Long Chain n-3 PUFA and Oleic Acid Modification Strategies to Enhance Fillet Quality in Tilapia, *Oreochromis* species

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Abstract

Tilapia are freshwater fish that have become important in aquaculture and as a stable global source of seafood due to their ability to thrive in different environments. However, tilapia are sometimes considered nutritionally undesirable due to their high *n*-6 to *n*-3 fatty acid ratios. A market study was conducted first to determine fatty acid compositions in tilapia fillets in different US markets. Then a research was conducted to enhance nutritional value of tilapia by improving the *n*-3 and oleic acid contents in fish fillets without compromising fish growth or feed conversion ratios. Feeds were formulated with combinations of high and low *n*-6, *n*-3, and oleic acid levels using soybean oil, fish oil, algae oil, and high-oleic sunflower oil. Then 12 diets, including a commercial diet, were assigned to 24 tanks, each with 25 tilapia per tank. A Recirculating Aquaculture System (RAS) was used to grow the fish for 8 weeks. Fatty acid compositions of tilapia fillets were determined and samples were vacuum packed and stored at -10^{o}C and -20^{o}C to test oxidative degradation and fatty acid compositional changes. The market survey data showed that there were significant differences in fatty acid composition, lipid content, and *n*-6:*n*-3 fatty acid ratios depending on the country of origin. Samples from USA had ideal *n*-6:*n*-3 ratios (1.3 ±0.85) while samples from Southeast Asia had higher *n*-6:*n*-3 fatty acids ratio (6.6 ±0.54). Algae oil incorporation significantly increased DHA level while fish oil incorporation significantly increased both EPA and DPA. High-oleic sunflower oil based diets improved oleic acid levels and reduced linoleic acid compared to the soybean oil based diets. Sensory evaluation indicated that lipid source did not significantly impact preference or overall fillet quality, including texture.

Interestingly, a survey showed people were interested in value-added tilapia, and would pay up to 30% more for nutritionally enhanced fish compared to the $5.00/lb fresh fillet price currently available in supermarkets. There was no observable oxidation during long term frozen storage. The oxidation study proved that value-addition would not be compromised during the long term storage conditions, even under temperature abuse. It is possible to improve tilapia nutritional quality through diet to provide consumers with value-added products that maintain quality during frozen storage.

Abstract for General Audience

Tilapia are high quality food with high levels of protein. However, many consumers are skeptical about tilapia due to their undesirable lipid composition containing high levels of omega 6. Therefore initially, the market study was developed to identify the lipid composition of tilapia US consumers were exposed to. Then the experiment was designed to create high omega 3 tilapia by improving the diets using mixture of fish oil, algae oil, high-oleic sunflower oil, and soybean oil. The 8 week tilapia growth trial was conducted with 12 experimental diets. Then the fish fillets were collected and analyzed for their fatty acid composition using GC/MS. Also fillet's shelf-life study was conducted in 2 frozen storage conditions up to 9 months. The market study proved that the omega 6 to omega 3 ratio was favorable especially in USA sourced tilapia. However it also showed wide variation in omega 6 to omega 3 ratio depending on the country of origin. Tilapia from USA had an ideal omega 6:omega 3 ratio (1.3 ±0.85) while tilapia from Southeast Asia had higher omega 6:omega 3 ratio (6.6 ±0.54). The experimental study showed that the algae oil addition significantly improved DHA level while fish oil addition improved EPA and DPA. High-oleic sunflower oil addition improved omega 9 compared to the soybean oil based diets. Sensory evaluation indicated that different lipid sources had no impact on consumer preference. Interestingly, the survey showed that the people were interested in healthier tilapia, and were willing pay up to 30% more to the $5.00/lb of supermarket fresh fillet price. There was no observable degradation during long term frozen storage. The shelf life study proved that value-addition would not degrade during the long term storage conditions. Therefore, development of high quality tilapia through diet manipulation is possible that does not degrade over 9 months of frozen storage.

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Chapter 1. Introduction

Unlike agriculture, which has been developing extensively since the dawn of civilization, aquaculture at industrial level is a relatively recent method of food production. Even though there has been recorded history of aquaculture dating back to 2000 B.C. in China, the expansion of aquaculture at an industrial level really took off in the 1700's to 1900's (Rabanal 1988). It is still unclear why, where, and how aquaculture technologies have been developed. Not only is the record of aquaculture fragmented, but most aquaculture is assumed to have been for ornamental purposes. Some records from China indicate there were developments of pen and cage culture in Han dynasty (2300 – 2100 B.P.), and fingerling production in Zhou dynasty (2300 B.P.), although the function and the uses are still unclear (Beveridge and Little 2002). Regardless of aquaculture history, aquaculture has been a relatively small endeavor until recently, in part, because the demand for seafood products have been mostly satisfied by wild catch fisheries.

The increase in seafood production mostly came from the increase in production level of aquaculture while the fisheries have remained relatively consistent in recent years. Importance of aquaculture in replacing fisheries can be highlighted by current state of fisheries. Fisheries and wild catch still account for an average of 90 million metric tons, however, the fisheries have shown stability in production with insignificant increase within the industry for last decade (FAO 2014). Latest tally showed, in 2014, global fisheries produced 93.4 million metric tons compared to the aquaculture at 73.8 million metric tons (FAO 2016). At higher production levels compared to aquaculture production, importance of fisheries cannot be overlooked. However, the problem is that the wild catch industry has not been able to answer the increased demand for seafood products. Improvement of technology can minimize by-catch, increase production efficiencies, and improve

processing, but these measures have not been able to solve issues regarding decrease in wild fish populations. Some researchers have looked at the issue with the current harvest method of fisheries, and created prediction models to prove we have started extinction process of some of species since 1950s (Burgess and others 2013). The problem is extended by the slow recovery of overfished species (Neubauer and others 2013). Therefore, development of more sustainable and economical aquaculture system is the possible answer to the increased demand for seafood products.

The solution to the current fisheries industry has been partially met by development of aquaculture of both freshwater and marine species since the early 1960s. Expansion of aquaculture has allowed humans to consume more fish than ever before. And to meet the demand, the production levels have been increasing parallel to the diet preference and human populations. The FAO Fisheries and Aquaculture Department has reported that since 1960s global fish consumption has increased from 9.9 kg per capita to 20.1 kg per capita in 2014, along with the increase in population to over 7.3 billion people (FAO 2016; FAO 2014). And in order to supply the increasing demand for seafood products, total fish and shellfish production has increased from approximately 20 million metric ton in 1950s to 167.2 million metric ton in 2014 (FAO 2016; FAO 2014). Although fisheries production has maintained steady over the decade, aquaculture production have exploded through improvement of technologies and culturing methods. In 2014, aquaculture production had surpassed 73.8 million metric tons with 47.1 million metric tons from inland and 26.7 million metric tons from marine aquaculture (FAO 2016). Fish species available to be cultured are still limited as more research is required to understand fish culturing specific to different fish species. Compared to the fisheries that fished 70 principal wild caught species with 150,000 metric tons production or more per species, aquaculture has only encompassed 31 principal species with 140,000 metric tons production or more per species in 2012 (FAO 2014).

Even though the aquaculture industry has come a long way in a short period of time, there is still much to be understood when culturing new species.

A feed conversion ratio (FCR) is important in any form of animal nutrition. It is calculated by pounds of feed intake over pounds of weight gain by the animal. Having a good food conversion ratio second to chicken, fish are important part of human diets. Not only do fish contain high levels of protein, they are also full of other nutrients not easily obtained through terrestrial animals. The most highly regarded nutrient of fish products are *n*-3 polyunsaturated fatty acids (PUFA). The most researched *n*-3 fatty acids are eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3). Many case studies of long term consumption of *n*-3 fatty acids such as EPA and DHA have showed *n*-3 fatty acids lowers the risks of coronary heart disease (Durrington and others 2001; Hu and others 2002). In one study, subjects were scaled on an *n*-3 index which measured concentration of EPA and DHA in red blood cell membranes. This index was inversely related to coronary heart disease with greatest cardio-protection at 8% or greater in red blood cell membranes (Harris and von Schacky 2004). It was also reported that DHA was related to visual functional development in early ages by affecting the retinal phospholipid composition (Uauy and others 1992). As brain is composed of structural lipid at 60%, mostly of arachidonic acids and DHA, DHA plays an important role in function and development of brain at early stages of growth (Crawford 1993). Therefore, it is highly recommended and nutritionally important for people of all ages to consume proper levels of long chain *n*-3 PUFA only practically found in marine food products.

Currently, the fatty acid composition of tilapia is a problem. According to the FAO, global tilapia production was second to carps by 2012, at 0.71 million metric ton wild caught and 4.51

million metric tons aquacultured. Not only is tilapia production second largest, its aquaculture production has doubled in only 6 years, with expected continual growth in the future (FAO 2014). FAO further identifies carps, catfish and tilapia aquacultures to account for 60% of total aquaculture industry by 2025 (FAO 2016). However, tilapia's economic success is now overshadowed by its fatty acid composition, specifically *n*-6 to *n*-3 fatty acids ratios. Proinflammatory activities are related to *n*-6 fatty acids and *n*-6 fatty acids have associated with inflammatory diseases such as diabetes, cardiovascular disease and more (Patterson and others 2012). A study observed that lower *n*-6 to *n*-3 ratios are associated with a reduction of breast cancer risk in women, reduced rectal cell proliferation with colorectal cancer patients, suppressed inflammation in rheumatoid arthritis patients, and beneficial effect on asthma. This shows that a lower *n*-6:*n*-3 ratios are important for improving the health of people consuming a Western diet, that currently has a 15:1 to 16.7:1 *n*-6:*n*-3 ratio (Simopoulos 2002). A study by Weaver and others (2008) highlighted tilapia's high *n*-6:*n*-3 ratio. Not only that, the paper stated, "the inflammatory potential of hamburger and pork bacon is lower than the average serving of farmed tilapia," which further strengthened the argument that the value of tilapia is worse than bacon (Weaver and others 2008). Although, the impact of Weaver and others (2008) cannot be fully understood, medias utilization of the paper in recent years have had negative impact on the industry and the first quarter of 2016 US saw 14% reduction in tilapia import, by volume, compared to the same time year before (Globefish 2016). The stagnation of tilapia sales in US is concerning and therefore, improving tilapia fatty acid composition through diet will allow consumers to have access to nutritionally improved premium quality tilapia.

Research Objectives

A consumer perception of nutritionally undesirable tilapia fillet lipid quality have been challenging to the tilapia industry, therefore, improving the lipid quality in tilapia can have positive impact on the seafood consumers. Although previous research has looked at improving *n*-3 fatty acids through agricultural based ingredients, poor conversion to long chain *n*-3 fatty acids made the diets ineffective. This research was designed to determine the lipid quality and fatty acid composition of tilapia products currently available in US supermarkets and designed the experiment to create value added tilapia fillet through nutritionally enhanced diets while determining the lipid autoxidation and degradation of long chain *n*-3 fatty acids during frozen storage conditions. Objective 1 was to determine production information, lipid quality, and fatty acid composition of tilapia products currently available to US consumers. Objective 2 was to formulate enhanced diets to increase *n*-3 PUFA from algae and fish oils, and oleic acid from high-oleic sunflower seed oil while reduce *n*-6 PUFA. Objective 3 was to evaluate the fatty acid composition of tilapia fillets produced from the enhanced diets. Objective 4 was to evaluate the differences in sensory characteristics of texture, aroma, and flavor, and gather demographics, seafood purchasing and consumption behaviors. And the last objective was to evaluate change in fatty acid composition and lipid autoxidation in fish fillet during long term storage in -10^{o}C and -20^{o}C cold storage.

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Chapter 2. Review of Literature

1. Tilapia

Tilapia is the common name for fish species in Family *Cichlidae*. There are well over 1600 different species under Cichlids and over 30 species under *Oreochromis*. Of them, the most commonly aquacultured and fished species are Nile tilapia (*Oreochromis niloticus*) and Mozambique tilapia (*Oreochromis mossambicus*) (Bender 2016). Tilapia are scaled, freshwater fish with compressed body that inhabit warm water rivers and lakes (Rakocy 2005). Tilapia are favored in the aquaculture industry for their ability to adapt to different environments. Tilapia are highly adaptable as they are eurythermic (wide range of temperature tolerance) and euryhaline (wide range of salinity tolerance) fish that performs well in various different feed formulations (El-Sayed 2006). Tilapia can reproduce multiple times a year, and can produce up to 1,500 eggs per kg fish (Rakocy 2005). Tilapia's ability to survive in different environments, reproduce exponentially, grow quickly, and provide a good source of protein allowed them to become a favorite within the aquaculture industry around the world. The strength of tilapia production is also highlighted by tilapia's omnivorous behavior that allows for inexpensive feed and crowding behavior suitable for intensive culturing (Watanabe and others 2002).

Since the introduction of tilapia into different part of the world from the Nile River, the tilapia aquaculture has steadily grown. Tilapia aquaculture started with 515,000 tons of Nile tilapia in 1984 (Fitzsimmons 2000). However, in 2012, 33% of 710,535 metric tons of cichlids that were wild captured were Nile tilapia, and additionally, 3,197,330 metric tons of Nile tilapia were aquacultured (FAO 2014). The world-wide production of tilapia at 3.20 million tons in 2012 has doubled in only 6 years (Rakocy 2005; FAO 2014). Currently China is the largest producer of

tilapia with Egypt, Philippines, Thailand, and Indonesia following behind (Rakocy 2005). In the United States, tilapia were first introduced in the 1950s for aquariums and research, but currently they are aquacultured mostly in recirculating aquaculture systems (Fitzsimmons 2000). Unlike other fish that attracts high price tags, tilapia are considered a cheap fish product. According to Globefish, in 2014 salmon averaged approximately 5.10 USD per kg of fresh fish (Globefish 2014). And in 2015, frozen tuna loins and frozen salmon fillets averaged 9.50 USD per kg, while frozen tilapia fillets were only 4.60 USD per kg, even with a 4.9% increase since 2013 (Globefish 2015b; Globefish 2015a). In US supermarkets, tilapia are commonly sold at or around 5.00 USD per lb fresh fillet, with price dropping significantly for frozen fillets sold in bulk. Because tilapia are mass produced at lower price, the industry has ability to grow higher quality product and still increase the price without affecting the consumer's willingness to buy tilapia products. With increase in new generation consumers, the food industry have seen abrupt changes in the demands for transparency, less processes and sustainable products (Topper 2015). Not only that, consumers are increasing premium brand purchases that indicates potential growth even for tilapia industry (IRI 2016). The growth in premium market has be evident in organic products where products with organic label are fetching higher price tag compared to same product without organic label (Jonas and Roosen 2005).

Although tilapia are known as robust species in aquaculture, they still require proper maintenance of environmental factors in order to minimize stress and susceptibility to pathogens. Along with proper nutrients, the factors involved in growth of any fish species include proper levels of salinity, water temperature, pH, dissolved oxygen (DO), alkalinity, ammonia, nitrite, and nitrate. Although tilapia are naturally found in freshwater, many studies have looked at adaptability of tilapia to different salinities. The studies have found that the tilapia have tolerant salinity level

of 15 parts per thousand (ppt), can reproduce in 29 ppt, but failed to adapt to 35 ppt (Balarin and Haller 1982; Alfredo and Hector 2002). Tilapia species' ability to adapt to saline water explains their ancestry as a marine species (Myers 1938). Generally, recirculating aquaculture system (RAS) requires 60% saturation or 5 parts per million (ppm) of dissolved oxygen (DO) for culturing fish; however, tilapia are able to survive in 0.1 to 0.5 ppm for short duration and even at 0 ppm with access to surface air (El-Sayed 2006). A study reported that tilapia had respiration rates of 0.74 to 0.97 ppm/hr at 37 to 42 ^{o}C (Franklin and others 1995). Ammonia is produced by fish through the gills by respiration. Toxicity from the excreted materials poses more of problem to fish than to terrestrial animals. Although the ionized form of ammonia (NH_4^+) is nontoxic to fish, unionized ammonia (NH_3) can be detrimental to fish health (El-Sayed 2006). For juvenile Nile tilapia, El-Shafai and others (2004) observed growth issues at concentration of 0.07 to 0.14 ppm with 0.14 ppm as lowest observable chronic toxicity (El-Shafai and others 2004). With adult Nile tilapia, sub-lethal level was observed at 2 ppm by gill, liver and kidney damage at six week exposure (Benli and others 2008). Although Nile tilapia seem to respond with more resistance to ammonia toxicity than salmonids, it is still important to maintain low levels of unionized ammonia (Benli and others 2008). For more sensitive salmonids, Westin (1974) recommended safe levels to be 25 to 35 ppm for nitrate, and 0.012 ppm for nitrite as they are 1/10 level of 10% lethal concentration. This suggestion was developed by determining the 10% mortality would be the upper allowable limit in hatchery facilities and analyzed by 7 day lethal concentration study at 10% mortality of the population (Westin 1974).

Bioenergetics is important in aquaculture. In tilapia farms, it is common for the farmers to use the majority of its funds for buying feeds. As an expensive factor in farms (up to 50% of operation cost), it is important to maximize feed efficiency and minimize feed waste (El-Sayed

2006). Proper ratios of nutrients in the feed is important in maximizing feed efficiency and fish growth while maintaining proper fish health. A food conversion ratio (FCR) is important parameter in aquaculture as FCR measures amount of feed fish consume compared to amount of weight fish gain, and therefore, used in many aquaculture feed research to determine the efficacy of the diet. Also, fish require different levels of various nutrients during different life stages. For instance, fry require more protein than adults (El-Sayed 2006). In fry, more proteins allowed better growth, higher survival rate, but seems to be insignificant over 40% protein diet; this seemed to be inversely related to the FCR (Al Hafedh 1999). In different study, fry had best growth with diets containing 45% protein, but it is more important to have appropriate protein to energy ratio of 110 mg protein per kcal to maximize growth and feed utilization efficiency (El-Sayed and Teshima 1992). Although 40% protein diets were best suited to fry; young tilapia with initial weight of 40 g had best growth and FCR on 30% protein diets (Siddiqui and others 1988). Unlike adult tilapia that require lowest amount of protein at 20-30%, juvenile tilapia requires 30-40% while larval stage require >50% protein diet (El-Sayed 2006). Proper protein requirement has direct impact on the growth performance of tilapia.

Amino acids impact the growth and reproduction of fish by providing essential components for new protein synthesis or maintenance of existing protein (Wilson 2002). Much like other fish and land animals, tilapia are known to require 10 essential amino acids: 4.20% arginine, 1.72% histidine, 3.11% isoleucine, 3.39% leucine, 5.12% lysine, 2.68% methionine, 3.75% phenylalanine, 3.75% threonine, 1.00% tryptophan, and 2.80% valine (Santiago and Lovell 1988). This agrees with a similar study that reported amino acid requirement of 4.10% arginine, 1.5% histidine, 2.6% isoleucine, 4.3% leucine, 1.3% methionine, 3.2% phenylalanine, 3.3% threonine, 0.6% tryptophan, and 3.0% valine, 2.1% cysteine, and 1.6% tyrosine (Fagbenro 2000). A study looking at the

digestibility of different protein sources reported that apparent amino acid of 87.7% (Köprücü and Özdemir 2005). This was similar to apparent digestibility of protein at 87.1%. The apparent protein digestibility, therefore, was an indication of apparent amino acids digestibility, which fluctuated from 71 to 90.5% and 70.6 to 91.2%, respectively (Köprücü and Özdemir 2005).

Lipids are important component for fish growth as lipids provide high levels of energy. Although some observed that excessive levels of lipids can retard growth, suppress immune system, and enhance lipid oxidation, proper levels of lipids in the diet provides hormone precursors, cell membrane structure, fat soluble vitamins, and growth (Gatlin III 2002; El-Sayed 2006). Currently, a common practice among the tilapia farmers is using diets with lipid at 3-7% of total diet composition, dry weight basis (El-Sayed 2006). Fish require different levels of essential fatty acids (EFA) and the species specificity also is complicated because the lipid requirements are different, depending on the life stage. Tilapia are warm water freshwater fish that accumulate fatty acids depending heavily on what they eat, unlike cold water and marine fish that requires high *n*-3 polyunsaturated fatty acids (PUFA) for survival. Multiple studies have showed that *n*-6 PUFA is the major lipid source required for tilapia for best growth performance (Kanazawa and others 1980; Stickney and others 1982). Although *n*-6 PUFA is essential in healthy fish growth, research also showed that feed efficiency was greatest in fish fed cod liver oil, along with greatest growth performance in fish fed combination of cod liver oil and corn oil (Chou and Shiau 1999). Another study that looked at the ratio of carbohydrate to lipid in *Tilapia zillii* saw diets containing 30% protein and 2% lipid had significantly worse growth performance compared to diets containing 30% protein and 5, 10, or 15% lipid, while observing no significant impact in carbohydrates at different concentrations on growth (El-Sayed and Garling Jr 1988). This study showed that lipid level at 5, 10, and 15% in total diet did not have significant differences in growth and FCR, and it is also

important that the diets contained both soybean oil and cod liver oil providing sufficient *n*-6 and *n*-3 PUFA. Another study that looked at the effect of fish oil diets compared to the blended vegetable oil diet with higher levels of α-linolenic acid and lower levels of long chain *n*-3 saw insignificant differences between the diets in growth performance of both genetically improved farmed tilapia (GIFT) and Red hybrid tilapia (Teoh and others 2011).

Research looked at the increase in *n*-3 PUFA through addition of flaxseed in diet in Nile tilapia fillet at 0, 10, 20, and 30 days saw significant improvement of *n*-6:*n*-3 ratios at day 30, but looking at the actual composition of *n*-3 PUFA, there were insignificant improvement of *n*-3 PUFA such as DHA and EPA (Justi and others 2003). 18:3*n*-3 needs to go through complex multi-step desaturation and elongation to become EPA and DHA. However these reaction pathways are competed with linoleic acid (18:2*n*-6). Research has shown that regardless of different type of diet, tilapia was not able to metabolize α-linolenic acid (18:3*n*-3) to DHA and EPA at significant concentrations (Karapanagiotidis and others 2007). It is also interesting that the study looking at the digestibility of fatty acids in GIFT tilapia compared to Red hybrid tilapia saw high digestibility of long chain *n*-3 PUFA along with 18:2*n*-6, 18:3*n*-3, and 18:1*n*-9 at over 95% regardless of the oil source (Teoh and others 2011). The same study also found that although diet contained high 18:3*n*-3, it was not converted to DHA or EPA in significant quantity compared to the diet containing fish oil with high DHA and EPA (Teoh and others 2011).

The reduction in growing time increases fish output as well as reduction in feed use. There are few ways to control proper growth conditions of tilapia. The success of tilapia aquaculture is also possible because of the homogenization of gender through masculinization by sex-reversal (Phelps and Popma 2000). The new technology of sex reversal allows for more uniform fillet size

and reduction in discard fish due to small size. Also overcrowding can cause stunting in mixed sex culture due to uncontrolled reproduction (Phelps and Popma 2000). Various research reported sex-reversal through genetic modification and hormonal control yielded faster growth and higher fillet yields compared to mixed-sex tilapia (Pandian and Sheela 1995; Mair and others 1995). The sex reversal technique that gives all male fish allows aquaculture farmers to improve marketable products even further. Also eliminating females in ponds can help create more intensive culture because reproduction is eliminated. There was no need to worry about having fry in the pond along with marketable fish (Mair and others 1995). Methods of sex reversal vary from steroid injection, steroid feed additives, to steroid in water immersions of fry stage (Gale and others 1999; Bhandari and others 2006). The most effective means of sex reversal is the steroid, 17-α-methyltestosterone (MT), added to feeds fed to the recently hatched fry with undifferentiated gonads (Megbowon and Mojekwu 2014). It has become a popular procedure in tilapia farms and is incorporated into the diets for fry at 40 to 60 ppm for up to 30 days (Penman and McAndrew 2000). Although the concern for consumption of MT exists for consumers, it has been shown that the less than 1% of MT in MT-incorporated diets exists in tilapia after 21 days (Goudie and others 1986). MT is readily metabolized in tilapia and 97-99% of MT is excreted readily (Curtis and others 1991). Many studies vary the days for depuration, and they agree that MT is excreted quickly and some even observed 100% elimination from carcass (Pandian and Kirankumar 2003; Khalil and others 2011). Although there is lack of research on environmental factors resulting from MT run off, it should be treated as any other chemical and hormone additives and treated similarly (Macintosh 1982; Khalil and others 2011).

Aquaculture is still at its infancy compared to other meat industries. Food industries have always been striving to meet the world's demand for food consumptions through genetic

modification and genetic engineering through gene selection, selective breeding, transgenic and other methods. Tilapia is a general term that covers *Oreochromis* and *Sarotherodon* spp. In 2002, 10 different species were aquacultured with approximately 85% of total tilapia production coming from *Oreochromis niloticus* (Nile tilapia) and *Oreochromis mossambicus* (Mozambique tilapia) (El-Sayed 2006). Nile tilapia is favored in many production facilities due to their ability to adapt well to different environments, grow fast, and provide high consistent yield in semi-intensive and intensive culture.

2. Recirculating Aquaculture System (RAS)

Around the world, aquaculture is gaining popularity for having high economical potential. RAS has many characteristics that are favorable in current economic and environmental states compare to open net pen mariculture or pond reared aquaculture systems. Unlike mariculture systems, RAS is environmentally friendly and can control environmental impact and pollution outputs by easily employing waste water management and monitoring systems. RAS can also closely regulate the water quality in the tanks for maximum control over fish health and stress factors as well as fillet quality. Unlike pond systems, RAS can be adapted to different environments. Unlike wild catch fish with steady production levels since 1990s at around 90 million metric tons, production level of aquaculture have been rising rapidly from 24 million metric tons in 1990s to 66.6 million metric tons in 2012 (FAO 2014).

The reduction in feed costs, efficiency in FCR, improvement in manufacturing methods and other cost reducing techniques allow for improving profitability of aquaculture. However, RAS is not without problems. RAS requires that the culturing methods use high density intensive aquaculture for the system to be profitable (Losordo and Westerman 1994). RAS also requires

relatively higher initial investment cost compared to the other aquaculture systems. Unlike other aquaculture systems, RAS requires more stringent management techniques, and expensive equipment.

Because the RAS is an enclosed, looped design, there are many possibilities for filters and sanitizers to generate reusable water. Solid waste removal is required to maintain proper level of DO and minimize production of ammonia and other toxic gases such as hydrogen sulfide (Masser and others 1999). DO in fish tanks is important in optimizing fish health. However, DO level is also important that the nitrification reactor where nitrification of ammonia to nitrate occurs with assist from microorganism. The nitrification reactor must have minimum of 2 ppm of DO for nitrifying bacteria (Masser and others 1999).

Sustainability of RAS is also questioned as it is a relatively new technology. Although it is highly effective way to utilize limited supplies of freshwater and saltwater, it is still not entirely independent and requires replacement of 5 to 10 percent of water volume per day (Masser and others 1999). The water replacement is necessarily due to the evaporation and the buildup of nitrate levels. Recently, there is more and more research focusing on RAS sustainability and the results are promising. Newer designed systems can achieve up to 99% less water use compared to older RAS (Nazar and others 2013).

3. Nitrification Cycle

The nitrification cycle is one of the closely monitored aspects of aquaculture because of its importance in maintaining good water quality in aquaculture systems. Nitrification is important because it allows detoxification of ammonia, a waste by-product of fish, through metabolism of

ammonia to nitrite and nitrate. The nitrification is predominantly controlled by *Nitrosomonas spp.* and *Nitrobacter spp.*, and water replacements in RAS.

RAS rely on stringent monitoring of the water quality due to the nature of the system's ability to recycle used water. Water quality in aquaculture involves ammonia, nitrate, nitrite, pH, temperature, turbidity, dissolved oxygen and other factors. However, nitrification is one of the most important factors in water quality of aquaculture. It is less of a problem in extensive and open flowing systems such as coastal open pen, which maintain good water quality using natural movement of water. In pond systems, pond sediments and presence of phytoplankton interacts with nitrogenous compounds such as ammonia, nitrite and nitrate (Hargreaves 1998). Also aquaculture systems using seawater has less problem because chloride from salt interferes with the biological activity of nitrite. However, in a closed looped system with high stocking density, increase in levels of ammonia, nitrite or nitrate can affect fish health.

The toxic effects of these compounds have been studied. It has been noticed that size of fish, density in tanks and other factors are involved with the toxicity level of nitrogenous compounds. In a 96 hour median lethality test Atwood and others (2001) observed that the smaller Nile tilapia had higher tolerance at 81 mg nitrite/L while bigger Nile tilapia tolerated only to 8 mg nitrite/L; thus smaller fish were more tolerant to the nitrite (Atwood and others 2001). Similar studies saw that, in different species, larger fish showed higher sensitivity to nitrite than smaller fish (Palachek and Tomasso 1984a; Lewis Jr and Morris 1986; Almendras 1987). Although metabolic rates and differences in size would predict the smaller fish to be more prone to nitrite toxicity, no solid explanation was concluded from the research. Also it seems like the nitrite toxicity relating to fish size is species dependent as other studies on grass carp, channel catfish,

and largemouth bass either found no differences nor found smaller fish to be more nitrite sensitive (Alcaraz and Espina 1995; Palachek and Tomasso 1984b).

It is important to understand the physiological characteristics of ammonia, nitrite and nitrate toxicity acutely and chronically in order to manage fish health more effectively. Ammonia is the most toxic compound compared to nitrite and nitrate. However, lethality of ammonia is affected by ionized form, lethal concentration, and sublethal chronic effects against fish health are still being studied. Unlike lethal levels of ammonia, sublethal toxicity have shown to affect tilapia by negatively reducing growth rate and FCR in water containing 0.144 mg unionized ammonia/L along with physiological disturbances compared to the control water containing 0.004 mg of unionized ammonia/L (El-Shafai and others 2004). In one study, *Tilapia aurea* exhibited tolerance to ammonia at 2.4 mg/L of unionized ammonia with 50% mortality in 48 hours when acclimatized to low dose of 0.43-0.53 mg/L for 35 days compared to complete mortality at 2.5 mg/L of unionized ammonia in 72 hours (Redner and Stickney 1979).

Therefore, minimizing the level of ammonia, nitrite, and nitrate is imperative. There are different ways in which aquaculture systems can maintain low levels. A major nitrification cycle takes place biologically using microbes such as *Nitrobacter spp*. and *Nitrosomonas spp*. These microorganisms break down ammonia and nitrite which are considered major nitrogenous wastes of fish metabolism. Biological filtration of toxic materials such as ammonia and nitrite are only as efficient as the microorganisms' make-up in the biological filtration system.

The solid waste also can produce additional ammonia during decomposition, and can act as physical attachment sites for nitrifying microorganisms (Masser and others 1999). There are also possibilities of disease treatment or other changes in water quality that can affect the

microorganism population in the biological filters (Masser and others 1999). The pH is another key factor in the nitrification cycle as microorganisms involved are inhibited below pH 6.8, thus maintaining pH level at 7 to 8 through the use of sodium bicarbonate or other alkaline buffers can maximize performance of nitrifying bacteria (Masser and others 1999). It is important for the microorganisms involved in nitrification cycle to be at an optimal pH, as pH also dictates the concentration of unionized (NH_3) and ionized (NH_4^+) forms of ammonia. Studies conducted by Two similar research found that rainbow trout and fathead minnows were more susceptible to ammonia toxicity at lower pH and saw similar effects of total nitrite against rainbow trout (Thurston and others 1981; Russo and others 1981).

4. Lipids

Lipids are group of compounds that are soluble in organic solvents and immiscible in water (McClements and Decker 2008). Although the hydrophobicity identifies lipid compounds, some are known to be surface-active and amphiphilic (Belitz and others 2009). Lipids in food are often divided into two main categories of oil and fats determined by the source and nature of the physical state. Lipids are nutritionally important to humans because of their high caloric nature as well as fat soluble vitamins and essential fatty acids required for normal physiological activities (Belitz and others 2009). In foods, lipids also play important roles as pleasant taste, aroma and as emulsifier (Belitz and others 2009). Six major lipid component include fatty acids, phospholipids, sterols, acylglycerols, sphingolipids, and waxes. Although most fatty acids are naturally found as glycerol after esterification, fatty acids as aliphatic chain are major component of lipids (McClements and Decker 2008). Fatty acids are classified as saturated or unsaturated depending on its chain length, number of double bonds, and occurrence of functional group (O'Keefe 2008).

Saturated fatty acids (SFA) are classified as short to medium chain fatty acids with no double bonds in its aliphatic chain. Although there are some branched chain fatty acids such as pristanic and phytanic acids, these fatty acids are rare in nature (Belitz and others 2009). The risk of high consumption of lipids have always interested human nutrition and health. According to the dietary guideline provided by USDA in 2010, Americans are over-consuming SFA; and suggests that people should limit the fat consumption to less than 10% of total daily calorie intake all while replacing SFA with monounsaturated fatty acids (MUFA) and/or PUFA in order to reduce cardiovascular diseases (USDA 2011). Although definitive health implications are often difficult to state categorically, a controlled dietary study saw reduction in SFA resulted in reduction of plasma total and LDL cholesterol, and concluded the dietary SFA had significant impact on heart health (Ginsberg and others 1998). Many research also observed a negative impact of saturated fatty acids on blood cholesterol concentration, and total and low density lipoprotein cholesterol (Bonanome and Grundy 1988; Hegsted and others 1965; Keys and others 1965).

Unlike SFA, *n*-6 PUFA are long chain, unbranched and have methylene interrupted double bonds in *cis*- configuration (O'Keefe 2008). Under the USDA dietary guidelines, saturated fatty acids should be replaced by PUFA (USDA 2011). However, it is important to distinguish *n*-6 from n-3 PUFA. They are different in the position of double bonds so that γ-linolenic acid (18:3*n*-6) have double bonds at carbon 6, 9, and 12, while α-linolenic acid (18:3*n*-3) have double at Δ-carbon 9, 12, and 15 (O'Keefe 2008). This distinction is important because the placement and number of double bonds affect the biological activities of these compounds. PUFAs are yet important part of human nutrition nonetheless, because linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) are EFAs that are recommended to be consumed at least 4.44 grams/day and 2.67 grams/day, respectively, in 2000 kcal diet/day (Patterson and others 2012; Simopoulos and others 2000).

PUFA are of interest to human health because although they are essentially required for the metabolism of eicosanoids that are responsible for the dietary fat modulated immune responses in human bodies (Klurfeld 2008). *N*-6 PUFA are precursors of proinflammatory eicosanoids such as prostaglandin (PG) and leukotrienes. The eicosanoids are a cyclized derivatives created in human bodies through enzymatic steps from 20 carbon chain fatty acids such as ARA, EPA and dihomo-γ-linolnic acid (20:4*n*-6) (Klurfeld 2008). The proinflammatory effect of eicosanoids can result to chronic conditions of cardiovascular disease and diabetes. A high consumption of linoleic acid (18:3*n*-6) is followed by the increase in its derivative, arachidonic acid (20:4*n*-6), that leads to cardiovascular disease (Patterson and others 2012). End products of arachidonic acid metabolism, such as PGE_2 and PGI_2 (prostacyclin), are known to promote inflammatory, vasoconstrictor, atherosclerosis, and branchiospasm (Das 2007; Sellers and Stallone 2008; Kobayashi and others 2004). As a precursor of PGI_2, arachidonic acid also has been associated with promotion of adipogenesis unlike *n*-3 eicosanoids, and an increase in PGI_2 signaling can results in child obesity (Massiera and others 2003). Recent study also reported possible correlation in depletion of β-amyloid, a sign of onset of Alzheimer's disease, with *n*-6 PUFA (Whelan 2008). High levels of *n*-6 PUFA consumption in diet is concerning also because of its competitive behavior in suppressing *n*-3 α-linolenic acid metabolism into DHA and EPA (Liou and others 2007).

The metabolism of PUFA is important in understanding how metabolism of *n*-6 and *n*-3 PUFA can be competitively inhibited. Eighteen carbons, α-linolenic acid and linoleic acids are precursors of the 20 and 22 carbon EFAs (Sprecher and others 1999). Enzymes such as Δ6-desaturase, elongase, Δ5-desaturase, cyclo-oxygenase, and 5-lipoxygenase are responsible for metabolizing α-linolenic acid and linoleic acid to their metabolites such as DHA and EPA, and

arachidonic acid respectively (Kidd 2007). Because the metabolic pathway shares the enzymes, improper ratio of consumption can lead to unbalanced metabolism of PUFA. According to Gibson and others (2011), competition for these set of enzymes are also zero-kinetic metabolism, which means the process has linear and concentration independent rate metabolism; and the production of 20- and 22 chain EFA is then concentration dependent (Gibson and others 2011). Also saturated fatty acids may play a role in competitive behavior of PUFA metabolism as well (Garg and others 1989).

N-3 PUFA are structurally different from *n*-6 PUFA by having different positions of double bonds. They are also is different in bioactivities and effects on human health. Importance of consumption of long chain PUFA, especially EPA and DHA, has been highlighted for their health benefits. They has been identified to play an important role "in membrane fluidity, cellular signaling, gene expression, and eicosanoid metabolism" (McClements and Decker 2008). A study saw decreased gene expression responsible for inflammatory and atherogenic (plaque forming) response by increasing EPA and DHA intake in healthy elderly subjects (Bouwens and others 2009). A recent clinical research have shown that EPA and DHA have possible protective activities against *n*-6 PUFA in brain in mood disorders (Freeman and others 2006). Although *n*-3 PUFA are essential in human diets, they are consumed at inadequate levels. Complete removal of linoleic acid is dangerous as it is EFA, however, a study reported that the reduction of *n*-6 to *n*-3 PUFA ratio from 15:1-16.7:1 in Western diet to below 10:1 saw suppression of inflammation with rheumatoid arthritis, and in asthma patients through reducing proliferation of leukotrienes and prostaglandins that are responsible for the inflammatory processes in human cardiovascular system (Simopoulos 2008).

N-3 PUFA, especially DHA and EPA, have also been known to benefit infants and pregnant women by affecting the nervous system. As case studies often have uncontrolled variables, many studies will report conflicting results. A study looking at the infant's behaviors when mothers had high DHA level at delivery saw positive correlation between high DHA level at birth and development in infant's cognitive abilities (Colombo and others 2004). On the other hand, a different study saw that the long term effect of increasing DHA in breast milk on neurological development in infants of least 12 weeks was not observed (Gibson and others 1997). Also, a study looking at the infants fed infant formula with fish oil at DHA:EPA (5:1) saw negative effect on growth of males during the 1st 6 months (Ryan and others 1999). In another study, however, looking at the visual acuity with the relationship of DHA saw that high DHA intake is recommended for 1st year of infants for better neural development (Hoffman and others 2004). Along with physiological benefits, some studies saw *n*-3 PUFA concentration in adipose tissue or phospholipids in red blood cells had indirect correlation with depression (Mamalakis and others 2002; Mamalakis and others 2004; Adams and others 1996). Although science behind major depressive disorder and the effect of DHA and EPA isn't unanimously positive, considering their overall health benefits, *n*-3 PUFA consumption is important part of human diet (Logan 2004).

Another group of fatty acids of interest is *n*-9 MUFA. *N*-9 MUFA may have 12 to 24 carbon atoms and have ethylene bond in position *n*-9 (Naudet 1996). Unlike *n*-6 and *n*-3 PUFA that share enzymes competitively, *n*-9 MUFA is a product of very specific desaturase (Thomas 2000). Oleic acid (octadecen-9c-oic acid, 18:1*n*-9) has single double bond in *cis*- configuration that is considered the most abundant fatty acid (Naudet 1996). Oleic acid is extensively studied for its effect in cancer and on human health, especially in the Mediterranean diet, and concludes that high oleic diet's health benefit resulted from replacing *n*-6 PUFA consumption (Ip 1997). However,

although oleic acid is commonly thought to be "neutral" in the human body, there was an *in-vivo* rat study that saw changes in metabolic effect by oleic acids through inhibition of glucose production (Obici and others 2002). This study could potentially explain research observing high oleic acid diets improved anti-atherogenic effect (Ryan and others 2000; Carluccio and others 1999; Parthasarathy and others 1990).

Another *n*-9 MUFA that needs to be observed is mead acid (eicosa-5, 8, 11-trienoic acid; 20:3*n*-9). The production of mead acid is observed with the oleic metabolite 8,11-eicosadienoic acid as probable intermediate (Fulco and Mead 1959). It is important in meat industry because presence of mead acid in meat products results from essential fatty acid deficiency. The presence of mead acid can indicate compromises in fish health. Because eicosanoids, linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) inhibit mead acid synthesis, under insufficient level of eicosanoids to produce long chain PUFA, the organisms will produce mead acid instead (Adeyeye and others 2013; Sargent and others 1995). The production of mead acid is not been exclusive to mammals. Normally, trout require both *n*-3 and *n*-6, trout fed fat-free diet reported elevation of mead acid production and otherwise improving fatty acid composition of diet inhibited mead acid production (Castell and others 1972). Therefore, production of mead acid in fish can indicate that the fish are being fed with insufficient amounts of essential fatty acids.

5. Soybean Oil

Soybean oil is extracted from soybeans through solvent extraction using hexane, or expelled/pressed. Soybean oil is used mostly in US as cooking oil while other countries use canola oil or palm oil as of cooking oils. However, soybean oil still accounts for majority of global oil production at 53.6 million metric tons in 2016, with 18.8% production accounted for by US

(IndexMundi 2016). Also, there has been big push in the aquaculture industry to break its reliance on fishmeal and fish oil. The driver of this change is the increase in cost, decrease production, and ability for tilapia to adapt to agricultural based ingredients. Not only does tilapia have good growth performance when using agricultural feed ingredients, *n*-6 fatty acids were identified as essential for tilapia (El-Sayed 2006). Therefore, soybean meal and oil has been utilized extensively in tilapia aquaculture feeds. The American Soybean Association stated the utilization of soybean meal and oil in replacing traditional animal and fish based ingredients has been responsible for quadrupling the China's freshwater aquaculture production to more than 20 million MT (Cremer 2011).

6. Sunflower Oil

Sunflower oil, holding gold-yellow hue, is a product of solvent or expeller pressing extraction of sunflower seed, *Helianthus annuus*. The majority of the world's consumption has been in food and cosmetics industries, with an increase in consumption by the biofuel industry since 1991 (FAO 2010). According to the FAO, sunflower seed is 4th most produced oilseed and accounts for 8% of total oilseed production worldwide (FAO 2010). USDA defines standards for agricultural products including sunflower oil. This lists standards for following sunflower oil: industrial mid-oleic, linoleic (less than 60%), linoleic (partially hydrogenated), linoleic (approx. 65%), and high oleic (70% and over). Linoleic sunflower oils differ from high oleic sunflower oil in that oil is made up of approximately 2/3 *n*-6 PUFA and 1/3 of MUFA (Thomas 2000). High oleic sunflower oil is sunflower oil that contains high levels of MUFA, mostly *n*-9 fatty acids. USDA standard defines high oleic sunflower oil as 100 grams of oil containing 100g total lipids, 41.08 mg α-tocopherol, 5.4 μg phylloquinone, 9.859 g total SFA, 83.689 g of total MUFA, 3.798 g total PUFA (USDA 2014).

It's been long understood that *n*-6 PUFA is pro-inflammatory, *n*-3 PUFA is anti-cardiovascular disease, and *n*-9 MUFA as neutral. Contrary to the common thought on MUFA, a case study saw reduction in plasma total cholesterol and plasma low density lipoprotein-cholesterol comparable to a PUFA diet, refuting the idea that MUFA has no effect on plasma cholesterol (Mattson and Grundy 1985). Other studies also saw similar patterns in replacing saturated fatty acid diet with MUFA through reduction in low density lipoprotein cholesterol, triglycerides, and VII coagulant activity (Allman-Farinelli and others 2005; Cater and others 1997). With single methylene interrupted double bonds, MUFA is more resistant to lipid oxidation compared to PUFA, and does not break down as easily as PUFA in human body (Mattson and Grundy 1985). This means MUFA is more shelf stable than PUFA, which in turn could improve the quality of food product. A study looking at vegetable oil PUFA oxidative stability through mixing high oleic sunflower oil saw good oxidative stability and flavor stability (Frankel and Huang 1994). A study evaluating the sunflower oil characteristics reported that high-oleic sunflower oil had better resistance to oxidative deterioration compared to the untreated sunflower oil, and saw that high oleic acid and low 18:2*n*-6 in high-oleic sunflower oil were responsible for the good oxidative stability (Purdy 1986). Replacing a SFA-rich diet with MUFA-rich diet gave oilier and softer animal carcass with the increase in MUFA to SFA ratio in the meat (Rhee and others 1988). The composition and quality of meat can therefore be affected by the SFA, PUFA, and MUFA contents.

A study examined the effect of high oleic sunflower oil compared to herring oil in Coho salmon and rainbow trout diets, and observed twice as much oleic acid deposition with high oleic sunflower oil diet with maximum oleic acid deposition only after 2 weeks of feeding (Skonberg and others 1994). A parallel study compared the effect of high oleic sunflower oil diet to herring

oil diet in Coho salmon and rainbow trout saw that the diets did not have significant impact on the growth or the composition of fillet (Skonberg and others 1993). In a feeding trial looking at the effect of sunflower oil replacing fish oil in salmon diets saw that with increase in sunflower oil from 0 to 100% of lipid source saw increase in 18:2*n*-6 from 1.6 to 28.2 g per 100 g total fatty acids, increase in total MUFA 19.0 to 24.3 g per 100 g total fatty acids, decrease in EPA from 11.8 to 2.9 g per 100 g total fatty acids, and decrease in DHA from 30.2 to 14.3 g per 100 g total fatty acids (Bransden and others 2003). The trial, although will not reflect the similar outcome in tilapia study, shows that addition of high oleic sunflower oil will have impact on the *n*-6 PUFA in fish fillet. The study also did not see significant differences in the growth among different diets containing different levels of *n*-3 and *n*-6 fatty acids.

Tilapia is one of the most cultured freshwater fish. Due to tilapia's feeding behavior as omnivores, tilapia consume feeds containing high levels of *n*-6 fatty acid. Therefore, it's been long understood that *n*-6 is essential fatty acids by tilapia. So there is a question about the effect of reducing *n*-6 PUFA with high oleic sunflower oil on fish health. Although some variants of sunflower oil are good sources of *n*-6 PUFA such as linoleic acid, high oleic sunflower oil contains less than 4% (w/w) of total PUFA.

7. Algae Oil

Algae oil is extracted from various microalgae species grown with addition of sugar and other energy sources. Different species are grown for different functions such as astaxanthin from *Haematococcus* or β-carotene from *Dunaliella salina* (Spolaore and others 2006). However, for microalgae oil, the primary focus has been in production of biofuel. In recent years, algae meal and oil has been produced for feed ingredients and human food supplements for high DHA and

EPA (Spolaore and others 2006; Doughman and others 2007). *Schizochytrium* sp. is most widely used for oil production for human consumption as the deodorized oil contains high levels of EPA (>12% w/w) and DHA (>24% w/w) (TGA 2014). The biggest detrimental factor in algae oil is its high production cost (Spolaore and others 2006). Toxicology screening showed that *Schizochytrium* sp. does not product any toxins such as domoic acid (a neurotoxin) and prymnesium toxin (from algal blooms) which could suggest safety for human consumption and is considered to be GRAS (Barclay and others 2010).

8. Lipid Oxidation

Oxidation is important in food product because it induces change in quality, flavor, and aroma profile as well as degradation of food product. Lipid peroxidation is induced by the presence of enzymes that often results in numbers of volatile and nonvolatile compounds by oxidation of allyl groups into hydroperoxides and their subsequent decomposition (Belitz and others 2009). Lipid autoxidation is different from peroxidation because autoxidation can occur without the presence of enzymes by hydrogen abstraction at allylic position in presence of hydroxyl radical (•OH) (McClements and Decker 2008; Kamal-Eldin and others 2003). Oxidation of lipid leads to the secondary oxidation products of aldehydes, ketones, alcohols, acids, and lactones resulting in off flavors, and off texture in foods (Kamal-Eldin and others 2003). Oxidation of lipid depends on many factors from "length of lag phase…temperature, oxygen concentration, degree of fatty acid unsaturation, activity of prooxidants, and increasing concentration of antioxidants." (McClements and Decker 2008). Also it can be seen that autoxidation rate and behavior change depending the type and the concentration of different fatty acid profiles (Belitz and others 2009).

The mode of lipid autoxidation has been extensively researched for over 70 years regarding the reaction of triplet oxygen with fatty acids (Kim and Min 2008a). Two different oxygen states, diradical triplet oxygen and nonradical singlet oxygen, have been identified to be involved in lipid autoxidation (Kim and Min 2008a). Triplet oxygen has different total quantum spin compared to singlet oxygen; thus triplet oxygen reacts readily only with other radical compounds in foods, whereas singlet oxygen can be formed chemically, enzymatically, and photo-chemically regardless of temperature due its low activation energy of 0-6 kcal/mol (Kim and Min 2008a). In contrast, hydrogen abstraction from the methylene interrupted carbon starts initiation step for the triplet oxygen oxidation (McClements and Decker 2008).

Unsaturated fatty acid autoxidation therefore can be initiated by triplet oxygen. Chain reaction of initiation, propagation and termination reaction explains the PUFA autoxidation kinetic scheme (Kamal-Eldin and others 2003). It requires two components, unsaturated fatty acids and oxygen, that create unstable intermediate stage to form volatile compounds resulting off-flavors in lipids (Erickson 2008).

$$\text{Initiation: } In^{\bullet} + RH \rightarrow InH + R^{\bullet}$$

$$\text{Propagation: } R^{\bullet} + O_2 \rightarrow ROO^{\bullet};\ ROO^{\bullet} + RH \rightarrow R^{\bullet} + ROOH$$

$$\text{Termination: } 2ROO^{\bullet} \rightarrow O_2 + \text{R}O_2R;\ ROO^{\bullet} + R^{\bullet} \rightarrow \text{R}O_2R$$

where $In^{\bullet}$ is a radical initiator; *RH* is a fatty acid; $R^{\bullet}$ is a lipid-free radical; $ROO^{\bullet}$ is a lipid peroxyl radical; *ROOH* is a lipid hydroperoxide; and RO_2R is a lipid peroxide.

(Erickson 2008)

The initiation step involves abstraction of hydrogen to form alkyl radical. The oxidation rate increases with double bonds at twice the rate. This is due to the fact that allylic carbon-hydrogen bond has the lowest bond dissociation energy in the aliphatic carbon chain. This is further lowered as more double bonds are present on the chain. This allows for more radical formation of compound, thus making PUFA with more double bonds to autoxidize at faster rate (McClements and Decker 2008). Therefore, PUFA are as much as 10-40 times more susceptible compared to oleic acid with single double bonds (McClements and Decker 2008). However, the causation of initiation is still a question, because the initiation of lipid molecule requires activation of oxygen to the singlet state. This can be achieved by formation of singlet oxygen, formation of active oxygen-iron complexes, and activation of singlet oxygen by hydrogen peroxide, superoxide anion, or hydroxyl radical, or oxidation can start due to the involvement of enzymatic systems (Erickson 2008).

The propagation phase is the main step of lipid autoxidation. The general steps involved in propagation are radical coupling with oxygen, atom or group transfer, fragmentation, rearrangement, and cyclization (Erickson 2008). Commonly found oxygen is in the triplet oxygen state which can attach to the hydrogen abstraction site to form peroxyl radicals (McClements and Decker 2008). Once the high energy peroxyl radical is formed, it promotes oxidation of other carbon-hydrogen covalent bond sites (McClements and Decker 2008). The promotion of new alkyl radicals resulted from the hydrogen addition to peroxyl radical to form fatty acid hydroperoxides (McClements and Decker 2008).

The termination step ends the oxidation process by creating stable, nonradical compounds from two radicals through radical-radical coupling or radical-radical disproportionate (Erickson

2008). The termination process is observed in frying oil with low oxygen, where the alkyl radicals react and form fatty acid dimers and larger polymers (McClements and Decker 2008).

9. Antioxidants

A key problem of lipid autoxidation is that it can occur at low temperature and in absence of light (Hamilton 2009). The molecular structure of triplet oxygen in biradical state will interact directly with unsaturated fatty acids to form radical intermediates for further degradation steps (Yanishlieva and Marinova 2003). Antioxidants can slow or stop the free radical reactions (Hamilton 2009). Antioxidants are therefore important in fighting autoxidation of EPA and DHA. PUFA autoxidation has a long lag phase with slow oxidation rate initially, which quickly escalates exponentially (Kim and Min 2008a). Antioxidants functions by extending the lag phase with chelating agents and/or promote termination phase by radical scavengers (Choe 2008). Types of dietary oils, depending on its fatty acid composition, can have significant impact on the oxidative properties of the tilapia fillet. A study looking at long term storage effect of various dietary oil-based diets in fish fillet saw insignificant differences in water holding capacity and texture of the fillet but observed improved oxidative stability in palm-oil based diets and significant degradation of *n*-3 fatty acids after 30 weeks of frozen storage (Ng and Bahurmiz 2009). Therefore presence of antioxidants are critical in preserving long chain *n*-3 fatty acids from oxidative degradation.

Vitamin E is fat soluble antioxidant compounds of tocopherols and tocotrienol. Tocopherols are strong antioxidants in presence of oxygen and free radicals. However, not all tocopherols work equally, and the activities depends on the chemicophysical characteristics (Kim and Min 2008b). Tocopherols have 4 homologs with chroman ring (Kulas and others 2003). A thermodynamic property of 4 homologs, α-tocopherol has the highest radical scavenging activities

and fastest degradation compared to other homologs with lowest standard reduction potential (Kim and Min 2008b). Looking at the structure, the activities, in part, have to do with the number and position of methyl groups on the chromanol ring; where α-tocopherol has methyl groups at 5, 7, and 8 positions with R configuration (Kim and Min 2008b). α-Tocopherol is a phenolic compound with weak oxygen hydrogen bond that allows free radicals to attack it rather than the allylic carbon hydrogen bond on fatty acids (Brown and others 2012).

Tocopherols are found naturally in many organisms in order to protect fatty acids from oxidative degradation; however, it accumulates in fish only through consumption, and only α-tocopherol is present in wild marine fish (Kulas and others 2003; Brown and others 2012). Tocopherols intercept lipid radicals by donating phenolic hydrogen to peroxyl radicals (Kulas and others 2003). Activities of antioxidant's ability of retard the autoxidation process increases shelf-life of marine fish oil such as menhaden oil by increasing oxidative stability and protective quality (Hamilton 2009).

A problem with tocopherol's loss of antioxidative ability and activity as prooxidant has been observed (Kim and Min 2008b). A mechanism involved in prooxidation is still in question, but the significance of high levels of hydroperoxide shows that the peroxide abstraction of hydrogen from tocopherol results in alkoxyl radical that act as prooxidants in lipids (Kim and Min 2008b). Also prooxidation behavior is related to the concentration of tocopherols present in the system. The concentration has indirect relationship with antioxdiant activities, because the oxidized tocopherol increases alkyl, alkoxyl, and peroxy radicals that can further promote lipid oxidation (Kim and Min 2008b). Also the change in temperature from 50^{o}C to 0 or 20^{o}C in crude herring oil saw change from high hydroperoxide formation to hydroperoxide decomposition

(Aidos and others 2002). In Atlantic salmon, 150 ppm *d*-α-tocopherol acetate acted as prooxidant under iced storage while γ- and δ- tocopherol had no apparent antioxdative activities under frozen conditions (Berge and Lie 1998). The same study also saw a direct relationship between tocopherol in diet and the retention of tocopherol in the muscle at approximately 10%; and in another study using rainbow trout, there was linear, positive correlation in α-tocopherol level in the diet and in muscle (Berge and Lie 1998; Yildiz and others 2006).

Natural antioxidant activities exist in tilapia to protect them from oxidative stresses. Tilapia farms can improve fish health as well as fillet quality by addition of vitamin E in its feeds. A study observed that high concentrations of vitamin E oral administration to *Oreochromis niloticus* saw that vitamin E worked as chemoprotectant against induced cylindrospermopsin, a cyanotoxin formed by freshwater cyanobacteria spp. (Guzmán-Guillén and others 2015). When tilapia were cadium stressed, tilapia increased tissue glutathione-dependent enzymes and other antioxidant enzymes activities in order to protect against oxidative stresses (Basha and Rani 2003). Also tilapia contains antioxidant peptides in the gelatins of scales that was identified as antioxidant hydrolysate structure of Glu-Gly-Leu and Tyr-Gly-Asp-Glu-Tyr with high hydroxyl radical scavenging activities (Ngo and others 2010; Zhang and others 2012).

10. Sensory Characteristics of Fish Fillets

Tilapia consumption world-wide has been growing at increasing rates. The popularity of the fish in part has to do with the easier access for consumers and increase in production level. However, its popularity also attributed to the mild taste of tilapia (Young and Muir 2000). Tilapia is an omnivore that can thrive in many different environment, and therefore, is affected greatly by the location and the feed that can impart off-flavors from periphyton, cyanobacteria, and other still

unknown sources (Young and Muir 2000; Tucker 2000). Off-flavors are an indirect indicator of food quality, because food safety is not compromised by the presence of these compounds. However, people have associated foul odor to the actual quality of the product (Tucker 2000). The presence of these compounds, therefore can be detrimental to the producers, because the compounds renders the products as unmarketable. Many studies indicated that musty off-flavors are small problem outside the US catfish industry and even less of problem in cultures with saline environment (Lovell and Broce 1985; Lovell 1983). This was further proved by the polyculture system that saw less susceptibility of tilapia to build up same off-flavors as catfish grown in same pond (Torrans and Lowell 1987). In channel catfish, *Ictalurus punctatus*, a study saw increased off flavor compound, 2-methylisoborneol (MIB), in catfish with higher fat content, and consequently required longer purging period up to 72 hr for fish with over 6% muscle fats with MIB concentration of 20.8 µg/kg (Johnsen and Lloyd 1992). Although off-flavors are a smaller problem in the tilapia industry compared to catfish industry, off-flavors are still an economical problem nonetheless. In tilapia RAS, geosmin and MIB were found at detectable levels in water samples from various locations within RAS; but most importantly, geosmin and MIB were found in aerobic, organic rich environments such as nitrification reactor essential in operation of tilapia RAS (Guttman and van Rijn 2008). A study evaluated the off flavor compound, geosmin, in tilapia aquaculture systems concluded that geosmin concentration was lowest in tilapia muscle compared to other organs, however the elimination of muddy flavor from geosmin required up to 16 days of purging (Yamprayoon and Noomhorm 2000). These off flavor metabolites are only an issue in freshwater and becomes much less concern for brackish and marine system (Tucker 2000).

In aquaculture, depuration of off flavors can be achieved mainly through two methods: 1) displacing water in the system/relocation of fish to system free of chemicals or 2) to treat the water

for organisms responsible for the off flavor build up (Tucker 2000). Also depuration kinetics shows that the intensity of off flavor and time of purging is linearly correlated; and moreover, the concentration of off flavor in fish exponentially decrease as purging period increases (Howgate 2004). Commercially, it is commonly suggested to purge tilapia for 48 to 72 hours to depurate any off flavor compounds in the fillet (Fitzsimmons 2000).

Although most fish are either sold as whole fish or filleted, it is important to acknowledge that different parts of fish fillet have impact on the sensorial characteristics. A study reported that the rate of lipid oxidation is different among different sections of the fillet (Flick and others 1992). Lipid does not accumulate evenly throughout the fillet and different lipid sources can influence the location of lipid deposition in the fillet (Freeman and Hearnsberger 1994). Fatty acid composition has an indirect relationship with flavor of the product as concentration of flavor compounds can be determined by the lipid content in the product. Therefore, uneven deposition of lipids can ultimately affect the overall flavor characteristics depending on the location of the fillet. In high lipid fish such as Atlantic salmon, researchers saw differences in preferences in fillet containing different levels of lipid with increasing preferences to higher lipid concentration (Robb and others 2002).

Sensory characteristics of fish fillet are imparted by the fish feed, water quality, and fillet composition. Therefore, manipulation of feeds have direct impact on the composition of fillet. A study comparing the effect of herring fish oil diet with sunflower oil diet on sensory characteristics saw significant differences in aroma, and the panelists tasted more of fishy taste in fish that were fed herring oil diet than the sunflower oil diet, although the preference was indifferent. In comparison, in rainbow trout, there were significant differences in aroma as well as taste, and the

panelists preferred sunflower oil diet fed fish fillet compared to the herring oil diet (Skonberg and others 1993). Oil sources in diets had different fatty acids that results in different rate of lipid autoxidation (Kim and Min 2008a). Also a study looking at the sensory characteristics of refrigerated tilapia fillet saw positive relationship between sensory deterioration and storage time (Rong and others 2009). A different study looking at the long term frozen storage of tilapia saw no significant quality deterioration compared to the fresh tilapia in -18 ^{o}C storage over 8 months (Tokur and others 2004). Sensory characteristics then can be affected greatly by the condition and the length of the storage. However, there is another study that looked at the sensory characteristics comparing the sensory quality of spirulina fed tilapia sashimi to commercial diet fed tilapia sashimi saw no significant differences in sensory quality (Lu and Takeuchi 2002). A study looking at two different species of tilapia with same given diet saw significant difference in consumer preference, and reports that flavor is minimally impacted by the composition of the diet but rather by the metabolism of fatty acids (Garduño-Lugo and others 2007).

Change in food composition can cause change in flavor. Also tilapia is naturally a low-fat product with 1-3% fat (w/w) (USDA 2015). Therefore, the effect of lipid on flavor and aroma is small compared to other seafood such as salmon, mackerel, or tuna. Also because of the low fat content of tilapia, change in dietary lipid contents have little effect on the physico-chemical characteristics of the fillet such as texture and water holding capacity (Ng and Bahurmiz 2009) . Lipid can indirectly affect the flavor compounds by partitioning the affected compounds into different phases (McClements and Decker 2008). A study observed more off-flavor characteristics in tench, *Tinca tinca*, fed higher *n*-6 fatty acid diets using soybean oil compared linseed oil based diets (Turchini and others 2007). In a similar study comparing commercial diet to spirulina based diet fed tilapia in a RAS, the study saw differences in protein and lipid but no sensorial differences

in sashimi (Lu and Takeuchi 2002). However, beyond aroma and flavor, concentration and the molecule size of lipids also played important role in providing a positive mouth-feel of food products from reduced gritty and grainy texture and improved smoothness (McClements and Decker 2008). Overall, there is little research done on the area in sensory impact of dietary lipids in tilapia fillets.

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Chapter 3. Evaluation of Lipid Quality and Fatty Acid Composition of Tilapia, *Oreochromis* spp., Fillets Available in US Supermarkets

Abstract

Tilapia (*Orechromis* spp.) aquaculture is one of the fastest growing segment in the seafood industry, and shows strong growth trends. Tilapia is lean, low fat fish with minimal fishy-odor associated with fatty fish. Also, many researchers have observed a direct relationship between the nutritional composition of diets and tilapia fillet composition. Recently, tilapia have been criticized for having fatty acid compositions "worse than bacon." The objective of this research was to characterize the fatty acid compositions (FAC) in tilapia fillets in supermarkets in various regions of USA. Duplicate samples of tilapia products were collected from Florida (n=9), Texas (n=9), and Virginia (n=10). The evaluated tilapia samples included fillet and whole fish, frozen or fresh, and originated from 12 countries. Total lipid extractions were conducted using a modified Bligh and Dyer method. GC/MS was used to analyze the FAC using the AOCS method. The samples were analyzed by regions of origins: China, Southeast Asia, Central America, South America, and USA. Total lipids were 1.95, 2.88, 2.75, 2.06, and 1.11 g/100 g fillet; *n*-6:*n*-3 ratios of 4.27, 6.55, 4.89, 5.30, and 1.30; docosahexaenoic acids were 16.41, 10.44 16.01, 16.74, and 40.77 mg/g oil; and arachidonic acids were 13.46, 10.39, 10.44, 15.47, and 19.12 mg/g oil respectively. The effect of storage conditions (-10 ^{o}C and -20^{o}C) FAC was not statistically significant. Although there was wide variation in FAC in commercial tilapia fillets examined, FAC indicated that the tilapia is a healthy diet choice for the consumers.

KEYWORDS: Tilapia, polyunsaturated, arachidonic, *n*-3, *n*-6

Introduction

The aquaculture industry for the past decade has been increasing at an exponential rate in parallel to the increase in fish consumption and growth in human population (FAO 2014). Not only that, the constant increase in supplies from fish such as carps, salmon, shrimps, bivalve, catfish and tilapias contributed to the success of aquaculture industry (FAO 2016b). Aquaculture has become an important part of the global food supply due to the stagnation of wild capture fisheries industry over the last decade at approximately 90 million metric tons (FAO 2014). Improvement in aquaculture technologies and knowledge have played important roles in the growth of the aquaculture industry. In previous decades, tilapia has been a staple source of diet limited to African countries; but now tilapia has become one of major globally aquacultured fish at 3.2 million metric tons in 2012 (FAO 2014). In US retail sector, tilapia supplies have been constant due to continual fresh, unfrozen tilapia products from Central America while Asia was able to provide frozen tilapia products (FAO 2016b). Tilapia's ability to grow in various conditions, resistance to diseases, and fast growth contribute to the explosion of tilapia aquaculture.

Fish contains highly regarded nutrients such as long chain *n*-3 polyunsaturated fatty acids (LCn3), specifically, EPA (20:5*n*-3), DPA (22:5*n*-3), and DHA (22:6*n*-3). Long term consumption of LCn3 such as EPA, and DHA lower the risks of coronary heart disease (Durrington and others 2001; Harris and von Schacky 2004). Subjects were scaled on *n*-3 index, defined as content of EPA and DHA in red blood cell membranes, which were inversely related to coronary heart disease, had greatest cardio-protection at 8% (Hu and others 2002). It was also found that DHA was related to vision function in early ages by affecting the proper composition of retina phospholipids (Uauy and others 1992). As brain is composed of structural lipid at 60%, mostly ARA (20:4*n*-6) and DHA, DHA plays important roles in function and development of brain at early stages of growth

(Crawford 1993). Therefore, it was highly recommended and nutritionally important for people of all ages to consume proper levels of LCn3. Increasing consumption of seafood is therefore highly recommended. However, aquaculture and fish industry have recently seen a set back from negative image portrayed by the media and online health bloggers. Commonly believed ideas include high antibiotic uses, sewage like water quality, or lack of regulation resulting low quality of imported products to US markets. Although the aquaculture industry is not without its issues, it is important not to spread unreliable information, which can be highly detrimental to the industry and consumers. Especially, tilapia have been receiving negative media attention for being a freshwater fish and for having a lean fillet with low DHA and EPA levels.

Essential fatty acids (EFA) are extremely important to humans because EFAs are critical in development and growth during early stages of human life (Innis 2007; Innis and Friesen 2008). Humans readily convert 18 carbon fatty acids (FA) into *n*-6 polyunsaturated fatty acids (PUFA), but have limited ability to convert n-3 FAs to LCn3 such as EPA, DPA, and DHA. The activities of desaturase enzymes are dependent on the concentration of their products; therefore, with high levels of *n*-6 FAs, there is a decrease in activities for further biochemical conversion to DHA and EPA (Watkins and others 2008). Because humans already consume high levels of long chain *n*-6 FAs, competitive inhibition can be observed for LCn3 (Dumm and Brenner 1975; Simopoulos 2002). Therefore, although humans have minor ability to convert linolenic acids into EPA, DPA, or DHA, it still is critical for consumption of sufficient level of LCn3 to make up for the lack of biochemical conversion of linolenic acids.

By understanding current state of lipid quality in tilapia, the tilapia industry can better create targeted marketing by answering producers and consumers needs simultaneously. The objective of this research was to identify the current state of FAC of various tilapia fillet obtained

in the US supermarkets. Specifically, concentration of LCn3 levels and *n*-6 fatty acid such as ARA in tilapia fillet lipids were analyzed. The consumers are highly concerned with the current tilapia fillet quality and its aquaculture practices. Wrongful fear have resulted in the stagnation in tilapia consumption in recent years. It is thus important to provide consumers with scientific evidence to combat the concerns. Although tilapias are omnivorous fish with dietary need for *n*-6 fatty acids, overall lipid quality of tilapia fillet will be healthy options for the consumers due to overall balance of *n*-6 to *n*-3 fatty acid ratio. The proposed study will establish the lipid quality and FAC of tilapia fillet consumers are exposed to. Thus the study will provide greater insight of current state of impact of tilapia fillet on human health benefits that is currently ambiguous. The results will provide value information that can be used by the consumers to make better buying decisions and the producers to utilize the data to help with tilapia feed decisions.

Material and Methods

Samples

Tilapia samples were collected from multiple supermarkets in various regions of USA. Collected samples were frozen or fresh, and whole or filleted. Total of 10 samples were collected from southwest Virginia, 9 samples were collected from central Florida, and 9 samples were collected from central Texas. Same products from different regions were not used. All samples were duplicated at package level with total sample size of N = 56. The samples that were collected from fresh fish section and freezers in the supermarket and fresh market were duplicated at fish or fillet level. Products that were seasoned or breaded were not sampled for the analysis due to its inability to accurately identify true fillet lipid composition. All samples were transported to the lab in Styrofoam boxes with frozen icepacks then were quickly stored in cold storage at -20^{o}C until analysis.

Sample Preparation and Total Lipid Extraction

The total lipid extraction method was adapted from Bligh and Dyer (1959) (Christie 1982). Whole/gutted fish were thawed overnight in refrigerator at 2^{o}C prior to being manually filleted and cut into small chunks. Fillet products was taken out of the -20^{o}C and thawed over the counter for 2 hours then cut to small cubes. One hundred grams of fillets were weighed then placed into Waring Blender jars. Chloroform (100 mL, HPLC grade; 0.75% ethanol as preservative; Fisher Scientific, Fair Lawn, NJ) and methanol (200 mL, HPLC grade; Fisher Scientific, Fair Lawn, NJ) were added to the jar then homogenized for 4 min. The mixture was vacuum filtered (Whatman No.4; Buckinghamshire, UK) into a flask. An additional 100 mL of chloroform was added to wash the blender jar for remaining samples and then filtered. The mixture was then transferred to a

separatory funnel. Then 100 mL of 0.88% KCl (aq.) solution was added to the separatory funnel. After clear bilayer was observed, the chloroform layer was collected. Several grams of anhydrous sodium sulfate (certified ACS; Fisher Scientific, Fair Lawn, NJ) was added to remove water and samples were left for 40 minutes. The chloroform layer was filtered (Whatman No.1; Buckinghamshire, UK). The solvents were completely evaporated using a Rotavapor (Evapotec, Haake Buchler Instrument, Saddle Brook, NJ) at 50^{o}C and traces of solvent removed using vacuum with slight heating. Weighed oil extracts were transferred into clean centrifuge vial then nitrogen flushed. The extracted oil was then stored in dark -20^{o}C cold storage until further analysis.

Preparation of Methyl Esters of Fatty Acids (FAME)

The FAME method was adapted from AOCS (2009). For internal standard (IS), 25 mg of tricosanoic methyl ester (23:0; Crescent Chemical Co., Germany) was added to 25 mL volumetric flask that was made up to volume with isooctane (HPLC grade; Fisher Scientific, Fair Lawn, NJ). Then 1.0 mL of IS was added into the clean centrifuge vials, and isooctane was evaporated using nitrogen gas. Extracted oil samples (25.0 mg) were then weighed into centrifuge vials containing 1 mg of IS. Then, 1.5 mL of 0.5 M NaOH (certified ACS; Fisher Scientific, Fair Lawn, NJ) in methanol solution was added, then blanketed with nitrogen gas, capped, vortexed then heated at 100^{o}C for 5 mins. Once the centrifuge vials were cooled to touch, 2 mL 12% BF_3/methanol reagent (1.5 M; Acros Organic, Pittsburg, PA) was added. Centrifuge vials were then blanketed with nitrogen gas, capped, vortexed and heated at 100^{o}C for 30 minutes. Centrifuge vials were then taken off the heat and cooled to 30-40^{o}C. One mL of isooctane was added then blanketed with nitrogen gas, capped, and vortexed for 30 sec. Five mL of saturated NaCl aqueous solution was added immediately and then blanketed with nitrogen, capped, and vortexed. Centrifuged vials were

left to cool and until isooctane layer separated. Isooctane layer was then transferred to a clean centrifuge vial, then blanketed with nitrogen and capped. Water and methanol phases were extracted again with additional 1 mL of isooctane. Isooctane layer was then combined with previous extract then concentrated to approximately 1 mL using stream of dry nitrogen gas, then 500 µL was transferred into a vial with Teflon-lined silicon septum for gas chromatography-mass spectrometry (GC/MS) analysis.

Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

GC/MS (Shimadzu, GC-2010/MS TQ-8030) was used to separate and identify FMAE from extracted oil samples. An autosampler (AOC-20i+s) was used in conjunction with GC/MS with a polar column (Zebron, ZB-Wax plus, 60 m, 0.25 mm i.d., and 0.25µm film thickness). The helium (ultra-high purity, 99.999%) was used as the carrier gas at total flow rate of 63.5 mL/min, column flow rate of 1.22 mL/min, linear velocity of 30.0 cm/sec, and split ratio of 50:1. The GC inject port temperature was set to 250 ^{o}C. The temperature program of oven had 175 ^{o}C initial temperature with 5 min hold time, program rate at 2.0 ^{o}C/min, and 225^{o}C final temperature with 30 min final hold time. GC column MS temperature parameters were set to ion source temperature at 230^{o}C and interface temp at 200^{o}C. The MS scan acquisition mode was set from 40 m/z to 400 m/z with scan speed of 1250. The total GC/MS run time was 60 minutes. Total of 1.0 µL of each FAME samples were injected. The chromatography was then analyzed using equivalent chain length (ECL) as well as using library comparison to the mass spectra of FA.

Statistical Analysis

Statistical analysis was carried out with JMP Pro 11.0 (©2013 SAS Institute Inc., Cary, NC). All samples were duplicated. Total lipids per 100 grams of fillet, FA compositions were

quantified as % oil and analyzed for significance. PUFAs were quantified as mg/g oil using IS, and analyzed for significance. The sampling was total convenient sampling limited by the availability around the region at the time of purchase in late 2015. Analysis of variance (ANOVA) at $\alpha = 0.05$ was used to evaluate the significance of the data. Multiple comparison was carried out using Tukey's HSD to identify significant samples at p-value $\alpha = 0.05$.

Results and Discussion

Market Evaluation

Tilapia is now a common fish product that can be found in grocery stores around the US as fresh fillets, live fish, frozen fillets, and frozen fish. Of the samples we obtained, none were identified as wild caught. This is to be expected as they are native to rivers and lakes in Africa, and no real wild catch operation can sustain global demands. This was reflected in the supermarket samples. Most tilapia products were processed foods: 11% were sold gutted and as whole fish with bones and head compared 89% of filleted products. Also, 39% were sold as fresh product compared to 61% of frozen products. There were no live fish obtained for this study; however, live tilapia products can be obtained in fish retail departments in international markets and in metropolitan areas. Also, this research was limited to southwest Virginia, central Florida, and central Texas as well as limited with the sample size (n=28) that does not truly represent the entire US supermarket. However, samples were collected from the urban and rural areas of three different regions of USA – mid-Atlantic, Southeast, and Southwest. So although, it does not have comprehensive representation of US tilapia products, the samples do represent the diverse area. Also although, metropolitan cities such as New York or LA is not represented, the supermarkets sampled include Kroger (largest grocery chain by retail sales), Albertsons, Publix, Walmart and 8 other supermarket chains that are located throughout the USA (Statista, 2015).

The following countries of origin (COOs) were identified: China, USA, Ecuador, Honduras, Peru, Colombia, Costa Rica, Mexico, Taiwan, Vietnam, Indonesia, and Thailand. The top ten tilapia-producing countries in 2014, by quantity (per ton), were China, Indonesia, Egypt, Bangladesh, Vietnam, Thailand, Philippines, Taiwan, Colombia, and Mexico (FAO 2016a). It is

interesting that samples from Bangladesh and Egypt were not found. To better analyze the given information, the country of origins were grouped into the COOs: USA, China, Central America (Mexico, Costa Rica, Honduras), South America (Colombia, Peru, and Ecuador), and Southeast Asia (Vietnam, Indonesia, Taiwan, and Thailand). The most samples evaluated were from China at 43%, which by ratio, was similar to the global tilapia production. Chinese tilapia production in 2013 was 1.6 million metric tons out of 3.2 million metric tons of global tilapia production (Globefish, 2015). Compared to China, 18% of tilapia products were from Southeast Asia, 32% from Central and South America, and 7% were from within the USA.

Thirty-two percent had certification (Best Aquaculture Practice, Guiding Stars, and/or HACCP) labeled on its packaging. Most likely explanation is to gain trust from the consumers of tilapia quality. Required by Food and Drug Administration (FDA), all seafood products must be processed in HACCP certified processing plants to ensure the safe handling and sanitary processing of the seafood products. Therefore, all seafood products are HACCP certified, but because the consumers may not be aware of it, companies placed HACCP labels on its packages to promote their products. Much like HACCP, Best Aquaculture Practice (BAP) certification is a third party program administered by the nonprofit organization Global Aquaculture Alliance to promote worker safety, social responsibility, and sustainable practices in aquaculture industry. Lastly, guiding star is designed to help consumers make easy choices when making product purchasing decisions based on nutritional quality of the particular product.

The average price of tilapia products was 1.64 USD per 100 g of product weight. Prices per 100 g tilapia products by purchase area were 1.28 ± 0.19 USD in southwest Virginia, 1.83 ± 0.20 USD in central Florida, and 1.82 ± 0.17 USD in central Texas. The differences in prices from

region to region can be attributed to the fluctuation determined by the farm gate value, location (metropolitan city, small town, or rural) and the supermarket characteristics. However, tilapia have been observed to have low price volatility, especially compared to other seafood products. This is due to the constant strong supply and increases in production. From 2014 to 2015, fresh and frozen tilapia imports increased by 1.4% and 27% by quantities, respectively (FAO 2015).

Total Lipid Contents

Tilapia is popular as a lean fish. It is high in protein and low in fat. USDA's standard of identity defines raw tilapia fillet as containing at least 1.70% (w/w) lipids (USDA 2015). Of all samples that were analyzed, the average total lipid contents of tilapia fillets from whole and filleted products were 2.21 ±1.08% (w/w). Because there is no standard operation in place for tilapia aquaculture, the high variability resulted from different COOs was interesting. The fish, even if they were from same farm, can have differences in fat deposition in fillet from fish to fish. Improving the number of samples analyzed could provide a stronger statistical power, yet the current study still presents valuable information about the current market state. It also provides insight as to the types of tilapia diets in the farms around the world. Many researchers looked at the impact of the diet on fish, and proved that the quality of the diets have a direct impact on the quality of tilapia fillets (Justi and others 2003; Zenebe and others 1998; Steffens 1997).

Wide range of crude lipid contents was observed when the samples were analyzed based on its ROO. The total lipids of fillet based on ROO were statistically significant with p-value of 0.014. The total lipids were highest in fish from Southeast Asia with an average of 2.87% (w/w) and lowest in USA with an average of 1.10% (w/w) (Table 2). This is another indication that different parts of the world utilize different sources of ingredient as tilapia diets. As Chou and

Shiau (1996) indicated the different levels of lipid in tilapia diets can result in different levels of lipids in fillets.

Fatty Acid Composition

The FAC of a specific fish is a result of the diet quality . This is due to the direct relationship between the fatty acid compositions of diet lipids and the fatty acid composition in the fish muscle tissues. The composition of fatty acids varied widely from fillet to fillet (Table 1). Trace levels of various fatty acids were also obtained but most were observed at insignificant ($p<0.05$). These included 13:0, 18:1n-5, 20:3n-9 and others. These compounds were found at lo concentration of FAC (<0.1%) or did not have good resolution to identify with confidence. The identification of compounds was difficult as most papers do not normally identify all compounds and rather are selective. For these samples, each individual peaks' mass spectra were compared to the library data, and we also compared equivalent chain length using peanut oil FAME under isothermal GC/MS conditions. The FAC in the tilapia fillet lipids were 33.53 ± 2.90 % SFA, 28.71 ± 4.14% MUFA, 24.93 ± 3.22 % *n*-6 PUFA, and 6.68 ± 2.43 % *n*-3 PUFA for all samples.

Interestingly, SFAs were found on average at 33.61% of oil in all samples and constant regardless of COOs, processing, or storage conditions (Table 2). This means that regardless of the diets or conditions of aquaculture practices, all tilapia yielded similar SFA concentrations. However, other fatty acid groups, MUFA, *n*-3 PUFAs, and *n*-6 PUFAs were significantly different between the COOs. The most significant pattern was observed in fish from Southeast Asia to USA samples as differences in MUFA concentrations (38.6 and 20.1% w/w) were opposite to the differences in *n*-3 PUFA concentrations (3.9 and 17.3% w/w). Some researchers reported that both *n*-3 and *n*-6 fatty acids are required for best growth performance (El-Sayed and others 2005; Chou

and Shiau 1999). El-Sayed (2006) suggested that essential fatty acids were therefore species-specific (El-Sayed 2006). Our data also support the idea that there is a wide variation in *n*-3 fatty acids and even some fillets were found to have high *n*-3 PUFA concentration at healthy level for human consumption (El-Sayed 2006).

Many health advocates forget a very important factor that not all *n*-3 fatty acids have same bioactivities in human body, and therefore, not all *n*-3 fatty acids are of equal importance. This is a key importance because 18:3*n*-3, α-linolenic acid, does not have same health impact as EPA, DPA, or DHA. This is because of the poor conversion of 18:3*n*-3 to LCn3 (Chavan 2015). Many researchers have attempted to improve overall *n*-3 PUFA concentrations in tilapia fillets through increasing 18:3*n*-3, with minor improvement in LCn3, such as EPA, DPA, and DHA (de Souza and others 2008; Visentainer and others 2005; de Souza and others 2007). This makes little sense from a nutritional stand point. Therefore, the long chain PUFAs, specifically ARA, EPA, DPA, and DHA were analyzed separately (Figure 2). Surprisingly, when looking at the long chain PUFAs, the ARA ratio to EPA, DPA, and DHA (ARA:LCn3) ranged from 0.33 to 1.17. Considering western diets have 20-25:1 *n*-6:*n*-3 ratios, tilapia can be considered healthy substitute to most protein sources (Simopoulos 1999).

The research also evaluated the impact of the frozen fillets compared to the fresh fillets on FAC. A statistical analysis determined that SFA (mg/g), MUFA (mg/g), *n*-6 PUFA (mg/g), *n*-3 PUFA (mg/g), and *n*-6:*n*-3 ratios were not different. This analysis provided an insight that the lipid quality was not affected by the storage conditions.

The major goal of our research was to identify the actual lipid contents and FAC that US consumers are obtaining from consumption of tilapia. A study conducted in 2008 reported a

potentially alarming FAC of tilapia products in US (Weaver and others 2008). Weaver and others (2008) evaluated various species of fish and grouped them into 3 categories based on the EPA and DHA levels (>500 mg, between 150 to 500 mg, and <150 mg per 100 g fish), and categorized farmed tilapia in category 2 (150 to 500 mg/100 g fish). Weaver and others (2008) reported an average of 135 mg/100 g fillet of ARA with some samples having >300 mg/100 g fillet. Our results did not agree. From multiple sales locales, COOs, and processing and storage conditions, we did not observe ARA concentration higher than 4.04% (w/w) of the fatty acids in oil. The ARA on mg/g oil basis only ranged from 7.63 to 29.9 mg/g oil in all samples. However, our results were similar to that of another study conducted in Poland that found 2.0 ± 0.6% lipid while our samples had 2.21 ± 1.00% lipid (Usydus and others 2011). The same study also found *n*-6:*n*-3 ratio of 2.0 while our study found *n*-6:*n*-3 ratios ranged from 1.30 to 6.55, depending on the COOs (Usydus and others 2011).

The study observed 4.2 ±3.4% of total lipid as LCn3. However, compared to other seafood, tilapia contains very little LCn3. According USDA Nutrient Data Laboratory, LCn3 (excluding DPA) in edible portion of fish is 0.9-1.83 g/g oil for Atlantic salmon, 0.98 -1.70 g/g oil for sardines, 0.84-0.98 g/g oil for rainbow trout, 0.34-1.57 g/g oil for mackerel, 0.24-1.28 g/g oil for tuna, and 0.15-0.2 g/g oil for catfish (Kris-Etherton and others 2002). Our study reported LCn3 (including DPA) of 0.42 ±0.0.34 g/g oil in tilapia fillet. Considering tilapia's lipid content is much lower than other fish, overall tilapia had high quality lipid in its fillets.

Conclusions

This research confirms the variation in total lipid content in commercial tilapia fillets, and the wide variability within the fatty acid profile of tilapia fillets. Interestingly, there was no

differences between fresh fillets and frozen/packaged fillets' fatty acid profiles suggesting the lipid quality is not compromised. This research also identified the small variation in lipid quality among different COOs and found that the tilapia products from the USA had the highest levels of LCn3 PUFA compared to other COOs. USA products were leaner with 80 mg/serving of LCn3 compared to 61 mg/serving, 45 mg/serving, 44 mg/serving, and 36 mg/serving in Central American, Chinese, South American, and Southeast Asian products, respectively. This research shows that overall tilapia fillets contain a healthy ratio of *n*-6 to *n*-3 FA. Also, as tilapia are inexpensive aquaculture products, there is a room to expand the "value-added" segments, including improved fillet quality through increased *n*-3 PUFA.

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Chapter 4. Impact of Enhanced Long Chain n-3 PUFA and Oleic Acid Diets in Tilapia Fillets

Abstract

The tilapia aquaculture industry has had sensational growth globally over the past 40 years. However, growth has slowed in recent years due to consumer misconceptions of tilapia's health benefits. In the 21st century where consumers demand clean labeling, less processing and wholesome products, the tilapia industry has not been able to satisfy consumers' changing demands. In order to adapt to the change in consumer behaviors and improve product quality of tilapia, research was conducted to create value-added premium tilapia through improved diets. Tilapia were raised in a recirculating aquaculture system over 8 weeks with pelleted diets. Total of 11 Diets were created iso-nitrogenous and iso-caloric, including 1 commercial diet (CD) used as a reference. All formulated diets shared same major ingredients. Oil mixture was added to all diets at 5% (w/w) in mixture of fish oil (FO), algae oil (AO), high-oleic sunflower oil (SFO), and soybean oil (SO). A diet had addition of 0.02% (w/w) α-tocopherol. Aquaculture parameters, growth performance, fillet lipid and fatty acid compositions were analyzed along with sensory analysis. Tilapias that were fed high AO diet contained high levels of DHA while tilapia that were fed high FO contained high levels of EPA and DPA. SFO based diets were able to improve tilapia fillet oleic acid contents by 50% compared to CD. The most favorable long chain *n*-3 fatty acid to arachidonic acid ratio was obtained from tilapia that were fed high AO and low SO mixed diet and high FO and low SO mixed diet. There were no differences (p-value > 0.05) in sensory preference or fillet texture analysis of high FO and low SO diet and high FO and low SFO diet fed tilapia. Thus texture and the preference were not affected by the type of lipid in tilapia diets. The surveyed participants also indicated a willingness to pay up to 30% additional in final purchase price for a

premium tilapia fillet. Therefore, customization of lipid content in the diet will be able to create value added premium tilapia.

KEYWORDS: Tilapia, polyunsaturated, *n*-3, *n*-6, DHA, oleic

Introduction

At 3.2 million metric ton in 2012, growth in aquaculture production has been exponential since the 1960s (FAO 2014). Since the birth of industrial aquaculture practices, tilapia industry has become an important part of the global food chain, and its importance has been increasing. Improvements in technologies have helped push aquaculture production beyond wild capture fisheries (FAO 2014). However, just like other food industries, aquaculture has seen a setback due to a negative image portrayed by the media, such as contamination of water sources, antibiotics use, unsustainable practices, or repulsion to certain feed ingredients. This has been a cause of stagnation of tilapia consumption in recent years (Fitzsimmons 2017). Negative images of tilapia are mostly drawn up and recycled talking points that are not only inflammatory, but scientifically disproven (Young 2009). In order to combat negatively portrayed images, the tilapia industry is employing new technologies and installing new certification programs to ensure high quality products that consumers can trust. However, the negative image portrayed online is still a recurring problem that doesn't seem to go away.

The increase in easy access to vast amounts of information has had beneficial but also had negative impact on the consumers (Morahan-Martin 2004). Improper or insufficient internet search practices by the consumers often leads to contradicting or scientifically unproven sources of information. In the 21st century, availability of misinformation has become a big problem, where consumers become unable to discern scientifically correct information and misinformation (Neumann 2003). New technologies and availability of information have also led to a huge change in consumer buying patterns. People are interested in transparency in product ingredients, less-processed foods, and sustainable practices by the food industry (Topper 2015). Another big increase in consumption has been seen in the "premium" product market, as more and more people

are willing to pay premium price for premium quality (Loureiro and McCluskey 2000; IRI 2016). The increase in the premium market is observed in organic foods, where choices of organic foods with premium price tags had grown around the world (Jonas and Roosen 2005).

Tilapia is high quality protein fish that is relatively inexpensive. Tilapia fetched 4.03 USD/kg for frozen products in December of 2015 in the US (FIS 2017). Compared to other seafood products that are already considered premium, tilapia has a price flexibility in its ability to enter into the premium market. According to the USDA standard reference, tilapia already has a high quality amino acid composition and 20% (w/w) protein content with low 1.7% (w/w) lipid content (USDA 2015). However, as tilapia is naturally an herbivore/omnivore, tilapia perform well on low-tropic level feeds (El-Sayed 2006). This was evident in studies that showed *n*-6 fatty acid as an essential fatty acid in tilapia diets (Teshima and others 1982). However, it has also been seen that dietary fatty acid composition can impact the fillet fatty acid composition (Olsen and others 1990; Visentainer and others 2005). Improvement in diets has been shown to improve tilapia fillet quality. However, many have tried to improve tilapia using agricultural based ingredients, which are often high in α-linolenic acid (ALA; 18:3*n*-3). Because 18:3*n*-3 and linoleic (LA; 18:2*n*-6) acids share biochemical pathways, there is competitive inhibition of their metabolism (Sprecher and others 1999; Kidd 2007). Not only that, the production of 20- and 22- chain fatty acids (such as eicosapentaenoic acid (EPA; 20:5*n*-3), docosapentaenoic acid (DPA; 22:5*n*-3)) and docosahexaenoic acid (DHA; 22:6*n*-3) are concentration dependent (Gibson and others 2011). This means that in order for humans to see the benefits from the long chain *n*-3 fatty acids (LCn3), humans either have to consume high concentration of 18:3*n*-3 fatty acids along with low 18:2*n*-6 or consume EPA, DPA, and DHA directly. Also oleic acid, (OA; 18:1*n*-9) has been known to reduce LDL cholesterol and, by replacing LA, can have a beneficial effect on the human body

(Mattson and Grundy 1985; Ip 1997). In addition, OA has better oxidative stability compared to polyunsaturated fatty acids (PUFA) (Mattson and Grundy 1985).

Tilapia are inexpensive seafood widely available to consumers in variety of forms from frozen to fresh fillets and ready-to-eat to live products. However, the high levels of *n*-6 fatty acids in tilapia concerns many consumers. Thus, improving LCn3 can create value-added tilapia health benefits. This research was designed to evaluate the lipid content and the fatty acid composition in nutritionally enhanced diet fed tilapia fillet. Diet strategies were developed with mixture of high quality lipid source such as fish oil, algae oil, high-oleic sunflower oil compared to soybean oil based diet to determine optimal diet for the tilapia fillets to contain high levels of *n*-9 and *n*-3 fatty acids while minimizing *n*-6 fatty acids. The studies were conducted to determine the impact of enhanced dietary strategies in fish growth performance, fillet lipid quality, and sensorial characteristics.

Methods

Diet Formulation

There were 11 treatment diets and 1 commercial diet (Production 35, 4.0 mm, Rangen, Buhl, ID) used in our research. Each of 12 diets were given randomized two digits and ranked from low to high. Then 12 tanks in block 1 (RAS 1 and 2) and block 2 (RAS 3 and 4) tanks were distributed random numbers. Tanks were then ordered from low to high and assigned to the diets with same rank to completely randomize to minimize tank to tank variabilities (Table 9). Diet formulation included soybean meal (SM; Hi-Pro Solvent Extracted Soybean Meal, ADM Alliance Nutrition Inc., Quincy, IL), wheat (WT; Quality Feed, Southern States, Richmond, VA), meat and bone meal (MBM; Smithfield Farmland, Smithfield, VA), fish meal (FM; Special Select, Omega Protein, Houston, TX), various oil sources, vitamin premix (VP; TestDiet, St. Louis, MO), mineral premix (MP; TestDiet, St. Louis, MO), mixed tocopherol (TOC; Sigma-Aldrich Inc., Milwaukee, WI), L(+)-ascorbyl palmitate (AP; Acros Organics, Fair Lawn, NJ), and carboxymethyl cellulose (CMC; CP Kelco, Atlanta, GA).

The diets were created to maximize the growth and food conversion ratio (Table 4). The feeds were formulated as iso-nitrogenous at 35% (w/w) and iso-lipid at 7% (w/w). Commercial diet (CD, negative control) was added to the experiment to compare the efficiency of formulated diets to a commercially available feed. Positive controls were formulated using soybean oil (SO; 100% Pure vegetable Oil, Admiration, Englewood, NJ) at 100% of added oil (SO100) and another using high-oleic sunflower oil (HOSFO; Sunflower Oil – High Oleic, JEdwards International, Inc., Braintree, MA) at 100% of added oil (SFO100). Different oil sources were used to enrich LCn3 with base oil of either SO or HOSFO. HOSFO was used to evaluate the effect of replacing high

levels of *n*-6 PUFA in SO with *n*-9 fatty acids. Fish oil (FO; Virginia Prime Gold, Omega Protein, Houston, TX) and algae oil (AO; Schizochytrium DHA-Rich Algal Oil, Source-Omega, LLC, Mason, OH) were used to enrich omega 3 PUFA in the feeds. SFOAO15 diet was made in ratio of 85:15 HO-SFO:AO; SFOAO85 diet was made in ratio of 15:85 HO-SFO:AO; SFOFO15 diet was made in ratio of 85:15 HO-SFO:AO; SFOFO85 diet was made in ratio of 15:85 HO-SFO:FO; SOAO15 diet was made in ratio of 85:15 SO:AO; SOAO85 diet was made in ratio of 15:85 SO:AO; SOFO15 diet was made in ratio of 85:15 SO:FO; SOFO85 diet was made in ratio of 15:85 SO:AO; and lastly HT diet was made with oil ratio of 15:85 HO-SFO:FO with addition of *dl*-α-tocopheryl acetate (Sigma-Aldrich Inc., Milwaukee, WI) at 0.02% to the total diet. The oil mixtures were made up of 5% of total diet (w/w), but the final formulation had higher lipid content due to oils from different raw ingredients.

In total, 55 kg of pelleted feed was created per diets. For each batch, dry ingredients (SM, WT, MBM, FM, VP, MP, and CMC) were weighed and added into a bucket. The ingredients were then mixed using V-mixer for 5 minutes. Then the mixed ingredients were moved into a planetary mixer. Another 5 kg batch of dry ingredients were processed and added to planetary mixer. Then liquid ingredients (AP, TOC, and oil mixture) for 10 kg batch were poured into the planetary mixer. Lastly, approximately 800 mL of water was slowly added and then mixed for additional 5 minutes. Then the 55 kg of mixture was processed through a flat pellet mill with 4 mm diameter (72A, 10 HP, Lawson Mills Biomass Solution, Hartsville, PE, Canada) and dried over the counter overnight. The pelleted diets were then bagged and stored in -20 ^{o}C cold storage.

Tilapia Aquaculture

The tilapia aquaculture and the following analysis were approved by the Institutional Animal Care and Use Committee prior to the start of the growth trial (Appendix D). Genetically Improved Farmed Tilapia, *Oreochromis* sp. (GIFT and sex reserved, Spring Genetics, Akvaforsk Genetics Center, Miami, FL), were fed a commercial diet (Bronze floating, 3.0 mm, Ziegler Inc., Gardners, PA) until a weight of 206.1 ± 6.1 grams per fish. Then, each tank was assigned 1 of 12 diets, in duplicate, for a total of 24 tanks, and fish were fed for 18 hours daily using automatic belt feeder (Aquatic Eco-system^{TM}, Pentair, Apopka, FL). The entire feeding trial continued for 8 weeks with final weight of 557.7 ± 37.6 grams per fish. A total of 25 tilapia were added to each tanks with the final fish density per tank to below 60 kg/m^3. Each week, tilapia were weighed per tank and feeds were allocated by changing percent body weight per day (%BW/d), adjusted weekly based on the weight gain (Table 1). Food conversion ratio, mortality, morbidity, and other behavior and physical signs were recorded.

Once the fish reached marketable size at the end of week 8, fish were individually euthanized with buffered MS-222 (200 mg/L water; Tricaine-S, Western Chemical, Inc., Ferndale, WA) then euthanasia confirmed by cervical dislocation. Fillets from 2 tilapia were harvested per tank in order to ensure 100.0 g of fillet for lipid extraction. Tilapia fillets from both sides were collected in the vacuum packs (3 mm standard barrier, nylon/PE, Prime Source Vacuum Pouches, USA) and nitrogen flushed. The packs were then vacuum sealed by vacuum sealer (Multivac A300/16, Multivac Inc., Kansas City, MO), and flash frozen using dry ice and iso-propanol. All fillets were stored at -20^{o}C until further analysis.

The samples for sensory evaluation were collected separately as MS-222 is not allowed for human consumption. RAS were purged for 2 days with 100% water replacement over 2 days with reduction of feed to 1% BW/d. The purge technique was used to minimize off-flavor compounds in fillets that could interfere with sensory evaluation. SOFO85 and SFOFO85 fed tilapia were used for the sensory evaluation. Total of 8 fish per diets (4 fish per tanks) were stunned with ice water then euthanized by cervical dislocation. Tilapia were then filleted on both sides. Fillets were put in zip-lock bags and placed on ice until cooked on the same day.

Recirculating Aquaculture System Parameters

The RAS were used to culture the tilapia for the research with the water exchange rate of approximately 3.25 L/hr. The Municipal water source, containing chloramines, was filtered using activated carbon filter. There were 4 independent RAS with sand filter (S180T, Pro Series, Hayward, Rockville, MD), bead filter (BBF8000 Aquaculture Systems Technologies, New Orleans, LA), UV filter (Smart UV Sterilizer, 02130, 130 Watt, Emperor Aquatics, Inc., Pottstown, PA), nitrification reactor (including fluidized media bed for biofiltration), heat exchanges (SP-210Kti, Alfa Heat Exchangers, Tampa, FL), and 6 fish tanks (1 meter diameter tanks, 265 L/each) per system. The systems were backwashed daily to maintain nitrate levels and low physical filtrate loads. The temperatures were set to 29 ^{o}C for all RAS using heat exchange units and monitored daily using thermometer. Each tank was equipped with aeration unit individually. Dissolved oxygen (DO) was monitored daily using DO meter (HQ40d, Hach, Loveland, CO). Ammonia, and nitrite were monitored every other day using spectrophotometer (DR-2800, Hach, Loveland, CO). Nitrate using spectrophotometer (DR-2800, Hach, Loveland, CO), pH using pH meter (AB15, Accumet Basic, Fisher Scientific, Fair Lawn, NJ) and alkalinity using titration method (method

8221, Hach, Loveland, CO) were monitored weekly. Sodium bicarbonate was added daily as needed to maintain alkalinity range between 100 to 250 mg/L. All data were recorded and monitored for any significant changes.

Lipid Extraction

The method was adapted from (Bligh and Dyer 1959) that is highly efficient in system with less than 2% lipid with 80% moisture (Christie 1982). Because fillets from 2 tilapias were harvested per tank, the fillets were chopped into small pieces, mixed and weighed to 100 g. The 100 g was homogenized using a Waring Blender with the addition of 100 ml chloroform (HPLC grade, Fisher Scientific, Fair Lawn, NJ) and 200 ml methanol (HPLC grade, Fisher Scientific, Fair Lawn, NJ) for 4 minutes. Then the extracts were filtered through Whatman No. 4 filter paper into 2 L Büchner flask under a vacuum. Then an additional 100 mL chloroform was poured into the blender jar and onto the filter paper. The filtered solution was then transferred to 2L separation funnels. Then, 100 ml 0.88% KCl (aq) was added to the separation funnel. After the filtrate were separated into the biphasic state, the chloroform layer containing lipids were collected. Several grams of anhydrous sodium sulfate was added to remove water then filtered using Whatman No. 1 filter paper. The chloroform layer was transferred to round bottom flasks and the lipids were concentrated using a rotavapor (Büchi Rotavapor R-3000) in 50 ^{o}C water bath. Oils were transferred to centrifuge tubes, then was blanketed with nitrogen gas. The tubes were then stored in a -20 ^{o}C freezer until further analysis.

Fatty Acid Composition by GC/MS

The production of lipid into fatty acid methyl esters (FAME) and analysis by GC/MS was conducted using the official AOCS method Ce 1b-89 (AOCS 2009). Internal standard (IS) was

prepared by weighing 25 mg of C23:0 methyl ester (Acros Organics, Fair Lawn, NJ) into a 25 mL volumetric flask. The flask was then filled with isooctane. One mL was then transferred to glass vial to be evaporated using nitrogen gas. The IS was utilized to quantify long chain fatty acids. Twenty five mg of purified fillet lipid sample was transferred to test tube with IS. Then, 1.5 mL of 0.5 M NaOH in methanol was added to the test tube. It was then blanketed with nitrogen gas and capped tightly. The solution was mixed then heated to 100 ^{o}C for 5 minutes. Once cooled, 2 mL of 12% BF_3 in methanol (Acros Organics, Fair Lawn, NJ) was added, blanketed with nitrogen gas, capped, mixed, and then heated at 100 ^{o}C for 30 min. It was then cooled to 30-40 ^{o}C. One mL of isooctane was added, blanketed with nitrogen, capped, and vortexed for 30 seconds. Immediately after vortexing, 5 ml of saturated sodium chloride solution was added, blanketed with nitrogen, capped, and mixed thoroughly. The isooctane layer was moved to GC vials and capped. Another 1 mL of isooctane was added to the test tube, blanketed with nitrogen gas, capped and vortexed. Isooctane layer was then removed once again into the same GC vial.

A GC/MS (Shimadzu, GC-2010/MS TQ-8030) was prepared by setting the injection port to 250 ^{o}C, initial oven temperature at 175 ^{o}C (5 min hold time) with program rate at 2.0 ^{o}C/min, and final oven temperature at 225 ^{o}C (30 min hold time). The column flow rate was set to 1.22 mL/min and linear velocity of 30 cm/sec with high purity helium as a carrier gas with split injection mode with split ratio of 50:1. The ion source temperature was set to 230 ^{o}C with interface temperature at 200 ^{o}C. A polar column was used to separate the fatty acid methyl esters (ZB Wax-plus, 60.0m x 0.22 mm i.d., 0.25 µm film thickness). The IS was used to quantify OA, ARA, EPA, DPA, and DHA.

Sensory Analysis

Product Preparation. The sensory analysis was conducted using a 2 sample preference test. To minimize participant fatigue, two diet fed groups were chosen: SFOFO85 and SOFO85. Individual fillets were wrapped in aluminum foil then placed on a cooking pan. Fillets were cooked for 20 minutes in a preheated oven (190.6 ^{o}C) until a minimum internal temperature of 62.8 ^{o}C. Then the fillets were placed in plastic bags and flaked to create a uniform texture. Bags of flaked tilapia were then placed in a water bath (45 ^{o}C) to maintain homogenous temperature of the fillets until served. The cooked samples after 2 hours in the baths were thrown out and repeated cooking process for a new batch. Samples (approximately 5 grams) of fillet were placed in a coded (3 digit number) 4 oz. plastic cup and sealed with a lid just prior to serving to panelists.

Participants. Participants (n=68) were recruited from the university community, especially from within the Food Science and Technology Department and Biological Systems Engineering Department, which are housed within the same building as the VT Sensory Evaluation Laboratory. The analysis was conducted with the approval of the Institution of Review Board for human subjects (Appendix C). No prescreening was completed. However, all participants gave a consent prior to the participation, and they were given option to quit as needed at the beginning or during the analysis. Each panelist was assigned an identification number to protect privacy.

Sample Preparation. Each sample was presented in plastic cups (4 oz.), coded with 3-digit codes in a balanced presentation order. Instructions and preference questions were displayed and answered electronically using touch screen computers with sensory software SIMS (SIMS 2000, Sensory Computer Management, Morristown, NJ). Demographic information, seafood products

purchasing and consumption behaviors were collected in form of 6 questions. Panelists were given a demographic questionnaire prior to the product tasting as a hard copy to fill out.

Texture Analysis

The texture of cooked SFOFO85 and SOFO85 fillets was also analyzed following a previously developed procedure (Felice and other 2011). Max shear force and work of shearing force were measured in quadruple per diets. Thickest part of the fillets were used. The fillets were cooked in an oven until an internal temperature of 62.8 ^{o}C (oven temperature of 190.6 ^{o}C for 20 minutes) then cooled to room temperature prior to analysis. The measurements were recorded by a texture analyzer (TA XT_plus Stable Micro System, Texture Technology Corp) with a 5 blade Kramer Shear cell.

Statistical Analysis

The statistical analysis was performed to determine significance in growth of fish, FCR, concentration of fatty acids, lipid content in fillet, and sensory analysis. Significances were determined based on the diet types (in duplicate) using ANOVA at α=0.05 (JMP Pro 13, 2016 SAS Institute, Inc., Cary, NC). Tukey's HSD analysis were used for mean separations. The sensory analysis results were analyzed using t-test at significance level $\alpha = 0.05$.

Results and Discussion

Proximate Analysis of the Experimental Diets

The proximate analysis were conducted by a third party laboratory (Midwest Laboratories, Inc., Omaha, NE). The analysis were done using AOAC methods. The proximate analysis reported the moisture of 10.08 ±1.51 % (w/w), dry matter of 89.92 ±1.51 % (w/w), crude protein of 41.2 ±1.22 % (dry weight), crude fat of 7.32 ±1.34% (dry weight), fiber of 7.31 ±1.76% (dry weight), ash of 7.39 ±0.24% (dry weight), and total digestible nutrient of 82.38 ±2.07% (dry weight) (Table 5). The diets were similar in their lipid content except for the experimental diets. The proximate analysis showed a low crude fat content in commercial diet at 3.88% DW compared to the average of experimental diets at 7.63% DW. Also, the crude fat in the diets was extracted using a petroleum/ether method. The extraction method affected the efficiency of extruded diet and therefore, differences were observed from the Bligh and Dyer (1959) method that was used in-house. Compared to the proximate analysis result, in-house extraction method yielded 8.93% DW for commercial diet and 7.39% DW for experimental diets.

Growth Performance

All 11 experimental diets performed well compared to the commercial diet on tilapia growth. Not only that, the performance was statistically not significant in weight gain, feed intake and FCR (Table 3). The feeds were adjusted weekly based on tilapia's growth rates and the consumption of feed from previous week. The weekly feed adjustments were designed to maximize growth and FCR (Table 1). There were no observable differences in the physiological effect from the diets. Behavioral abnormalities such as bullying did not result in mortality and/or morbidity. Out of 600 fish in all tanks, 2 fish were taken out of their respective tank and euthanized

before the end of the growth trials. A fish from SFOFO15 and SOAO85 diet tanks were each removed. One fish was found dead in the tank at week 4, and another was found on week 7 to have white cyst on the mandible area and was removed. The mortality did not have any indication of death due to the system or diet. Therefore, the mortality rate was 0.3% for the entire study. The RAS worked efficiently with high consistency. The water quality shows there were minor fluctuations in water quality parameters (Table 2). The growth performance was interesting because some research showed that different levels of fatty acids can have significant impacts on the growth performance in various tilapia species (Santiago and Reyes 1993; Kanazawa and others 1980; Stickney and others 1982). However, with the tilapia diets varying in their fatty acid compositions, there were no significant differences in the growth performance. The mean growth of tilapia did not have significant differences throughout the entire 8 week trial (Figure 1). No impact from different oil sources in the growth performance of GIFT tilapia and red hybrid tilapia were observed in another study (Teoh and others 2011). This suggests that when the tilapia reaches adult size, the relationship between fatty acid composition and the growth performance might be negligible compared to different stages of life.

Diet Lipid Content and Fatty Acid Compositions

The formulated experimental diets were analyzed for total lipid content as well as the fatty acid composition. While lipid concentration was not different at 8.4 with a p-value of 0.57, the fatty acid compositions varied widely from diet to diet (Table 6). The fatty acid composition analysis showed that all the diets had significant differences at $\alpha=0.05$ in all major fatty acids such as OA, LA, ARA, EPA, DPA, and DHA (Table 6). The SFO100 diet was able to improve OA concentration in the feed by up to 495 mg/g oil compared to 135 mg/g oil in commercial diet or

229 mg/g oil in SO100 diets. In addition, the HOSFO diets reduced LA compared to the commercial and SO diets. DHA levels improved significant when high levels of AO was used in the diet, at 187 mg/g oil in SFOAO85 and 170 mg/g oil in SOAO85 compared to 16.5 mg/g oil in CD. Also, EPA and DPA improved significantly using FO in the diets at 49.4 mg/g oil and 8.4 mg/g oil in SFOFO85 and 42.7 mg/g oil and 7.5 mg/g oil in SOFO85, respectively. The dietary formulations were able to achieve their purpose. HOSFO based diets were able to improve OA and reduce LA and ARA while SO based diets were reflective of cheap agricultural diet with high levels of LA and ARA. When improving *n*-3 fatty acids, FO improved diets were able to increase EPA and DPA while AO improved diets were able to increase DHA concentration in the diets.

Another interesting result was the relatively high quality of the commercial diet. The overall *n*-6:*n*-3 fatty acid ratio was 2.4 with ARA of 2.4 mg/g oil and EPA, DPA, and DHA of 22.2, 1.1, and 16.5 mg/g oil, respectively. For the experimental diets, *n*-6:*n*-3 fatty acid ratio for SOAO85 SFOAO85, SFOFO85 were highly favorable with the ratio below 1 at 0.73, 0.68 and 0.89; while SO100, SOFO15, SFO100, SFOAO15, and SFOFO15 were least favorable with the ratio of 4.8, 6.4, 7.6, 5.4, and 6.5, respectively. There were increases in OA content when HOSFO replaced SO. The reason for improving OA had two purposes. Because it has less methylene interrupted double bonds, the OA has very good oxidative stability compared to PUFA (Frankel and Huang 1994). Also, HOSFO has been known to reduce prevalence of cardiovascular disease that can provide health benefits to the consumers (Cater and others 1997; Allman-Farinelli and others 2005).

Tilapia Fillet Lipid and Fatty Acid Compositions

The fatty acid composition in tilapia fillets were highly correlated to the diets consumed by the tilapia (Table 8). Tilapia fillet lipids were no different ($p > 0.05$) and ranged from 1.2% to 1.75% fillet lipid (w/w). There were no correlations between different diets and the tilapia fillet lipid concentrations. Quantification using total fatty acid composition, % area, showed that OA was highest in SFO100 diet fed tilapia fillet at 36.80% (Table 8). This was parallel to the diet fatty acid composition as SFO100 diet contained the highest levels of OA (Table 6). Highest concentration of ARA was found in CD diet fed tilapia fillet at 3.25%. EPA and DPA were found highest in HT diet fed tilapia fillets at 2.0% and 3.8%, respectively. DHA was found highest in SOAO85 diet fed tilapia fillets at 17.68%, which was 316% greater than CD fed tilapia fillets. In all cases, comparing performance of algae oil vs fish oil based diet, algae oil provided higher levels of DHA. Except SFOAO85 diet fed tilapia fillets compared to SFOFO85 diet fed tilapia fillets, other comparisons showed that DPA and EPA were found at higher concentrations in fish oil based diet fed tilapia fillets compared to the algae oil based diet fed tilapia fillets.

Unlike the lipid concentrations, fatty acids were all statistically significant except for ARA, which approached significance (p-value = 0.0589). Highest level of ARA was found in SO100 fed tilapia fillets at 22.5 mg/g oil and lowest was found in SOAO85 fed tilapia fillets at 11.0 mg/g oil. The *n*-6:*n*-3 ratios ranged from 0.86 in HT to 4.0 in SO100. SO100, CD, and SFO100 diets fed tilapia fillets were significantly different from the rest of the diets fed tilapia fillets. Most interestingly, tilapia fillet fed SOFO85, SFOAO85, SOAO85, and HT diets were had *n*-6:*n*-3 ratios below 1:1 ratios. This is important because Americans consume high *n*-6:*n*-3 ratio of 10:1 (Simopoulos and others 1999). Consumption of high *n*-6 fatty acid levels been associated with cardiovascular disease (Simopoulos 2008). Therefore having higher consumption of *n*-3 fatty acids

is critical in current American diet and the high fish oil and high algae oil diet fed tilapia were able to achieve appealing ratios for consumers. ARA has been associated with cardiovascular disease due to its proinflammatory responses in the human body while long chain *n*-3 eicosanoids such as EPA have been associated with anti-inflammatory responses. (Patterson and others 2012). Therefore the ARA to LCn3 (EPA, DPA and DHA) ratio was also calculated for the tilapia fillet lipids. ARA:LCn3 ratios ranged from 0.11 to 0.73. Tukey analysis showed there were minor differences between the fillets however, CD, SFO100, and SO100 had highest ratios of 0.44, 0.52, and 0.73, respectively; while SOAO85 had lowest ratio of 0.11.

Another interesting observation was made when the fatty acid composition of the tilapia fillet lipids at the end of growth trials were compared to the fatty acid composition of the tilapia fillet lipids before the beginning of experiment. The ARA, EPA, DPA, DHA and OA concentrations (in mg/g oil using IS) of tilapia fillet lipid at the end of 8 week growth trial were compared to tilapia fillet lid pre-trial using Tukey HSD analysis at α=0.05. The analysis showed no significant differences in OA and ARA. However, there were clear differences in EPA, DPA, and DHA in some diets fed tilapia fillet compared to the pre-trial tilapia fillet lipid. For EPA concentration, SOFO85, SFOFO85, HT, and SFOAO85 were significantly different from pre-trial tilapia fillet lipid. For DPA, SOFO85, HT, SFOAO85, and SFOFO85 were significantly different from pre-trial tilapia fillet lipid. For DHA, SOAO85, SOFO85, and HT were significantly different from pre-trial tilapia fillet lipid.

The science behind the impact of diet on fillet quality is clear (Tonial and others 2009; Karapanagiotidis and others 2007). Our experiment also proved that the fatty acid composition of tilapia fillet is affected by the lipid quality of the diet. An interesting pattern was observed from

the tilapia specie used for the experiment. Total lipid content of the fillet averaged 1.5 g of lipid per 100 g fillet. Even at high rate of 5% inclusion of lipid in the diet with average total feed lipid content of 8.4% (w/w), the tilapia were extremely lean with even lower lipid content than the USDA standard of reference of 1.7% (w/w) (USDA 2015). Considering normal tilapia feeds are formulated to be 3-5% (w/w) lipid, the high feed lipid content was expected to yield fatter fish (El-Sayed 2006). This could indicate that genetics can play an important role in tilapia's ability to store large quantity of lipid rather than the lipid content of the feed they consume.

Sensory Analysis

The preference test was conducted to determine if lipids in the feed affected the cooked fish fillet in a way that created an obvious preference. Because the objective of research was to develop value added product, this secondary objective was necessary to determine whether the enhanced feed strategies can negatively impact product perception by the consumers on tilapia fillets. The tilapias that were fed SFOFO85 and SOFO85 diets were compared. SOFO85 was used as the control as soybean oil and fish oil mixture would be the most commonly used ingredient for the producers. No significant preference was found (Figure 3). This is important as it can be extrapolated to diets containing different oil mixture. Differences in FAC within the fillet, associated with the different fish diets, did not contributed to ta preference between the two treatments.

We did not conduct an overall acceptability test because of the manner of preparation. Most tilapia preparation recipes involved flavoring and/or seasoning, which could mask the subtle differences created by changes in FAC in tilapia fillet. Tilapia are lean fish with low fat content; however, because they are grown in freshwater, off-flavors can be a problem for producers. This

is partly because off-flavor compounds (OFC), such as geosmin and 2-methylisoborneol, are fat soluble and highly detectable by humans (Yamprayoon and Noomhorm 2000). Therefore, the purge method was employed to reduce OFC presence in the sensory analysis that can interfere with the preference test. Although fatty acid composition is not important to the susceptibility of OFC absorption by tilapia, 2 day purge was necessary conducted. The purge was limited to minimize the impact of purge technique on the metabolism of fats during purging period in fish. Also because our RAS was not set up to do flow through where continuous freshwater replaces tank water, purge procedure was limited as stated.

The impact of FAC on OFC was evident as the participants showed no significant differences in detection of off flavors in the sensory analysis, which confirms that FAC does not have impact on the off-flavor compound absorption (Figure 3). Although it is an economically significant problem, evidently, off-flavor problems in tilapia aquaculture cannot be resolved with change of feed strategies currently. Further research can help understand the importance of various purge techniques and impact of these purge techniques on the OFC presence in the fillet and also the loss of fillet lipids during the purge.

As part of the sensory analysis, demographics, seafood consumption frequency and consumption and purchasing behaviors were determined (Figure 2). Demographics within the participants, 54% were male and 46% were female; 72.1% were white, 4.4% were Latin American, 2.9% were African American, 16.2% were Asian, and 4.4% selected others; and 2.9% were less than 20 years old, 69.1% were between 20-29, 11.8% were between 30-39, 4.4% were between 40-49, 5.9% were between 50-59, 2.9% were over 60 and 2.9% did not selection. US census shows that male to female ratios of 49.2:50.8 male to female was close (Census, 2015). The age

distribution showed that our data were much more skewed to younger population with only 2.9% that were over 60 while US population has 14.9% of population over age of 65 (Census, 2015). The race distribution also represented US population to some degree as US population is made up of 77.1% white, 17.6% Latin American, 13.3% Black or African American and 5.6% Asian (Census, 2015).

The seafood consumption frequencies were also surveyed with 30.9% stated they consume 1-3 seafood meals per week, 60.3% stated they consume 1-3 seafood meals per month, and 8.8% stated they consume only a few seafood meals per year. A small percentage, only 2.9% of participants, either reported 'do not like seafood' or did not answer, while 11.8% consume seafood for health reason, 42.6% like the taste of seafood, 5.9% consume because they grew up with it, and 36.8% consume seafood because of the combination of the three reasons. Because of the convenience sampling within the university environment, 55 out of 68 participants were considered to be millennials. This was interesting because out of all participants, 29.4% of the participants were willing to pay $0.50 more and 25% of the participants were willing to pay $1.50 more for a premium quality product, assuming the tilapia fillet costs $5.00/lb. This was important because it shows that the consumers are wanting a premium products and are willing to pay for that premium quality. Parallel to our finding, another surveys reported that with the entrance of millennials into the financial independence, the market saw increase in consumption of premium products in the supermarkets (Topper 2015; IRI 2016).

Texture Analysis

The texture analysis was conducted to determine the possible impact of oil in overall texture quality of the cooked tilapia. Texture can be an important contributor to the overall

characteristics of the food product. Often, lipids are important in giving fuller mouth feel. When smoked and cooked Atlantic salmon with varying lipid content from 2.9 to 10.7% (w/w) were analyzed for texture and flavor, higher lipid content resulted in higher preference by the sensory panelists (Robb and others 2002). Not only that, tilapia fillet had (2.6% fat w/w) lower firmness and higher flakiness compared to salmon (13.5% fat w/w) (Felice, 2011). It shows that differences in lipid content can affect the texture of the fillets. Beyond sensory analysis, different feed types can have huge impact on the physical characteristics. In a feeding trial looking at the impact of raw Spirulina diet fed tilapia fillet saw increase in springiness compared to commercial diet fed tilapia fillet (Lu and Takeuchi 2002). However, the texture analysis proved that diets has negligible impact on the overall texture quality of the tilapia. Considering that tilapia are lean fish, overall composition of tilapia fillet is affected little by the dietary oil composition. No significant differences were observed in work of shearing and max shear force at $\alpha = 0.05$ (Figure 4). Differences in texture quality can affect overall liking of the product by the consumers. So it is important to determine sensorial qualities of new products. The results indicates that consumers will not be able to discern differences in lipid quality of tilapia fillets through texture.

Conclusions

A direct relationship between the fatty acids of the feed and tilapia fillets was observed. The lipid quality of the feed has huge impact on the lipid quality of the fish fillet. Not only that, all diets provided good growth performance and FCR comparable to the commercial diets, which indicates the experimental diets can be used in the tilapia aquaculture industry without economic burden. The demand for high quality product by the consumer is present and can be a lucrative market for tilapia industry. It is important to discuss the economics of the feed in the aquaculture as the feeds are over 50% of total operating cost associated with aquaculture. However, this factor assumes that the price of tilapia is fixed; when the producers can start selling tilapia as value added product, it can fetch premium prices that can offset the cost of the production.

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Chapter 5. Evaluation of Lipid Content, Fatty Acid Composition, and Peroxide Value in Cold Stored (-10ºC, -20ºC) Fillets of Tilapia, *Oreochormis* sp. Grown in a Recirculating Aquaculture System

Abstract

Aquaculture has become an important food provider in the 21st century. Because seafood products are becoming critical parts of staple food sources, sustainable practices and nutritional quality of the seafood has become ever increasingly important. Because tilapia is highly adaptable and can be easily aquacultured, it has seen large production growth recently; however, tilapia is considered to be a lower quality seafood by many consumers. Therefore, improving tilapia fillet quality, especially lipid composition, can supplement consumers' desire for healthy diet. A commercial diet, two base diets (100% of lipid either in high oleic sunflower or soybean oil), one diet with an additional α-tocopherol, and the rest of 8 diets were made up with varying concentrations of high oleic sunflower, soybean, algae, and fish oil. All formulated diets were pelleted iso-nitrogenous and iso-caloric in-house. The 12 diets were fed in duplicate to 24 fish tanks containing 25 tilapia per tank over 8 weeks in 4 recirculating aquaculture system with 6 fish tanks each. Euthanized fish were filleted, and vacuum packed. Month 0 samples were stored at -20ºC prior to analysis, temperature abuse samples were stored in freezer with fluctuating temperature between -10 to -20ºC, and stable frozen samples were stored in -20ºC cold storage for 0, 3, 6, and 9 months. Lipid extraction followed by FAME production and GC/MS of fatty acid composition. Oxidative stability was observed using peroxide value. The results indicated that the differences in storage condition and in varying storage time did not significantly impact the degradation of long chain polyunsaturated fatty acids or oleic acid, nor increased the peroxide

value. There were no observable patterns in the loss of fatty acids. The findings of this research shows current dietary formulation and processing techniques are sufficient in providing high quality product to the consumers.

Keywords: Tilapia, Oxidation, Long Term Storage, PUFA

Introduction

The aquaculture industry enjoyed fast growth over last 50 years (FAO 2014). Due to the improvements in fish nutrition, aquaculture technology, and transport and storage technologies, the aquaculture industry has been able to reach bigger markets around the world. Tilapia industry has enjoyed fast growth to become second most consumed seafood globally and 4th in the US (Zajdband 2012). Although tilapia industry is one of the fastest growing sector in aquaculture industry, the industry have seen a recent setback due to the negative image portrayed in the media (Fitzsimmons 2017). Value-added products can help alleviate those negative images while providing healthier alternative to the consumers. Food industry, as a whole, has become consumer driven in recent years, and with the increase in consumer voice over their food choices, consumers are beginning to demand less processed, more transparent, more sustainable, and higher quality premium products (Topper 2015; IRI 2016). There have been attempts by the tilapia industry to create value-added products through frozen products and ready eat meals (Fitzsimmons 2016). However, the raw ingredient, tilapia fillet, have not had any increase in value. Tilapia has good quality protein; however, due to the omnivorous feeding preferences, tilapia have high *n*-6 fatty acids in the lipid (El-Sayed 2006). In normal tilapia aquaculture production, feed can take up to 50% of the operation cost (El-Sayed 2006). The production of tilapia then can be extremely costly venture with little room for a failure. Thus, producers often utilize agriculture based feeds, high in *n*-6 fatty acids, to cut costs. However, effectively utilizing premium ingredients in the feed, producers can sell the products as value added product for premium price. Therefore, improving *n*-3 fatty acids can have significant improvement in the overall tilapia quality for human consumption as value added premium product.

Improving *n*-3 fatty acids in tilapia is especially important for US consumers. Normal US consumers consume *n*-6:*n*-3 fatty acid ratio of 10:1; and this number can be higher in some pockets of the population (Simopoulos 2002). The problem is that the *n*-6 fatty acids, specifically arachidonic acid (ARA; 20:4*n*-6), has proinflammatory activities in humans that can exacerbate cardiovascular disease and other health problems (Patterson and others 2012; Adams and others 1996; Young 2009). Increased consumption of long chain *n*-3 fatty acids (LCn3) such as eicosapentaenoic acid (EPA; 20:5*n*-3), docosapentaenoic acid (DPA; 22:5*n*-3), and docosahexaenoic acid (DHA; 22:6*n*-3) that are known to have anti-inflammatory responses in human bodies are highly desirable (Patterson and others 2012; Kidd 2007). Not only are *n*-3 fatty acids highly beneficial to off-set high *n*-6 fatty acid consumption, the *n*-9 fatty acid oleic acid (OA; 18:1*n*-9) has been associated with positive heart health impact as well (Ryan and others 2000; Parthasarathy and others 1990).

Improving diets to create high quality tilapia have been observed before (Visentainer and others 2005; de Souza and others 2007).The biggest importance to the producers should be the improvement of product quality when the consumers receive them. And because feed costs are a significant portion of the aquaculture operation, it is important for the producers to reap the benefit of high cost associated with nutritionally enhanced feeds. One of the biggest challenges in improving *n*-3 fatty acids such as EPA, DPA and DHA is autoxidation of fatty acids. Long chain polyunsaturated fatty acids (PUFA) are highly susceptible to oxidative degradation (Miyashita and others 1993). Specifically under long term storage, degradation of long chain PUFA can be observed along with increase in malondialdehyde production (Ng and Bahurmiz 2009). However, research does not indicate the level of antioxidant addition in the feed, as it would normally be observed in commercially available feeds. Previous studies have observed that dietary inclusion

of tocotrienols and tocopherols were deposited in the fish fillet, and as such could have antioxidative effect on the long chain PUFA (Ng and others 2004; Wang and others 2006). Thus, in real world application, inclusion of antioxidants as a part of the diet is important in determining the degradation of long chain PUFA in tilapia fillet over time during the transportation and during the storage.

LCn3 have high oxidative instability, and can break down to hydroperoxide and other volatile compounds. Therefore, understanding the rate of autoxidation during storage and minimizing the autoxidation rate can have economic benefits to the producers while nutritionally benefit the consumers. The research was conducted to evaluate the lipid content, fatty acid composition, and peroxide value in temperature abused and -20^{o}C cold stored tilapia fillet. The objectives were to determine the rate of autoxidation, impact of autoxidation on lipid content and fatty acid composition; and whether -20^{o}C cold storage can better prevent autoxidation compared to the temperature abused condition over 9 months of storage.

Material and Methods

Diet Formulation

A total of 12 diets were used in the experiment. Eleven diets were formulated and pelleted in-house (Table 1). The raw ingredients were chosen and the diet formulations were created to ensure iso-nitrogenous and iso-caloric feeds to minimize non-essential ingredients affecting the experimental design. The proximate analysis of the diets were obtained from an external laboratory (Midwest Laboratories, Inc., Omaha, NE). Eleven diets contained same ingredients except for a diet strategy HT that contained additional 200 ppm of *dl*-α-tocopheryl acetate (aTOC; Sigma-Aldrich, Inc., Milwaukee, WI) in place of wheat of same amount (w/w). Otherwise, 11 diets had varying composition of lipid sources from high-oleic sunflower oil (HOSFO; Sunflower Oil – High Oleic, JEdwards International, Inc., Braintree, MA), soybean oil (SO; 100% Pure vegetable Oil, Englewood, NJ), high DHA algae oil (AO; Schizochytrium DHA-Rich Algal Oil, Source-Omega, LLC, Mason, OH), and fish oil (FO; Virginia Prime Gold, Omega Protein, Houston, TX).

Besides 5% of varying mixture of oils, all diets were formulated with 58.500% soybean meal (SBM; Hi-Pro Solvent Extracted Soybean Meal, ADM Alliance Nutrition, Inc., Quincy, IL), 25.794% wheat (WT; except for HT diet which was formulated with 25.774%; Quality Feed, Southern States, Richmond, VA), 3.000% meat and bone meal (MBM; Smithfield Farmland, Smithfield, VA), 6.5% fish meal (FM; Special Select, Omega Protein, Houston, TX), 0.100% vitamin premix (VP; TestDiet, St. Louis, MO), 0.100% mineral premix (MP; TestDiet, St. Louis, MO), 0.0005% mixed tocopherol (TOC; Sigma-Aldrich, Inc., Milwaukee, WI), 0.001% L(+)-

ascorbyl palmitate (AP; Acros Organics, Fair Lawn, NJ), and 1.000% carboxylmethyl cellulose (CMC; CP Kelco, Atlanta, GA).

A commercial diet, (CD; Production 35, 4.0 mm, Rangen Inc., Buhl, ID) was implemented as a reference diet. In house formulated diets had addition of 5% lipid (w/w) from the oil sources. Two base diets were chosen with single lipid sources each as high oleic sunflower oil (SFO100) and soybean oil (SO100). A SFOAO15 diet contained 4.25% (w/w) HOSFO and 0.75% (w/w) AO. A SFOAO85 diet contained 0.75% (w/w) HOSFO and 4.25% (w/w) AO. A SFOFO15 diet contained 4.25% (w/w) HOSFO and 0.75% (w/w) FO. A SFOFO85 diet contained 0.75% (w/w) HOSFO and 4.25% (w/w) FO. A SOAO15 diet contained 4.25% (w/w) SO and 0.75% (w/w) AO. A SOAO85 diet contained 0.75% (w/w) SO and 4.25% (w/w) AO. A SOFO15 diet contained 4.25% (w/w) SO and 0.75% (w/w) FO. A SOFO85 diet contained 0.75% (w/w) SO and 4.25% (w/w) FO.

Fifty-five kg of each in-house formulated diets were made. Diets were made in 5 kg batch. Dry ingredients (soybean meal, wheat, meat and bone meal, fish meal, vitamin and mineral premix and CMC) were weighed and added to a V mixer. Once thoroughly mixed, 2 batches of dry ingredients were transferred into a planetary mixer. During mixing, the 2 batch worth of liquid ingredients (oils, TOC, aTOC, and AP) were slowly added into the mixer and mixed for 5 minutes. An additional 800 grams of water was added and the homogenized ingredients were pelleted using mill pellet machine with 4 mm diameter (72A, 10 HP, Lawson Mills Biomass Solution, Hartsville, PE, Canada). The pellets were dried over the counter overnight at a room temperature. Dried diets were bagged and stored in -20^{o}C cold storage until used for the tilapia growth out phase.

Tilapia Aquaculture

Genetically Improved Farmed Tilapia, *Oreochromis sp.*, (GIFT and sex-reversed, Spring Genetics, Miami, FL) with initial average weight of 100.7 ±2.6 grams were brought into the recirculating aquaculture system (RAS). Tilapia were fed a commercial diet (Bronze floating, 3.0 mm, Ziegler Inc., Gardners, PA) until the tilapia were adapted to the system and then were distributed evenly to all tanks. In total of 600 fish, 25 fish per tank to 24 tanks were distributed. Tilapia were then switched over to the 12 experimental diets at the average weight of 206.1 ±6.1 grams. Four RAS were blocked into two groups and within each group, twelve experimental diets were randomized to the tanks to eliminate tank location and system variability. Once a day, feeds were loaded on to the automatic feeders operated over 18 hours daily. Tilapias were fed daily for 8 weeks. Once a week, weight of the total number of fish in individual tanks were measured to adjust and optimize food conversion ratio (FCR) for optimal feeding rate for the following week.

Each RAS had an UV filtration (Smart UV Sterilizer, 02130, 130 Watt, Emperor Aquatics, Inc., Pottstown, PA), a sand filter (S180T, Pro Series, Hayward, Rockville, MD), a beads filter (BBF8000 Aquaculture Systems Technologies, New Orleans, LA), a nitrification unit (including fluidized media bed for biofiltration), a heat exchanger (SP-210Kti, Alfa Heat Exchangers, Tampa, FL), 6 fish tanks (1 meter diameter tanks, 265 L/each) and aerators. Incoming municipal water was filtered using activated carbon. Water quality parameters measured were total ammonia nitrogen (TAN), NO_2^-, NO_3^-, pH, water temperature, dissolved oxygen, and alkalinity in each RAS throughout the experiment. Temperature and DO were measured using DO meter (HQ40d, Hach, Loveland, CO). TAN, NO_2^-, and NO_3^- were measured using spectrophotometer and Hach kit (DR-2800, Hach, Loveland, CO). pH was measured using pH meter (AB15 Accumet Basic, Fisher

Scientific, Fair Lawn, NJ). Alkalinity was measured using Hach kit (method 8221, Hach, Loveland, CO) and maintained by adding sodium bicarbonate to maintain alkalinity concentration of 100 to 250 mg/L in each RAS. Backwash was conducted daily to maintain high water quality.

Tilapia Sampling and Storage Protocol

Tilapia were collected in sets of 2. These samples were pooled later to create homogenized sample, as lipid content is not uniformly distributed within tilapia fillets. All sampling was done in one day. Fourteen tilapia were collected from each tank. A total of 336 fish were euthanized by overdose of buffered MS-222 (Tricaine-S, Western Chemical, Inc., Ferndale, WA) and confirmed by cervical dislocation. Then the tilapia were filleted on both sides, vacuum packed and flash frozen using dry ice and iso-propanol and stored in -20^{o}C until analysis.

The month 0 samples were stored at -20 ^{o}C cold storage until further analysis. For month 3, month 6 and month 9 samples, they were stored for 90 days, 180 days, and 270 days in temperature abused conditions and in walk-in -20^{o}C cold storage. The temperature abuse was induced by reducing the freezer (Whirlpool ET22RK/ET22RM, Whirlpool Corporation, Benton Harbor, MI) temperature down to -10 ^{o}C. Then after the specified time, the samples were moved to -20^{o}C freezer until further analysis was conducted.

Lipid Extraction

Bligh and Dyer (1959) total lipid extraction method was used to extract lipid from the fillets (Christie 1982). The stored samples were taken out of storage and thawed for 2 hours then cut to small chunks. One hundred grams of samples were blended in blender jar with 100 mL of

chloroform (HPLC grade; 0.75% ethanol as preservative; Fisher Scientific, Fair Lawn, NJ) and 200 mL of methanol (HPLC grade; Fisher Scientific, Fair Lawn, NJ) for 4 min. Then the blended mixture was filtered with filter paper (Whatman No.4; Buckinghamshire, UK) into a flask under a vacuum. Remaining samples in the blender jar were washed with addition 100 mL of chloroform and filtered. The solution was then moved to separatory funnel. 100 mL of 0.88% KCl (aq.) solution was then poured into the separatory funnel. The solution was left uninterrupted for 2 hours for bilayer to form. The chloroform layer containing lipid was collected. Remaining moisture in the chloroform layer was removed using several grams of anhydrous sodium sulfate (certified ACS; Fisher Scientific, Fair Lawn, NJ). Then anhydrous sodium sulfate was removed (Whatman No.1; Buckinghamshire, UK). The chloroform was removed completely through Rotavapor (Evapotec, Haake Buchler Instrument, Saddle Brook, NJ) at 50^{o}C. The remaining lipid in the round bottom flask was then weighed and transferred into a clean glass test tube. The tube containing lipid was then flushed with nitrogen gas. The extracted tilapia fillet lipid was stored in -20^{o}C freezer until further analysis.

Fatty Acid Methyl Esters Analysis by GC/MS

The fatty acid methyl esters (FAME) were analyzed with AOCS Ce 1b-89 (AOCS 2009). First, internal standard (IS) was prepared by weighing 25 mg of tricosanoic methyl ester (C23:0; Acros Organics, Fair Lawn, NJ) into 25 mL volumetric flask with isooctane. Then 1.0 mL of IS solution is transferred into a glass test tube, and isooctane was evaporated using nitrogen gas.

The preparation of FAME was conducted by weighing about 25 mg of extracted lipids into the test tube containing 1 mg of IS. One and five tenth mL of 0.5 M NaOH/MeOH (certified ACS; Fisher Scientific, Fair Lawn, NJ) was added and heated at 100^{o}C for 5 mins. Then 2 mL 12%

BF_3/methanol reagent (1.5 M; Acros Organic, Pittsburg, PA) was added into cooled sample then heated at 100^{o}C for 30 minutes. The test tube was then cooled to 30-40^{o}C, 1 mL of isooctane added and vortexed for 30 sec. Immediately 5 mL of saturated NaCl aqueous solution. The isooctane layer separated and collected into a GC vial. Lastly, additional 1 mL of isooctane was added to the solution to separate remaining FAME. Throughout the entire procedure, tubes and vials were nitrogen flushed capped and mixed or vortexed.

FAME samples were then analyzed by injecting 1.0 µL into a GC/MS (Shimadzu, GC-2010/MS TQ-8030) with autosampler (AOC-20i+s). The carrier gas was helium (ultra-high purity, 99.999%) at total flow rate of 166.5 mL/min, column flow rate of 1.22 mL/min, linear flow of 30 cm/sec, and split ratio of 50:1. The inject port temperature was set to 250^{o}C, and the oven temperature program with initial temperature of 175^{o}C for 5 min hold time, program rate at 2.0^{o}C/min, and 225^{o}C final temperature with 30 min final hold time. A GC polar column (Zebron, ZB-Wax plus, 60 m x 0. 0.25 mm i.d., 0.25µm film thickness) was used. MS was set to ion source temperature at 230^{o}C and interface temp at 200^{o}C. The mass spectra of the peaks in the chromatogram were analyzed using NIST14 library software and calculated equivalent chain length (ECL).

Peroxide Value

Peroxide value of tilapia fillet lipid samples were measured using the International Dairy Federation standard method, 74A:19991, spectrophotometry analysis adapted by Shantha and Decker (1994). An iron (II) chloride solution was prepared by dissolving 80 mg of barium chloride dehydrate (ACS certified, Fisher Scientific, Fair Lawn, NJ) in 10 mL of deionized water. Then the solution was added slowly with constant stirring to 100 mg of iron (II) sulfate heptahydrate in

10 mL of deionized water. 0.4 mL of 10 N hydrochloric acid (Certified 10 N, Fisher Scientific, Fair Lawn, NJ) was added to resulting solution in constant stir. Then the solution was filtered through a filter paper (Whatman No. 1, Buckinghamshire, UK) into a brown glass vial, and stored in the dark. The ammonium thiocyanate solution was prepared by dissolving 7.5 g of ammonium thiocyanate in deionized water, and made up to the volume in 25 mL volumetric flask. The ammonium thiocyanate solution was then transferred into brown glass vial and stored in the dark until the analysis. Two solutions were prepared fresh before month 0, 3, 6, and 9, each.

Fe^{3+} concentration to absorbance standard curve was created prior to the analysis by dissolving 0.5 g iron powder in 50 mL of 10 N hydrochloric acid. Then 1 to 2 mL of 30% hydrogen peroxide solution was added to the hydrochloric acid. The solution was then boiled for 5 min and cooled to room temperature. The solution was then diluted to 500 mL with deionized water. The standard curve was created containing iron (III) concentration of 1 to 40 µg. To analyze under the spectrophotometer, 4.9 mL of chloroform/methanol solution (7:3 v/v) was added to the test tube. Then 25 µL of ammonium thiocyanate solution was added. Fe (II) standard solution was added, lightly vortexed, rested for 5 mins, and measured for absorbance at 500 nm. Slope was calculated using the standard as 17.936 (Figure 1). To calculate for milliequivalents of peroxide/kg of sample (meq/kg), the formula used:

$$\text{P. V.} = \frac{(A_S - A_b) \times m}{55.84 \times m_0 \times 2};$$

where A_s is absorbance of the sample, A_b is absorbance of the blank, m is slope, m_0 is the weight of the sample, 55.84 is atomic weight of iron, and 2 is the constant factor.

To measure peroxide value in meq/kg, 10 to 300 mg of fillet lipids were weighed and transferred into the glass test tubes. 4.9 mL of chloroform/methanol (7:3 v/v) was pipetted into the test tube. The solution was vortexed until the lipid was completely dissolved. Then 25 µL of ammonium thiocyanate solution was added and vortexed for 2-4 sec. It was followed by 25 µL of iron (II) chloride solution was added and vortexed for 2-4 sec. The test tubes were left under dark for 5 min to complete the reaction. Then the samples were ran at 500 nm in spectrophotometer. Blank containing no lipid sample was also created and measured in the spectrophotometer before every sample.

Statistical Analysis

Analysis of variance (ANOVA) and multiple comparison (Tukey's HSD test) at $\alpha = 0.05$ was carried out using JMP Pro 13.0 (©2016 SAS Institute Inc., Cary, NC). Lipids are not deposited into fish fillet uniformly, therefore, 2 tilapia fillet were pooled to create homogenous sample. Total lipids per 100 grams of fillet, and fatty acid compositions were quantified as % oil (w/w) and analyzed for significance. Long chain PUFA were quantified as mg/g oil using IS, and analyzed for significance. Standard linear regression was used to analyze the slope and the p-value of the time effect on the fatty acids. Peroxide values as meq/kg were also tested for significance.

Results and Discussion

Fatty Acid Composition of Tilapia Fillet Lipids

The lipid extracted from experimental diets fed tilapia fillets ranged between 0.53 to 1.745 g per 100 g fillet, on average. Only samples within -20 ^{o}C cold storage over 3 months had significant differences in total lipid extracted from tilapia fillet at p-value of < 0.001 (Table 4). There were also significant differences within the storage duration and conditions when individual groups of diets were analyzed. Tilapia fed diets SFO100, SFOAO15, SFOAO85, SFOFO85, SOAO15, SOAO85, SOFO15, and SOFO85 had p-values less than 0.05 (Table 9). Although the differences within the diets were significant there were no observable patterns. Also the interaction effect of storage duration and temperature conditions were analyzed under two factor experimental design; and results indicated no statistical significance of interaction was observed. Rate of autoxidation, dependent on the presence of oxygen radicals, temperature, and duration, would indicate 9 month storage would have greater degradation of lipid, and temperature abused conditions would have more effect on the autoxidation of lipid. However, these were not observed.

Diets did not differ in other ingredients except for the different levels of SO, HOSFO, FO, and AO (Table 1). HT diet had addition of 200 mg/kg of aTOC. HT diet was designed to determine whether the addition of aTOC retards lipid autoxidation during the storage; because aTOC is a predominant form of TOC in muscle tissue and has been associated to have better radical scavenging activities in the biological systems than other tocopherols (Kim and Min 2008; Brown and others 2012). For the rest of the diets, antioxidants were added in the diets to improve the stability of the feeds during the storage and the growth trial. The fillets did not yield discernable

differences in its fatty composition resulting from the storage duration and conditions (Figure 3, 4, 5, 6, & 7). Hydroperoxide is a product of autoxidation by oxygen radicals (Belitz and others 2009). Lipids can be oxidized to produce secondary oxidation product that can result in off-flavors and off textures in foods (Kamal-Eldin and others 2003). Therefore, minimizing lipid autoxidation is important to the product quality. Not only that, the oxidation of long chain PUFA renders the bioactivity of those fatty acids negatively; which means that enhancing diets to improve LCn3 can be undermined by the lipid autoxidation as observed in highly oxidized cod liver oil (Fisher and Wishner 1968). In salmon and trout, concentration of antioxidants in the feed had positive correlation within the fish fillet (Berge and Lie 1998; Yildiz and others 2006). Fish are able to store antioxidant in its muscle tissues that can aid in prevention of LCn3 autoxidation in fillet lipid.

For instance, OA in SFOAO15 diet fed tilapia fillet, which had highest levels of OA on average, were 195.2, 219.64, 239.85, 208.58, 229.28, 198.48, and 212.76 mg/g oil for month 0, month 3 and temperature abused, month 6 and temperature abused, month 9 and temperature abused, month 3 and -20^{o}C storage, month 6 and -20^{o}C storage, and month 9 and -20^{o}C storage, respectively (Figure 3). Considering oxidative stability is more stable compared to long chain PUFA, OA stability was not surprising. However, other LCn3 also exhibited no significance. ARA in CD diet fed tilapia fillet, which had highest levels of ARA on average, were 19.76, 20.66, 18.90, 23.70, 24.76, 16.55, and 22.79 mg/g oil for month 0, month 3 and temperature abused, month 6 and temperature abused, month 9 and temperature abused, month 3 and -20^{o}C storage, month 6 and -20^{o}C storage, and month 9 and -20^{o}C storage, respectively (Figure 4). EPA in SOFO85 diet fed tilapia fillet, which had highest levels of EPA on average, were 11.56, 1077, 5.05, 10.30, 10.41, 9.68, and 12.01 mg/g oil for month 0, month 3 and temperature abused, month 6 and temperature abused, month 9 and temperature abused, month 3 and -20^{o}C storage, month 6 and -20^{o}C storage,

and month 9 and -20°C storage, respectively (Figure 5). DPA in SOFO85 diet fed tilapia fillet, which had highest levels of DPA on average, were 24.83, 22.68, 11.07, 21.13, 19.52, 20.47, and 22.91 mg/g oil for month 0, month 3 and temperature abused, month 6 and temperature abused, month 9 and temperature abused, month 3 and -20°C storage, month 6 and -20°C storage, and month 9 and -20°C storage, respectively (Figure 6). DHA in SOAO85 fed tilapia fillet, which contained highest levels of DHA on average, were 94.32, 103.71, 112.82, 82.75, 110.76, 132.10, and 135.59 mg/g oil for month 0, month 3 and temperature abused, month 6 and temperature abused, month 9 and temperature abused, month 3 and -20°C storage, month 6 and -20°C storage, and month 9 and -20°C storage, respectively (Figure 7).

The data was also analyzed using standard linear regression for the time effect (Table 11). For the temperature abused samples, the significant pattern was observed in OA with slope of 2.38 (HT) and -3.43 (SOFO85); ARA with slope of 0.29 (SFOAO15) and 0.72 (SOAO85); EPA with slope of 0.22 (SOAO85); DPA with slope of -1.23 (SFOAO85), and 0.73 (SFOFO85); and DHA with slope of 6.14 (SFOAO85) and 4.05 (SOAO85). For the -20°C stored samples, the significant pattern was observed in OA with slope of -5.89 (SFOFO15); ARA with slope of 0.6 (SOFO85); EPA with slope of -0.55 (SFOAO85) and 0.18 (SOAO85); DPA with slope of -1.23 (SFOAO85) and 0.73 (SFOFO85); and DHA with slope of 6.14 (SFOAO85) and 4.05 (SOAO85). Although significant patterns were observed in the change of fatty acids in tilapia fillets, the negative slope was what we were interested in. Positive slope means that there was significant increase in fatty acids from month 0 to 9. However, metabolism of these fatty acids in frozen fillet cannot occur, which means the fish to fish variation caused these change in fatty acids found in the tilapia fillets. The possible reason for this trend would be due to the fact that water binding capacity of fillet change over storage period (Ng 2009). Therefore, when fillets were thawed, weight of the fillet

could have be affected by the loss of moisture and resulted in this trend. Negative slope indicated that the fatty acids degraded over time. Overall, the logical trend was not observed. For instance, SFOAO85 diet fed tilapia fillets exhibited both negative (-0.55 for EPA and -1.23 for DPA) and positive (6.14 for DHA) that were statistically significant. Because EPA, DPA, and DHA are all LCn3 with highly oxidative methylene interrupted double bonds, trend is expected to be have similar patterns. This was not the case for many tilapia fillets analyzed. Thus, the pattern cannot be determined from the analysis. Therefore, although slopes with significant patterns were observed in some diets, we cannot conclusively state that degradation of OA, ARA, EPA, DPA, or DHA occurred in any of the diets.

The results from total lipid, fatty acids grouped by either the diets or the storage conditions all indicated no significant patterns were present. Expected degradation of fatty acids over time was not observed nor did temperature abused samples had stronger impact on the lipid autoxidation compared to the -20^{o}C stored samples.

Peroxide Value

There were no observable patterns associated with peroxide value (Figure 2). Also looking at the specific fatty acids concentration, OA, ARA, EPA, DPA, and DHA showed no significant patterns (Figure 3-7). Research looking at the effect of TOC and AP in cod liver oil saw mixture of TOC and AP added cod liver oil had significantly lower levels of peroxide value compared to the no added antioxidants or TOC only added cod liver oils over 14 weeks of storage at 25 ^{o}C (Olsen and others 2005). This could indicate why tilapia fillets did not have significant increases in peroxide values over 9 months of storage. Also under frozen storage conditions, spontaneous

decomposition cannot happen due to the bond dissociation energies; and requires metal ions, such as heme and nonheme iron (Erickson 1997).

Tilapia fillet lipids were analyzed to determine whether storage condition had an effect on the autoxidation of lipids (Table 10). Few diets had significant differences in peroxide values. However, the importance is whether there were patterns or not. Further analysis using Tukey's HSD determined that there were no statistical significance due to the storage duration or the storage conditions. Peroxide value showed that -20^{o}C cold storage did not perform better than temperature abused conditions. It was known that inclusion of antioxidants can significantly enhance shelf life in the fish oil (Hamilton 2009). However, it does not determine the impact of antioxidants on the fish fillet quality. In another study, tilapia fillet hamburger with addition of α-tocopherol in the diet and/or filleted products had significantly reduced production of malondialdehyde, a secondary oxidative product, over 90 days of -18 ^{o}C cold storage; albeit 200 mg of dietary tocopherol addition performed the best regardless of aTOC addition on filleted products (dos Santos Fogaça and Sant'Ana 2007). Experimental diet in this study contained 50 mg/kg TOC, 10 mg/kg AP, and 200 mg/kg aTOC (just for HT diet) in comparison to the previous studies that used 0, 100, and 200 mg of aTOC additions in the diets. Contrary to some of the previous studies with different fish species, that saw initial and minimum temperature had remarkable impact on the rate of lipid autoxidation, the experiment saw good prevention of lipid autoxidation even at -10 ^{o}C cold storage (Hwang and Regenstein 1989; Bilinski and others 1981). The storage duration showed that 9 months of storage did not affect lipid autoxidation any more than 0, 3, or 6 months stored samples. Therefore, proper vacuum packaging under subdued light and sufficient amount of antioxidants in the feeds could have had much more significant impact in retarding lipid autoxidation; thus further investigation

must be conducted to determine the single effect and the interaction effect of vacuum packaging, presence of light, and antioxidants in the feed.

The results of insignificant level of lipid autoxidation was not expected, but regardless gives us insight to the stability of the tilapia fillets under the various conditions. The autoxidized products in tilapia fillet can be the source of oxidative radicals in humans when consumed (Erickson 1997). Therefore, regardless of the diets, tilapia fillet lipids strongly resisted autoxidation over long period of time. This indicates, tilapia can be a great product for the consumers even after long periods of frozen storage.

Conclusion

There were initial concerns with stability of PUFA during long term storage. The diets were designed to create high quality tilapia through increase in LCn3. As the addition involved increase in feed cost associated with more expensive raw ingredients, it was extremely important to identify the lipid autoxidation and determine the availability of LCn3 when consumers receive them. The standard procedures of including antioxidants in the feeds and proper vacuum packaging were able to create an oxygen free environment that preserved the lipid quality of the tilapia fillets. Therefore, although the experiment did not observe differences in autoxidation of the fatty acids, experiment was able to determine that the addition of antioxidants, proper vacuum packaging under subdued light conditions created a stable environment for the tilapia fillet up to 9 months of storage.

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Chapter 6. Conclusion and Future Direction

The current research had three objectives. One was to determine the lipid quality and fatty acid composition of tilapia fillets available to consumers. Consumers have been subjected to negative images of tilapia which could have had, in part, impacted the reduction of tilapia consumption seen recently. The supermarket samples have been found that the fatty acid quality of tilapia was excellent in US produced samples. In fact, *n*-6:*n*-3 fatty acid ratio was highly desirable compared to what American consumers are currently exposed to in their regular diets. Although the tilapia products contained good levels of LCn3 and low levels of ARA, value-added modification could bring healthier options to the consumers that seek higher quality products.

Therefore, the second objective was to further improve the lipid quality and fatty acid composition in order to create value added premium tilapia. Because tilapia are considered to be an inexpensive product, improving fatty acid composition could create value-added products that gives consumers healthy options. The results from the research indicated that the algae oil and the fish oil improved LCn3 while high oleic sunflower oil was able to provide increased level of oleic acid (18:1*n*-9) while reduced level of linoleic acid (18:2*n*-6) in tilapia fillets. Therefore, creating value added premium tilapia for consumers that demand nutritionally higher quality products was achieved.

The third objective was to determine the impact of long term storage on the lipid autoxidation. Because these raw ingredients increased the production cost, value addition from the oil sources have to be maximized when consumers receives the product. Nine months storage have shown that the consumers are able to receive high quality product with minimal lipid autoxidation.

This means the consumers get tilapia fillet without quality degradation from oxidative products, and reduction of healthy LCn3.

This research also suggested that much more research needs to be conducted to better understand the rate of fatty acid deposition in tilapia fillet from the diets. As the ingredient costs for feed productions are high, more efficient usage of the raw ingredients can better serve the producers by allowing them to create economically friendly value added tilapia products. Also, understanding the impact of these oils on tilapia gut health and physiology can better improve diet formulation without compromising fish health. As third objective proved, lipid autoxidation is highly resisted over 9 months in various temperatures under vacuum package. However, further evaluation of unfrozen fillet's lipid autoxidation rate can provide better answer for the shelf life of tilapia fillet. Better understanding of TOC and AP in the diet's effect on the tilapia fillet could provide insight into studying storage conditions not only in tilapia, but also in different seafood products as well. Lastly, sensory analysis of long term stored tilapia fillet can help determine if there are change in overall characteristics during the long term storage. Although fatty acid composition did not change and the deterioration of LCn3 did not occur, there can be differences in preference affected by different diets.

Appendix A. Tables and Figures for Evaluation of Lipid Quality and Fatty Acid Composition of Tilapia, *Oreochromis* spp., Fillets Available in US Supermarkets

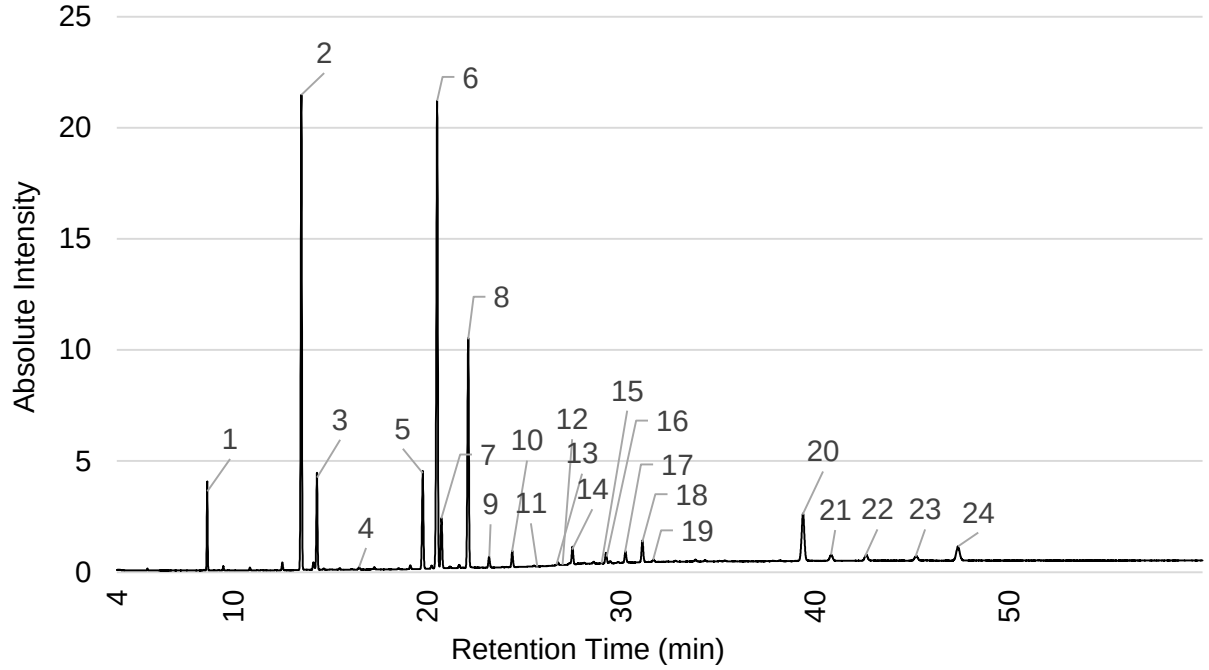

Figure 1. Average Chromatogram of FAME Samples in GC/MS Analysis

Typical GC profile of FAME obtained from the extracted lipid (1=14:0, 2=16:0, 3=16:1*n*-7; 4=17:0, 5=18:0, 6=18:1*n*-9; 7=18:1*n*-7, 8=18:2*n*-6, 9=18:3*n*-6, 10=18:3*n*-3, 11=18:4*n*-3, 12=20:0, 13=20:1*n*-9, 14=20:2*n*-6, 15=20:3*n*-3, 16=20:4*n*-6, 17=20:4*n*-3, 18=20:5*n*-6, 19=20:5*n*-*3*, 20=23:0 (Internal Standard), 21=22:4*n*-6, 22=22:5*n*-6, 23=22:5*n*-3, and 24=22:6*n*-3.

Table 1. Total Identifiable Fatty Acid Composition (% oil) by COOs

	China	Southeast Asia	Central America	South America	USA	P-value	Pooled error
12:0	0.30	0.63	0.63	0.34	0.34	0.0683	0.069
14:0	2.5^{B}	2.6^{AB}	3.2^{A}	2.3^{B}	2.7^{AB}	0.0383	0.11
15:0	0.47^{A}	0.14^{B}	0.16^{B}	0.12^{B}	0.43^{AB}	0.0043	0.045
16:0	23	23	24	23	24	0.1625	0.29
16:1*n*-7	0.53	0.53	0.55	0.54	0.53	0.978	0.012
17:0	0.44^{AB}	0.17^{B}	0.15^{B}	0.15^{B}	0.86^{A}	0.0018	0.053
17:1*n*-8	0.17	0.12	0.10	0.11	0.25	0.0917	0.017
18:0	6.3	6.4	6.3	6.7	7.0	0.3727	0.13
18:1*n*-11	0.2	0.21	0.19	0.19	0.17	0.1604	0.010
18:1*n*-9	25^{B}	33^{A}	29^{AB}	28^{AB}	14^{C}	<0.0001	0.62
18:1*n*-7	3.8^{B}	2.9^{C}	3.0^{C}	3.2^{BC}	4.3^{A}	<0.0001	0.067
18:2*n*-6	16^{A}	13^{AB}	15^{A}	15^{A}	7.5^{B}	0.0013	0.56
18:3*n*-6	0.94^{A}	0.78^{AB}	0.69^{B}	0.71^{B}	0.68^{B}	<0.0001	0.024
18:3*n*-3	2.0^{A}	0.73^{C}	0.98^{BC}	0.76^{C}	2.0^{AB}	<0.0001	0.11
20:0	0.2^{A}	0.21^{AB}	0.16^{B}	0.29^{AB}	0.15^{B}	0.0008	0.006
20:1*n*-9	1.0^{C}	1.6^{A}	1.2^{BC}	1.5^{AB}	0.56^{D}	<0.0001	0.042
20:2*n*-6	0.79^{A}	0.68^{AB}	0.75^{A}	0.89^{A}	0.44^{B}	0.0027	0.029
20:3*n*-6	0.13^{B}	0.24^{A}	0.23^{A}	0.28^{A}	0.15^{AB}	<0.0001	0.012
20:3*n*-3	0.92	0.87	0.84	1.1	0.97	0.1134	0.028
20:4*n*-6	2.3^{BC}	1.8^{BC}	1.7^{C}	2.8^{AB}	4.0^{A}	<0.0001	0.13
20:4*n*-3	0.21^{A}	0.03^{B}	0.029^{B}	0.012^{B}	0.36^{A}	0.0004	0.026
20:5*n*-3	0.36^{B}	0.07^{C}	0.11^{BC}	0.1^{BC}	1.3^{A}	<0.0001	0.042
22:0	0.13^{A}	0.098^{B}	0.099^{B}	0.1^{AB}	0.11^{AB}	0.0066	0.005
22:4*n*-6	0.79	0.86	0.72	0.96	1.2	0.0828	0.045
22:5*n*-6	1.2^{AB}	0.99^{AB}	0.79^{B}	1.6^{A}	1.2^{AB}	0.0242	0.083
22:5*n*-3	0.89^{B}	0.43^{B}	0.47^{B}	0.48^{B}	3.9^{A}	<0.0001	0.072
22:6*n*-3	2.8^{B}	1.8^{C}	2.6^{BC}	2.9^{BC}	8.8^{A}	<0.0001	0.22

Fatty acids as % oil; Fatty acids less than 0.01% of FAC were excluded from the table; superscript letters indicate significantly different by Tukey's HSD test ($P<0.05$) within a row.

Table 2. Total Lipid (g per 100 g fillet w/w) and Composition of Fatty Acids (% oil) in Tilapia Fillet by COOs

	China	Southeast Asia	Central America	South America	USA	P-value	Pooled error
Total Lipid	2.0^{AB}	2.9^{A}	0.27^{AB}	2.1^{AB}	1.1^{B}	0.014	0.16
Total SFA	33	3	35	33	35	0.283	0.46
Total MUFA	31^{B}	36^{A}	34^{AB}	34^{AB}	20^{C}	<0.0001	0.63
Total *n*-6	26^{A}	22^{B}	24^{AB}	26^{AB}	22^{AB}	0.0074	0.51
Total *n*-3	7.2^{B}	3.9^{C}	5.0^{BC}	5.3^{BC}	17^{A}	<0.0001	0.38
n-6:*n*-3 ratios	4.3^{B}	6.6^{A}	4.9^{AB}	5.3^{AB}	1.3^{C}	<0.0001	0.27

Total lipid is measured in grams of crude lipid per 100 g fillet (w/w); Fatty acids are measured as % oil; superscript letters indicate significantly different by Tukey's HSD test ($P<0.05$) within a row.

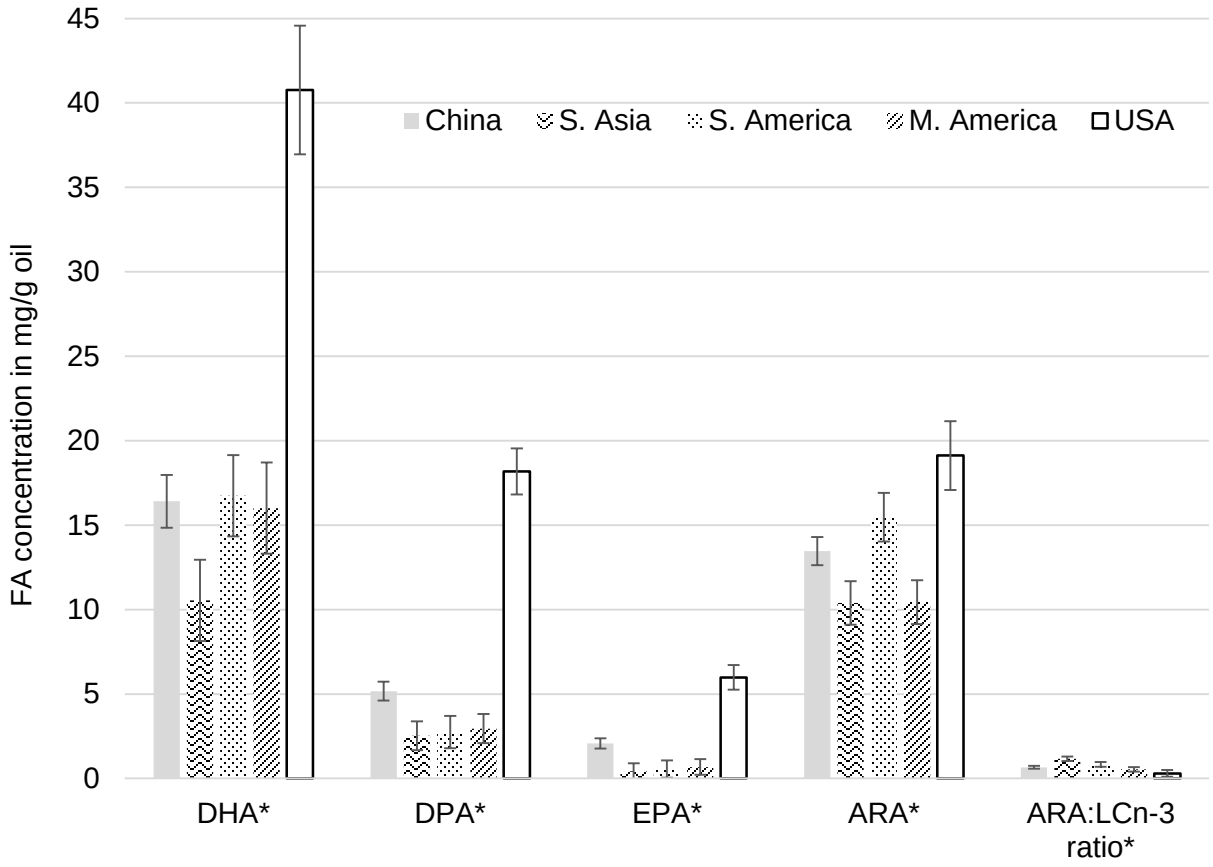

Figure 2. Concentration of long chain polyunsaturated fatty acids of COOs.

Internal standard (C23:0) to calculate mg/g oil. ARA:LCn3 ratio involves ARA compared to sum of DHA, DPA, and EPA. * signifies statistical significance at $\alpha=0.05$. Error bar represents standard error for each fatty acids COOs.

Appendix B. Tables and Figures for Impact of Enhanced Long Chain n-3 PUFA and Oleic Acid Diets in Tilapia Fillets

Table 1. % Body Weight per Day Used for Weekly Feed Calculation

WEEKS	%BW/DAY
0	3.5
1	3
2	3.25
3	2.5
4	2.15
5	2
6	1.9
7	1.8
8	1.6

Table 2. The Average Water Quality Parameters over 8 Week Growth Trial in RAS

	# OF MEASUREMENTS	SYS. AVG (±SE)
Temperature (ºC)	n = 64	29.3 ±0.45
pH	n = 12	7.8 ±0.07
Alkalinity (mg/L)	n = 64	141.1 ±14.4
TAN (ppm)	n = 33	0.44 ±0.10
Nitrite (ppm)	n = 33	0.14 ±0.042
Nitrate (ppm)	n = 17	16.7 ±3.5
DO (ppm)	n = 64	4.8 ±0.29

Sys. Avg = Average value of RAS over 8 weeks with standard error. n = data point for each RAS.

Table 3. Total Fish Grow-out Parameters per Fish Tank based on the Diets Consumed

DIET	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
FINAL AVG. WEIGHT PER FISH (in g)	578	561	531	582	570	553	582	560	544	505	590	559	0.46	6.82
FCR	1.43	1.46	1.6	1.35	1.49	1.48	1.4	1.5	1.51	1.74	1.41	1.41	0.40	0.028
SURVIVAL RATE (%)	100	100	100	100	100	98	100	100	100	98	100	100		

*FCR (Feed Conversion Ratio) was calculated by dividing total feed given over total weight gain over 8 weeks.

Table 4. Pelleted Diet Formulation of 11 Enhanced Diet Strategies in percent

INGREDIENT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	HT
SOYBEAN MEAL	58.5	58.5	58.5	58.5	58.5	58.5	58.5	58.5	58.5	58.5	58.5
WHEAT	25.794	25.794	25.794	25.794	25.794	25.794	25.794	25.794	25.794	25.794	25.774
MEAT AND BONE MEAL	3	3	3	3	3	3	3	3	3	3	3
FISH MEAL	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
SO	0	0	0	0	0	5	0.75	4.25	0.75	4.25	0
HO-SFO	5	0.75	4.25	0.75	4.25	0	0	0	0	0	0.75
FO	0	0	0	4.25	0.75	0	0	0	4.25	0.75	4.25
AO	0	4.25	0.75	0	0	0	4.25	0.75	0	0	0
VITAMIN PREMIX	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
MINERAL PREMIX	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
VIT. E. (TOCOPHEROL)	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
A-TOCOPHEROL	0	0	0	0	0	0	0	0	0	0	0.02
ASCORBYL PALMITATE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
CMC	1	1	1	1	1	1	1	1	1	1	1
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 5. Proximate Analysis of Diets in percent

DIETS	MOISTURE (%)	DRY MATTER (%)	CRUDE PROTEIN (DW)	CRUDE FAT (DW)	FIBER ACID DETERGENT (DW)	ASH (DW)	TOTAL DIGESTIBLE NUTRITION (DW)
CD	9.11	90.89	38.30	3.88	10.20	8.01	77.30
HT	9.09	90.91	41.70	7.71	7.45	7.39	82.35
SFO100	9.50	90.50	41.30	7.59	9.15	7.61	81.55
SFOAO15	9.10	90.90	40.55	6.94	5.90	7.51	82.05
SFOAO85	10.36	89.65	40.60	7.68	7.95	7.29	84.95
SFOFO15	11.34	88.67	40.70	7.67	5.95	7.33	83.00
SFOFO85	7.86	92.14	41.30	7.34	9.15	7.43	81.40
SO100	8.68	91.32	42.20	6.37	6.20	7.17	81.40
SOAO15	9.49	90.52	40.75	7.37	5.65	7.19	84.40
SOAO85	12.51	87.49	42.95	8.10	8.90	7.36	82.10
SOFO15	12.06	87.95	41.35	9.75	6.75	7.21	85.05
SOFO85	11.87	88.13	42.70	7.45	4.50	7.22	83.00

Moisture and dry matter was measured as is and the rest were measured in dry weight basis.

Table 6. Lipid Concentration (% fillet w/w) and Fatty Acid Composition (% oil and mg/g oil) of the Experimental Diets

	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
total lipid (% w/w)	9.93	6.97	10.8	7.22	7.63	8.45	7.25	9.92	9.75	8.15	7.20	7.80	0.5772	0.41
% lipid														
n-6:n-3	2.36	1.07	7.57	5.39	0.67	6.52	0.89	4.80	3.10	0.73	6.42	1.06	0.0123	0.38
18:0	3.89^{AB}	4.03^{A}	3.28^{CDE}	3.25^{DE}	2.95^{E}	3.15^{DE}	4.10^{A}	3.49^{BCD}	3.42^{CD}	2.95^{E}	3.71^{DE}	4.12^{A}	<0.0001	0.023
18:1n-9	22.9^{AB}	19.1^{AB}	60.4^{A}	52.9^{AB}	15.8^{B}	26.8^{AB}	18.9^{AB}	36.2^{AB}	32.5^{AB}	11.3^{B}	34.3^{AB}	14.5^{B}	0.0132	2.2
18:2n-6	23.4^{C}	16.8^{F}	19.1^{DE}	19.9^{D}	14.9^{G}	18.9^{DE}	14.2^{G}	35.1^{A}	32.4^{B}	17.7^{EF}	33.1^{B}	18.2^{EF}	<0.0001	0.085
18:3n-3	2.31^{AB}	2.54^{AB}	1.29^{B}	1.71^{B}	1.87^{B}	1.86^{B}	2.04^{AB}	6.15^{A}	5.42^{AB}	2.35^{AB}	2.78^{AB}	3.29^{AB}	0.0138	0.22
20:4n-6	0.41^{BC}	0.83^{A}	0.10^{D}	0.03^{D}	0.52^{B}	0.09^{D}	0.85^{A}	0.05^{D}	0.06^{D}	0.38^{BC}	0.22^{CD}	0.84^{A}	<0.0001	0.013
20:5n-3	3.77^{B}	7.21^{A}	0.53^{C}	0.58^{C}	1.04^{C}	0.77^{C}	7.57^{A}	0.53^{C}	0.62^{C}	0.99^{C}	1.63^{C}	7.29^{A}	<0.0001	0.065
22:5n-3	0.18^{BC}	0.63^{ABC}	0.04^{C}	0.19^{ABC}	0.04^{C}	0.14^{C}	1.29^{A}	0.04^{C}	0.08^{C}	0.09^{C}	0.23^{ABC}	1.27^{AB}	0.0032	0.057
22:6n-3	2.80^{BCD}	5.03^{B}	0.58^{D}	2.08^{BCD}	27.95^{A}	0.64^{D}	5.26^{B}	0.54^{D}	4.45^{BC}	28.32^{A}	1.24^{CD}	5.03^{B}	<0.0001	0.19
TOTAL SFA	26.4^{AB}	31.8^{A}	15.1^{C}	19.1^{C}	28.2^{A}	20.9^{BC}	32.9^{A}	17.0^{C}	19.5^{C}	29.1^{A}	20.3^{BC}	32.9^{A}	<0.0001	0.34
TOTAL MUFA	39.2^{C}	31.9^{E}	63.1^{A}	55.5^{B}	19.2^{G}	56.4^{B}	32.1^{E}	40.4^{C}	36.3^{D}	14.8^{H}	39.8^{C}	27.6^{F}	<0.0001	0.14
TOTAL n-3	10.1^{C}	17.3^{B}	2.6^{D}	4.7^{CD}	31.6^{A}	3.6^{D}	18.2^{B}	7.3^{CD}	10.8^{C}	32.3^{A}	6.3^{CD}	18.9^{B}	<0.0001	0.32
TOTAL n-6	24.1^{C}	18.4^{F}	19.3^{DEF}	20.7^{DE}	21.3^{D}	19.0^{EF}	16.1^{G}	35.2^{A}	33.3^{B}	23.7^{C}	33.5^{AB}	20.1^{DEF}	<0.0001	0.094
mg/g oil (using is)														
OLEIC	135^{BC}	129^{BC}	495^{A}	379^{AB}	106^{BC}	180^{BC}	123^{BC}	229^{ABC}	205^{ABC}	67.5^{C}	226^{ABC}	84.8^{BC}	0.0028	15
ARA	2.40^{CD}	5.57^{A}	0.85^{DEF}	0.20^{F}	3.48^{BC}	0.60^{DEF}	5.52^{A}	0.30^{EF}	0.39^{DEF}	2.29^{CDE}	1.45^{CDEF}	4.91^{AB}	<0.0001	1.0
EPA	22.2^{B}	48.6^{A}	4.37^{C}	4.13^{C}	6.97^{C}	5.19^{C}	49.4^{A}	3.33^{C}	3.90^{C}	5.97^{C}	10.8^{C}	42.7^{A}	<0.0001	0.55
DPA	1.12^{B}	4.11^{AB}	0.38^{B}	1.33^{AB}	0.27^{B}	0.95^{B}	8.40^{A}	0.24^{B}	0.46^{B}	0.55^{B}	1.47^{AB}	7.45^{AB}	0.0054	0.37
DHA	16.5^{B}	33.9^{B}	4.73^{B}	15.6^{B}	187^{A}	4.30^{B}	34.3^{B}	3.38^{B}	28.1^{B}	170.0^{A}	8.21^{B}	29.5^{B}	<0.0001	1.9
ARA: LCn3	0.06	0.06	0.09	0.027	0.018	0.029	0.060	0.038	0.012	0.013	0.071	0.062	0.0487	0.0045

*The statistical analysis was conducted using ANOVA at $\alpha=0.05$; superscript letters indicate post hoc analysis using Tukey's HSD.

Table 7. Lipid and Fatty Acid Composition of Tilapia Fillets Collected Prior to 8 week Growth Trial

	AVERAGE	STD. DEV
TOTAL LIPID (% w/w)	1.45	0.22
***n*-6:*n*-3**	2.09	1.34
% LIPID		
18:0	7.40	1.51
18:1 *n*-9 (OA)	20.03	2.43
18:2 *n*-6 (LA)	10.09	8.59
18:3 *n*-3 (ALA)	1.03	0.15
20:4 *n*-6 (ARA)	2.96	0.25
20:5 *n*-3 (EPA)	0.60	0.22
22:5 *n*-3 (DPA)	1.16	0.63
22:6 *n*-3 (DHA)	6.07	0.52
TOTAL SFA	34.74	2.18
TOTAL MUFA	30.09	4.21
TOTAL *n*-3	9.57	1.15
TOTAL *n*-6	19.6	11.4
mg/g oil		
OLEIC	129.2	15.8
ARA	19.3	3.64
EPA	4.0	1.61
DPA	7.8	4.44
DHA	40.1	10.5
ARA: LCn3	0.39	0.10

The sample size (n=3) were collected prior to the beginning of the growth trial; *n*-6:*n*-3 ratio includes all *n*-6 fatty acids and *n*-3 fatty acids (not listed on table 7); LC *n*-3 is sum of EPA, DPA, and DHA.

Table 8. Lipid Concentration and Fatty Acid Composition of Tilapia Fillet after 8 Week Growth Trial

	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85		
% w/w													p-value	Pooled error
TOTAL LIPID	1.46	1.32	1.49	1.75	1.70	1.46	1.61	1.29	1.75	1.70	1.46	1.24	0.5173	0.05
***n*-6: *n*-3**	2.79^{AB}	0.86^{C}	2.78^{AB}	1.52^{BC}	0.90^{C}	1.84^{BC}	1.32^{BC}	3.99^{A}	2.03^{BC}	0.88^{C}	2.24^{BC}	0.95^{C}	<0.001	0.086
% LIPID														
18:0	7.40^{AB}	8.07^{A}	6.40^{B}	6.32^{B}	7.25^{AB}	6.45^{B}	7.48^{AB}	6.88^{AB}	6.78^{AB}	7.18^{AB}	6.95^{AB}	6.62	0.017	0.078
18:1 *n*-9	22.1^{CDE}	17.4^{DE}	36.8^{A}	33.2^{AB}	18.8^{DE}	32.8^{AB}	19.4^{DE}	27.2^{BC}	23.3^{CD}	16.2^{E}	23.7^{CD}	18.2^{DE}	<0.001	0.36
18:2 *n*-6	17.4^{A}	10.4^{B}	12.1^{B}	10.8^{B}	9.9^{B}	10.9^{B}	10.0^{B}	17.1^{A}	17.0^{A}	11.1^{B}	16.4^{A}	11.3^{B}	<0.001	0.15
18:3 *n*-3	1.20^{C}	0.93^{CD}	0.63^{D}	0.64^{D}	0.89^{CD}	0.70^{D}	0.89^{CD}	2.01^{A}	1.97^{A}	1.02^{CD}	1.72^{AB}	1.29^{BC}	<0.001	0.023
20:4 *n*-6	3.25	3.18	3.23	2.33	2.65	2.76	2.30	2.46	2.62	2.07	3.16	2.13	0.0197	0.067
20:5 *n*-3	0.42^{B}	2.01^{A}	0.34^{B}	0.38^{B}	1.77^{A}	0.49^{B}	1.45^{A}	0.25^{B}	0.15^{B}	0.53^{B}	0.48^{B}	1.59^{A}	<0.001	0.036
22:5 *n*-3	1.32^{BC}	3.75^{A}	0.81^{BC}	0.85^{BC}	3.42^{A}	1.44^{BC}	2.38^{AB}	0.60^{C}	0.77^{BC}	1.07^{BC}	1.48^{BC}	3.41^{A}	<0.001	0.088
22:6 *n*-3	5.59^{CD}	11.80^{AB}	5.02^{CD}	9.03^{BC}	10.10^{BC}	6.31^{BCD}	6.62^{BCD}	2.83^{D}	8.24^{BCD}	17.68^{A}	6.22^{BCD}	9.36^{BC}	<0.001	0.34
TOTAL SFA	32.7	35.3	28.2	28.8	34.7	28.9	38.4	32.6	33.9	33.5	31.1	30.9	0.0494	0.53
TOTAL MUFA	32.7^{CD}	27.8^{DE}	44.7^{A}	40.8^{ABC}	29.7^{DE}	41.3^{AB}	30.9^{DE}	35.7^{BCD}	30.5^{ABC}	24.0^{E}	32.6^{CD}	29.6^{DE}	<0.001	0.42
TOTAL *n*-3	8.89^{Cd}	19.09^{AB}	6.95^{D}	10.93^{BCD}	16.82^{ABC}	9.22^{CD}	12.0^{BCD}	6.05^{D}	11.2^{BCD}	20.6^{A}	10.2^{BC}	16.4^{ABC}	0.0002	0.42
TOTAL *n*-6	24.7^{A}	16.3^{C}	19.1^{BC}	16.5^{C}	15.0^{C}	16.9^{C}	14.4^{C}	22.7^{AB}	22.3^{AB}	18.2^{BC}	22.9^{AB}	15.5^{C}	<0.001	0.24
mg/g oil														
OLEIC	133.9^{AB}	94.4^{B}	222.0^{AB}	195.2^{AB}	97.1^{B}	194.7^{AB}	160.0^{AB}	254.9^{A}	137.7^{AB}	87.2^{B}	142.3^{AB}	132.8^{AB}	0.0156	7.7
ARA	19.8^{AB}	17.3^{AB}	19.5^{AB}	13.6^{AB}	13.7^{AB}	16.6^{AB}	17.9^{AB}	22.5^{A}	15.3^{AB}	11.0^{B}	18.9^{AB}	15.5^{AB}	0.0589	0.57
EPA	2.53^{B}	10.9^{A}	2.05^{B}	2.21^{B}	9.14^{A}	2.87^{B}	11.0^{A}	2.10^{B}	0.82^{B}	2.86^{B}	2.88^{B}	11.6^{A}	<0.001	0.18
DPA	8.01^{CDE}	20.3^{B}	4.85^{DE}	4.97^{DE}	17.7^{B}	8.52^{CD}	16.6^{B}	4.91^{DE}	4.51^{E}	5.74^{CDE}	8.88^{C}	24.8^{A}	<0.001	0.20
DHA	34.0^{CD}	64.1^{B}	30.2^{CD}	52.6^{BC}	51.9^{BC}	37.4^{CD}	46.8^{BC}	23.8^{D}	48.1^{BC}	94.3^{A}	37.3^{CD}	68.1^{B}	<0.001	1.2
ARA: LCn3	0.44^{AB}	0.18^{B}	0.52^{AB}	0.23^{B}	0.17^{B}	0.34^{AB}	0.24^{B}	0.73^{A}	0.29^{B}	0.11^{B}	0.39^{AB}	0.15^{B}	0.0042	0.025

*The statistical analysis was conducted using ANOVA at α=0.05; superscript letters represents post hoc analysis using Tukey's HSD.

Table 9. The randomized distribution of diets to each tanks in RAS.

	RAS 1	**RAS 2**	**RAS 3**	**RAS 4**
Tank 1	SFO100	SOAO20	SOFO15	SFOAO85
Tank 2	CD	SFOFO85	SFOFO85	SFOAO15
Tank 3	SOFO85	SFOAO15	SFO100	SOFO85
Tank 4	SFOFO15	SOFO15	CD	SOAO15
Tank 5	SFOAO85	SO100	SO100	SFOFO15
Tank 6	SOAO85	HT	SOAO85	HT

Simple random sampling was used to randomize the diet distribution. RAS were blocked into RAS 1 + 2 and RAS 3 + 4.

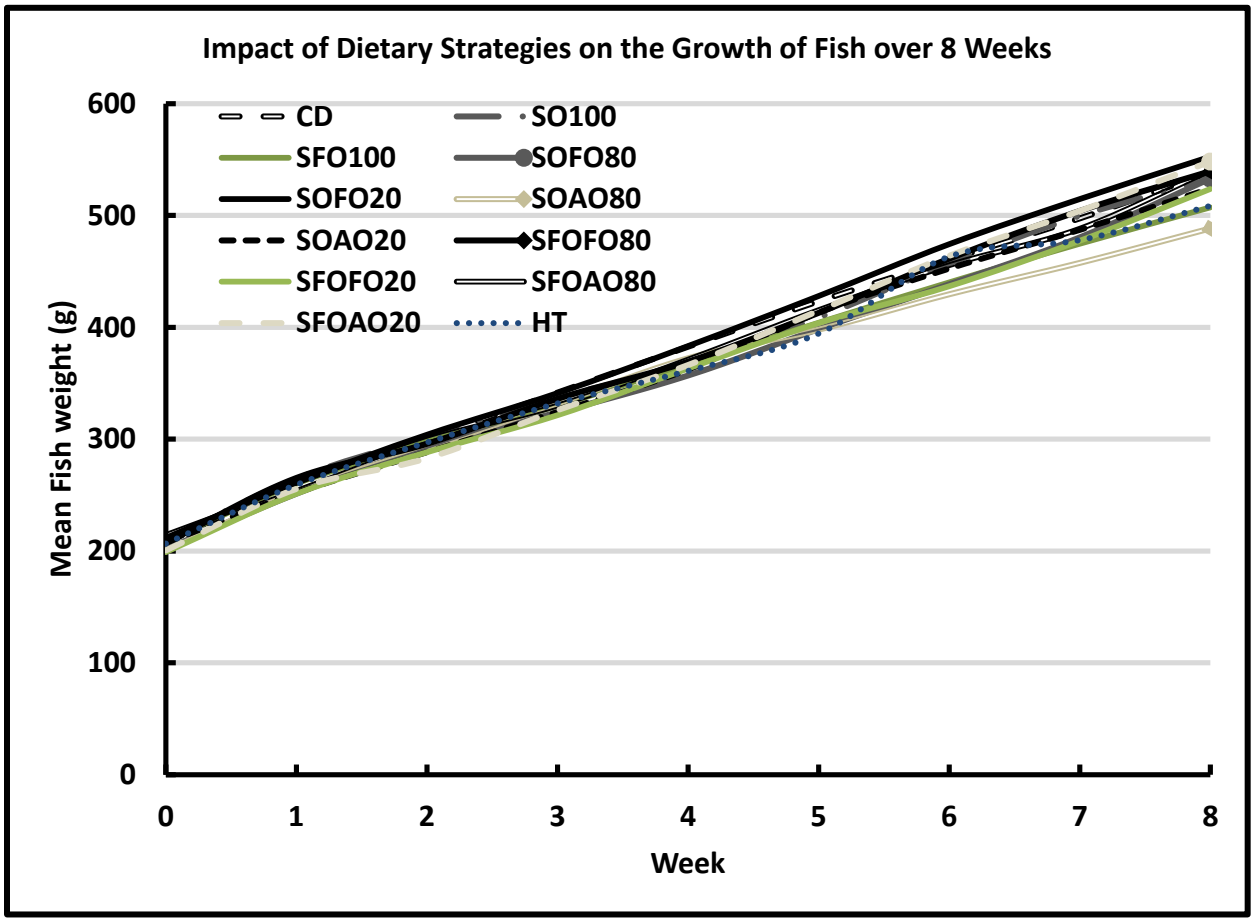

Figure 1. Mean fish weight per diet during the 8 week growth trial. Statistical significance was not observed at $\alpha = 0.05$.

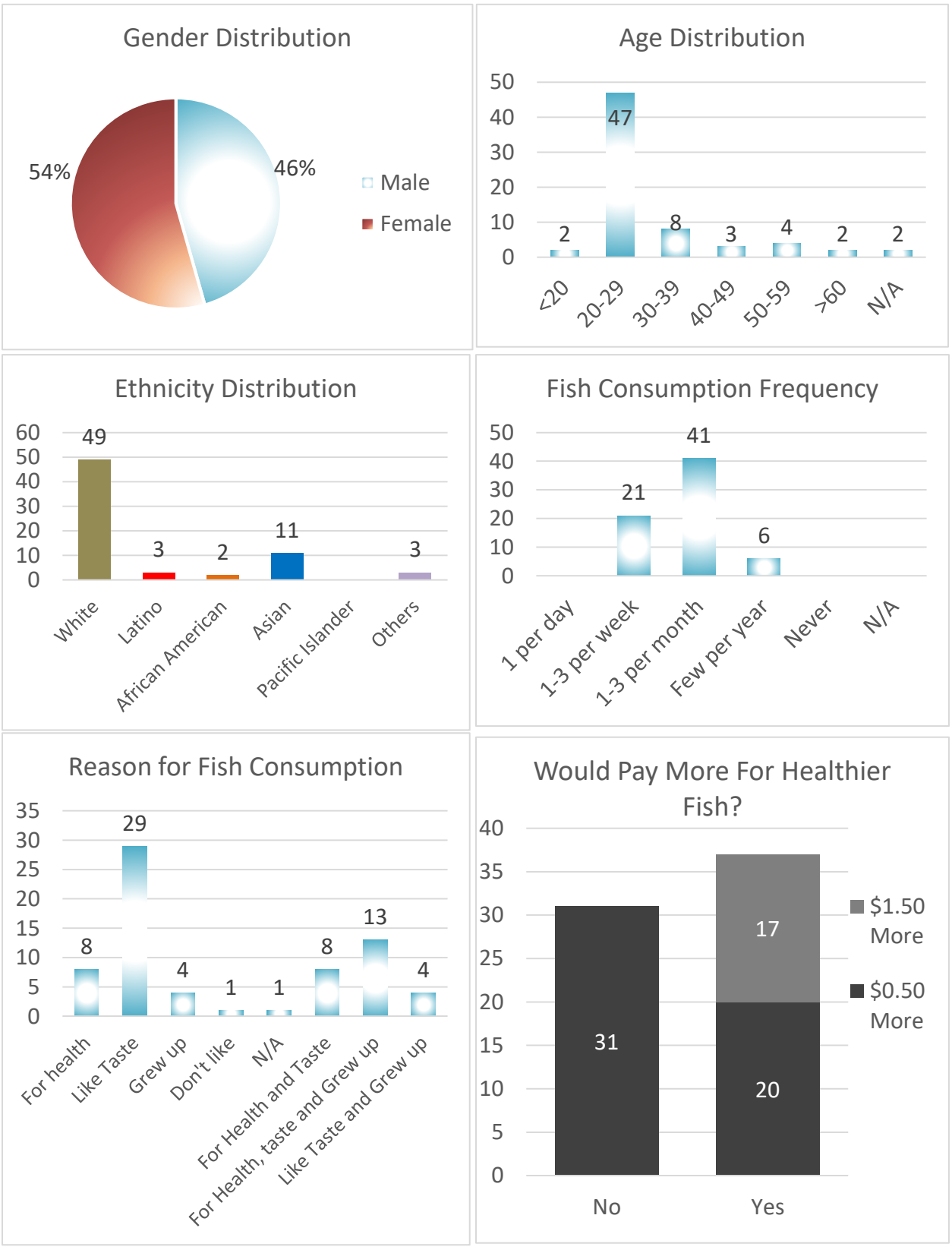

Figure 2. Demographic and Survey Questions Answered by Participants (n=68)

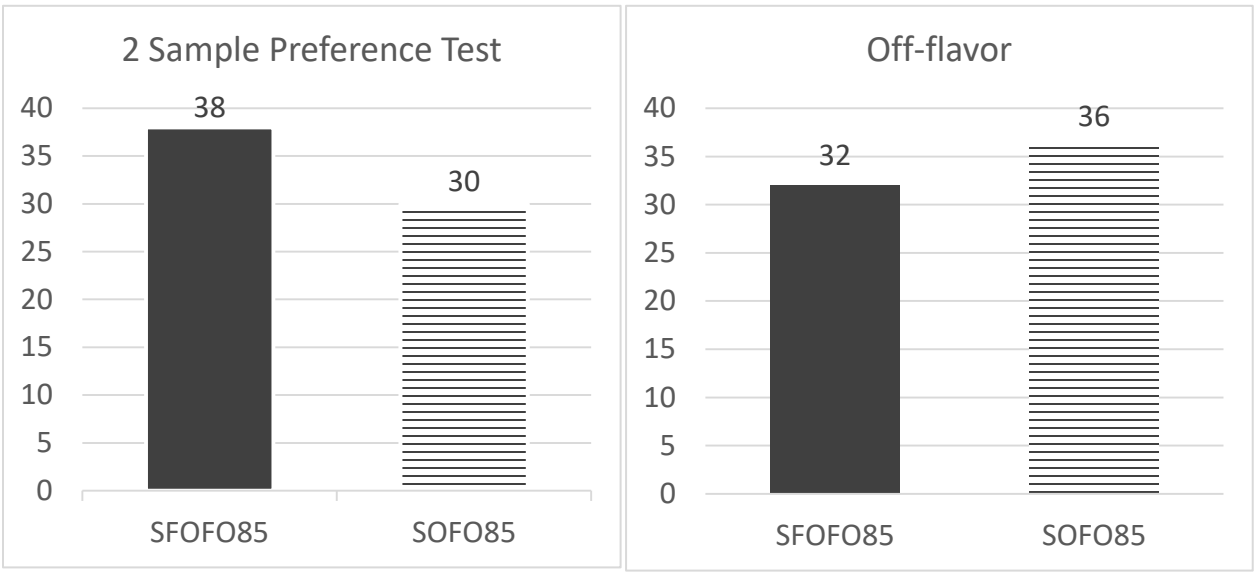

Figure 3. Sensory Analysis Result of 2-Sample Preference Test and the Detection of Off-flavor; values on top represents the number of participants (n=68)

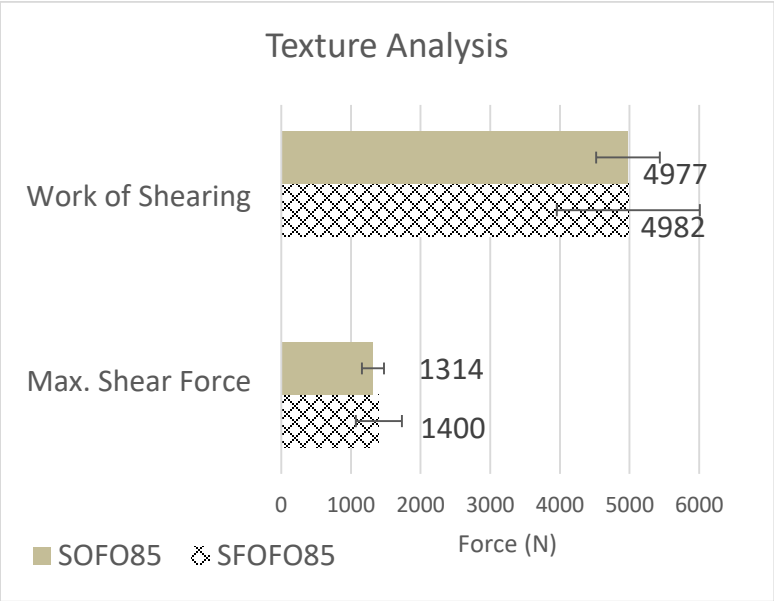

Figure 4. Texture Analysis of Oven Cooked Tilapia Fillet (n=4) from SOFO85 and SFOFO85 Diets Fed Fish.

Appendix C. Institutional Review Board Approval Letter

VirginiaTech

Office of Research Compliance
Institutional Review Board
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
540/231-4606 Fax 540/231-0959
email irb@vt.edu
website http://www.irb.vt.edu

MEMORANDUM

DATE: March 30, 2016

TO: Sean O'Keefe, Susan E Duncan, Kayla Moberg, Hyun Sik Chu, David D Kuhn, Tyler Robert Jeffery Stoneham

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires January 29, 2021)

PROTOCOL TITLE: Evaluation of Consumer Acceptability of Tilapia, Orechromis sp., fillet fed n-9 Fatty Acid Enhanced Diet

IRB NUMBER: 16-317

Effective March 30, 2016, the Virginia Tech Institution Review Board (IRB) Chair, David M Moore, approved the New Application request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

http://www.irb.vt.edu/pages/responsibilities.htm

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As:	**Exempt, under 45 CFR 46.110 category(ies) 2,6**
Protocol Approval Date:	**March 30, 2016**
Protocol Expiration Date:	**N/A**
Continuing Review Due Date*:	**N/A**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

Date*	OSP Number	Sponsor	Grant Comparison Conducted?

* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

Appendix D. Institutional Animal Care and Use Committee Approval Letter

VirginiaTech

Office of Research Compliance
Institutional Animal Care and Use Committee
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
540/231-2166 Fax 540/231-0959
email iacuc@vt.edu
website http://www.researchcompliance.vt.edu/iacuc

MEMORANDUM

DATE: January 4, 2016

TO: David D Kuhn

FROM: Virginia Tech Institutional Animal Care and Use Committee

IACUC NUMBER: **15-155 (FST) (New Application)**

TITLE: Long Chain n-3 PUFA and Oleic Monounsaturated Fatty Acids Improved Dietary Strategies to Enhance Fillet Quality in Tilapia, Oreochromis sp.

SUBJECT: Review of Research Protocol Involving Animals

The purpose of this memo is to verify that, on January 3, 2016, the Virginia Tech Institutional Animal Care and Use Committee (IACUC) reviewed and granted approval of the above described Protocol submission.

Period of Protocol Approval

This Research Protocol is approved for the following period:

Protocol Approval Date: **January 3, 2016**
Protocol Expiration Date: **January 2, 2019**

All protocols must undergo continuing review on an annual basis for as long as the protocol is active, even if the protocol is only active for a portion of the first year after approval. The principal investigator must submit an annual continuing review form when notified by the IACUC office.

If the research proposed under this protocol will continue to be conducted after the end of the three-year approval period, a new protocol must be submitted and approved prior to the three-year anniversary of the original approval date if uninterrupted work is desired to continue. The principal investigator is responsible for submitting all paperwork required to maintain IACUC approval.

Changes to Approved Protocols

Any changes in study personnel, animal numbers, species, procedures/treatments, or any other minor or significant change to your protocol must be submitted to the IACUC for review and approval before those changes are implemented. Failure to seek IACUC approval for amending approved protocol procedures may result in withdrawal of permission to conduct the research.

PI Responsibility for Adequate Staff Training

Federal laws and regulations require that research staff have the requisite training for humane care and use of animals, and are aware of risks inherent in handling of animals and their tissues. As the principal investigator, you are responsible for ensuring that your staff have sufficient training and expertise with the technical procedures that they are listed as performing in the protocol. You are required to ensure that they are proficient in the procedures, and will, as necessary, provide additional training to ensure their competency when performing procedures. You are also responsible for identifying needed PPE (Personal Protective Equipment) and ensuring its proper use by your staff, and, as appropriate, directing staff to EHS for additional training and monitoring.

Grant Comparison Information

The PHS Policy (IV, C, 1) requires that IACUCs review and approve all PHS-supported activities. The PHS Policy (IV, D, 1) also requires that all applications and proposals contain the five elements described therein. These two statements in the PHS Policy, and additional Guidance provided by OLAW (e.g., Lab Animal 32(9):33-36, 2003), impoly the need for comparison between the IACUC approved protocol and the PHS proposal.

The following chart indicates whether grant proposals are related to this IACUC protoocol, and which of the listed proposals, if any, have been compared to this IACUC protocol, if required:

Date*	OSP Number	Sponsor	Grant Comparison Conducted?

* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IACUC protocol is to cover any other grant proposals, please contact the IACUC office (iacuc@vt.edu) immediately.

Federal Compliance Assurance

All protocols involving the use of vertebrate animals are reviewed by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) to assure humane care and treatment of the animals involved.

Approved proposals comply with:

1. "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training", when applicable,

2. The Animal Welfare Act, As Amended,

3. The Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, when applicable,

4. "Virginia Tech Policies Governing the Use of Animals in Research and Teaching"

Virginia Tech has a written, approved Animal Welfare Assurance on file with the PHS Office of Laboratory Animal Welfare (OLAW). Virginia Tech's Animal Welfare Assurance number is A-3208-01, expiration date 7/31/2017.

Appendix E. Discussion of Economics of Oil Ingredients in Nutritionally Enhanced Feed

Analysis. The feed is expensive in aquaculture. It can take up to 50% of the cost associated with operation of aquaculture farms. The cost associated with nutritionally enhanced diet was analyzed to understand the price increase associated with the new raw ingredients. Because all other ingredients were same except for the oil mixture, oil prices were only accounted for in this section. The cost associated with increased benefit is miniscule. For instance, 100% replacement of soybean oil will add 0.05 USD per fish compared to the soybean oil based diet fed fish. However, this study indicated that majority of surveyed participants were willing to pay additional 0.50 to 1.50 USD per lbs of fish fillet. Therefore, estimated added feed cost compared to surveyed response showed that the benefit far outweighs the initial increase in cost associated with expensive raw ingredients. Although algae oil is still economically not feasible, commercialization of algae oil over time as demand increases can drive the cost down and make it more cost effective.

Table 1. Analysis of cost associated with utilization of high quality oil sources in the tilapia aquaculture over 8 week growth study.

Ingredients	USD Price/ton	Price of 5% addition to 1 ton feed (USD)	Added cost per fish (USD)
SO[1]	704.16	35.21	0.04
SFO[2]	936.78	46.84	0.06
FO[3]	1500	75	0.09
AO[4]	200,000	10,000	11.75

[1]Soybean oil: price was obtained from Commodity Price on IndexMundi.com (as of March 2017). [2]High oleic sunflower oil: price was obtained from Commodity Price on IndexMundi.com (as of March 2017) for regular sunflower oil. [3]Fish oil: price was approximated from the chart within the FAO The state of world fisheries and aquaculture 2016. [4]Algae oil: price was the commercially available price of our source. The added cost per fish was calculated based on the average diet consumed by fish in our study (1.175 kg of feed over 8 weeks per fish).

Appendix F. Tables and Figures for Evaluation of Lipid Content, Fatty Acid Composition, and Peroxide Value in Cold Stored (-10ºC, -20ºC) Fillets of Tilapia, *Oreochormis* sp. Grown in a Recirculating Aquaculture System

Table 1. Pelleted Diet Formulation of 11 Enhanced Diet Strategies in percent.

Ingredient	SO100	SOFO15	SOFO85	SOAO15	SOAO85	SFO100	SFOFO15	SOFO85	SOAO15	SOAO85	HT
SBM	58.5	58.5	58.5	58.5	58.5	58.5	58.5	58.5	58.5	58.5	58.5
WT	25.794	25.794	25.794	25.794	25.794	25.794	25.794	25.794	25.794	25.794	25.774
MBM	3	3	3	3	3	3	3	3	3	3	3
FM	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
SO	5	0.75	4.25	0.75	4.25	0	0	0	0	0	0
HOSFO	0	0	0	0	0	5	0.75	4.25	0.75	4.25	0.75
FO	0	4.25	0.75	0	0	0	4.25	0.75	0	0	4.25
AO	0	0	0	4.25	0.75	0	0	0	4.25	0.75	0
VP	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
MP	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vit. E	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
atoc	0	0	0	0	0	0	0	0	0	0	0.02
AP	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
CMC	1	1	1	1	1	1	1	1	1	1	1
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 2. Lipid Concentration and Fatty Acid Composition of Tilapia Fillet after 8 Week Growth Trial, Month 0

% w/w	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
Total Lipid	1.46	1.32	1.49	1.75	1.70	1.46	1.61	1.29	1.75	1.70	1.46	1.24	0.5173	
***n*-6: *n*-3**	2.79^{AB}	0.86^{C}	2.78^{AB}	1.52^{BC}	0.90^{C}	1.84^{BC}	1.32^{BC}	3.99^{A}	2.03^{BC}	0.88^{C}	2.24^{BC}	0.95^{C}	<0.001	0.086
% lipid														
18:0	7.40^{AB}	8.07^{A}	6.40^{B}	6.32^{B}	7.25^{AB}	6.45^{B}	7.48^{AB}	6.88^{AB}	6.78^{AB}	7.18^{AB}	6.95^{AB}	6.62	0.017	0.078
18:1 *n*-9	22.1^{CDE}	17.4^{DE}	36.8^{A}	33.2^{AB}	18.8^{DE}	32.8^{AB}	19.4^{DE}	27.2^{BC}	23.3^{CD}	16.2^{E}	23.7^{CD}	18.2^{DE}	<0.001	0.36
18:2 *n*-6	17.4^{A}	10.4^{B}	12.1^{B}	10.8^{B}	9.9^{B}	10.9^{B}	10.0^{B}	17.1^{A}	17.0^{A}	11.1^{B}	16.4^{A}	11.3^{B}	<0.001	0.15
18:3 *n*-3	1.20^{C}	0.93^{CD}	0.63^{D}	0.64^{D}	0.89^{CD}	0.70^{D}	0.89^{CD}	2.01^{A}	1.97^{A}	1.02^{CD}	1.72^{AB}	1.29^{BC}	<0.001	0.023
20:4 *n*-6	3.25	3.18	3.23	2.33	2.65	2.76	2.30	2.46	2.62	2.07	3.16	2.13	0.0197	0.067
20:5 *n*-3	0.42^{B}	2.01^{A}	0.34^{B}	0.38^{B}	1.77^{A}	0.49^{B}	1.45^{A}	0.25^{B}	0.15^{B}	0.53^{B}	0.48^{B}	1.59^{A}	<0.001	0.036
22:5 *n*-3	1.32^{BC}	3.75^{A}	0.81^{BC}	0.85^{BC}	3.42^{A}	1.44^{BC}	2.38^{AB}	0.60^{C}	0.77^{BC}	1.07^{BC}	1.48^{BC}	3.41^{A}	<0.001	0.088
22:6 *n*-3	5.59^{CD}	11.80^{AB}	5.02^{CD}	9.03^{BC}	10.10^{BC}	6.31^{BCD}	6.62^{BCD}	2.83^{D}	8.24^{BCD}	17.68^{A}	6.22^{BCD}	9.36^{BC}	<0.001	0.34
Total SFA	32.7	35.3	28.2	28.8	34.7	28.9	38.4	32.6	33.9	33.5	31.1	30.9	0.0494	0.53
Total MUFA	32.7^{CD}	27.8^{DE}	44.7^{A}	40.8^{ABC}	29.7^{DE}	41.3^{AB}	30.9^{DE}	35.7^{BCD}	30.5^{ABC}	24.0^{E}	32.6^{CD}	29.6^{DE}	<0.001	0.42
Total *n*-3	8.89^{Cd}	19.09^{AB}	6.95^{D}	10.93^{BCD}	16.82^{ABC}	9.22^{CD}	12.0^{BCD}	6.05^{D}	11.2^{BCD}	20.6^{A}	10.2^{BC}	16.4^{ABC}	0.0002	0.42
Total *n*-6	24.7^{A}	16.3^{C}	19.1^{BC}	16.5^{C}	15.0^{C}	16.9^{C}	14.4^{C}	22.7^{AB}	22.3^{AB}	18.2^{BC}	22.9^{AB}	15.5^{C}	<0.001	0.24
mg/g oil														
Oleic	133.9^{AB}	94.4^{B}	222.0^{AB}	195.2^{AB}	97.1^{B}	194.7^{AB}	160.0^{AB}	254.9^{A}	137.7^{AB}	87.2^{B}	142.3^{AB}	132.8^{AB}	0.0156	7.7
ARA	19.8^{AB}	17.3^{AB}	19.5^{AB}	13.6^{AB}	13.7^{AB}	16.6^{AB}	17.9^{AB}	22.5^{A}	15.3^{AB}	11.0^{B}	18.9^{AB}	15.5^{AB}	0.0589	0.57
EPA	2.53^{B}	10.9^{A}	2.05^{B}	2.21^{B}	9.14^{A}	2.87^{B}	11.0^{A}	2.10^{B}	0.82^{B}	2.86^{B}	2.88^{B}	11.6^{A}	<0.001	0.18
DPA	8.01^{CDE}	20.3^{B}	4.85^{DE}	4.97^{DE}	17.7^{B}	8.52^{CD}	16.6^{B}	4.91^{DE}	4.51^{E}	5.74^{CDE}	8.88^{C}	24.8^{A}	<0.001	0.20
DHA	34.0^{CD}	64.1^{B}	30.2^{CD}	52.6^{BC}	51.9^{BC}	37.4^{CD}	46.8^{BC}	23.8^{D}	48.1^{BC}	94.3^{A}	37.3^{CD}	68.1^{B}	<0.001	1.2
ARA: LCn3	0.44^{AB}	0.18^{B}	0.52^{AB}	0.23^{B}	0.17^{B}	0.34^{AB}	0.24^{B}	0.73^{A}	0.29^{B}	0.11^{B}	0.39^{AB}	0.15^{B}	0.0042	0.025

*The statistical analysis was conducted using ANOVA at α=0.05; superscript letters represents post hoc analysis using Tukey's HSD.

Table 3. Lipid Concentration and Fatty Acid Composition of Tilapia Fillets Stored in Temperature Abused Conditions for 3 Months

% fillet w/w	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
Total Lipid	0.81	0.60	0.97	0.91	0.86	0.86	0.92	0.99	1.23	0.88	0.92	0.95	0.1758	0.03
***n*-6: *n*-3**	2.96	0.98	3.20	1.73	0.84	2.03	0.98	3.06	2.30	0.97	2.45	1.00	<0.0001	0.06
% lipid														
18:0	8.38	8.74	6.59	6.63	7.60	7.35	7.98	7.71	7.23	8.32	7.62	7.45	0.0743	0.12
18:1 *n*-9	20.91	17.96	38.85	35.85	19.29	35.33	19.93	25.87	26.75	16.57	24.22	18.69	<0.0001	0.52
18:2 *n*-6	17.85	10.77	11.19	10.44	9.55	10.52	10.20	18.21	17.30	11.21	16.69	10.87	<0.0001	0.12
18:3 *n*-3	1.16	0.89	0.58	0.60	0.76	0.63	0.90	1.96	1.97	1.02	1.71	1.21	<0.0001	0.2
20:4 *n*-6	3.46	3.16	2.68	2.14	2.33	2.55	2.61	3.83	2.22	2.40	2.97	2.40	0.1411	0.11
20:5 *n*-3	0.46	1.78	0.28	0.34	0.62	0.46	1.79	0.35	0.32	0.67	0.49	1.63	<0.0001	0.03
22:5 *n*-3	1.31	3.55	0.83	0.76	1.09	1.40	3.50	1.13	0.70	1.05	1.57	3.43	<0.0001	0.04
22:6 *n*-3	5.80	11.59	4.14	8.31	18.45	5.98	9.75	5.20	7.37	17.27	5.78	9.47	<0.0001	0.33
Total SFA	34.38	35.21	27.88	28.98	34.09	29.74	35.58	29.00	29.73	35.10	31.82	35.76	<0.0001	0.23
Total MUFA	29.27	27.61	46.73	43.18	26.35	43.28	30.57	33.44	34.76	24.08	32.93	30.27	<0.0001	0.61
Total *n*-3	9.09	18.47	6.03	10.20	21.29	8.72	16.68	9.12	10.80	20.39	10.04	16.52	<0.0001	0.39
Total *n*-6	26.80	17.96	18.61	17.00	17.86	17.49	16.07	27.90	24.10	19.64	24.48	16.56	<0.0001	0.22
mg/g oil														
Oleic	123.72	102.61	229.62	219.64	111.68	201.92	120.40	154.69	181.62	99.32	140.73	123.44	0.0053	6.0
ARA	20.66	18.00	15.67	12.59	13.44	14.57	15.52	22.95	14.42	14.36	17.24	15.87	0.0922	0.58
EPA	2.75	10.07	1.65	1.99	3.58	2.61	10.62	2.12	2.07	3.99	2.87	10.77	<0.0001	0.10
DPA	7.79	20.17	4.88	4.52	6.29	8.01	20.78	6.76	4.51	6.32	9.17	22.68	<0.0001	0.21
DHA	34.46	65.84	24.10	48.80	106.66	34.23	57.61	31.19	48.08	103.71	33.68	62.49	<0.0001	1.6
ARA:LC*n*3	0.45	0.19	0.52	0.23	0.12	0.33	0.17	0.57	0.26	0.13	0.38	0.17	<0.0001	0.0074

*The statistical analysis was conducted using ANOVA at α=0.05.

Table 4. Lipid Concentration and Fatty Acid Composition of Tilapia Fillets Stored in -20ºC Cold Storage Conditions for 3 Months

% fillet w/w	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
Total Lipid	0.74	0.63	0.53	0.92	1.04	0.82	0.70	0.86	0.71	1.04	0.82	0.75	<0.0001	0.01
***n*-6: *n*-3**	2.63	0.98	2.67	1.78	0.77	1.94	1.00	3.03	1.98	0.89	2.46	0.98	<0.0001	0.02
% lipid														
18:0	8.63	8.65	7.51	6.74	7.84	7.56	8.27	7.81	7.54	7.56	7.38	8.10	0.0146	0.08
18:1 *n*-9	19.13	18.43	34.99	33.39	18.35	31.85	21.32	24.26	23.94	16.36	25.14	18.05	<0.0001	0.27
18:2 *n*-6	16.90	10.60	11.26	11.37	9.69	11.16	9.93	17.87	16.99	11.67	16.58	10.79	<0.0001	0.15
18:3 *n*-3	1.04	0.90	0.51	0.70	0.77	0.61	0.81	1.91	1.79	1.16	1.85	1.14	<0.0001	0.02
20:4 *n*-6	4.35	3.17	3.98	2.40	1.56	3.65	2.79	4.03	2.65	2.06	3.03	2.88	0.0242	0.13
20:5 *n*-3	0.51	1.85	0.37	0.40	0.73	0.60	1.66	0.37	0.46	0.63	0.46	1.83	<0.0001	0.01
22:5 *n*-3	1.67	3.41	0.99	0.82	1.05	1.62	3.38	1.11	1.04	1.03	1.43	3.44	<0.0001	0.03
22:6 *n*-3	6.99	11.16	5.81	8.17	19.34	7.22	9.96	5.38	8.72	18.83	5.77	10.49	<0.0001	0.15
Total SFA	33.52	34.83	28.10	28.96	34.72	29.26	34.99	30.58	30.18	33.99	31.16	35.94	<0.0001	0.14
Total MUFA	27.42	28.48	42.24	41.51	25.30	39.73	31.43	31.99	31.92	23.61	33.61	28.54	<0.0001	0.34
Total *n*-3	10.58	18.06	7.89	10.34	22.25	10.32	16.47	9.17	12.48	22.07	9.97	17.59	<0.0001	0.19
Total *n*-6	27.80	17.78	21.06	18.41	17.21	19.99	16.37	27.64	24.74	19.74	24.58	17.25	<0.0001	0.25
mg/g oil														
Oleic	108.77	107.86	222.04	229.28	114.69	200.86	123.63	150.42	145.64	98.01	152.40	102.36	<0.0001	2.5
ARA	24.76	18.62	25.19	16.46	9.28	23.02	16.21	24.88	16.15	12.36	18.39	16.41	0.0228	0.80
EPA	2.93	10.85	2.37	2.74	4.52	3.76	9.53	2.28	2.81	3.78	2.78	10.41	<0.0001	0.09
DPA	9.48	19.96	6.28	5.64	6.46	10.24	19.43	6.86	6.31	6.18	8.67	19.52	<0.0001	0.11
DHA	39.75	65.40	36.86	56.13	119.76	45.53	57.46	33.18	53.03	112.82	34.99	59.68	<0.0001	0.86
ARA:LC*n*3	0.47	0.19	0.55	0.26	0.07	0.39	0.19	0.59	0.26	0.10	0.40	0.18	<0.0001	0.0076

*The statistical analysis was conducted using ANOVA at α=0.05.

Table 5. Lipid Concentration and Fatty Acid Composition of Tilapia Fillets Stored in Temperature Abused Conditions for 6 Months

% fillet w/w	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
Total Lipid	0.95	0.94	1.00	0.92	1.02	0.86	1.04	0.99	1.36	0.80	0.86	1.14	0.0718	0.03
***n*-6: *n*-3**	2.92	1.58	3.04	1.79	0.83	2.27	0.97	3.83	2.53	0.93	2.78	1.64	0.0024	0.11
%														
18:0	7.99	9.11	6.32	7.48	7.04	9.12	7.30	5.02	6.84	9.22	8.80	9.28	0.7119	0.47
18:1 *n*-9	21.51	20.94	38.77	34.50	19.28	33.40	21.40	26.39	28.07	15.13	24.08	21.13	<0.0001	0.56
18:2 *n*-6	16.97	10.40	10.82	11.26	9.59	10.18	9.57	16.94	16.93	10.37	15.81	9.44	<0.0001	0.22
18:3 *n*-3	1.08	0.81	0.57	0.64	0.77	0.49	0.88	0.86	1.80	0.85	1.35	0.89	0.1819	0.08
20:4 *n*-6	3.15	2.19	2.51	2.40	2.17	2.71	2.29	3.54	1.86	2.86	3.16	1.93	0.5369	0.16
20:5 *n*-3	0.43	1.21	0.30	0.40	0.63	0.45	1.57	0.31	0.29	0.76	0.44	1.02	0.0099	0.06
22:5 *n*-3	1.41	2.28	0.91	0.84	1.06	1.17	3.44	1.01	0.76	1.16	1.20	2.23	0.0303	0.13
22:6 *n*-3	5.54	6.55	3.95	8.18	18.30	5.21	9.06	4.80	5.85	17.97	5.16	6.12	0.0003	0.46
Total SFA	35.40	39.94	28.60	29.19	34.69	33.05	35.78	30.62	31.14	36.28	33.83	40.92	0.0272	0.62
Total MUFA	29.89	32.52	46.95	41.55	26.14	41.76	32.54	35.49	36.17	22.25	33.28	33.32	0.0007	0.71
Total *n*-3	8.81	11.35	5.88	10.28	21.12	7.50	15.67	7.41	9.12	21.14	8.49	10.75	0.0043	0.65
Total *n*-6	25.40	15.52	17.83	18.38	17.56	17.03	15.23	25.88	23.05	19.68	23.62	14.21	<0.0001	0.31
mg/g oil														
Oleic	129.97	110.46	276.28	239.85	116.91	177.11	128.55	129.98	150.06	79.67	134.04	99.20	<0.0001	5.1
ARA	18.90	12.29	17.88	16.87	13.15	14.35	13.77	16.81	9.80	15.06	17.25	9.37	0.5037	0.90
EPA	2.59	6.81	2.14	2.81	3.79	2.40	9.44	1.50	1.54	3.99	2.42	5.05	0.0261	0.38
DPA	8.48	12.90	6.46	5.82	6.44	6.27	20.65	4.79	3.97	6.01	6.67	11.07	0.0507	0.82
DHA	33.27	37.35	28.14	57.01	111.01	27.68	54.41	22.51	30.89	92.75	28.31	30.46	0.0003	2.7
ARA:LC*n*3	0.42	0.23	0.49	0.26	0.11	0.40	0.16	0.64	0.27	0.15	0.47	025	<0.0001	0.014

*The statistical analysis was conducted using ANOVA at α=0.05.

Table 6. Lipid Concentration and Fatty Acid Composition of Tilapia Fillets Stored in -20ºC Cold Storage Conditions for 6 Months

% fillet w/w	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
Total Lipid	0.88	0.84	0.86	0.95	1.02	0.94	1.09	1.11	1.16	0.89	1.04	0.79	0.3239	0.03
***n*-6: *n*-3**	3.42	1.05	2.67	1.71	0.85	2.06	1.07	3.68	2.57	0.82	2.45	0.90	<0.0001	0.05
%														
18:0	7.98	9.01	7.63	7.18	7.63	7.55	7.58	7.36	6.84	7.87	7.42	9.80	0.0511	0.14
18:1 *n*-9	23.32	18.72	33.81	34.18	18.99	32.41	20.94	27.75	27.10	16.14	25.24	15.62	<0.0001	0.34
18:2 *n*-6	16.34	10.23	11.53	10.76	9.44	11.28	10.53	18.50	17.70	11.10	16.41	9.91	<0.0001	0.16
18:3 *n*-3	1.04	0.87	0.54	0.61	0.73	0.65	0.93	1.99	1.85	1.04	1.72	0.95	<0.0001	0.02
20:4 *n*-6	2.85	2.85	3.30	2.51	2.25	3.15	2.30	2.85	2.04	1.30	3.19	3.34	0.1023	0.12
20:5 *n*-3	0.33	1.53	0.39	0.36	0.65	0.53	1.54	0.28	0.31	0.71	0.45	1.78	<0.0001	0.02
22:5 *n*-3	1.10	3.36	1.00	0.81	1.11	1.43	3.45	0.82	0.73	1.07	1.50	3.76	<0.0001	0.02
22:6 *n*-3	4.29	9.49	5.66	8.63	17.89	6.62	8.40	3.74	6.06	19.54	5.99	12.18	<0.0001	0.02
Total SFA	36.08	37.41	29.51	29.21	35.25	29.96	35.45	30.39	30.48	34.78	30.93	37.40	<0.0001	0.19
Total MUFA	32.36	29.32	41.64	41.26	26.11	40.44	32.33	35.46	35.33	23.18	33.53	25.19	<0.0001	0.43
Total *n*-3	7.07	15.89	7.82	10.61	20.75	9.48	15.10	7.21	9.40	22.78	10.13	19.34	<0.0001	0.24
Total *n*-6	24.00	16.70	20.22	18.19	17.55	19.28	16.25	26.39	24.22	18.66	24.75	17.31	<0.0001	0.22
mg/g oil														
Oleic	135.12	102.81	199.43	198.48	108.67	193.73	120.99	165.09	163.87	91.61	148.13	85.51	0.0003	3.9
ARA	16.55	15.52	19.34	14.57	12.82	18.65	13.21	16.60	12.28	7.22	18.70	18.17	0.0438	0.60
EPA	1.91	8.31	2.29	2.09	3.70	3.15	8.89	1.65	1.89	4.06	2.64	9.68	<0.0001	0.08
DPA	6.43	18.37	5.87	4.70	6.36	8.48	19.86	4.78	4.40	6.09	8.77	20.47	<0.0001	0.18
DHA	25.01	51.60	32.89	50.08	102.47	39.25	48.34	21.75	36.60	110.76	35.09	66.30	<0.0001	1.2
ARA:LC*n*3	0.51	0.20	0.48	0.26	0.12	0.37	0.17	0.59	0.29	0.06	0.40	0.19	<0.0001	0.0079

*The statistical analysis was conducted using ANOVA at α=0.05.

Table 7. Lipid Concentration and Fatty Acid Composition of Tilapia Fillets Stored in Temperature Abused Storage Conditions for 9 Months

% fillet w/w	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
Total Lipid	0.77	0.89	1.04	0.76	0.71	1.07	0.79	0.94	0.93	0.74	0.74	1.04	0.1722	0.03
***n*-6: *n*-3**	2.79	0.92	2.40	1.75	0.77	1.94	1.16	3.27	1.80	0.79	2.05	0.95	<0.0001	0.06
%														
18:0	9.33	8.36	7.43	7.48	8.84	7.52	8.68	7.95	8.37	8.65	7.92	8.07	0.1473	0.13
18:1 *n*-9	11.47	19.58	33.33	31.67	15.78	30.88	20.57	25.61	21.20	13.42	22.43	18.11	0.0110	1.0
18:2 *n*-6	17.54	10.16	11.35	11.40	8.86	11.27	9.94	17.33	16.12	10.24	16.97	10.50	<0.0001	0.11
18:3 *n*-3	1.15	0.96	0.56	0.65	0.65	0.69	0.84	1.81	1.61	0.94	1.82	1.11	<0.0001	0.02
20:4 *n*-6	4.22	2.95	3.87	3.05	3.17	3.67	2.46	3.41	3.89	3.03	3.72	3.04	0.0016	0.06
20:5 *n*-3	0.45	1.84	0.46	0.39	0.85	0.55	1.43	0.35	0.50	0.85	0.65	1.76	<0.0001	0.03
22:5 *n*-3	1.57	3.63	1.22	0.77	1.07	1.58	2.97	1.03	1.14	1.21	1.87	3.61	<0.0001	0.04
22:6 *n*-3	6.87	10.80	6.53	9.32	21.56	7.48	7.82	4.58	10.92	22.16	7.94	11.26	<0.0001	0.24
Total SFA	30.15	34.49	27.65	28.91	33.85	28.85	37.03	30.29	29.68	33.19	29.39	34.07	<0.0001	0.23
Total MUFA	30.75	30.31	41.19	39.32	22.30	39.31	32.48	34.35	28.74	20.36	30.77	29.09	<0.0001	0.45
Total *n*-3	10.37	17.99	8.99	11.36	24.46	10.58	13.72	8.20	14.65	25.58	12.82	18.46	<0.0001	0.30
Total *n*-6	28.02	16.57	21.24	19.78	18.81	20.46	15.77	26.37	26.31	20.18	26.23	17.55	<0.0001	0.17
mg/g oil														
Oleic	66.18	115.58	157.60	208.58	84.86	182.63	116.49	148.46	113.47	79.73	138.02	106.58	0.0101	6.0
ARA	23.70	17.40	18.51	20.09	16.96	21.71	14.04	19.77	20.88	17.97	22.81	17.75	0.0338	0.45
EPA	2.52	10.85	2.24	2.56	4.52	3.25	8.21	2.03	2.70	5.07	4.04	10.30	<0.0001	0.19
DPA	8.74	21.43	5.82	5.10	5.74	9.36	17.01	5.92	6.10	7.21	11.48	21.13	<0.0001	0.31
DHA	38.36	63.80	31.38	61.47	115.25	44.36	44.74	26.41	58.44	132.10	48.71	65.71	<0.0001	1.8
ARA:LC*n*3	0.49	0.18	0.48	0.29	0.14	0.38	0.20	0.59	0.31	0.13	0.36	0.19	<0.0001	0.011

*The statistical analysis was conducted using ANOVA at α=0.05.

Table 8. Lipid Concentration and Fatty Acid Composition of Tilapia Fillets Stored in -20ºC Cold Storage Conditions for 9 Months

% fillet w/w	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85	p-value	Pooled error
Total Lipid	0.90	1.08	0.88	0.98	0.88	1.05	0.71	0.84	0.69	0.66	0.66	0.61	0.1501	0.03
***n*-6: *n*-3**	2.70	0.94	2.41	1.73	0.74	1.76	0.85	2.95	1.75	0.78	1.98	0.85	<0.0001	0.02
%														
18:0	8.57	8.20	7.29	6.43	7.83	8.31	7.26	7.04	8.07	7.64	8.08	8.58	0.2687	0.16
18:1 *n*-9	19.90	17.70	33.38	34.91	17.71	30.04	20.04	25.84	22.09	15.20	21.53	15.85	<0.0001	0.45
18:2 *n*-6	16.70	10.85	11.97	11.15	8.94	10.64	9.63	18.53	16.54	10.33	17.01	10.32	<0.0001	0.15
18:3 *n*-3	1.09	0.98	0.60	0.67	0.72	0.59	0.91	2.09	1.73	0.98	1.67	1.03	<0.0001	0.02
20:4 *n*-6	4.44	3.28	3.76	2.39	2.55	4.06	2.56	3.43	3.29	2.52	4.16	3.58	0.0452	0.12
20:5 *n*-3	0.49	1.96	0.44	0.36	0.71	0.58	1.88	0.37	0.47	0.74	0.72	2.05	<0.0001	0.03
22:5 *n*-3	1.55	3.78	1.15	0.81	0.96	1.70	3.85	1.15	0.99	1.10	1.87	3.90	<0.0001	0.04
22:6 *n*-3	6.84	12.28	6.36	8.54	21.17	8.54	11.22	5.27	11.09	21.85	8.75	14.07	<0.0001	0.29
Total SFA	33.24	33.24	27.88	27.61	33.45	29.65	33.47	28.20	29.62	32.48	29.37	33.40	<0.0001	0.16
Total MUFA	28.40	27.77	41.41	42.69	24.40	37.72	30.84	33.99	29.26	22.13	29.63	25.64	<0.0001	0.55
Total *n*-3	10.28	19.76	8.77	10.61	23.88	11.69	18.71	9.40	14.75	25.05	13.53	21.78	<0.0001	0.33
Total *n*-6	27.68	18.34	21.16	18.38	17.78	20.34	15.97	27.70	25.83	19.55	26.78	18.34	<0.0001	0.23
mg/g oil														
Oleic	107.03	85.86	195.66	212.76	100.19	138.10	124.54	171.50	130.74	94.41	136.61	94.67	0.0013	4.6
ARA	22.79	15.50	21.87	14.51	14.29	19.06	15.69	22.69	19.46	15.62	26.40	20.90	0.0151	0.59
EPA	2.57	9.21	2.60	2.17	3.97	2.69	11.58	2.47	2.77	4.59	4.55	12.01	0.0010	0.12
DPA	8.16	18.04	6.76	4.93	5.40	7.93	23.82	7.62	5.85	6.84	11.85	22.91	<0.0001	0.36
DHA	35.66	57.61	37.29	51.96	119.18	40.08	69.04	34.89	65.37	135.59	55.49	82.36	<0.0001	1.45
ARA:LC*n*3	0.50	0.18	0.47	0.25	0.12	0.38	0.15	0.51	0.26	0.11	0.37	0.18	<0.0001	0.0061

*The statistical analysis was conducted using ANOVA at α=0.05.

Table 9. Total Lipid Extract, in g per 100 g fillet, in Tilapia Fillets Stored in -20°C Cold Storage and in Temperature Abused Conditions for over 9 Months

	Month 0	Month 3, -10	Month 6, -10	Month 9, -10	Month 3, -20	Month 6, -20	Month 9,-20	p-value	Pooled error
CD	1.47	0.81	0.95	0.77	0.74	0.88	0.90	0.0600	0.05
HT	1.32	0.60	0.94	0.89	0.63	0.84	1.08	0.0521	0.05
SFO100	1.49^{A}	0.97^{AB}	1.00^{AB}	1.04^{AB}	0.53^{B}	0.86^{B}	0.88^{AB}	0.0061	0.05
SFOAO15	1.75^{A}	0.91^{B}	0.92^{B}	0.76^{B}	0.92^{B}	0.95^{B}	0.98^{B}	<0.0001	0.02
SFOAO85	1.70^{A}	0.86^{B}	1.02^{B}	0.71^{B}	1.04^{B}	1.02^{B}	0.88^{B}	0.0004	0.03
SFOFO15	1.46	0.86	0.86	1.07	0.82	0.94	1.05	0.2499	0.06
SFOFO85	1.62^{A}	0.92^{B}	1.04^{AB}	0.79^{B}	0.70^{B}	1.09^{AB}	0.71^{B}	0.0051	0.04
SO100	1.30	0.99	0.99	0.94	0.86	1.11	0.84	0.4506	0.05
SOAO15	1.75^{A}	1.23^{AB}	1.36^{AB}	0.93^{B}	0.71^{B}	1.16^{AB}	0.69^{B}	0.0310	0.07
SOAO85	1.70^{A}	0.88^{B}	0.80^{B}	0.74^{B}	1.04^{B}	0.89^{B}	0.66^{B}	0.0003	0.03
SOFO15	1.46	0.92	0.86	0.74	0.82	1.04	0.66	0.0426	0.05
SOFO85	1.24	0.95	1.14	1.04	0.75	0.79	0.61	0.0247	0.06

*The statistical analysis was conducted using ANOVA at α=0.05; superscript letters represents post hoc analysis using Tukey's HSD. Month indicates the duration of storage while -10 indicated temperature abused conditions and -20 indicates -20°C cold storage found in commercial settings.

Table 10. Peroxide Value of Enhanced Diet Fed Tilapia Fillet

	Month 0	Month 3, -10	Month 6, -10	Month 9, -10	Month 3, -20	Month 6, -20	Month 9, -20	p-value	Pooled error
CD	1.7	2.9	1.1	0.72	0.96	0.55	0.65	0.0887	0.24
HT	0.79	1.0	0.73	0.95	0.93	0.44	0.16	0.1955	0.10
SFO100	0.49^{AB}	0.71^{AB}	0.71^{AB}	0.25^{AB}	0.78^{A}	0.16^{B}	0.28^{AB}	0.0219	0.07
SFOAO15	0.50	0.65	0.91	1.31	0.84	0.41	1.1	0.1369	0.10
SFOAO85	0.53^{BCD}	0.93^{ABC}	1.1^{AB}	0.37^{D}	0.48^{CD}	0.32^{D}	1.1^{A}	0.0021	0.09
SFOFO15	0.68	0.77	0.37	0.22	0.65	0.10	0.52	0.2276	0.08
SFOFO85	0.53	1.07	0.67	0.76	1.4	0.61	0.43	0.3629	0.12
SO100	0.47	0.89	0.64	0.32	0.59	0.53	0.31	0.2440	0.07
SOAO15	0.45	0.77	0.60	0.51	0.54	0.51	0.08	0.4425	0.07
SOAO85	0.77^{AB}	1.0^{A}	0.38^{B}	0.35^{B}	0.62^{AB}	0.63^{AB}	0.39^{B}	0.0194	0.07
SOFO15	0.36	0.97	0.63	0.28	0.49	0.47	0.16	0.0558	0.07
SOFO85	0.81^{AB}	0.97^{A}	0.53^{AB}	0.44^{AB}	0.49^{AB}	0.18^{B}	0.50^{AB}	0.0244	0.07

*ANOVA analysis was conducted at $\alpha=0.05$ for statistical significance; superscript letters represents post hoc analysis using Tukey's HSD. Peroxide values were measured in meq/kg sample. Month indicates the duration of storage while -10 indicated temperature abused conditions and -20 indicates -20^{o}C cold storage found in commercial settings. Superscript were determined by Tukey's HSD.

Table 11. Change (slope) of fatty acids concentration, in mg/g oil, of tilapia fillets over 9 month storage.

-10ºC	CD	HT	SFO100	SFOAO15	SFOAO85	SFOFO15	SFOFO85	SO100	SOAO15	SOAO85	SOFO15	SOFO85
OA	-6.56	2.38*	-4.88	2.01	-1.05	-2.03	-4.08	-11.47	-3.48	-1.4	-0.65	-3.43*
ARA	0.33	-0.18	-0.02	0.29*	0.32	0.51	-0.45	-0.48	0.4	0.72*	0.39	0.0083
EPA	-0.0064	-0.11	0.036	0.058	-0.46	0.028	-0.31	-0.027	0.17	0.22*	0.0985	-0.32
DPA	0.098	-0.13	0.15	0.059	-1.19*	0.023	0.032	0.037	0.14	0.14	0.17	-0.75
DHA	0.4	-0.98	0.25	1.16	6.47*	0.48	-0.32	-0.033	0.47	3.41	0.97	-1.31
-20ºC												
OA	-1.81	-1.02	-3.38	0.73	0.11	-5.892*	-3.63	-7.85	-0.088	0.51	-0.71	-4.37
ARA	0.028	-0.28	0.047	0.027	0.18	0.11	-0.33	-0.26	0.28	0.29	0.76	0.6*
EPA	-0.03	-0.25	0.053	-0.0295	-0.5455*	-0.041	0.041	0.016	0.17	0.18*	0.16	0.022
DPA	-0.086	-0.284	0.18	-0.033	-1.23*	-0.12	0.73*	0.2	0.072	0.11	0.3	-0.16
DHA	-0.32	-1.51	0.572	-0.27	6.14*	0.064	1.92	0.72	1.18	4.05*	0.78	1.65

* indicates statistical significance at $\alpha = 0.05$ using standard square linear regression. Negative slope indicates reduction in the fatty acids over time while positive slope indicates increase in the fatty acids over time.

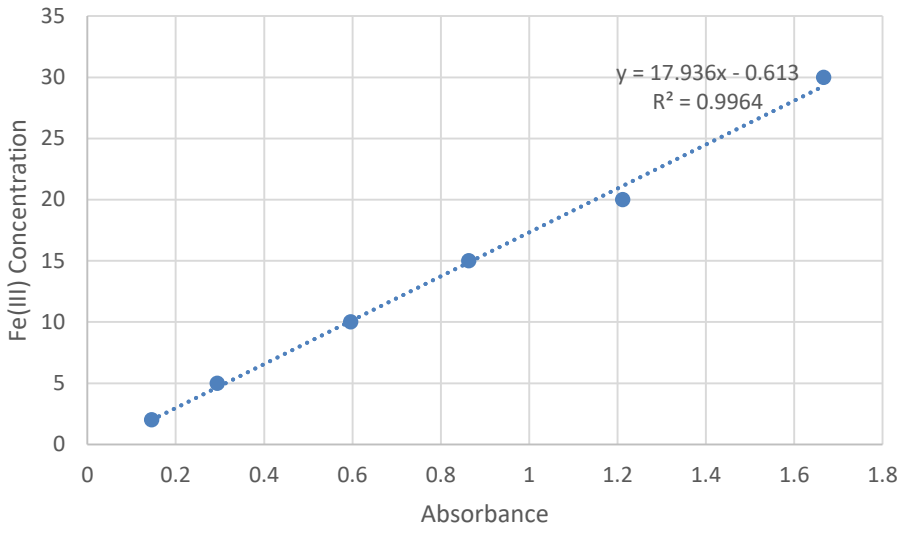

Figure 1. The standard curve of spectrometric analysis of peroxide value at 500 nm.

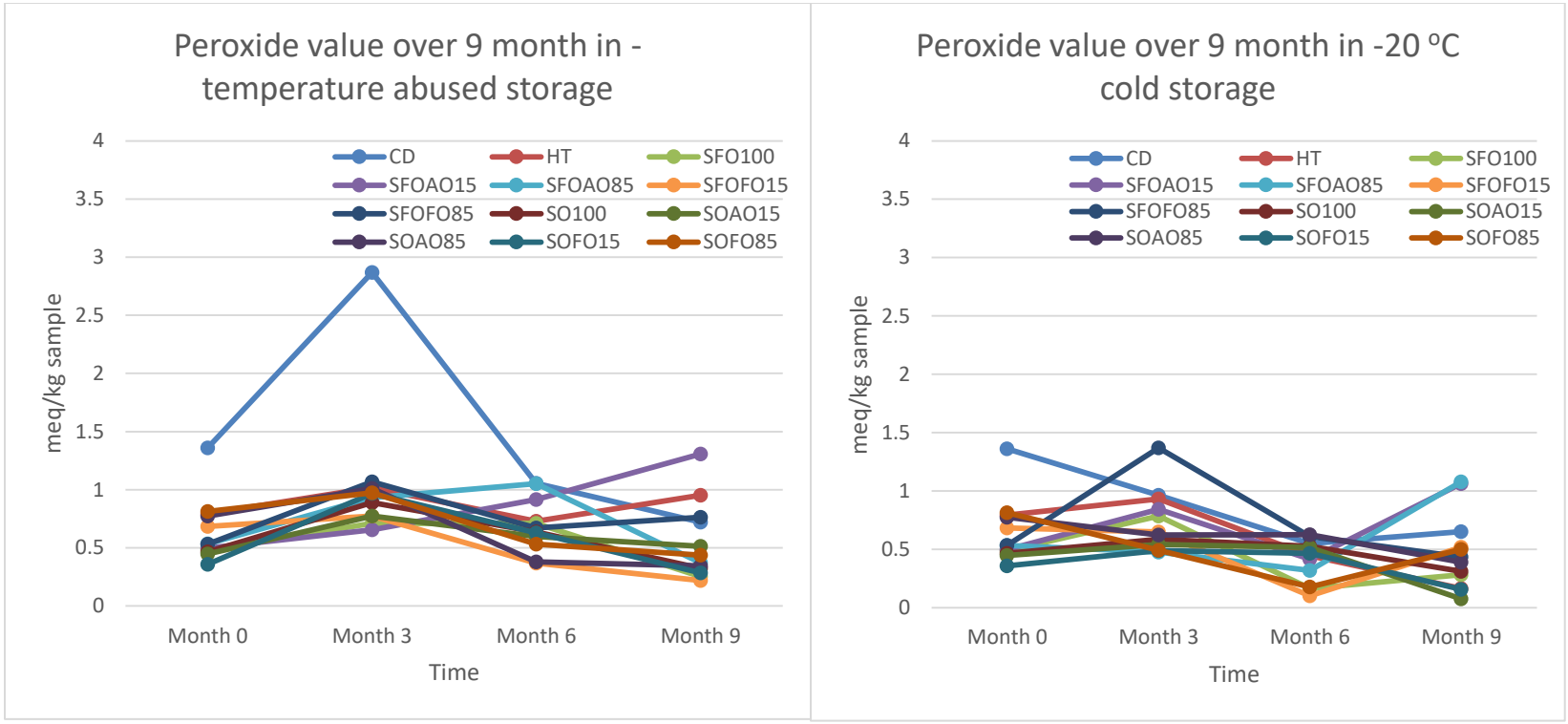

Figure 2. The peroxide value, in meq/kg sample, were observed over 9 month period in temperature abused freezer and static -20°C cold storage.

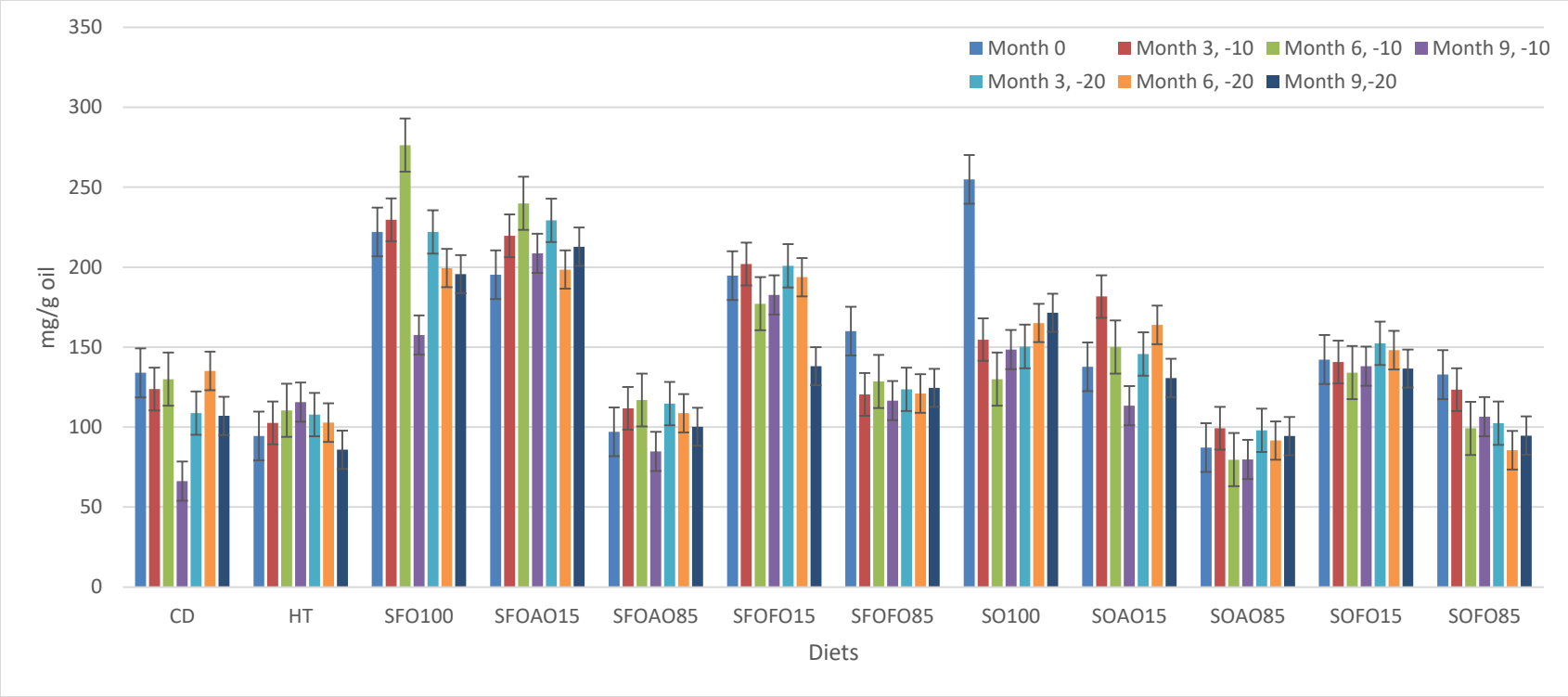

Figure 3. Change in Oleic Acids of Tilapia Fillets Stored in Temperature Abused Condition and -20^{o}C Cold Storage Condition Over 9 Months.

*Error bar is represented by the standard error within each time and storage conditions; Month indicates the duration of storage while -10 indicated temperature abused conditions and -20 indicates -20^{o}C cold storage found in commercial settings.

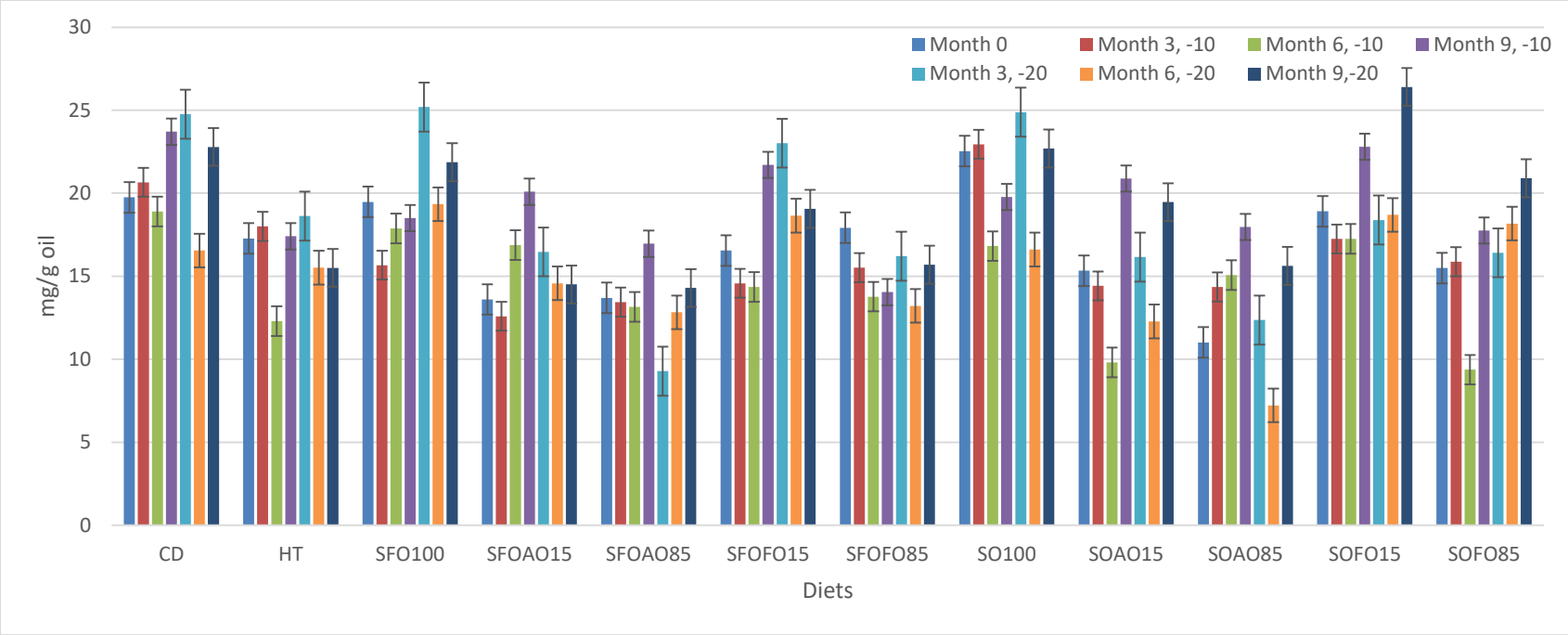

Figure 4. Change in Arachidonic Acids of Tilapia Fillets Stored in Temperature Abused Condition and -20^{o}C Cold Storage Condition Over 9 Months.

*Error bar is represented by the standard error within each time and storage conditions; Month indicates the duration of storage while -10 indicated temperature abused conditions and -20 indicates -20^{o}C cold storage found in commercial settings.

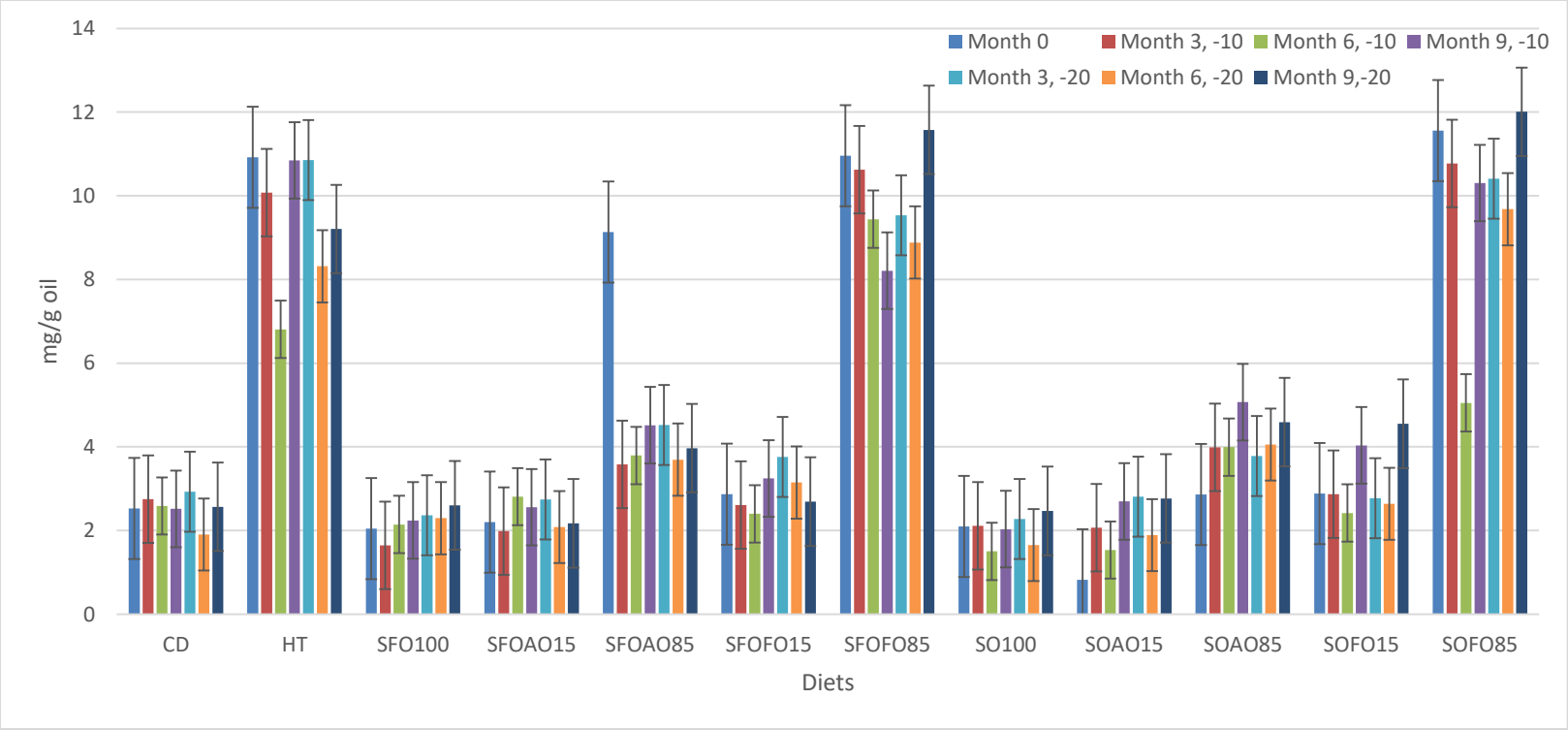

Figure 5. Change in Eicosapentaenoic Acids of Tilapia Fillets Stored in Temperature Abused Condition and -20^{o}C Cold Storage Condition Over 9 Months.

*Error bar is represented by the standard error within each time and storage conditions; Month indicates the duration of storage while -10 indicated temperature abused conditions and -20 indicates -20^{o}C cold storage found in commercial settings.

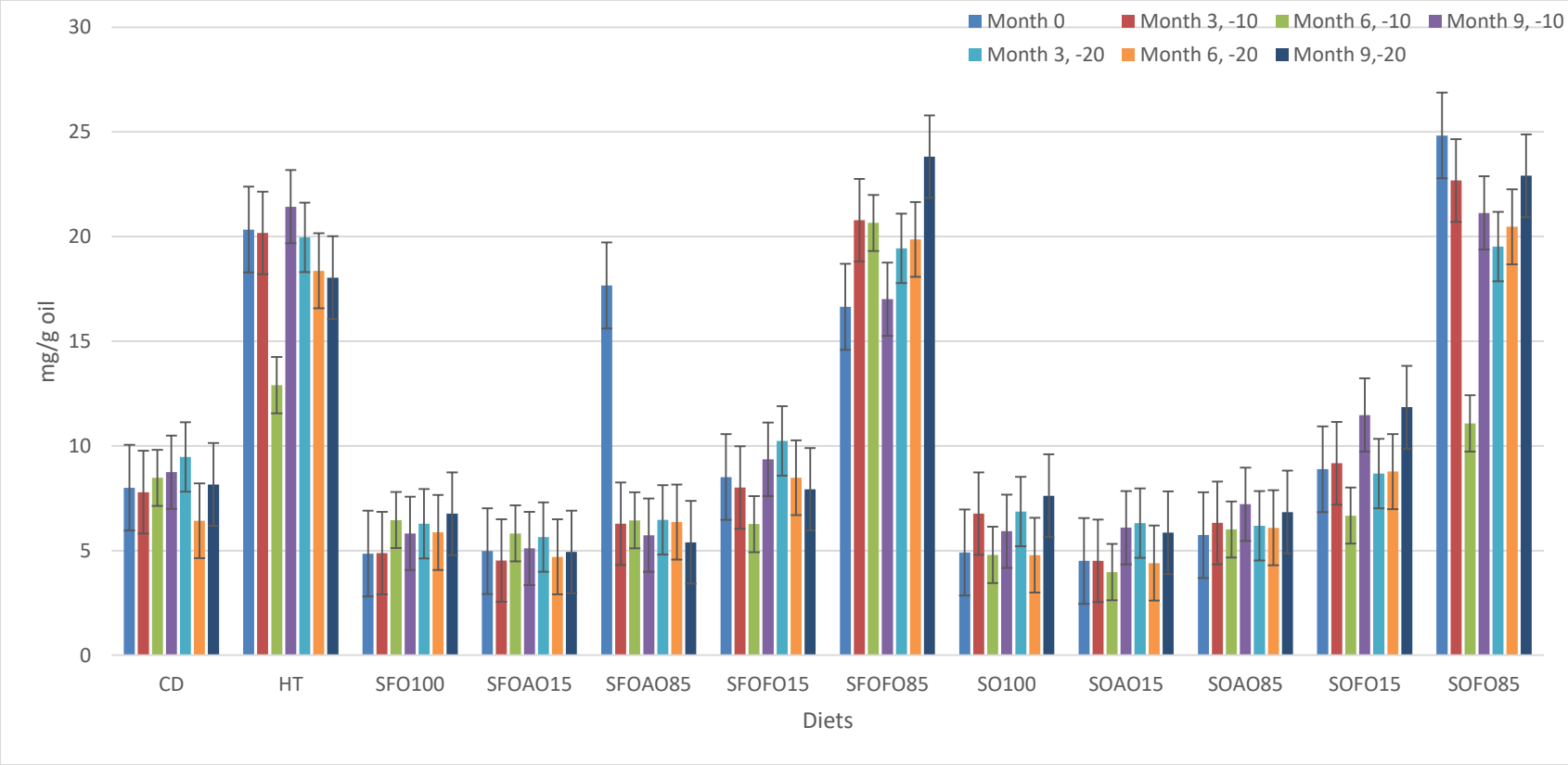

Figure 6. Change in Docosapentaenoic Acids of Tilapia Fillets Stored in Temperature Abused Condition and -20^{o}C Cold Storage Condition Over 9 Months.

*Error bar is represented by the standard error within each time and storage conditions; Month indicates the duration of storage while -10 indicated temperature abused conditions and -20 indicates -20^{o}C cold storage found in commercial settings.

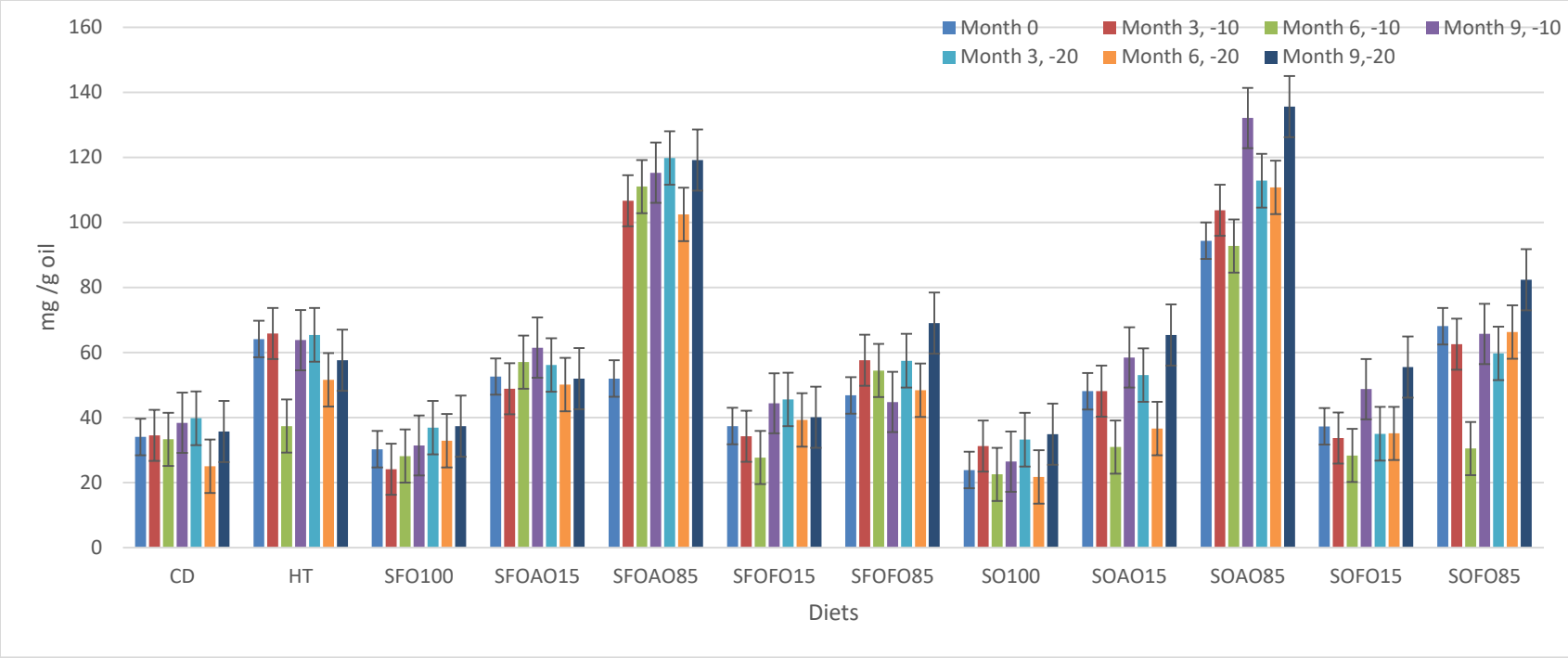

Figure 7. Change in Docosahexaenoic Acids of Tilapia Fillets Stored in Temperature Abused Condition and -20^{o}C Cold Storage Condition Over 9 Months.

*Error bar is represented by the standard error within each time and storage conditions; Month indicates the duration of storage while -10 indicated temperature abused conditions and -20 indicates -20^{o}C cold storage found in commercial settings.