and marked the scorecard, pass the samples and scorecard through the hatch.

Sample Code	**Check the Odd Sample**

Comments:

Thank you for your participation. Please come into the hallway to receive candy as a token of appreciation.

<div style="border:1px solid black; padding:10px;">
<center>

SCORECARD
PAIRED COMPARISON TEST

</center>
</div>

Panelist Number_____

You will receive three sets of samples for you to evaluate by tasting. Each set will be presented independently.

There are two samples in each sample set. Taste the samples from left to right. Determine which sample has more oxidized flavor. You must make a choice. When you have identified the more oxidized sample and marked the scorecard, pass the samples through the hatch. The next set of samples will be passed through to you. Repeat for the third set of samples.

SET 1:

        Sample Codes   _____   _____

        Which sample is more oxidized? _____

SET 2:

        Sample Codes   _____   _____

        Which sample is more oxidized? _____

SET 3:

        Sample Codes   _____   _____

        Which sample is more oxidized? _____

Comments:

Thank you for completing this sensory experience. Please come into the sensory lab to receive candy as a token of appreciation.

SCORECARD
MULTISAMPLE DIFFERENCE TEST

Panelist Number_____

You will receive one set of samples for you to evaluate by tasting.

Taste the samples from left to right and note the intensity of the oxidation. Rate each sample on the following scale:
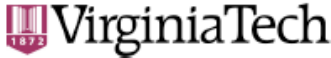

1
2   Imperceptible
3

4
5   Slightly perceptible
6

7
8   Moderately perceptible
9

10
11   Strongly perceptible
12

13
14   Extremely perceptible
15

Sample Code	Sample Rating

Comments:

Thank you for completing this sensory experience. Please come into the sensory lab to receive candy

APPENDIX D
IRB 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
www.irb.vt.edu

FWA00000572(expires 1/20/2010)
IRB # Is IRB00000667

DATE: March 19, 2008

MEMORANDUM

TO: Susan E. Duncan
             Margaret Orders
             Kim M. Waterman

FROM: David M. Moore

SUBJECT: **IRB Exempt Approval:**   "Sensory Evaluation of Mozzarella Cheese Supplemented
             with Algal Oil" , IRB # 08-157

I have reviewed your request to the IRB for exemption for the above referenced project. I concur that
the research falls within the exempt status.   Approval is granted effective as of March 18, 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.

cc: File

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