Yogurt as a Vehicle for Omega-3 Fatty Acid Enrichment

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Abstract

Consumer interest in supplementation with healthy omega-3 fatty acids (ω3 FA) has led to increased research in fortification of popular foods with these healthy fats. Yogurt, which is already popular, offers a functional food matrix to fortify with ω3 FA. Fish oil, a major source of two important long chain ω3 FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is an excellent source of ω3 FA enrichment into foods but brings problems of oxidation and off-flavors or odors when added to foods. Encapsulation, deodorized fish oil, and flavoring have been investigated to reduce these off-flavors and odors in food products while producing a fish oil-fortified yogurt.

Discrimination of butter, fish or oxidized fish oil at 0.5% (wt/wt) levels was investigated in unflavored low-fat (1%) yogurt using untrained panelists (n=31) and sensory triangle tests. Five sensory attributes (lime, sweet, heat, acid, oxidized) were analyzed by experienced sensory panelists (n=12) in chile-lime flavored yogurts with butter, fish or oxidized fish oils added at low (0.43%) and high (1% wt/wt) levels. Analytical analysis for composition, fatty acid profile, and volatile chemistry of the yogurts was conducted. Consumer acceptance of a low-fat (1.5%) chile-lime flavored yogurt enriched with fish oil was investigated using a 9-point hedonic scale (1="dislike extremely", 9="like extremely").

Untrained panelists (n=31) were unable to differentiate 0.5% (wt/wt) levels of fish and butter oils in unflavored yogurts but were able to detect oxidized fish oil compared to butter or fish oil under in the same conditions. Experienced panelists (n=12) found significant differences ($p<0.05$) in lime and acid attributes in chile-lime flavored yogurts containing 1% (wt/wt) oxidized fish oil compared with 0.43 and 1% (wt/wt) butter and fish oil yogurts and 0.43% (wt/wt) oxidized fish oil yogurts. Oxidized attributes were determined as significantly different ($p<0.05$) by experienced panelists in chile-lime yogurts with 1% (wt/wt) fish oil, 0.43 and 1% (wt/wt) oxidized fish oil added. The acceptance of a fish oil-enriched chile-lime flavored yogurt was neutral ("neither liked nor disliked") by consumers (n=100) but 44% rated the product "like slightly" (6 of 9) or greater. A successful chile-lime flavored yogurt offering a novel savory flavor was formulated from pre-pasteurization addition of fish oil to deliver more than 145 mg DHA+EPA/170 g serving of yogurt.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Terms

ALA	α-linolenic acid
AHA	American Heart Association
BO	butter oil
Car/PDMS	Carboxen-Polydimethylsiloxane
CFR	Code of Federal Regulations
CLA	conjugated linoleic acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FAME	fatty acid methyl ester
FDA	Food and Drug Administration
FO	fish oil
FST	Food Science and Technology
GC	gas chromatograph
GCMS	gas chromatography-mass spectrometry
GLC	gas-liquid chromatography
GRAS	generally recognized as safe
LDL	low density lipoprotein
NIST	National Institute of Standards and Technology
oxFO	oxidized fish oil
PUFA	polyunsaturated fatty acid
ω3 FA	omega-3 fatty acid(s)
SIMS	Sensory information management system (software)
SPME-GC/MS	solid phase micro-extraction coupled to gas chromatography/mass spectrometry
US	United States

Chapter 1: Introduction

Omega-3 fatty acids (ω3 FA) have substantial health benefits when consumed regularly in the human diet, as shown in numerous research studies. Major sources of the primary ω3 FA in the US diet include fatty fish and some vegetable oils; fatty fish oils provide more docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), while vegetable oils rich in ω3 fatty acids provide α-linolenic acid (ALA). DHA provides critical structures for the proper development of brain and retinal function in infants. DHA is highly concentrated in the adult brain as well and affects the functions of inflammation, neurotransmitters, membrane fluidity, enzyme regulation, gene expression and oxidative stress.[1] DHA assists in reducing inflammation and oxidative stress while increasing membrane fluidity in the body, which contributes to reduced blood pressure and reduced risk of coronary heart disease.[2] In 2004, the Food and Drug Administration (FDA) began allowing foods and dietary supplements containing EPA and DHA to carry the qualified health claim the product 'may be beneficial in reducing coronary heart disease'.[3]

Public awareness of the health benefits of omega-3 fatty acids (ω3 FA) has increased from 63% to 72% between 2006 and 2008 in the US,[4] with an associated increase in consumer interest in dietary sources of these beneficial lipids. Based on the recommended daily intake suggested by the American Heart Association (AHA)[5] and World Health Organization (WHO)[6], the daily serving used in this project is 145 milligrams of DHA+EPA. Due to the fact that the current intakes of EPA and DHA in the United States are estimated at four times lower than the recommended levels[7], the food industry seeks to incorporate ω3 FA into popular foods to increase health benefits and diversify food sources.[8] This presents opportunities for functional foods, described as 'food and food components that provide a health benefit beyond basic nutrition (for the intended population)'.[9]

Yogurt has been recognized as a functional food because of the health benefits associated with "live and active" culture bacteria used in fermentation as well as the added nutrition from milk proteins and vitamins. When enriched with ω3 FA, yogurt has even greater health-promoting properties. The United States (US) dairy industry continues to benefit from large annual growth in the yogurt segment. In 2003, the US yogurt market grew 2.5% with an overall increase from 1999-2003 of 13.7%.[10] A larger proportion (44.2%) of refrigerated yogurt consumers self-classified themselves as 'heavy users' meaning they regularly made six or more

yogurt purchases over a 30 day period.[11] The US functional food market also is growing, with an increase of 7.2% in 2004 and generating 18.9 billion dollars, with $5 billion in dairy products alone.[12] In 2001, reports indicated dairy represented 6% of the US functional food sales but 65% of European sales.[13] This demonstrated significant room for new products in the US functional dairy market. Of all functional foods and beverages, yogurt reported the highest sales, $3.3 billion, for the year ending October 2008.[4] The functional food market is expected to generate approximately $43 billion dollars by 2013.[4] The addition of ω3 FA into dairy foods, for the purpose of providing heart health functional benefits, also increases risk of oxidation in these biologically important lipids, leading to altered sensory (odor, flavor) quality and health value.

The daily amount of essential ω3 FA needed for the proposed health benefits is usually more than the amount fortified into products. Currently 32 mg of DHA+EPA qualifies a product as an "excellent source" of ω3 FA[4]; this amount is lower than the suggested dose of 145 mg/day, but is the level to which many food products are fortified. One reason for a lower level of ω3 FA addition to dairy products is that fish oil introduces commonly associated fishy odors and flavors to the dairy product odor and flavor profile. In addition, oxidation produces undesirable odors and flavors in the fish oil and yogurt and do not provide the same health benefits as fresh. Research on the oxidation of ω3 FA in yogurts gives conflicting results; work by Kolanowski et al reported that additional precautions during processing of these oils are needed to prevent oxidation of fish oil added to a yogurt matrix.[14] Contradicting work by Neilson et al found lower oxidation of fish oil in yogurt than in milk[15] and that iron-induced oxidation was not possible in drinking yogurt[16]. Encapsulation of fish oils provides a protective barrier to prevent oxidation of the unsaturated fatty acids.[17]

This research project investigated the incorporation of an ω3 FA-rich ingredient and an innovative flavoring system into a set-style low-fat yogurt base to produce a functional dairy food source of ω3 FA. This product was formulated to deliver the suggested daily health-promoting amount of essential ω3 FA (145 mg of DHA+EPA) in one 6 oz serving. Fish oil was added to yogurt as the ω3 FA source and chile-lime flavoring was added in an attempt to compliment or mask any off flavors introduced by the ω3 FA oil source. The levels of total fat (fish oil and dairy fat) between 0.5% and 2% fat overall in this yogurt product were targeted for the "low-fat" yogurt category.[18] Microcapsules of chitosan-starch encapsulated fish oil were

evaluated for functionality at different pH levels and processing temperatures to evaluate potential for application in a beverage or yogurt system.

The specific questions answered by this research are:

- Can we deliver 145 mg DHA+EPA in one serving (170g) of yogurt?
- What levels of fish oil addition to plain yogurts are detectible? Is fish oil fortified at these levels detectible in a chile-lime flavored yogurt?
- Are consumers willing to consume a chile-lime flavored yogurt?
- How does the addition of fresh or oxidized fish oil change the fatty acid profile of yogurts? Is the calculated amount of ω3 FA really delivered into the final product?
- Can trained panelists detect differences in low or high levels of oil addition in a chile-lime flavored yogurt product? What is the flavor interaction with oxidized fish oils in chile-lime flavored yogurts?
- Would the encapsulated fish oil be physically compatible in acidic or neutral food matrices at varied processing temperatures (30, 63, 68, 85°C)?

The primary research objectives of this project were:

1. Determine important attributes and acceptable levels of fortification in chile-lime flavored omega-3 fortified yogurt products using sensory evaluation and analytical techniques.
2. Investigate the viability of using chitosan-starch encapsulated fish oil in a dairy matrix.

The projects that supported these objectives were:

- Evaluate detectible levels of oils and chile-lime flavoring by untrained panelists using sensory triangle tests in a low-fat yogurt matrix;
- Measure five sensory attributes of chile-lime flavored low-fat yogurt with three oil sources at low and high levels using an experienced sensory panel and rating scales;
- Quantify if formulated amounts of ω3 FA were delivered in final yogurt product
- Assess consumer acceptance of fish oil enriched chile-lime flavored low-fat yogurt products using a consumer sensory panel;
- Investigate the effect of temperature (30, 63, 68, and 85°C) and pH (4.5, 5.5, 6.5) on the reconstitution of chitosan-starch encapsulated fish oil (60:40 chitosan starch wall-material, 1:2 fish oil:wall-material wt/wt);
- Explore the addition of chitosan-starch fish oil microcapsules into a yogurt matrix.

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Chapter 2: Literature Review

Background, Synthesis, and Sources of Omega-3 Fatty Acids

Omega-3 fatty acids (ω3 FA) are a family of polyunsaturated fatty acids with the last carbon-carbon double bond meeting the third carbon from the methyl end of the fatty acid (Figure 2.1). These chains are known as polyunsaturated fatty acids because of the multiple (more than one) double bonds found in the structure; all double bonds in this fatty acid group are in the *cis* conformation, which allows for easier transformations in nature and all bonds are methylene interrupted.

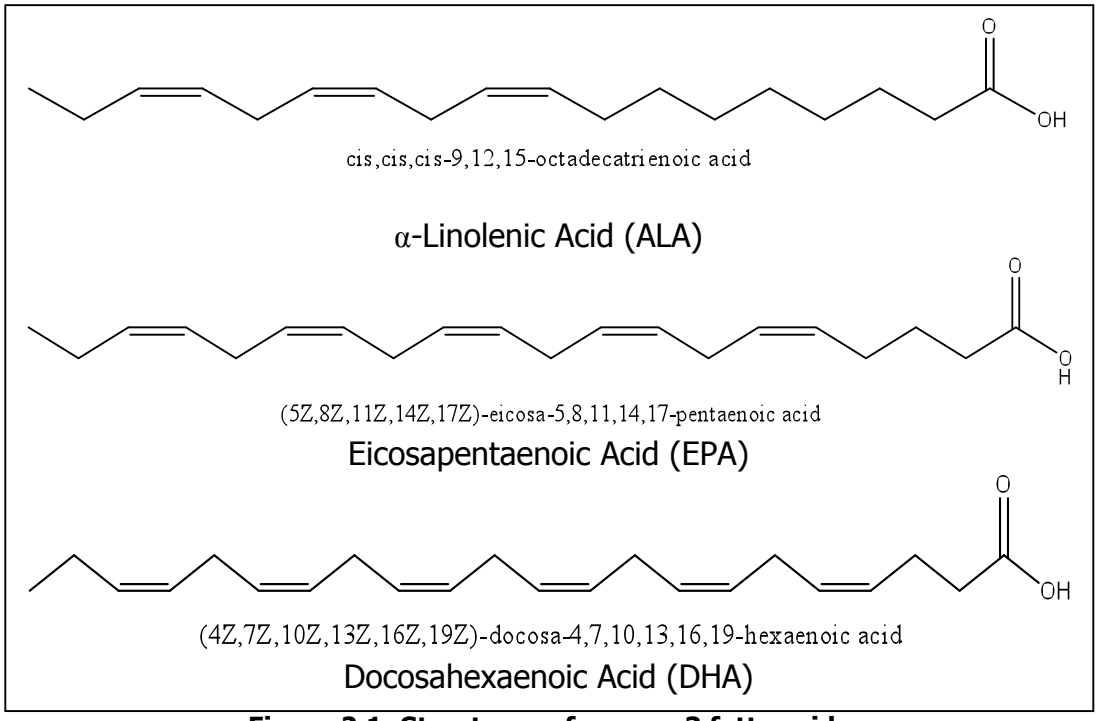

Figure 2.1: Structures of omega-3 fatty acids

The chemical name for α-linolenic acid (ALA) is *cis,cis,cis*-9,12,15-octadecatrienoic acid, also written as 18:3ω-3 signifying the eighteen carbons with three double bonds and the first double bond from the methyl end at the third carbon, indicating an omega-3 fatty acid. ALA is found naturally in many seed oils such as flaxseed, canola, soy, and walnut oil. Eicosa-5,8,11,14,17-pentaenoic acid (EPA) also can be abbreviated 20:5ω-3 indicating an omega-3 fatty acid with twenty carbons and five double bonds. EPA is found naturally in fish oils and breast milk or can be synthesized from algae. EPA also can be found as an intermediate when ALA is converted to docosahexaenoic acid (DHA; 22:6ω-3; Docosa-4,7,10,13,16,19-hexaenoic acid). DHA, like EPA, is found primarily in fish or algae oils.

These polyunsaturated fatty acids are essential to the human diet because they cannot be easily synthesized. Limiting factors for synthesis include the long carbon chain (18:0 or longer), which requires appropriate desaturation and elongation enzymes present for formation.[1] It is suggested that Δ6-desaturase, the enzyme that converts ALA to 18:4ω3, is the rate limiting enzyme because it begins the conversion pathway to EPA and DHA.[2] The eighteen-carbon fatty acid ALA can be used to form longer chained (20 and 22 carbon) fatty acid in the body (Figure 2.2) but does so with low conversion rates (<20% for EPA, <9% for DHA)[2-3]. Low efficiency for conversion of EPA and DHA from ALA suggests that it is more efficient to obtain these fatty acids directly from food sources. It is reported that, on average, the body can only convert 5% of ALA to DHA and EPA.[3] Women convert at rates approximately 2.5 times greater than men, possibly because of the developmental need to supply DHA to the fetus during pregnancy.[3] Research by Burdge compares the conversion rates of ALA to EPA at 8% and DHA the highest estimate 4% in men while women are able to convert 21% of ALA to EPA and 9% to DHA.[2] This study also reports that ALA concentration in cell membranes and blood lipids is less than 0.5% of total fatty acids while DHA can be up to 50% of the fatty acids in the brain and retina.[2]

ALA is converted to EPA, which then may be converted to DHA, through removing hydrogen molecules to form additional double bonds (desaturation) and addition of carbon atoms (elongation) (Figure 2.2).

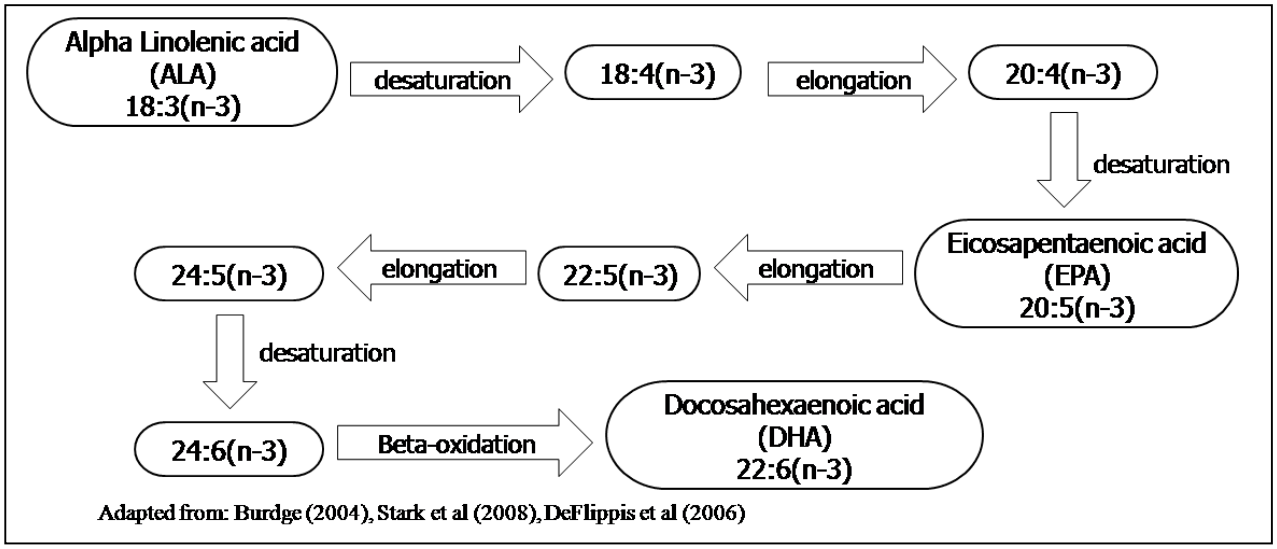

Figure 2.2: Conversion of ALA to EPA and DHA

Omega-3 fatty acids come from many sources; flax seed and fatty fish are two major dietary sources of ω3 FA. Plant sources tend to have larger amounts of ALA than DHA or EPA; unfortunately, since ALA needs to be converted to EPA or DHA for providing health benefit and there is a low biological conversion rate, plant sources are not the optimum dietary source of ω3 FA. Algae, another large source of ω3 FA, are used in the main pathway for fish to obtain long-chain ω3 FA. Marine phytoplankton show high levels of 16:0, 16:1 and EPA while marine macroalgae show higher levels of 16:0, 20:4 and varied amounts of EPA and 18 carbon unsaturated fatty acids.[4] Fish cannot synthesize long chain fatty acids because they lack desaturase enzymes. Algae are an increasingly popular dietary source of ω3 FA because they can be used for vegetarian supplementation but literature continues to debate the comparative amounts of DHA and EPA provided from algae sources. Fatty fish, such as mackerel, herring, and salmon, provide higher levels of DHA and EPA than other fish sources and higher levels than plant sources.[5] It is suggested that one third of all harvested fish are used in production of fish oils or nutraceuticals and fish meal; substituting algae as an ω3 FA source also would allow the demand for fish as sources of omega-3 rich ingredients to be lowered so they can be used in other areas of the food chain.[6] ω3 FA also can be derived from marine mammalian oil; Barrow and Shahidi report that the human body may absorb these essential fatty acids more quickly from mammalian oil than from fish oil.[7] They also suggest the triacylglycerol structure and distribution of mammalian oils provide better resistance to oxidation than fish oil.[7]

Health Benefits of Omega-3 Fatty Acids

Scientific research documenting the diverse health benefits of ω3 FA suggests a broad range of levels necessary to experience these benefits. The US Food and Drug Administration (FDA) regulate label claims for health benefits associated with food components on foods and supplements. In 2004, the FDA began allowing foods and dietary supplements containing EPA and DHA to carry the qualified health claim the product 'may be beneficial in reducing coronary heart disease'.[8] The FDA also regulates food additives and maintains menhaden oil as a generally recognized as safe (GRAS) substance even when used as a direct human food ingredient.[9] The FDA does not regulate fish oil supplements, allowing them to contain varied amounts of EPA and DHA and even very low levels of mercury.[10] The FDA also suggests that consumers not exceed 2 grams per day of ω3 FA from dietary supplements or a total of 3 grams

a day from food, probably due to possibilities of mercury contamination in some fish, though instances have been rare in the United States.[8, 10] Many other nongovernmental agencies have reviewed research evidence associated with ω3 FA health benefits; MedlinePlus, a webpage by the National Institute of Health, documents 'strong scientific evidence' that fish oil supplementation can help lower high blood pressure, reduce blood triglyceride levels, and help to prevent secondary cardiovascular disease risks.[11] They also include other health benefits of fish oil backed by scientific evidence, relating to rheumatoid arthritis, infant brain and retinal development, reduced inflammation, age-related macular degeneration, asthma, atherosclerosis, attention deficit hyperactivity disorder, cancer prevention, immune function, inflammatory bowel disease, and many other health conditions.[11]

Substantial health benefits of ω3 FA are shown in developing children, especially infants, because DHA and EPA are essential for proper brain and retina development. For this reason prenatal vitamins contain these essential fatty acids. DHA is found in high concentrations in human cellular membranes, especially the brain, so it is needed in high concentrations for these tissues to form. Infants show elevated levels of DHA in the brain during prenatal life and the first months of life; research indicates that deficiencies during this time can lead to delayed cognitive development of the brain, visual impairments or behavioral problems as the child grows.[12] This developmental need for DHA is augmented by the fact that children under the age of two are unable to synthesize DHA or EPA from other fatty acids.[6] Research by Prescott and Dunstan also suggests that increasing the intake of ω3 FA in the early years of development can prevent the onset of allergic disease because of its importance in immune system development.[13]

DHA is found in high concentrations in the adult brain as well and affects the functions of inflammation, neurotransmitters, membrane fluidity, enzyme regulation, gene expression and oxidative stress.[7] These important functions suggest that DHA levels in the brain may be linked to mood swings and memory loss with age. Fish oil rich in DHA and EPA has been linked to reducing symptoms of lung diseases such as exercise-induced bronchoconstriction and asthma due to competitive inhibition of pro-inflammatory biomolecules. These same properties allow for ω3 FA to be used in treatment of joint tenderness due to rheumatoid arthritis or obesity.[6] ω3 FAs provide relief to those who suffer from inflammatory diseases such as arthritis; Barrow and Shahidi summarize studies that use different methods of an ω3 FA oil supplementation but all report reduced pain from stiffness and joint tenderness in patients receiving supplements.[7]

Research by Weaver et al reveals the reasons behind ω3 FA effectiveness in treating inflammatory diseases, showing the correlation of ω3 FA in regulation of the expressions of signal transduction genes and those for proinflammatory cytokines in humans.[14] Evidence also indicates that ω3 FAs have antiaging effects, most likely due to their role in human tissue structure and their high concentration in the brain. In a twelve year cohort study, researchers found that higher levels of ω3 FA intake caused participants to be less likely (30%) than others studied to develop age-related diseases of the eyes such as age related macular degeneration or central geographic atrophy.[15]

Strong evidence for reduced risks of coronary heart disease, hypertension and atherosclerosis have been found from increased amounts of ω3 FA in the diet, providing the basis for the qualified health claim. ω3 FA reduce the risk of heart disease by lowering blood viscosity, reducing plasma fibrinogen to inhibit platelet aggregation, reducing fasting serum triglyceride levels, and decreasing blood pressure. It also has been shown that omega-3 supplementation can reduce total cholesterol in the body.[6]

A nutritional deficiency of these essential fatty acids can be linked to a host of health problems including restrictive growth, irregular fatty acids in body tissue and damaged reproductive systems.[6] Other sources have found a correlation of low ω3 FA levels in red blood cells to neurological disorders such as attention deficit disorder and hyperactivity, Alzheimer's disease, and depression but research has not been conducted to prove that ω3 FA can improve symptoms of these diseases.[7]

Dietary Recommendations for Omega-3 Fatty Acids

The evidence for the relationship of health benefits to dietary intake levels of ω3 FA is still growing. Although recommendations related to fish intake are provided by several organizations the dietary recommendations of omega-3 fatty acids for preventing coronary heart disease remain highly debated. The American Heart Association suggests 2 servings of fatty fish per week[16]; the World Health Organization recommends eating 1-2 servings of fish with each serving supplying 200-500 mg of DHA and EPA per week[17]; the British Nutritional Foundation Task Force on Unsaturated Fatty acids recommends 8-10 g of DHA and EPA per week equivalent to 2-3 medium portions of oil-rich fish per week[18]; and the Australian Nutrient Reference Values set the suggested intake of DHA, EPA and DPA at 90 mg/day for women and

160 mg/day for men[19]. Table 2.1 provides a summarized list of the recommendations and a comparison of the suggested mg/day of DHA and EPA for health benefits. The level of supplementation also differs based on the benefits desired; a higher intake is necessary for some health improvements such as those already suffering from coronary heart disease or inflammatory diseases. The reported intake of total ω3 FA in the United States in 2000 was 1.6 g/day with approximately 0.1-0.2 g EPA and DHA.[5] Consumer concern about the increased intake of heavy metals, such as mercury, with increased fish consumption can influence consumer choices about dietary sources of ω3 FA.[10]

Table 2.1: Suggested dietary intake of DHA and EPA as reported in literature

Recommending Organization	**Suggested Serving**	**mg/day of DHA+ EPA**
American Heart Association (AHA)	2 serving fatty fish/week[16]	not reported
World Health Organization (WHO)	200-1000 mg DHA & EPA/week[17]	28-143[a]
British Nutritional Foundation Task Force on Unsaturated Fatty Acids	8-10g DHA & EPA/week[18]	1142-1428[a]
Australian Nutrient Reference Values	90-160 mg DHA, EPA, DPA/day[19]	90-160
Minister of National Health and Welfare Canada	1.2-1.6 g/day ω-3[20]	not reported
Essentiality of and Recommendations for Omega-6 and Omega-3 Fatty Acids, Simopoulos	0.65 g/day DHA+EPA[21]	650
Committee on Medical Aspects (COMA)	0.2 g/day long chain PUFA[22]	200

[a] calculated

Functional foods enriched with ω3 FA are increasing in popularity in the United States. There is no legal definition of a functional food because the FDA is still in the process of reviewing regulatory requirements for such foods. Various organizations have provided definitions in order to assist the public and scientific communities with understanding this growing segment of the food industry. The Institute of Food Technologists (IFT) Expert Food Panel (US) has defined functional foods 'as food and food components that provide a health benefit beyond basic nutrition (for the intended population)'.[23] It is defined similarly by the International Food Information Council (IFIC), American Dietetics Association, and the Institute of Medicine of the US National Academy of Sciences.[24] ω3 FA enrichment in foods primarily comes from powdered or oil forms of fish, algae, or flax seed oils, resulting in more functional food options to consumer interested in alternative ways to increase dietary intake of ω3 FA. One

gram of fish oil provides approximately 300 mg EPA + DHA to food products[5] but this can vary based on processing technique and source of fish. Many foods currently on the market are enriched with ω3 FA, including cereals, orange juice, milk (cow and soy), butter substitute spreads, yogurt, cheese, ice cream, breads, infant food and formulas, marshmallows, chocolate, spaghetti sauce and eggs. Some foods that traditionally are used as the main portion of meals such as hamburgers, pizzas and peanut butter, are enriched with ω3 FA.[25]

Potential Food Safety and Quality Problems Associated with Omega-3 Fatty Acid Enrichment

Omega-3 fatty acids, with a higher proportion of unsaturated sites, are more susceptible to oxidation, or the creation of free radicals through the oxidation of unsaturated fatty acids. The oxidation process has three steps: initiation, propagation and termination (Figure 2.3). The oxidation reaction must be catalyzed to begin (initiate) the oxidation process; catalysts (metals, thiols, heme compounds, light energy, and other compounds that react to form free radicals) may induce an autoxidation pathway or cause a photochemical reaction sequence. In photosensitized oxidation, light interacting with a singlet oxygen causes initiation; in autoxidation, a catalyst such as metals, thiols, heme or iron containing compounds produce a superoxide anion that attacks the lipid. This reaction forms free radicals. Once started, the oxidation process is very difficult to stop because the reaction self-propagates by creating and using every new free radical formed. For termination of an oxidation reaction to occur, two free radicals, two lipid peroxyl radicals, or one of each must combine to form non-radical products to prevent self-propagation. The free radicals produced from oxidation can undergo scission reactions to form aldehydes, ketones, or alcohols; these products can be volatile and alter flavor composition, contributing unwanted flavors and odors.

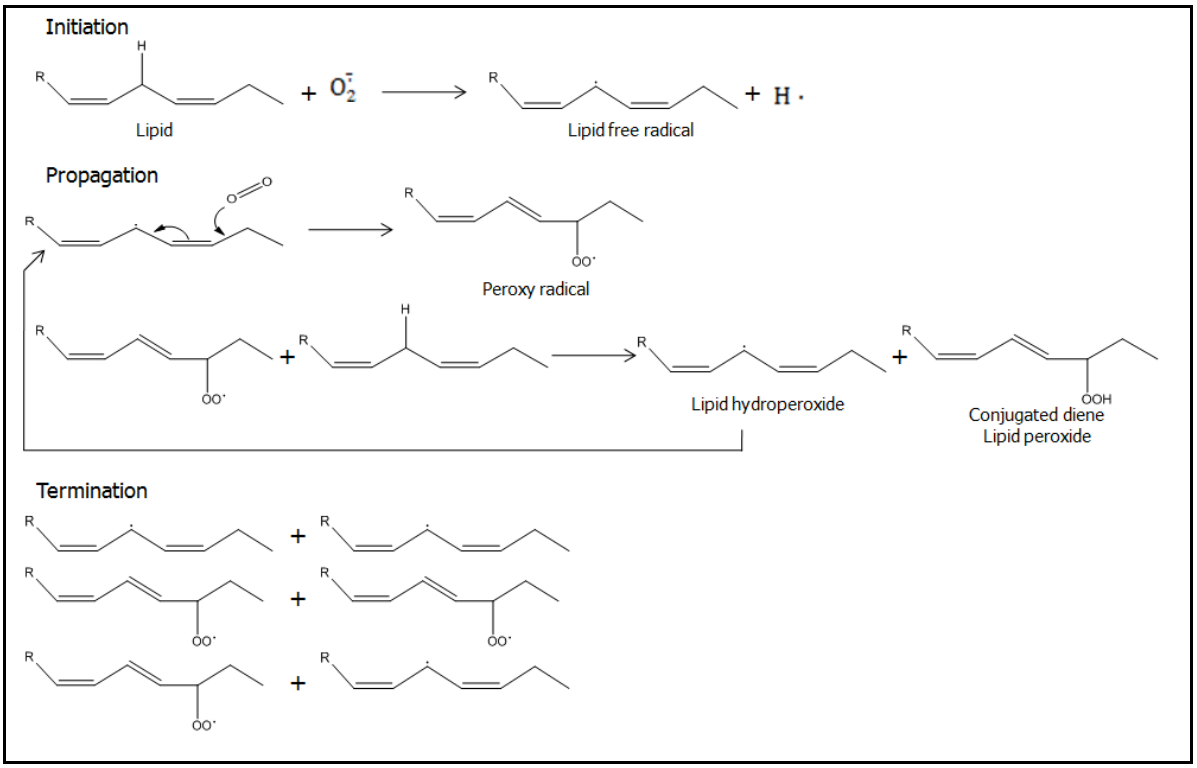

Figure 2.3: Autoxidation pathway of lipids

Oxidized products can be toxic to animals; in a research study where mice were fed oxidized sardine oil, the oxidized oil was proposed to have caused tumors in the mice.[6] Peroxides formed during the break down of ω3 FA can be toxic and possibly cancer causing.[26] Deodorizing and refining of fish oil, using microencapsulation techniques, or adding antioxidants can help reduce oxidation of fatty acids.[6] Proper packaging and storage of enriched food products and fish oils also can prevent oxidation.[26] Appropriate processing, formulation, and packaging technologies for protecting the biological value of these essential fatty acids are needed to deliver the intended health benefit.

Fish oils and other highly unsaturated food oils are susceptible to autoxidation. Dairy products, which contain relatively low levels of highly unsaturated fatty acids, are susceptible to light-induced oxidation in dairy products. Riboflavin has been known to induce light oxidation in dairy products and other porphyrin compounds, such as chlorophyll, may also contribute to this type of reaction.[27] Iron and copper have been associated with initiating metal-induced autoxidation in dairy products, mostly when the product has been processed on older equipment (not stainless steel). Incorporating ω3 FA into dairy products may increase the risk of oxidation from autoxidation and cause new challenges associated with photoxidation.

Protection of Omega-3 Fatty Acids by Encapsulation with Chitosan

Spray drying is an effective technique for encapsulating fish oil and potentially protecting fish oil from oxidation. Kolanowski et al studied the protection of fish oil from oxidation by microencapsulation of the oil with modified cellulose.[28] Based on peroxide values, microencapsulated fish oil experienced significantly less oxidation than bulk fish oil. The maximum oil load retained using cellulose and spray dried encapsulation was 400 g oil/kg of microcapsules.[28] Shaw et al documented that a multilayer emulsion of chitosan, lecithin, and menhaden oil encapsulated with corn syrup solids added gave evidence that the encapsulation prevented oxidation even when reconstituted.[29] This research suggests that spray drying encapsulation technologies to protect oil from oxidation and deliver ω3 FA in food would work well in functional foods. Klinkesorn et al also concluded that spray drying fish oil provided a more oxidatively stable powder for use in foods.[30] Spray drying tuna oil in a multilayer lecithin-chitosan coating resulted in a more stable product than bulk oil. They also found that at higher water activities this spray dried product was more stable to oxidation, possibly because Maillard reaction products acted as antioxidants. The addition of EDTA or mixed tocopherols to the emulsions also increased oxidative stability.[30] Hannah demonstrated that spray drying a chitosan and starch blend provided some protection of deodorized fish oil from oxidative changes over time.[31]

Chitosan Background and Properties

Chitosan, also known as β-(1,4)-linked-D-glucosamine or poly-(β-(1,4)-linked-2-amino-2-deoxy-D-glucose) comes from the shellfish waste product, chitin. Chitin is a cationic polysaccharide made of units of β (1,4) linked *N*-acetyl-D-glucosamine. The most common polymorphic form of chitin is α that consists of repeated links of two N,N-diacetyl-chitobiose units in antiparallel arrangements.[6] The α arrangement provides excellent thermodynamic stability due to a large proportion of intermolecular hydrogen bonds; this also contributes to the insolubility of chitin in water and most organic solvents.[32] Chitin is found abundantly in nature and is second only to cellulose as the most abundant biopolymer found in nature.[7] Chitin comes from harvested crustacean exoskeletons, and cell walls of fungi, insects or marine diatom. The most prevalent source of this natural polymer comes from marine shellfish and crustaceans such as shrimp heads and shells, crabs, squid and krill but it also is found in algal and fungal cell

walls. The shell waste of crustaceans contains an average of 20-30% chitin but can vary with season and species.[33] Processing of shell waste to chitin takes place through three steps; demineralization, deproteinization, and bleaching.[6] The estimated amount of chitosan produced globally in the year 2000 was 2000 tons, with 75% of the chitosan coming from chitin.[34] The estimated total global potential for chitin production is 76,000 tons annually.[6]

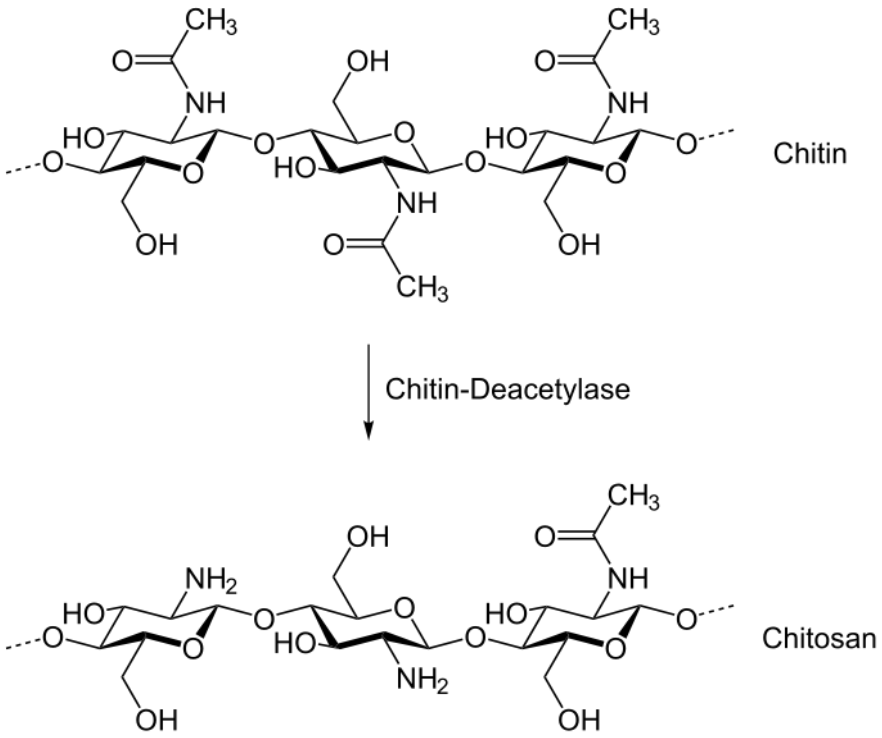

Figure 2.4: Structures of chitin and chitosan

Chitin, when deacetylated, forms chitosan (Figure 2.4). This natural biopolymer was discovered by C. Rouget in 1859, but scientists were not interested in possible uses of chitosan until the 1930's.[6] The process of converting chitin to chitosan involves adding 30-60% (w/v) sodium or potassium hydroxide to chitin at 80-140 ^{o}C, drying to produce flakes that are purified by dissolving in dilute acetic acid and reprecipitating with alkali; finally the product is washed and dried. Increases in heat during processing or increasing particle size of chitin will cause an increase in the amount of deacetylation of chitosan. Enzymes offer an alternative production method for converting chitin to chitosan without using the harsh chemicals listed in the process above.[6] Chitosan possesses chemical properties of a cationic polyelectrolyte that is insoluble in pure water and organic solvents but soluble in weak inorganic acids such as hydrochloric and nitric. Some organic acids also may dissolve chitosan; these include formic, acetic, propionic,

ascorbic, lactic, malic, citric acids and acidic amino acids.[35] Some of these acids also are found naturally at low levels in yogurt and may influence the functionality of chitosan in a yogurt matrix. Chitosan is categorized by its molecular weight and degree of deacetylation. The degree of deacetylation, ionic strength, and pH of a solution containing chitosan all can affect the viscosity of the solution. Chitosan is positively charged at an acidic pH.

Beneficial Uses of Chitosan

Chitosan has many suitable applications because of its cationic charge and multiple reactive functional groups. Chitosan also has a benefit to the current food industry trends because it can be labeled as natural.[33] Chitosan is found in food, as antioxidants and antimicrobials, or used in medical, biotechnology, and water treatment fields. In food, chitosan can act as an antioxidant agent, by chelating with metal ions that catalyze lipid oxidation, to prevent or decrease the oxidation process.[33] In meats chitosan has been observed to chelate iron and prevent the initiation of oxidation.[36] It can act as an antimicrobial by disrupting the outer membrane of bacteria by ionic interactions and also inhibits fungal growth. Chitosan has antimicrobial effects against several food borne pathogens such as *Bacillus cereus, Clostridium perfringens, Listeria monocytogenes,* and *Salmonella typhimurium.*[6] Chitosan also has been shown to disrupt *Lactobacillus* spp. and *Saccharomyces* spp., which could prevent proper fermentation of yogurt. Agullo et al suggest that the ability of chitosan to be protonated at an acidic pH allows interaction with cell walls of *Salmonella typhimurium,* causing cell wall disruption and injuring the cell.[33] Other mechanisms are suggested as to how chitosan works as an antimicrobial.[36] No et al demonstrated that antimicrobial properties of chitosan increase with decreased pH and the minimum inhibitory concentration of chitosan was 0.05% to >0.1% but this can vary based on the targeted bacteria and molecular weight of the chitosan.[37] Auser et al investigated the ability of chitosan to be used in cheese making; they suggest it is possible to use chitosan in fermented dairy products without detrimental effects to *Lactobacillus bulgaricus or Streptococcus thermophilus*.[38] This study showed that chitosan used as an antimicrobial and added to milk did not inhibit bacterial growth as occurred in enrichment broth, possibly due to the interactions of the chitosan with casein. The Auser research group successfully produced a cheese-like product using high molecular weight chitosan as a coagulating agent; the chitosan

created milk coagula, indicating that a fiber-enriched cheese could be produced in this manner but with different physical characteristics than a control cheese.[38]

The use of chitosan in preparation of edible films also allows enhancement of food products by creating gas (oxygen, carbon dioxide) barriers or antimicrobial and antifungal coatings on foods. The interaction of positively charged chitosan with other negatively charged molecules, lipids, proteins and starches also can allow it to be used as a thickening, emulsifying, or clarifying agent in foods. When used as an emulsifier, chitosan creates emulsions that are stable to flocculation and coalescence under temperature changes and aging but emulsification properties can change with the degree of deacetylation.[33]

Recent research has investigated the nutritional and health function of chitosan to reduce low density lipoprotein (LDL) cholesterol levels and act as fat scavengers in the digestive tract. The proposed mechanism involves the emulsifying properties of chitosan to entrap oil in the small intestines through aggregation and allow it to pass unabsorbed through the large intestines and leave the body through feces.[33] A study by Beysseriat et al suggests that increasing dietary fiber in food products can lead to a lower caloric intake of foods.[39] The study showed that in emulsions of chitosan and lipid, the chitosan inhibited lipase from hydrolyzing fat for absorption into the body due to the flocculation of the chitosan particles around the fat. Other uses for chitosan include use as a drug delivery system and in skin healing agents in the pharmaceutical industry or removal of lead, copper, mercury, and negatively charged contaminants in water treatment.[6] Glucosamine, made from fully hydrolyzed chitosan, is suggested for use as a supplement to ease joint pain and illustrates another potential functional ingredient from chitin and chitosan.

Health and Regulatory Status of Chitosan

The regulation and approval of chitosan in foods differs by country. The US has not yet approved chitosan for use in foods. Primex (Siglufjordur, Iceland), the company that makes Chitoclear™, has applied and withdrawn twice an application for GRAS (generally recognized as safe) status in the US. The Korea Food Additives Code includes chitosan as an approved natural food additive and provides identification procedures for it in food.[40] Japan allows for the use of chitosan in food as a natural thickener or stabilizer.[41] In 1992, Japan also recognized chitosan as

a functional food ingredient. The only FDA approved use of chitosan in the US is as a dietary supplement.[7] The FDA recognized chitosan for use in livestock feed in 1983.[42]

The ability of chitosan to block fat absorption is suggested in the research. As an ingredient for encapsulation of ω3 FA, it would be important that chitosan would not interfere with the absorption of these fatty acids into the body. Park et al explored the effect that chitosan encapsulation, by freeze-drying encapsulation techniques, had on emulsified lipids in mice digestion.[43] Mice were fed different diets containing fat and chitosan. Body organ and blood analysis showed that diets caused no adverse effects to the mice and no overall differences were found in fat absorption when comparing treatment diets. The researchers concluded that there is no digestibility impact from encapsulating fat using chitosan, but they also acknowledged that humans metabolize fat differently than mice.[43] For the body to absorb the beneficial fatty acids in this research matrix, the chitosan-starch wall material must be broken down during digestion. A study by Zeng et al, investigating the effect of the absorption of chitosan solutions in mice, suggests that pH, molecular weight and water-solubility all have strong correlations to the ability of mice to absorb chitosan in the intestines.[42] Increased water-solubility and decreased molecular weight both gave an increase in absorption of chitosan in the intestines. As pH reached the 6-7 range, the higher molecular weight chitosan would precipitate and gelation would occur, causing fat to be trapped in the gel and not absorbed to the system.[42] Klinkesorn and McClements showed that chitosan separately added to tuna oil-in-water emulsions can protect lipids from oxidation and allow release of fatty acids by pancreatic lipase.[44] Their results illustrated that chitosan in food emulsions protected the triacylglycerol structure and caused a lower release of fatty acids than emulsions without chitosan, but they also claimed that pancreatic lipase digested the chitosan, releasing glucosamine.

Not all research indicates that lipids are absorbed into the body when chitosan is present. Mun et al reported less access of pancreatic lipase to lipids in lecithin-chitosan emulsions than emulsions containing lecithin or lecithin-chitosan-pectin droplets.[45] While a chitosan-lecithin-pectin emulsion revealed similar amounts of free fatty acids as a lecithin emulsion, the researchers suggested this result was due to chitosan and pectin interacting at a pH of 7 and desorbing from the lipid surface during the simulated digestion process. Research conducted on the ability of chitosan to inhibit or allow absorption of lipid into the human body suggests the need for further studies before decisive conclusions should be made.[45]

The protection of ω3 FA from oxidation by microencapsulation in a chitosan-starch matrix may provide additional health benefits conferred through a functional food. Yogurt is a product that may be a good vehicle for omega-3 enrichment. A chitosan-starch encapsulation matrix may provide physical (texture) property functionality within yogurt, as well as health value.

Yogurt Background and Functionality

Yogurt is a popular food product around the world and has been consumed for thousands of years. In ancient times yogurt was prescribed as remedies for stomach and intestinal pain and liver ailments[46]; it continues to be recognized as a food that delivers nutrition and health benefits. In the United States, the FDA's Code of Federal Regulations (CFR), Title 21, Volume 2 (21CFR131.206) regulates what may be labeled as yogurt with certain specifications concerning particular ingredients.[47] The code specifies that culture containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* must be used in conjunction with one or more dairy ingredients (cream, milk, partially skimmed milk, or skim milk). Before adding bacterial culture yogurt mix must be pasteurized or undergo heat treatment necessary to destroy pathogenic and most spoilage microorganisms, thereby extending shelf life and providing an environment for culture bacteria to flourish. This regulation also specifies optional ingredients, sweeteners, flavoring and color additives, and identifies stabilizers that may be used. To label a yogurt as "low-fat", it must contain either 0.5, 1, 1.5, or 2% milkfat before addition of bulky flavorings but not be less than 8.25 percent milk solids non fat.[47] The CFR states that select non-dairy ingredients may be added to yogurt and still permit labeling as yogurt. Fish oil is not a permitted 'non-dairy' ingredient for addition into yogurt as stated by the standard of identity[47]; yogurt with fish oil added must be given a descriptive name that reveals the basic composition of the product such as 'yogurt enriched with omega-3 fatty acids'.[48]

Yogurt is characterized as a functional food, primarily due to the live and active cultures that promote healthy digestion, boost the immune system, and provide other health benefits. The National Yogurt Association allows for voluntary classification of yogurt into the live and active culture category with proof that at least 100 million *Streptococcus thermophilus* or *Lactobacillus bulgaricus* cultures per gram of yogurt are present at the time of yogurt manufacture.[49] Many of the macro- and microcomponents inherent in milk, the primary ingredient of yogurt, also are

recognized for health benefits beyond basic nutrition and contribute to the recognition of yogurt as a functional food.

Health Benefits of Yogurt

Many of the beneficial nutrients in milk such as calcium, phosphorus, potassium, niacin, and folic acid, are found in yogurt at more concentrated levels than in milk because milk powder is added to increase the total milk solids during yogurt formulation. Lactic acid bacteria in yogurt production have been shown to increase concentrations of vitamins K and B_{12}, riboflavin, thiamine, and folate.[50] During the fermentation process of yogurt, bacteria break down lactose molecules for energy, creating a more favorable dairy product for lactose intolerant individuals.[51] The addition of probiotics or prebiotics allows yogurt to be used as a vehicle for gastrointestinal disturbances and to enhance the immune system. Yogurt enriched with probiotics assists in regulating the digestive system when eaten regularly and assisting with bacterial recolonization in the intestine during antibiotic therapy. Other benefits from increased yogurt consumption include declined serum cholesterol levels[46] and protection from foodborne microorganism infection.[48] Beneficial organic acids found naturally in yogurt include lactic, pyruvic, oxalic, succinic, formic, acetic, propionic, and butyric acids.[52] Enjoying low-fat or nonfat yogurt as part of a low-fat meal or snack provides health benefits by reducing fat in the diet.

Market Trends of Yogurt

The United States (US) dairy industry continues to benefit from large annual growth; the increasing popularity of yogurt is reflected in this growth. In 2003 the US yogurt market grew 2.5% with an overall increase from 1999-2003 of 13.7%.[53] In the global yogurt market, the US sold the second largest amount of yogurt in 2003.[53] The main US yogurt market competitors are the US company General Mills (selling Yoplait and Colombo) and the French company Danone (selling Dannon). In 2007, the per capita consumption of yogurt in US was 11.5 million pounds.[54] In 1995, the average per capita yogurt consumption in the US was 4.5 pounds per year.[55] A large proportion (44.2%) of refrigerated yogurt consumers self-classified themselves as 'heavy users', meaning they regularly made six or more yogurt purchases over a 30 day period.[56] Yogurt also is tracked in the packaged snack foods market; from 2004 to 2008 yogurt sales increased from 9.1 to 10.4% of this market.[57] The global growth of yogurt is exemplified

by the 903 new introductions of yogurt products in 2008 world wide.[57] While yogurt makes up a large share of the sweet snack market, there is potential grown for yogurt in the savory snack foods market.

The US functional food market also is growing; in 2004, this market increased 7.2% with a value of 18.9 billion dollars. The functional dairy market accounted for 5 billion dollars of the functional foods market in 2004.[58] In 2001, dairy foods made up 6% of the US functional food sales while, in Europe, this segment held 65% of functional food sales.[59] This indicates significant room for growth and introduction of new products to the US functional dairy market. Consumer actions and decisions primarily drive the functional food market. In 2001, 91% of US consumers claimed to use fortified foods.[59] Of all functional foods and beverages, yogurt is the highest in sales with $3.3 billion for the year ending October 2008.[60] Omega-3 fatty acids consist of one of the fastest-growing functional ingredients on the market, US consumers having knowledge of omega-3 fatty acids increasing from 63% to 72% between 2006 and 2008.[60] If growth of the functional food market continues as expected it will generate approximately $43 billion dollars by 2013.[60] Increasing consumer interest in ω3 FA fortified food products may help drive that growth.

Yogurt as the Ideal Vehicle for Omega-3 Fatty Acids

The health benefits from yogurt in combination with growing trends in yogurt consumption make yogurt an ideal vehicle to incorporate omega-3 fatty acids into a normal diet. Yogurt also offers a food that is not eaten exclusively by one population or at one meal; consumption of yogurt, as a snack and with meals anytime throughout the day offers broad appeal for its use as a functional food. The product is enjoyed by all ages and cultures, which also expands its functional capacity. Dairy products have a history of ω3 FA fortification. Milk enriched with ω3 FA was introduced into the global market as early as 1998 (Parmalat, Plus Omega-3 Milk, Collecchio, Italy).[59] Many yogurt products containing ω3 FA have already been developed; some yogurts with DHA have already reached the market, and many are targeted at children. Chee et al investigated the addition of algae oil emulsions to flavored yogurt for increased ω3 polyunsaturated fatty acids.[61] The study resulted in a strawberry yogurt that delivered 400 mg of ω3 polyunsaturated fatty acids (unspecified) per 272 g serving of yogurt, which the researchers suggested would meet the targeted levels of ω3 FA needed for health

benefits. This would be dependent on the concentration of DHA and EPA in the algal oil. The taste was not significantly different between products and rated as 'liked moderately' in control yogurts as well as yogurts with algae oil added pre- or post-homogenization by a consumer sensory panel (n=139) using a 9-point hedonic scale. The positive responses could be attributed to the masking properties of the fruit flavor.[61]

The possibility of ω3 FA oxidization or degradation during milk synthesis or processing is a concern for enriching yogurt using pre- or post-harvest methods. Dave et al reported that adding 2% wt fish oil to bovine diets did increase the ω3 FA concentration significantly ($p<0.05$) in raw milk and neither pasteurization of milk for yogurt nor the fermentation process had effects on conjugated linoleic acid (CLA) or ω3 FA composition in the yogurt.[62] Scientists suggest adding fish oil to products late in processing to minimize potential stresses and avoiding excess exposure to light, heat and oxygen.[63]

Adding fish oil to yogurt mix during formulation to increase the ω3 FA has also been studied. Kolanowski et al determined that yogurts enriched with fish oil up to 0.3% (0.1% DHA/EPA) were acceptable to a sensory panel.[64] Fish oil added to milk (0.15%) and flavored yogurt (0.3%) were compared to soy bean oil (1.5%), fat spreads (1.5%), an orange drink (0.3%) and apple-beetroot juice (0.15%); those with stronger flavor and sweetness were better at masking the fishy flavor and aroma. They also suggested that products of lower pH may require special considerations to prevent oxidation of the fish oil.[64] Kolanowski and WeiBbrodt reported that higher levels of fish oil fortification were possible in dairy products with higher levels of fat.[65] They were able to fortify spreadable cheese or butter (30 g serving) at a level that would provide 180-360 mg of omega-3 fatty acids consisting mostly of DHA and EPA.[65] A study on the bioavailablity of ω3 FA in humans reported that a fish oil supplemented yogurt drink supplied the fastest absorption of lipids into the blood, measured in composition of fatty acids in chylomicrons, compared to a fitness bar or hard fish oil capsule.[66] They attributed these findings to the fact that yogurt has preformed emulsions, which aid in absorption of lipids. No significant differences in conjugated dienes, as a measure of oxidation of ω3 FA, were found over time but higher levels of oxidation in blood after consumption of the fitness bar were observed. This increased oxidation effect was attributed to the baking or thermal processing of the fitness bar and could be improved or eliminated by encapsulating the fish oil or adding an antioxidant.[66]

Studies on fish oil-supplemented yogurt also have been conducted on other animals. Higuchi et al studied the effects of yogurt and fish oil-enriched yogurt diets compared to a control (no yogurt or fish oil) on mice.[67] Decreases in plasma triacylglycerol, plasma total cholesterol, phospholipid, and glucose concentrations as well as hepatic triacylglycerol content associated with the fish oil-enriched yogurt diet were found. The fish oil was added to the yogurt post-fermentation and the enriched yogurt was frozen and stored (< -40°C) to prevent oxidation. They found EPA and DHA only in the plasma and liver tissue of mice that were fed the fish oil-enriched diets and found the fish oil diets decreased the levels of palmitoleic acid and arachidonic acid in plasma and liver tissues as well. They concluded that fish oil and not yogurt lead to decreased plasma phospholipid and total cholesterol concentrations. They also suggested that ω3 FA may inhibit the synthesis of arachidonic acid from linoleic acid.[67]

While Kolanowski et al reported that products with lower pH, such as yogurt, may be more difficult to protect from oxidation[64], other authors have demonstrated that yogurt provides a more oxidatively stable matrix for fish oil. Nielson et al conducted a study on the oxidative stability of 1 wt% fish oil in fluid milk and in drinking yogurt.[68] The drinking yogurt had lower peroxide values (4 meq/kg) than milk after four weeks of storage (2°C, dark, 4 weeks). Sensory evaluation using descriptive analysis by nine trained panelists compared the two products and detected greater fishy aroma in milk than drinking yogurt (plain or added citric acid, pectin, glucono-delta-lactone, or strawberry syrup and sugar). The researchers suggested that the low pH of yogurt increased the metal ion repulsion from the oxidation interface, causing less oxidation in yogurt. They validated this by individually adding ingredients during yogurt processing to show that yogurt fermentation/pH was the main effect in lowering the oxidation, not other ingredients.[68] Other work by Nielsen et al discussed only the oxidative stability of strawberry flavored drinking yogurt enriched with fish oil.[69] They investigated if added antioxidants helped decrease oxidation of fish oil. They reported that Vitamin K and EDTA may reduce oxidation but citric acid would not. They also were unable to induce oxidation of fish oil in drinking yogurt by adding 50 µg of iron as an oxidation initiator. It was suggested that at the pH of yogurt, below the isoelectric point of milk, proteins may have formed a positive charge, repelling metal ions, or the lower pH may have decreased the solubility of free iron in the protein network, therefore reducing the oxidation rate.[69] A study comparing the addition of fish oil, with no added antioxidants, to salad dressings, milk, and yogurt (post-fermentation) concluded that

yogurts gave higher oxidative stability than milk or dressing, based on secondary volatile compounds (1-penten-3-one, (E)-2-hexenal, (E,E)-2,4-heptadienal) and peroxide values.[63] The researchers hypothesized this may be due to transition metals playing a smaller role as pro-oxidants in yogurt systems than the higher fat salad dressings.[63] Another way suggested to prevent oxidation in fish oil fortified foods would be to only enhance products that currently have a short shelf life.[26]

Competitors in Omega-3 Yogurt Products

Currently marketed yogurt products containing omega-3 fatty acids come in a variety of styles. The source of ω3 FA may be fish or algal oil and the oils are added in encapsulated or bulk oil forms. The ingredient form influences the physical properties of the ingredients and these changes may cause differences in delivery of DHA and EPA. Many of the current products on the market do not list the levels of DHA and EPA provided per serving. Some of the products use an encapsulated product (Life's DHA™, Martek Biosciences Corporation, Columbia, MD)[70], which also has been incorporated into breads, milks, butter and margarine spreads, eggs, cheeses, nutrition bars, fruit drinks, and many other foods that are common to not only American diets but international tastes as well. Table 2.2 provides a list of omega-3 enriched yogurt products currently on the US market with an estimate of the amount of ω3 FA delivered per serving. Other ω3 FA enriched yogurt products that were marketed only briefly included Breyers Smart! DHA omega-3 (North Lawrence, NY), Yoplait Kids (Minneapolis, MN), Rachel's Wickedly Delicious Yogurt (Aberystwyth, Ceredigion, UK), and Cardivia (Danone, Boucherville, Quebec, Canada).

Table 2.2: Omega-3 enriched yogurt products revealing source and amount of DHA delivered

Product, package size	Company, market	Omega-3 source	Amount of omega 3 advertised per serving	Concentration (mg) of DHA per 100 g serving	Servings needed to meet daily intake (145 mg)
ABC Infant yogurt, 100 g	Central Lechera Asturiana, Spain	Life's DHA™	not found	not able to calculate	not able to calculate
Danino, 100 g	Danone, Boucherville, Quebec, Canada & Europe	MEG-3®DHA/'refined fish oil'	40 mg DHA/ 100 g	40	3.6
Nogurt, 6 oz	Rich and Wholesome Foods Co, Boulder, CO, US	Life's DHA™ (algae oil)	32 mg DHA/ 6 oz	19	4.5
Vaalia, My First Yoghurt, 60 g	Parmalat Austrailia	Life's DHA™	36mg/60g	60	4.0
Vaalia for Toddlers, 90 g	Parmalat Austrailia	Life's DHA™	58mg/90g	64	2.5
Wegmans Food You Feel Good About Organic Super Yogurt, (6 oz)	Wegmans Grocery, Rochester, NY, US	anchovy and sardine fish oil	32 mg EPA/DHA	19	4.5
YoBaby Plus Fruit & Cereal with DHA, (4 oz)	Stonyfield Farms, US	Fish oil (anchovy, sardine, tilapia) and flaxseed	17 mg/ 100 calories	15	8.5
Yo on the Go, (237 mL, 8 oz)	Whitney's Foods Inc, Jamacia, NY, US	Life's DHA™	not found	not able to calculate	not able to calculate

Based on the recommended daily intake suggested by the AHA and WHO, a daily serving is approximately 145 milligrams of DHA and EPA. Differences in concentration of DHA and EPA among products (Table 2.2) illustrate the difficulties in achieving the suggested targeted dietary intake of these fatty acids. Most of these products are targeted to infants and toddlers. Very few yogurt products with ω3 FA are currently available for children, teenage, or adult markets. The current average adult serving size of yogurt in the US is 6 ounces (170 grams) and it would be necessary to consume at least several servings to achieve the desired daily intake of omega-3 lipids.

Flavoring in Yogurt

Flavoring in yogurt has been shown to decrease the fishy off-flavor associated with fish oil as a source of omega-3 enrichment in yogurt. Jacobsen et al showed that strawberry jam

stirred into premade yogurt with fish oil could make the fish oil less noticeable than in milk.[71] They also measured fewer volatiles and less oxidation degradation, as indicated by peroxide values, in yogurt with strawberry jam than in unflavored yogurts. The researchers believe the lower pH of yogurt contributes to metal ions being less attracted to the fat membrane where autoxidation primarily occurs.[71]

Innovation in flavoring expands product lines and maintains consumer interest in products, such as in yogurt. Popular flavors of yogurt in the United States include strawberry, raspberry, vanilla, strawberry-banana, peach, blueberry, lemon-lime, mixed berry, and cherry.[48] Most companies are constantly looking for new or culturally popular flavors to add to their yogurts. Öztürk and Öner studied the effect of adding concentrated grape juice, a common grape product in Turkey, to milk before yogurt production to increase iron content in yogurt.[72] They reported that the grape juice increased the fermentation time, decreased the viscosity, and lowered the pH due to increased lactic acid bacteria compared to the control (non-flavored) yogurt. They suggested that the lower pH of the fruit yogurt made the casein more robust to syneresis.[72] Atasoy studied carob juice concentrates added to yogurt because of popularity of carob juice in Turkey and the compositional and health benefits, such as providing additional carbohydrates and minerals along with an antidiarrhoeal effect.[73] Addition of carob juice extended yogurt fermentation time and decreased the number of viable organisms and viscosity compared to control yogurts. Another observation was that lower pH provided stability against whey separation. The decreased yogurt bacteria counts for the flavored yogurt were suggested to be due to the increased carbohydrate levels in the carob juice.[73] Celik et al also reported lower viscosity and increased syneresis in yogurt when adding cherry paste, another popular flavor in Turkey.[74] Trends show that consumers often desire experiences from flavors and that traditional flavors, including capsaicin, blend well in functional foods to disguise the functional ingredient, which often contributes a negative sensory aspect to food.[75]

A savory flavor in yogurt might be a bit different to the US market but it could provide a product for making healthy dips, salad dressings, and sauces for foods. Yogurt makes healthier dips because it has the consistency to replace sour cream or mayonnaise in many of their applications in foods. In the culinary world and many non-US cultures, yogurt is made with savory flavors. A few dairy products with savory flavors have been marketed in the US. For example, Quark cheese from Marin French Cheese Company (Petaluma, CA) has herb, garlic,

jalapeno and triple onion flavors. Some savory-flavored yogurt products have been released to market but then withdrawn such as a Stonyfield Farms (Londonderry, NH) vegetable garden yogurt, or IncreDiples (Blue Bunny, Le Mars, Iowa) fajita lime, spicy buffalo and taco fiesta flavored yogurt based dips.[76] Cottage cheese products with flavors such as Rachels Wickedly Delicious (Aberystwyth, Ceredigion, UK) cucumber-dill also are found on the US market.

Use of Encapsulation in Yogurt

Encapsulation techniques have been used successfully to protect probiotics in yogurt. Krasaekoopt et al describe an encapsulation procedure using alginate beads coated in chitosan to protect *Lactobacillus acidophilus* (probiotic).[77] The encapsulated cells showed survival rates of one log higher than free cells during refrigerated storage of yogurts. Researchers believed that a chitosan coating added on the encapsulation beads caused a slower interaction of inhibitory compounds to *L. acidophilus*, causing an improved survival rate. The chitosan-coated beads or probiotics were added aseptically post-fermentation to the yogurts.[77] Iyer and Kailasapathy researched the effect of chitosan coatings in protecting alginate-encapsulated probiotic bacteria using *in vitro* acidic conditions.[78] A 0.6 log cell count increase of prebiotics studied resulted with chitosan-coated beads compared to alginate- or poly-L-lysine-coated beads. The gel interaction of chitosan at low pH values was attributed for increased probiotic survival. When tested in a yogurt matrix, added immediately prior to fermentation, the chitosan-coated co-encapsulated probiotics increased the counts of *Lactobacillus* spp. compared to non-coated encapsulated probiotics.[78] This indicates that the positive effects of encapsulation in combination with chitosan as protection for bacteria in yogurt.

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Chapter 3: Analytical and Sensory Evaluation of Fish Oil-Enriched Chile-Lime Flavored Yogurt

Abstract

The acceptance of a fish oil-enriched chile-lime flavored yogurt was examined using sensory evaluation and analytical techniques. Untrained panelists (n=31) were unable to differentiate 0.5% (wt/wt) levels of fish and butter oils in unflavored yogurts but were able to detect 0.5% (wt/wt) oxidized fish oil added to unflavored yogurt. Experienced panelists (n=12) found significant differences ($p<0.05$) in lime and acid attributes in chile-lime flavored yogurts containing 1% (wt/wt) oxidized fish oil compared with 0.43 and 1% (wt/wt) butter and fish oil yogurts and 0.43% (wt/wt) oxidized fish oil yogurts. Oxidized attributes were determined as significantly different by experienced panelists in chile-lime yogurts with 1% (wt/wt) fish oil, 0.43 and 1% (wt/wt) oxidized fish oil added. Consumers (n=100) deemed the chile-lime flavor in yogurt was "neither liked nor disliked" on a 9-point hedonic scale (1="dislike extremely", 9="like extremely") but a majority of consumers (71%) indicated they would consume an omega-3 fortified dairy products at least once per week. A chile-lime flavored yogurt delivering levels of ω3 FA within the recommended daily values per serving of yogurt (145 mg DHA+EPA/170 g) was successfully formulated from the pre-pasteurization addition of fish oil.

Introduction

Scientific research documents the diverse health benefits of omega-3 fatty acids (ω3 FA). Increased ω3 FA consumption can help lower blood pressure, reduce blood triglyceride levels and help prevent secondary cardiovascular disease. These healthy fatty acids are found in high concentrations in the cellular membranes of the body and have central roles in infant brain and retinal development, reducing age-related macular degeneration and inflammation, and helping improve symptoms of many health conditions including rheumatoid arthritis, asthma, atherosclerosis, attention deficit hyperactivity disorder, cancer, and inflammatory bowel disease. The United States (US) Food and Drug Administration (FDA) has determined there is sufficient scientific evidence that ω3 FA can help maintain heart health, allowing foods and dietary supplements containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to carry a qualified health claim that the product 'may be beneficial in reducing coronary heart disease'.[1] Most of the US population do not consume the suggested levels of these ω3 FA (200-1428 mg/day[2]), naturally found in fish and fish products, and continue to search for alternative ways to incorporate these healthy fats in their diets. Foods fortified with ω3 FA provide alternative choices for people with dietary restrictions or adverse reactions to fish products.

The international popularity of yogurt and the health promoting properties associated with probiotics, minerals (i.e. calcium), vitamins, and milk proteins in yogurt, create a healthy and popular vehicle to deliver ω3 FA to the consumer. The expansion of research investigating the addition of ω3 FA to yogurt from fish or algae oil sources[3] and number of current products on the market (US and international) containing ω3 FA exemplify the importance of this field of study. The problems faced when enriching yogurt with ω3 FA include fortification with amounts to help the consumer receive the recommended levels needed for experiencing potential health benefits, prevention of oxidative degradation of these highly susceptible ω3 FA lipids, and prevention of off-flavors and odors associated with ω3 FA sources (usually algae or fish oil). None of the currently marketed products in the US found provide even half of our targeted daily amount of DHA+EPA (145 mg/day) in one serving. This project investigated the effects of enriching a savory yogurt product with fresh and oxidized fish oils compared to butter oil at levels close to the recommended daily value per serving (145 mg DHA+EPA/170 g yogurt).

The primary research objective of this project was: To determine important attributes and acceptable levels of fortification in chile-lime flavored omega-3 fortified yogurt products using sensory evaluation and analytical techniques.

The studies supporting this objective were:

Study 1: Discrimination of different oils (butter, fish, oxidized fish) and flavors (chile-lime, lime, none) in low-fat yogurt by sensory evaluation;

Study 2: Description of attributes (lime, sweet, heat, acid, oxidized) in chile-lime yogurt with different oil sources (butter, fish, oxidized fish) at two levels (high, low) by an experienced sensory panel;

Study 3: Consumer acceptance of chile-lime flavored low-fat yogurt products fortified with fish or butter oil.

Materials and Methods

General Processing and Analytical Methods

Low-fat chile-lime yogurt enriched with butter, fish, or oxidized fish oils was manufactured and analyzed in the laboratory to be used for and compared with sensory evaluation results.

Yogurt Manufacture

Raw milk, obtained from the Virginia Tech dairy farm, was heated (60°C) and separated into skim and cream using a pilot scale cream separator (The Creamery Package MF; model # P50STT0, Chicago, IL) in the dairy pilot plant (Food Science and Technology Department, Virginia Tech, Blacksburg, VA). Yogurt was formulated (Table 3.1) with skim milk, cream, sugar, low-heat non-fat dry milk (Franklin Farms East, Inc, Asbury, NJ), stabilizer (Germantown Crown, Danisco, Copenhagen, Denmark; consisting of modified food starch, mono and diglycerides, carageenan and carob bean gum), natural chile-lime and organic lime flavoring (Gold Coast Ingredients, Inc, Commerce, California), and oil (clarified butter oil (BO), DenOmega fish oil (FO) (Gamle Fredrikstad, Norway), or oxidized fish oil (oxFO)). Clarified butter oil was prepared in the dairy laboratory by manufacturing butter from fresh cream, then melting and separating into oil and aqueous phases. The oil phase was collected and used as butter oil in this project. Oxidized fish oil was prepared by exposing approximately 1 L of fresh fish oil, opened to air at room temperature, to fluorescent light for seven days to induce auto-and

photo-oxidation. All oils were flushed with nitrogen and stored at -15°C in the dark throughout the study to protect against any additional oxidization. Peroxide values of fish oils, determined by the titration method,[4] were 0 meq/kg for fresh fish oil and 3.9 meq/kg for oxidized fish oil. These levels compare with low levels of oxidation in literature, although the oxidative aroma of the oil was quite strong. Antioxidants (mixed tocopherols, lecithin, ascorbyl palmitate and rosemary extract) were listed on the fish oil by the manufacturer and probably limited the oxidative degradation from light and air exposure. The targeted yogurt fat content for all products was 'low-fat' (between 0.5 and 2% fat).

Table 3.1: Formulations (% wt/wt of ingredients) for omega-3 fortified yogurt evaluated in three different sensory studies

	Average wt/wt % of formulation			
Study: Method (n=number of samples evaluated) **Ingredient**	**Study 1: Triangle discrimination (flavor)** (n=4)	**Study 1: Triangle discrimination (oil)** (n=3)	**Study 2: Descriptive rating experienced panel** (n=3)	**Study 3: Hedonic rating consumer panel** (n=2)
Cream (~40% fat)	2.37[1]	1.34	1.04	1.04
NFDM[2]	3.29	3.29	3.29	3.29
Skim Milk	92	92	94	94
Sucrose	1.60	1.60	1.60	1.60
Stabilizer[3]	0.50	0.50	0.50	0.50
Oil-low level[4]	NA	NA	0.43	NA
Oil-high level[4]	0.50	0.51	1.00	1.00
Chile-Lime flavor[5]	varied	none	0.11	0.12
Lime flavor[5]	varied	none	0.06	0.05

[1] cream was determined to be a lower % fat so more was added for final 1% fat formulation
[2] NFDM=non-fat dry milk
[3] Germantown Crown, Danisco, Copenhagen, Denmark
[4] oils (butter, fish, or oxidized fish), low (0.43% wt/wt), high (1% wt/wt)
[5] Gold Coast Ingredients, Inc, Commerce, California

Ingredients were mixed together with an immersion hand blender (Kitchen Aid, St. Joseph, MI), heated (54°C), then homogenized in two stages (3.4/17.2 MPa (500/2500 psi)) using a laboratory homogenizer (model 15MR, APV Gaulin, Inc., Everett, MA). Yogurt mix was vat pasteurized at 85°C for 30 minutes. After cooling to 40°C, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* cultures (Ultra gro UG-55, Cargill, Minneapolis, MN) were added (0.04%/g yogurt) and yogurt was incubated at 40.9°C to a final pH of 4.3 (4.1 for yogurts in Study 2). Yogurt was removed from the incubator, cooled and stored at 4°C for the duration of testing.

Characterization of Yogurt Composition

Analyses of yogurt composition and characterization were completed on all products based on dairy standard methods[5] for pH (Accumet XL 15 pH meter, Fisher Scientific, Waltham, MA), titratable acidity, total solids and moisture content by convection oven, the presence of coliforms on Petrifilm (3M, St. Paul, MN), total fat content using a modified Pennsylvania Babcock method, protein content (DC Protein Assay by BioRad, Hercules, CA), and soluble solids (Abbe Mark 2 Digital Refractometer, Reichert, Depew, NY). Physical, compositional, and chemical measurements were completed on all treatments within four days of manufacture.

Volatile Chemistry

Headspace solid phase micro-extraction coupled to gas chromatography/mass spectrometry (SPME-GC/MS) was used to measure volatile chemistry of yogurt samples. On day four after yogurt manufacture, ten grams of yogurt were put into 20 mL amber vials and sealed with headspace aluminum caps each fitted with a PTFE septum; samples were prepared in duplicate. An 85 µm Carboxen-Polydimethylsiloxane (Car/PDMS) fiber (Supelco, Bellefonte, PA) was conditioned according to the manufacturer's suggestions (300°C, one hour). Sample vials were held in an autosampler (CTC Analysis CombiPal, Leap Technologies, Carrboro, NC), heated at 50°C for 15 seconds, and the Car/PDMS fiber was exposed 22 mm for two minutes with agitation. The fiber was automatically injected (44 mm) into the injection port of a HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA) and desorbed for ten minutes at 250°C. Desorbed compounds were separated using a HP 5MS Crosslinked 5% PH ME siloxane (Hewlett Packard, Palo Alto, CA) column (30m x 0.25mm x 0.25µm), with a run time of 51 minutes and helium as a carrier gas (1mL/min). The temperature program began at 35°C for seven minutes, increased to 120°C at 2.5°C/min, increased to 220°C at 20°C/min and held for five minutes. The oven was cooled to 35°C before the next sample was injected. Detection of volatile compounds used a HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA), set at 280°C. Volatiles of interest were identified using an internal library and external standards (1-penten-3-one, pentanal, propanal, limonene).

Fatty Acid Analysis

Lipids in the yogurt were extracted from freeze dried yogurts with a modified Soxtec extraction using methylene chloride.[6] Remaining yogurt was frozen (-70°C) four days after manufacture then placed in shallow glass dishes, covered with cheese cloth and placed in a freeze drier (LabConco, Kansas City, MO) until all moisture was removed. Freeze dried yogurts were weighed into thimbles and placed in the Soxtec System (HT 1043, Tecator, FOSS, Laurel, MD). Thimbles were submerged into cups of methylene chloride, heated to 90°C for 1.5 hours and then removed for a 30 minute rinse in the same environment. Solvent and wash were transferred to round bottom flasks and evaporated to 1-2 mL under vacuum (Evapotec, Haaken Buchler). Remaining solvent was transferred to test tubes and evaporated using a stream of nitrogen gas until only oil remained. Oils were stored flushed with nitrogen in the freezer (-15°C) until all samples were extracted. An internal standard (C23:0 methyl ester, 1mg dissolved in 1 mL isooctane) for calculation of percent fatty acid from the gas chromatograph (GC) was added to approximately 25 μg extracted oils and the oils were esterified as described by Maxwell and Marmer.[7] This method was chosen because low amounts of free fatty acids were expected in the lipids from dairy or fish oil and the method provides fewer artifacts than acid catalyzed reactions. To esterify samples, 1 mL of isooctane was added to oils containing the internal standard along with 200 μL 2N KOH in MeOH. Samples were mixed (vortexed) and centrifuged and the lower layer discarded. Aqueous saturated ammonium acetate (0.5 mL) was added, sample was mixed again, centrifuged, and again the lower layer discarded; this process was repeated with 0.5 mL deionized water. Finally, a small amount of sodium sulfate was added, the mixture sat undisturbed for 5 minutes, then was centrifuged and the top layer was removed to a clean test tube for injection into the GC.

Samples were stored flushed under nitrogen in the freezer (-15°C) until manual injection (1 μL) into the GC. A modified method for fatty acid composition by capillary column gas-liquid chromatography (GLC)[7] was used to determine fatty acid methyl ester (FAME) profiles as well as calculate levels of DHA and EPA in the final products.

For analysis, a Shimadzu CG-17A gas chromatograph (Kyoto, Japan) with a SP-2560 (nonbonded biscyanoproply polysiloxane, Supelco, Sigma-Aldrich, St. Louis, MO) capillary column (100m x 0.25mm x 0.2μm) with split (1:20) injection, helium carrier gas and a linear flow velocity of 35cm/min were used to separate the fatty acids. An oven program of 130°C,

raised to 240 at 2°C/min and held for 10 min with a total run time of 65 min was used with injector and detector port temperatures set to 270°C. A quadrupole mass spectrometer (Shimadzu QP5050A, Kyoto, Japan) was used with Gas Chromatography Mass Spectroscopy (GCMS) Real Time Analysis (Version 1.1, Shimadzu, Kyoto, Japan) the National Institute of Standards and Technology (NIST) 2005 Mass Spectral Database to identify chromatographic peaks.

Sensory Analyses

All sensory studies were approved by the Virginia Tech Institutional Review Board (IRB 09-726, Approved September 9, 2009, Appendix A) and followed appropriate protocols as described in Meilgaard et al.[8] All sensory testing took place in the Food Science and Technology Department (FST) sensory laboratory at Virginia Tech and collected information using touch computer screens and sensory software (Sensory Information Management System (SIMS) 2000, Version 6, Morristown, NJ).

Study 1: Discriminating Savory Flavoring and Fish Oil Addition in Yogurt

A preliminary experiment was conducted to determine levels and blends of chile-lime flavor addition, examine if panelists could detect fresh and oxidized fish oils in unflavored yogurt, and screen for panelists with discriminating ability.

Yogurt Processing and Analyses

Yogurt was manufactured as previously described based on the formulations presented in Table 3.1 and analyzed for standard composition and characterization. Chile-lime flavored yogurts with varied levels of flavoring added were manufactured for sensory triangle testing. For flavor triangle tests, flavor was formulated into yogurt at 0.09% chile-lime flavor, 0.09% chile-lime + 0.065% lime, 0.09% chile-lime+0.085% lime, 0.11% chile-lime, and an unflavored (no flavor added) yogurt served as a control. Unflavored yogurts were manufactured for a separate triangle test sensory investigating discrimination of oils using 0.50% (wt/wt) of clarified butter, fish, or oxidized fish oil.

Determination of Flavoring Levels and Oil Addition in Yogurt

Triangle tests were used to define levels of flavoring for subsequent sensory evaluation studies. Flavoring comparisons included 1) no flavor vs 0.09% chile-lime flavor; 2) 0.09% chile-lime flavor vs 0.09% chile-lime + 0.065% lime; 3) 0.09% chile-lime vs 0.09% chile-lime+0.085% lime; and 4) 0.11% chile-lime vs 0.09% chile-lime+0.085% lime. Thirty-two panelists were recruited from the FST Department and classes at Virginia Tech, each panelist completed four triangle tests in one session. Yogurt samples (approximately 28 g each), portioned into 1 oz portion cups and sealed with lids, were identified by random 3-digit codes and presented at 4°C in a balanced order. To reduce fatigue, panelists were instructed to rinse their mouths with filtered water and eat an unsalted cracker between test sets; they were forced to wait one minute between triangle test sets. A demographic survey followed completion of the four triangle tests (Appendix B). All information was collected with touch computer screens using sensory software (Appendix B).

On a subsequent date, thirty-one panelists completed three sets of triangle tests in one session to determine if addition of fish oil or oxidized fish oil was detectable in unflavored yogurts. Samples were manufactured as described above with 0.5% fat (butter oil (control), fish oil, and oxidized fish oil) added. Testing was conducted as described above and a demographic survey was completed (Appendix B).

Results were used to identify potential panelists who could discriminate between changes in yogurt with different levels and variations of chile-lime flavoring and types of lipid. Panelists with four or more correct responses of the seven triangle tests on flavor and oil were invited to participate in training for Study 2.

Statistical analysis was performed using α=0.05, β=0.30, p_d=30% and methods described in Meilgaard et al.[8] Panelists represented multiple replications.

Study 2: Changes in Flavor Profile of Chile-Lime Flavored Yogurt with Varied Oil Sources and Levels

Chile-lime flavored (0.11% chile-lime + 0.60% lime flavor) yogurts with two lipid levels (low (0.43%) and high (1%)) of clarified butter oil, fresh fish oil (DenOmega, Gamle Fredrikstad, Norway), or oxidized fish oil were manufactured as previously described using formulations shown in Table 3.1. Yogurt was fermented to a pH endpoint of 4.1 in this study.

Products were analyzed for composition, fatty acid analysis, and volatile chemistry and sensory evaluation of attributes (lime, sweet, heat, acid, oxidized) using an experienced panel.

Panelist Training

Twelve panelists (four males, eight females) with intermediate levels of prior sensory experience in yogurt and oxidation of dairy products were selected to participate in further training, based on performance on the triangle test studies (Study 1) and willingness to participate. Panelists underwent training for six predetermined attributes (lime, sweet, heat, acid, fishy, and oxidized) in six one-hour sessions. Reference standards for each attribute were used to introduce panelists to the attributes of interest (Appendix C, Table C.1). Training goals were defined as developing terminology related to the product attributes of interest and learning the scaling method using four levels of each attribute in different matrices (water, milk, and yogurt).[8] Appendix C, Table C.2 briefly describes training activities for each session.

Panel Validation

The next step in training was to validate if panelists could replicate their individual performance on the six attributes they learned in training. This was done by mixing six samples of plain low-fat yogurt (Kroger, Cincinnati, OH) with three or more attributes each (Appendix C, Table C.3) and having each panelist rate the samples on unstructured 15 cm line scales. Samples (approximately 28 grams), portioned into 1 oz cups and sealed with lids were identified by random 3-digit codes and presented at 4°C in a balanced order. Sample tasting was standardized during training as were palate cleansing (Appendix C, Table C.2) and waiting times between samples (as during Study 1). The validations were conducted in individual sensory booths, using SIMS sensory software and panelists signed in by name for individual statistical analysis. Explicit directions given to panelists can be found in Appendix C. Samples were prepared in the same manner for two days of validation testing and the results (numerical translations measured from 15 cm line scales) of individual panelists were compared using a statistical t-test. Select panelists required refinement for oxidation as determined by the validation testing; this was achieved with additional practice (Appendix C, Table C.2) on the oxidized attribute in yogurt.

Validation led to the removal of the fishy attribute for sensory testing because panelists did not receive sufficient training to differentiate "fishy" and "oxidized" tastes. It was decided

that "oxidized" encompassed more of the fishy attribute and "fishy" was too difficult to separate with the amount of training time available for the project. During validation, only some panelists were able to replicate themselves on the five remaining attributes. Panelists completed additional training and testing on the oxidized attribute to refine their skills prior to the first replication (Appendix C, Table C.2). During the three replications panelists continued improving their abilities in discriminating for oxidized attribute by performing weekly duo-trio tests (Appendix C, Table C.2). Based on panel performance evaluation, the panel is subsequently described as "experienced".

Attribute Rating of Chile-Lime Flavored Yogurts with Fish Oil

For sensory evaluation of chile-lime flavored yogurts with high and low levels of butter, fish, and oxidized fish oils, experienced panelists (n=12) assessed each product for five attributes (lime, sweet, heat, acid, oxidized) on 15 cm unstructured line scales. Panelists tasted the yogurt samples (n=6) in the same manner as during validation (directions in Appendix C). Products (approximately 28 grams), portioned into 1 oz portion cups and sealed with lids were identified by random 3-digit codes and presented at 4°C in a balanced order. All six formulations were evaluated in one sensory session with one minute wait times in between each. Rating intensities were converted into numbers in the SIMS software by measuring the distance from left anchor on the line scale in millimeters (mm) with a maximum of 150 mm and these numbers were used in calculations.

Statistical analysis on the three replications of twelve panelists was completed with JMP Statistical Software (SAS, Cary, NC). Results from all replications of the line scales were tabulated and an ANOVA table with Tukey's LSD compared the six formulations for each attribute. For the experienced sensory panel a two-way Analysis of Variance (ANOVA) was conducted using the following model:

$$Y_{ij} = \mu + L_i + S_j + LS_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the ij^{th} observation; μ is the general mean; L_i (oil level; low or high) and S_j (oil source; BO, FO, or oxFO) are the main effects; LS_{ij} are their interaction; and ε_{ij} is the random error. Analytical testing on yogurt for the three replications was carried out in duplicate.

Study 3: Consumer Perception of Chile-Lime Flavored Yogurt as a Dietary Source of Omega-3 Lipids

Two formulations of chile-lime flavored yogurts were manufactured, as previously described (Table 3.1), for determining overall acceptance and flavor acceptance of the product using a consumer sensory panel. Formulations consisted of 1% (wt/wt) clarified butter oil or fresh fish oil. Yogurt was fermented at 40.9°C to a pH of 4.3, at which time the yogurt was stored at 4°C for the duration of testing.

Consumer Sensory Panel

Demographics and other questions pertaining to product use, general awareness, and interest in dietary source of dairy, fish, and omega-3 food sources were collected from 186 students, faculty, staff, and local community members at and around Virginia Tech using an electronically delivered survey (www.survey.vt.edu); 100 of these subjects were chosen to participate in the consumer sensory study based on willingness to participate, having no allergies or health conditions prohibiting participation, and being regular consumers of yogurt (consume 1-3 times per month or more). Panelists were from all age ranges and backgrounds and most had limited experience in participating in sensory panels. All responses to the screening survey (Appendix D) were included in the demographic information.

A consumer sensory panel (n=100) was conducted to determine overall acceptance and flavor acceptance, of chile-lime flavoring in yogurt using a nine-point hedonic scale (1="dislike extremely", 9="like extremely"). After completion of written consent forms panelists were given two chile-lime flavored yogurt samples (butter oil or fish oil-enriched, approximately 28 grams each), portioned into 1 oz cups and sealed with lids, identified by random 3-digit codes and presented at 4°C in a balanced order. In the first set, each sample was evaluated for overall acceptance; the second set of two samples (same formulations) specifically asked for evaluation of flavor acceptance. Score cards can be found in Appendix D. A predetermined value of 6.5 for overall liking and flavor preference were set to decide if further research was warranted with these products. All information was collected using touch computer screens and SIMS sensory software.

Statistical analysis was completed using Excel to tabulate results of surveys and questions, then calculate and report percentages. Hedonic rating scores were averaged and

compared using a statistical t-test. All statistical evaluation used JMP (SAS, Cary, NC) models, and a preset alpha of 0.05 for determining significant differences.

Results and Discussion

Line extensions for yogurt through new flavor innovations and health-improving formulations are critical for continued market growth in the dairy industry and meeting consumer desires for health and flavor. Savory flavor innovations, leading to strategic placement of yogurt into a main meal, condiment, or salad dressing, can expand opportunities. Many food companies currently employ trained culinary scientists to create innovation in their foods by creating products that stem directly from cuisine cooked for specific populations or regions. Already a popular flavor in many areas of the food industry, including dips and toppings, chile-lime has not yet been explored in yogurt. This project capitalizes on this opportunity by bringing together many flavors, including the original yogurt dairy flavor and the possible fish flavors from the ω3 FA source oil. An ω3 FA source oil from fish provides a larger fortification of EPA and DHA to products; since the body does not readily make these healthy long chain fatty acids it is best to start with the highest levels achievable. Chile and lime flavors already complement fish and seafood in many culinary recipes. Our intentions were that if fish flavor from the source oil was noticed, it may blend well with the chile-lime flavor. Chile-lime is not a traditional flavor in the dairy industry but lime in yogurt is already a popular flavor, adding capsaicin could capture a new section of the market. A chile-lime flavored yogurt targeted at the correct population with appropriate applications could succeed; it is possible that a chile-lime yogurt would not be eaten as a full 6 oz (170 g) serving but as a healthy alternative for toppings or bases for dips.

Discrimination Testing for Flavor and Oil Levels in Yogurt

Preliminary testing was conducted to help identify possible flavoring levels for use in further testing. Untrained panelists (n=32) could significantly ($p<0.05$) distinguish between chile-lime and unflavored yogurt and between chile-lime yogurts with added lime compared to chile-lime flavoring only (Table 3.2). Differences were not found when comparing increases in chile-lime flavoring alone. An untrained panel (n=31) could identify differences in 0.5% (wt/wt) addition of oxidized fish oil compared to butter or fresh fish oil in unflavored yogurt. Full panel description and demographic responses for each session are shown in Appendix H.

Table 3.2: Discriminating[1] flavor (chile-lime, lime, none) and oils (butter, fish, oxidized fish) in low-fat yogurt (4°C) using triangle tests with untrained panelists.

Flavor Comparisons (panelists, n=32) (0.50% wt/wt butter oil, 1.3% fat)	**p-value**	**Significant**
unflavored vs chile-lime flavor (0.09%)	0.0007	yes
chile-lime flavor (0.09%) vs chile-lime (0.09%) + lime flavor (0.065%)	0.0377	yes
chlie-lime (0.09%) + lime flavor(0.065%) vs chile-lime (0.09%) + lime flavor (0.085%)	0.0064	yes
chile-lime(0.09%) + lime flavor (0.085%) vs chile-lime flavor (0.11%)	0.2427	no
Oil Comparisons (panelists, n=31) (0.51% wt/wt source oil, 1.1% fat)		
butter oil vs fish oil	0.0589	no
fish oil vs oxidized fish oil	0.0111	yes
butter oil vs oxidized fish oil	0.0004	yes

[1] α=0.05, β=0.30, p_d=30%

Changes in chile-lime flavor level and additional lime flavoring indicated that untrained panelists could distinguish changes at relatively low flavor concentrations in a yogurt base. Based on this preliminary work and subsequent discussion, a final flavor formulation was identified at 0.11% chile-lime flavor + 0.06% lime flavor.

Pohjanheimo and Sandell evaluated sensory connections between yogurt, food choice motives, and acceptability of products.[9] They determined that consumers motivated by health trends enjoyed yogurts with vastly different characteristics than consumers motivated by price, convenience, mood, or familiarity.[9] Consumers motivated by health choices also were willing to consume yogurts with lower levels of sweetness. These authors encouraged development of yogurts and advertising campaigns that connect product attributes to consumer desires.[9] The omega-3 fatty acid fortified chile-lime flavored yogurt in this project could easily be marketed to a target population looking for heart health and lower sugar/reduced calorie health benefits. Research also shows that traditional flavors are becoming popular in non-traditional applications and can drive the consumers interest in purchasing functional foods.[10] Exotic flavors, such as the chile-lime combination used in this research, were a new flavor trend for new products in 2009.[10] While only 37% of consumers participating in this panel (n=32) responded as were "likely" or "highly likely" to consume products similar to the chile-lime flavored yogurt they tasted in this study, 51% were "likely" or "highly likely" to consume a savory (defined as full-flavored, not sweet) yogurt product (Appendix H). Fruits (40%), grains (18%) and meats (16%) were identified as the best food companions for the chile-lime yogurt tasted.

The addition of fish oil, as a source of ω3 FA, to yogurt was a concern because of potential 'fishy' flavor notes from the source oil. The goal was to add a sufficient level of oil to provide a significant contribution of ω3 FA into the daily diet. Panelists (n=31) could not differentiate between yogurt with butter oil (0.5% wt/wt) and fish oil (0.5% wt/wt), in unflavored yogurts with a total fat content of 1.8% fat. This level of fortification would have supplied 187mg DHA+EPA per 170 g serving of yogurt based on the fish oil source used in this study. When used in flavored yogurts, this level of fortification could possibly be increased without consumers distinguishing a difference in products. Research comparing plain and strawberry flavored drinking yogurt reported possible fortification of 153 mg DHA+EPA/170 g serving in flavored yogurt but only 120 mg DHA+EPA/170 g of unflavored yogurt; other dairy products such as spreadable fresh cheeses (unflavored and garlic), butter (unflavored), or processed cheeses (unflavored, garlic and vanillin flavored) were fortified up to 4000 mg DHA+EPA/170 g suggesting that increased fat and flavorings help mask fish taste in dairy products fortified with fish oil.[3c] Chee et al also suggested that fishy flavor in ω3 FA added to strawberry yogurt from an algae source was masked by the strawberry fruit base.[3a]

Nielson et al concluded from sensory evaluation of fish oil-enriched (1% wt/wt) milk and drinking yogurts by nine trained panelists, that yogurt provided a much better base to mask fishy flavors and odors and oxidation than did milk.[11] The fish oil enriched milk underwent oxidation more quickly than yogurts, possibly during emulsification and because of the higher oxygen content in milk. The low pH in yogurt may have contributed to repulsion of metal ions that catalyze oxidation and proteins that stabilize the matrix may have retarded the oxidative degradation of fish oils.[11]

Many participants in this panel (50%) reported that they supplemented their diet with ω3 FA through foods or supplements; 73% of panelists also indicated they would consume an ω3 FA-fortified dairy product 1-3 days per week or more often (Appendix H). This provides data that consumers are searching for novel and easy ways to incorporate omega-3 rich lipids into their diets and yogurt would provide a good food vehicle for delivering these lipids in the US diet.

Compatibility of Flavoring and Fish Oil in Yogurt: Descriptive Analyses

Culinary preparation of fish frequently includes citrus and spicy notes. Spices such as curry, dill, cilantro and others add complementary flavors to seafood dishes. We had already documented that 0.5% (wt/wt) fish oil added to unflavored yogurt did not create a discernible difference in the yogurt (Table 3.2). However, the interaction of fish oil with yogurt and chile-lime flavoring characteristics could change the flavor profile, especially with an increased proportion of highly unsaturated ω3 FA increasing the risk of oxidation.

Yogurts produced for this study had similar gross composition, differing only between low and high fat composition as intended (Appendix E). Fatty acid composition was quantified by comparison to tricosanoic acid (C23:0) methyl ester internal standard (Table 3.3).

Table 3.3: Mean[1] concentration (mg/g) of EPA[2] and DHA[2] in source oils (butter, fish, oxidized fish oil) and chile-lime flavored oil-supplemented yogurt products at two fat levels

	mg EPA /g oil		mg DHA /g oil		mg EPA+DHA/ g oil	weight % EPA+DHA	mg DHA+EPA delivered/170 g serving
Source oils	Mean	St dev	Mean	St dev			
fish oil[3]	99.55	±6.25[a]	99.74	±4.88[a]	199.3[a]	23.29[a]	NA
oxidized fish oil	105.5	±7.53[a]	105.6	±6.60[a]	211.1[a]	23.35[a]	NA
Butter oil	NA		NA		NA		
Yogurts							
butter oil-low[4]	NA		NA		NA		0
butter oil-high[4]	NA		NA		NA		0
fresh fish oil-low	38.15	±2.95[c]	37.32	±2.66[d]	75.48[d]	10.58[d]	150
fresh fish oil-high	65.92	±0.87[b]	65.26	±0.54[b]	131.2[b]	16.99[b]	357
oxidized fish oil-low	50.68	±2.61[c]	50.17	±2.47[c]	100.9[c]	13.77[c]	200
oxidized fish oil-high	69.18	±4.01[b]	68.67	±3.77[b]	137.9[b]	17.54[b]	375

[1] n=3 replications
[2]EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; calculated based on tricosanoic acid internal standard
[3]DenOmega
[4] low: 0.43% wt/wt, high:1% wt/wt
[a-d*] means within columns with different super script are significantly different ($p<0.05$)

Significant differences ($p<0.05$) in EPA and DHA concentrations between low and high levels of oil fortification were expected and were observed. Significant differences ($p<0.05$) were found between the low levels of oxidized oil and fresh fish oil. This could be due to a higher proportion of dairy fat decreasing the reported long chain fatty acids or other fatty acids present that decrease the area percentages of the EPA and DHA. The fish oil used for this study

was reported by the manufacturer (DenOmega, Gamle Fredrikstad, Norway) to contain a minimum of 22 % DHA+EPA; 23.3% (Table 3.3) was found with the methods in this study.

Further identification of chromatographic peaks was done using molecular weights and an external standard (37 component FAME mixture, Supelco, Bellefonte, PA). Labeled chromatographs for the external standard and source oils are shown in Appendix F.

A full fatty acid profile of source oils (butter, fish, oxidized fish) and yogurt formulations used in Study 2 is given in Appendix F. Due to the large number of fatty acids in the products, peak areas less than 1% were not examined. Investigation of the fully fatty acid profile and the graph in Figure 3.2 show that levels of all fatty acids identified were higher in oxidized fish source oil than fresh fish oil.

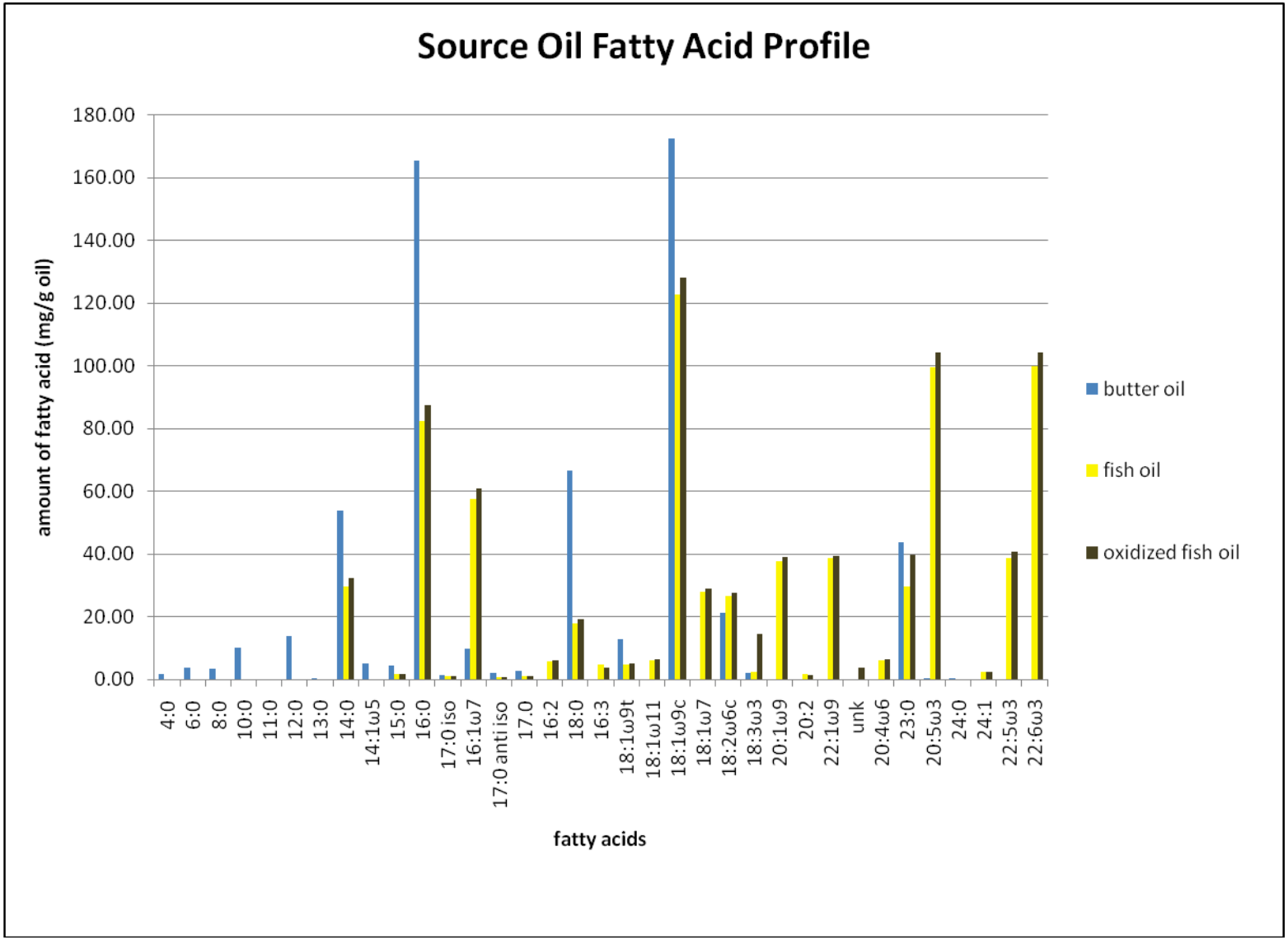

Figure 3.1: Concentration of fatty acids in source oils (butter, fish, oxidized fish) as determined by FAME-GC/MS, n=3 replications

Explanations for increased amounts of fatty acids in oxidized compared to fresh fish oil are unknown, but difficulties in the method of fat extraction or esterification may have contributed to these differences. During fat extraction and esterification components other than fatty acids (riboflavin, lactoferrin, antioxidants) present in the extracted oils could have contributed to the weight calculations being incorrect. The fish oil did contain antioxidants (mixed tocopherols, lecithin, ascorbyl palmitate and rosemary extract) that most likely remained dissolved in the oil after extraction and esterification steps and may have contributed to the total recorded weight of fish oil, thus affecting the calculations. Another error could have occurred during rinsing in the esterification process; oil could have been lost from the isooctane phase due to repeated removal steps of the aqueous phase via pipette; this is a problem because the weight of oil is used in the calculation.

Research reports fatty acids (>8 carbons) found in butter oil at levels greater than 1 wt% include 10:0, 12:0, 14:0, 15:0, 16:0, 16:1ω3, 18:0, 18:1ω9, 18:2ω6, and 18:3ω3.[12] Yogurts formulated in this project identified these milk fat containing fatty acids and as exemplified by increased peak areas in fish oil yogurt compared with bulk fish oil (with the exception of 16:1ω7). Fatty acids in cod liver oil greater than 1 wt% include 14:0, 16:0, 16:1ω7, 18:0, 18:1ω9, 18:1ω7, 18:2ω6, 18:4ω3, 20:1ω11, 20:1ω9, 20:5ω3, 22:1ω11, 22:5ω3, and 22:6ω3.[13] This study did not find 18:4ω3, 20:1ω11, or 22:1ω11 but they could have been mislabeled or less than 1 weight % in chromatograms and thus not reported. Fatty acids 14:0, 16:0, 18:0, and 18:1ω9 were higher in yogurts with low fish oil added, most likely due to the higher proportion of dairy fats in these yogurts since dairy fat has high levels of these fatty acids. All other fatty acids listed as high in fish oil were found in increased amounts in yogurts with high fish oil addition compared to low fish oil addition, signifying an increase in these fatty acids was directly due to addition of fish oil (Appendix F).

Targeted levels of ω3 FA fortification were 145 mg DHA+EPA/170 g serving of yogurt. Calculations from FAME analysis showed that fish oil and oxidized fish oil yogurts were fortified with greater than this amount even in low levels of oil addition (Table 3.3). Levels of fortification achieved in this yogurt are in line with levels reported in the literature. Chee et al reported strawberry flavored yogurt fortified with 400 mg ω3 FA/272 g serving;[3a] a break down of ω3 FA proportions was not given for this oil. Kolanowski et al reported a range of 4-40 mg of omega-3 long chain polyunsaturated fatty acids (PUFA) from fish oil powders fortified into a

single serving of instant foods; powdered milk-rice or wheat kids flavored breakfasts were fortified up to 40 mg of long chain PUFA, but not many other foods supported this level.[14] The researchers concluded that instant foods with milk or higher fat levels as well as flavors could be fortified at higher levels of ω3 FA.[14] It also has been shown that milk was not palatable at fish oil fortification of 0.15% wt (0.05% EPA+DHA) but flavored yogurt could support 0.3% wt fish oil (170 mg DHA+EPA/170 g serving yogurt).[3b] Kolanowski and Weiβrodt reported overall sensory acceptance in strawberry flavored drinking yogurt fortified at 153 mg DHA+EPA/170 g serving but only 102 mg DHA+EPA/170 g serving of unflavored drinking yogurt.[3c]

Headspace solid phase micro-extraction (SPME) provides a quick and solvent-free detection system for the volatile analysis of substances. In foods SPME can identify flavors, freshness, or off-odors present.[15] Fresh fish oil did not generate many peaks in the SPME volatile chemistry analysis of oils and oxidized oils generated few additional peaks (Appendix G, Figure G.1). Over 100 peaks were reported in some chile-lime samples, for this reason, these peaks were not all quantified but comparing the flavor volatiles to the fish oil volatiles explains why flavored products help mask fish off-odors and permit higher levels of ω3 FA fortification than unflavored products (Appendix G, Figure G.2). Limonene, the large peak eluting around 21.5 minutes was not quantified due to its magnitude and the inability to integrate the whole peak.

Retention times for various volatiles were determined using external standards (propanal,1-penten-3-one, pentanal, and 2-heptanone) and identification using the mass spectrometer. Compounds eluting between 1.4 and 2.8 minutes were difficult to identify due to the large peak from ethanol used as the base for the chile-lime flavoring. Only a few volatile peaks were reported by SPME analysis in the oxidized fish oils. Four volatile secondary oxidation products (propanal, RT=1.61 min; 1-penten-3-one, RT=3.20 min; pentanal, RT=3.33 min; and 2-heptanone, RT=12.27 min) were chosen and compared between chile-lime yogurt formulations used in Study 2 (Figure 3.2). With the exception of propanal, oxidized fish oils had slightly higher levels than fish oil and high levels of oil addition increased the peak areas. 2-Heptanone is identified as an oxidation product in fish oil enriched milk over 14 days of storage[16] but also could be attributed to the yogurt matrix.[17]

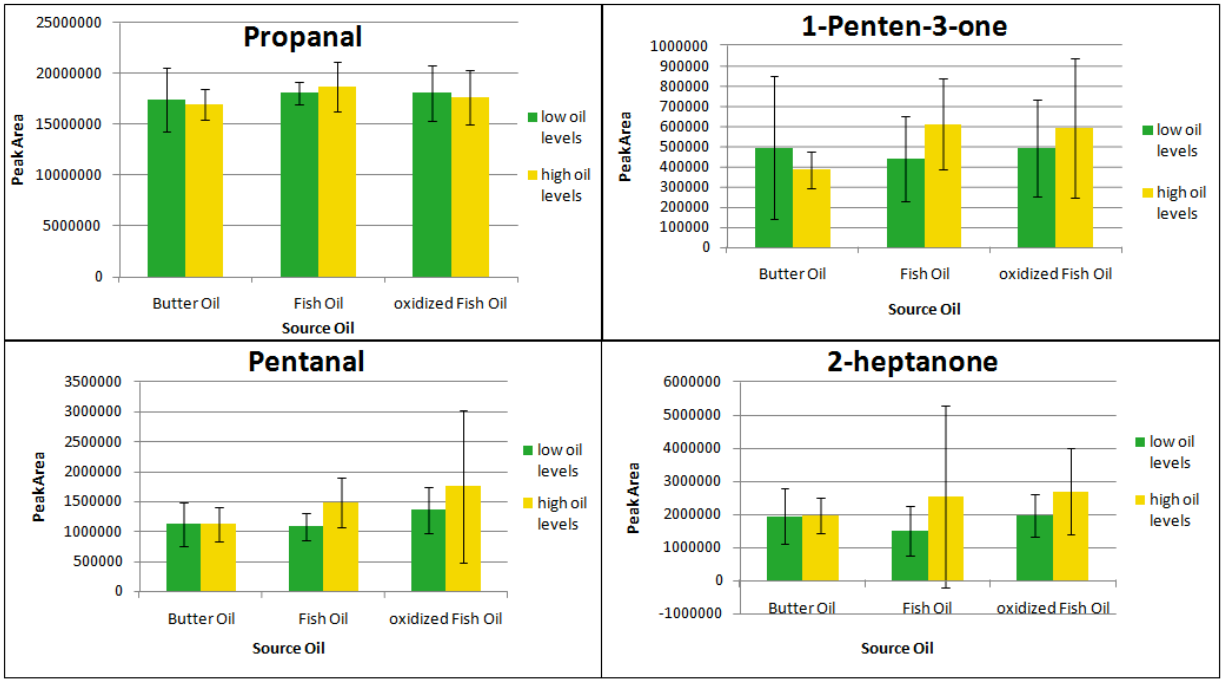

Figure 3.2: Peak areas for volatile compounds with standard deviations of chile-lime flavored yogurts as determined by SPME-GC/MS comparing oil levels (0.43=low or 1% wt/wt=high), n=3 replications

SPME has been used to study oxidation volatiles in fish oil-enriched yogurts in literature as well. Let et al reported that fish oil-enriched yogurt had higher oxidative stability than salad dressings or milk; the yogurt had lower levels of the volatile compounds 1-penten-3-one, 2-hexenal, and 2,4-heptadienal.[18] This volatile headspace analysis was supported by sensory evaluation (n=9-13 panelists trained in fishy off-flavors).[18] In drinking yogurt fortified with rapeseed oil, cod liver oil, or a blend, low levels of oxidation volatiles were reported over four weeks of storage.[19] Research by Pan et al reported 51 volatiles formed due to oxidation of cod liver oil through autoxidation combined with photosynthesized oxidation; these volatiles included the compounds highlighted above with the exception of 2-heptanone.[13] Propanal was increased more dramatically by addition of ferrous chloride to cod liver oil compared to rose bengal addition paired with light induced oxidation.[13]

The fish oil used in this study combined with detection limits and repeatability of the procedure were not sufficient to quantify volatiles in the yogurts. It is suggested that further work be done on the method for more precise quantification using SPME-GC/MS for detection of fish oil and oxidation volatiles.

Evaluation of Yogurt Attributes

Significant differences in oxidized, lime, and acid attributes were found in yogurt with different levels (low, high) and different types of oil (butter, fish, oxidized fish) (Table 3.4). Lime and acid flavors were perceived as significantly ($p<0.05$) lower in yogurt with a high level of oxidized fish oil compared to other formulations; this can be attributed to the oil source, and not the oil levels (Table 3.4).

Table 3.4: p-values: Effects of oil source (butter, fish or oxidized fish) and oil level (0.43 or 1%) of chile-lime flavored yogurt products evaluated by an experienced panel (n=12; 3 replications)

effects / attribute	All formulations	Oil source	Oil level	Interact source*level
acid	0.0220	0.0113	0.8671	0.1194
heat	0.2902	0.6676	0.1396	0.2037
lime	0.0017	0.0310	0.1264	0.0056
oxidized	<.0001	<.0001	0.0003	0.0193
sweetness	0.4840	0.5443	0.4337	0.2681

Oxidized oils typically contain higher levels of volatile aldehydes, ketones, and other small molecular weight compounds that may mask or interfere with the perception of lime flavor. Oxidized flavor characteristics were higher, as anticipated, in the high oxidized fish oil yogurt formulation and significantly different ($p<0.05$) from yogurts with low levels of oxidized fish oil and high levels of fresh fish oil formulations (Figure 3.3). Yogurt with low amounts of fresh fish oil and both butter oil formulations were significantly ($p<0.05$) lower in oxidized flavor from the other formulations. Research demonstrating that sensory data does not always correlate with analytical methods for oxidation in food products[20] supports the panelists finding a difference in oxidized flavors in the yogurts even though SPME analysis of volatile chemistry did not find large differences.

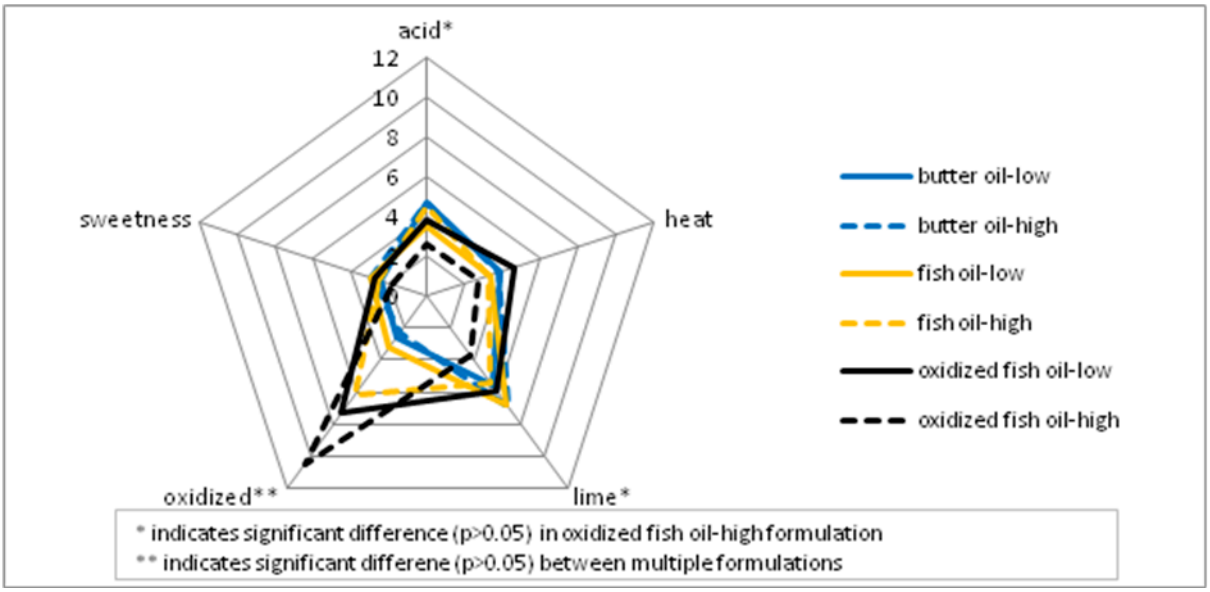

Figure 3.3: Mean levels of attributes in chile-lime flavored yogurt product (4°C) by formulation (varied oil sources and levels, 0.43 or 1%) as determined by experienced sensory panel (n=12 panelists) using a 15cm line scale; n=3 replications.

During training, panelists had difficulty separating 'fishy' characteristic from 'oxidized' character and the decision to discard the term 'fishy' from the evaluation was made. It is possible that panelists still confused oxidized and fishy notes in the yogurt containing high fish oil content, identifying the 'fishy' notes associated with the fresh fish oil as 'oxidized' (Figure 3.3). High levels (1%) of fish oil fortification in a chile-lime flavored yogurt product can be distinguished from 0.43% levels of fish oil by an experienced sensory panel but low levels (0.43%) of fish oil in a chile-lime flavored yogurt product cannot be distinguished from formulations made using butter oil at 0.43 or 1%.

Consumer Acceptance of Chile-Lime Yogurt

Study 3 employed a consumer panel to evaluate acceptance of two chile-lime flavored yogurts, fortified with butter or fish oil (1% wt/wt; total fat 1.2, 1.5%, respectively). Subjects (186 respondents) were recruited from the Virginia Tech campus and local community using an online survey (www.survey.vt.edu, Appendix D). The largest sections of demographics were female (66%), age 18 to 35 (75%), and self-described as Caucasian/white (81%). Responses to investigate knowledge of yogurt and omega-3 fatty acid (ω3 FA) health benefits also were collected. Respondents (47%) reported having general food and nutrition knowledge, such as a high school or college level introduction food and nutrition class, and only 12% reported having

very limited knowledge. When asked about dairy and ω3 FA health benefits, 87% reported they were generally or very aware of the potential health benefits associated with regular consumption of milk or dairy products but only 64% were generally or very aware of the benefits of the consumption of ω3 FA. Most respondents (65%) reported that they attempted to supplement the levels of ω3 FA in their diet naturally, with fortified foods or supplements, in contrast to 35% that claimed they make no attempts to increase the ω3 FA intake in their diets. Demographic surveys are shown in Appendix D and full demographic results are reported in Appendix H.

The consumer panel consisted of 100 panelists selected from the above 186 subjects based on interest in participating, regular consumption of yogurt (1-3 servings per month or more) and interest in exploring new flavors in traditional foods. This subgroup of consumers were 66% female, mostly age 18-25 (66%), and mostly (84%) Caucasian/white (full demographics in Appendix H). Participants responded to additional survey questions after completing the sensory testing; 38% of the panelists were unlikely to consume our chile-lime yogurt on a regular basis while 39% were likely to consume it on a regular basis. The most highly suggested food complements for the chile-lime flavored yogurt sampled were fruit (35%) and grains (28%) given choices of: nothing, eat it alone; beverage; meat; fruit; vegetable; grain; and other.

Chee et al enriched a strawberry fruit yogurt with 400 mg ω3 PUFA per 272 g of yogurt; the product was rated as "liked moderately" (mean=7 on a 9-point scale) by a 239 person consumer sensory panel.[3a] The mean hedonic scores for chile-lime yogurt were lower than those reported by Chee et al[3a] and lower than our minimum present target goal of 6.5. Hedonic test averages are shown in Table 3.5.

Table 3.5: Hedonic ratings (9 point scale, 1="dislike extremely", 9="like extremely") for consumer sensory panel (n=100) on chile-lime flavored yogurt product

formulation	hedonic acceptability
butter oil overall	4.85[a]
fish oil overall	4.68[a]
butter oil flavor	5.40[b]
fish oil flavor	4.82[c]

*letters connected by different letters are significantly different (α=0.05)

Significant differences in product flavors were found (p=0.0017), but overall differences in yogurt formulations were not significant. It also should be noted that distributions of ratings

were not normally distributed as shown in Figure 3.4. Distribution of responses for overall flavor less than "neither liked nor disliked" (5 of 9) were 49 and 50% for butter oil and fish oil, respectively, compared to 46 and 44% over 5 (butter oil, fish oil). Flavor responses for butter and fish oil less than 5 were 35 and 45% respectively, and 55 and 44% for greater than 5. This demonstrates that in flavor responses the fish oil sample was more evenly distributed but the butter oil formulation seemed to be liked slightly more even though in overall responses it seemed to be liked slightly less.

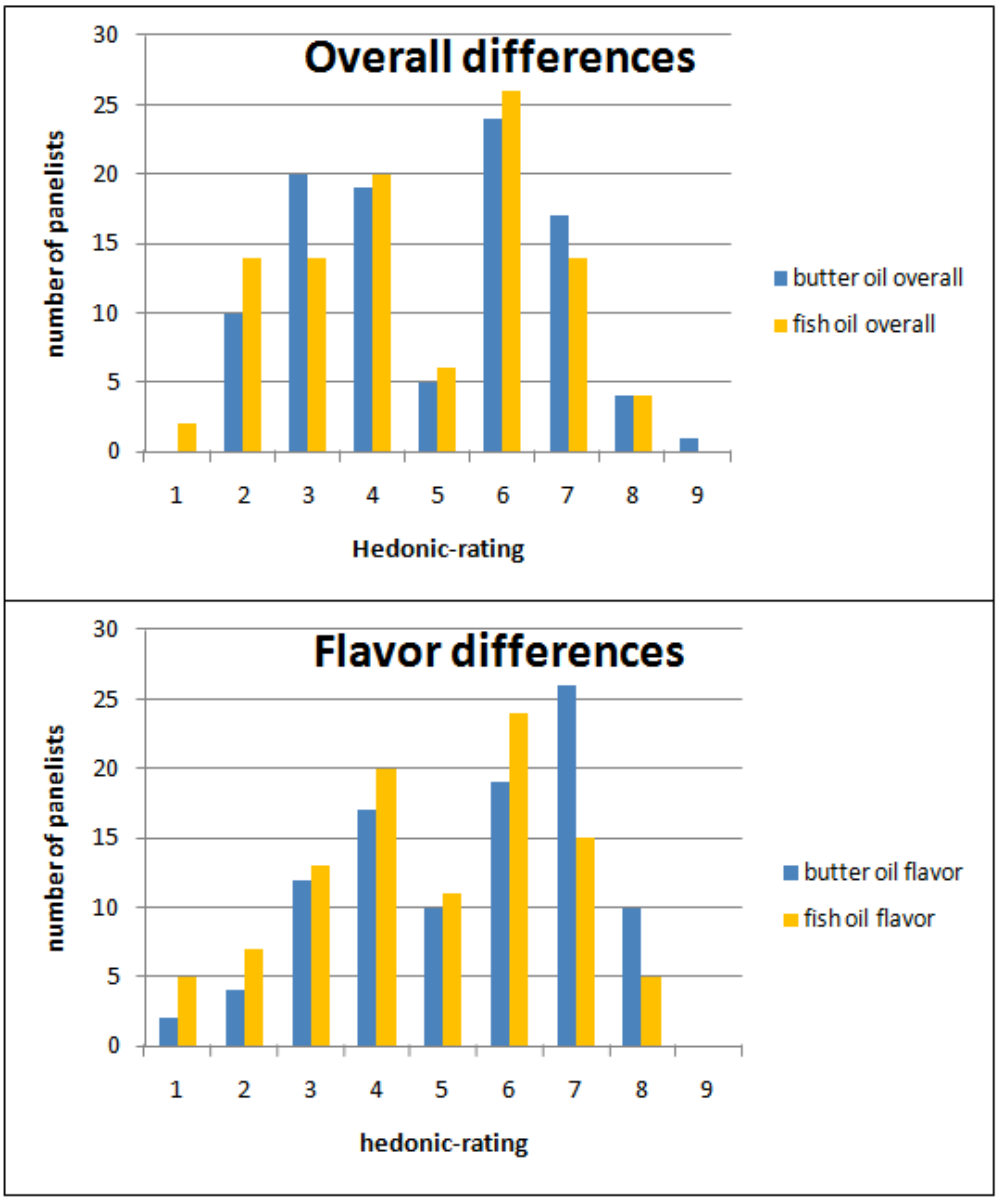

Figure 3.4: Distribution of hedonic ratings (9 point scale, 1="dislike extremely", 9="like extremely") from consumer sensory panel (n=100) of chile-lime flavored yogurt product

Comments provided by consumer respondents revealed very mixed responses and helped explain the bimodal distribution; some found the chile flavor and aftertaste unappealing and many commented they felt the yogurt was too bitter or sour. Attributes often marked as liked included creaminess and sweetness level or not too sweet. Common word descriptors that often were used for both like and dislike responses were related to aftertaste, texture, and consistency. The panelists were not told the flavor of the product but did identify it as citrus and often commented on the lemon flavor. Identifying the flavor to the panelists and screening for panelists that enjoy spicy foods could have prevented dislikes of the aftertaste. A spicy burning sensation would not be pleasant if a sweet fruit flavor is expected in yogurt, and would probably be seen as a defect. The texture of the yogurt was a bit grainy and not well blended compared to a commercial yogurt because production took place in the pilot plant with no blending apparatus; this could have led to dislikes in the texture of the product compared to expected yogurt textures.

Most of the dislike comments from panelists who rated samples as 4 or less fell into the categories of undesirable textures or criticisms of too acidic, sour or bitter and unpleasant aftertastes; a few panelists commented that there was not enough flavor but most who gave the product a low rating, did not like the spiciness or flavor. The comments associated with high hedonic ratings (6 or higher) were associated with aroma, decreases in harsh, sour or bitter tastes (assumed from previous sample(s) tasted), as well as refreshing, spicy or citrus flavor or light (amounts) of flavor characteristics and sweetness levels. Negative comments by panelists who rated yogurts at 6 or higher were primarily addressing aftertaste. The chile flavoring in this product did evoke a heat sensation on the palate and for some this seemed appealing, but others identified it as an unpleasant attribute. While many enjoyed the spicy flavor, it seems that these formulations were a bit too spicy for the population from which we sampled. The triangle test performed at the beginning of the study showed that significant differences between butter oil and fish oil formulations of yogurt were not apparent but a few panelists in the consumer study did add comments that they detected fishy flavors. This could be due to a higher proportion of fish oil being used, compared to study 1, but most panelists did not indicate detection of fishy flavors and a general consumer would probably not be able to detect the fishy flavor.

Chile-lime is not a flavor expected in yogurt in the US and this could have affected the results of the consumer sensory study. It cannot be assumed that the fish oil-enriched yogurt was not liked because of the fish oil addition since so few panelists reported noticing such a taste.

The hedonic flavor scores and comments suggest that the flavor is the reason the yogurts were not rated highly.

Research by Brennan et al found in a consumer panel (n=120) of 11-14 year olds that a lemon-lime yogurt with thick texture was preferred when compared to thin textured lemon-lime yogurt, while strawberry flavor acceptance was not affected by texture.[21] By concluding that yogurt thickness can significantly influenced flavor liking, it is possible that chile-lime flavored yogurt would be more preferred in a thicker yogurt; this could be achieved by using a different type or increased amount of stabilizer, fat, or total solids in the product. They also reported that yogurt color significantly increased flavor-liking intensities;[21] adding a green-tint to this also product could give an indication of what the consumer should expect in flavor.

One change to note during production of yogurts for the consumer panel was that different proportions of flavorings had to be used, due to a product shortage. The two flavorings in question were a chile-lime ethanol based flavoring (#341308) and a chile-lime oil based flavoring (#337649) both from Gold Coast Ingredients, Inc (Commerce, California). The butter oil formulation had 40:60% ethanol to oil base and the fish oil product had 67:33% ethanol to oil base. The same amount of additional lime (0.07% wt/wt) was added to each product. Surplus yogurt from the consumer panel was used for sensory testing in a sensory laboratory class demonstrating difference test methods, to determine if the flavoring changes resulted in similar products. A triangle test for similarity was conducted using a balanced order of presentation and 37 panelists. Twenty panelists were able to correctly identify the odd sample; with an alpha of 0.05 this concludes the samples were significantly different. A simple difference test with the same panelists and one set of samples presented to each panelist gave a calculated chi^2 of 3.71 compared to the tabulated chi^2 of 3.84 for an alpha of 0.05, with the conclusion that no significant difference between the samples existed. A paired comparison test, again with the same panelists, asking if the lime flavoring in the samples was different, concluded that no significant difference existed in lime flavor between samples using an alpha of 0.05 ($z=1.808<1.960$). The results of these small tests would suggest that the different proportions of flavoring were not the same but did not show significant differences in lime flavor or overall product to a small panel. These tests are relatively low in power because of the small number of panelists used but allow the results of the consumer sensory to be accepted.

Chile-Lime Yogurt Overview

From the experienced sensory panel, no significant differences were found between levels of butter oil in any attribute suggesting that the level of oil (<1% wt/wt) addition when using butter makes no difference on yogurt samples.

The low and high levels of fresh fish oil fortification differed only in the oxidized attribute; this is most likely due to the fishy aroma the panelists confused with oxidation during training. This confirms that fish oil fortification at 1% (wt/wt) could be differentiated based on taste by an experienced panel, but results from untrained panelists in the initial study suggest that overall differences were not noted. A consumer panel did not rate significant differences in overall acceptability of yogurts fortified with 1% fish or butter oil. These studies suggest that, with further investigation, fortification at 1% fish oil could be acceptable to consumers.

Oxidized fish oil added to yogurts, even at low levels of oxidation, is detectable by both trained and untrained panelists. This detection is beneficial to manufacturers interested in fish oil fortification because consumers would be able to detect products that have oxidized during processing or storage and research shows that consuming oxidized lipids could be a safety hazard because it does not provide benefit to the body and consumption can be toxic to animals.[22] Most products would also not have to worry about oxidized oil because and ideally oils would not oxidize during processing and yogurt has been suggested as a good matrix to prevent oxidation of fish oil post-manufacture; this project was specifically comparing oxidized oils.

Conclusions

A chile-lime flavored yogurt delivering more than 145 mg DHA+EPA/170 g serving of yogurt could be formulated from the pre-pasteurization addition of fish oil. The shelf life of fish oil addition to chile-lime yogurt was not investigated during this study and should be studied for further application of this product. Chile-lime flavoring in yogurt did not appeal to consumers and less than 40% of participants were willing to consume the chile-lime yogurts again. Pairing the flavor with grains or fruits and continuing research for a savory yogurt flavor should investigated because greater than 50% of participants claimed to be willing to consume a savory yogurt. Fish oil added to yogurt provides elevated levels of both long and short-chain fatty acids, indicating an increase in the health benefits from both. The number of volatile peaks from the chile-lime flavor in yogurts demonstrates how flavor disguises off-odors well. Consumers

indicate a willingness to supplement their diets with ω3 FA, indicating an opportunity for market expansion in dairy products delivering ω3 FA.

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Chapter 4: Reconstitution of Chitosan-Starch Encapsulated Fish Oil

Abstract

The effect of temperature (30, 63, 68, and 85°C) and pH (4.5, 5.5, 6.5) on the reconstitution of chitosan-starch encapsulated fish oil (60:40 chitosan starch wall-material, 1:2 fish oil:wall-material wt/wt) was investigated in a magnesium phosphate dairy blank solution. Particle size analysis was used to determine the success of reconstitution of the particles mentioned. Median particle size was significantly different ($p<0.001$) for each pH change and significantly different ($p<0.05$) between 30 and the three higher temperatures (63, 68, 85°C). Chitosan-starch fish oil microcapsules were successfully reconstituted at pH 4.5 and temperatures above 63°C as well as 85°C at pH 5.5. With further research fish oil microcapsules could be used in acidic beverages to increase the amounts of omega-3 fatty acids even at ambient temperatures. A second study evaluating the addition of chitosan-starch fish oil microcapsules into a yogurt matrix indicated that microcapsule addition prevented proper fermentation possibly due to an acetic acid residue on the microcapsules or an interaction of the casein and chitosan.

Introduction

Fish oil is an important nutraceutical in the food industry due to the concentrated amounts of the healthy omega-3 fatty acids (ω3 FA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Dietary sources of EPA and DHA provide health benefits such as reducing coronary heart disease, lowering blood pressure, and reducing inflammation.[1] Fish oil provides higher levels of EPA and DHA than other polyunsaturated fatty acid sources but is not used much in food products because of the associated off-flavors and odors.

Microencapsulation of fish oil has been studied for food use to prevent oxidation and decrease off-flavors and odors. Gharsallaoui et al defines microencapsulation as 'a process in which tiny particles or droplets are surrounded by a coating, or embedded in a homogenous or heterogeneous matrix to give small capsules with many useful properties'.[2] Microencapsulation can be used to protect the core, which is material surrounded a the coating, from damaging heat, light, or chemicals in the environment. This technology also can offer controlled release of active ingredients, mask tastes, and allow for extended storage or dilution of a specific ingredient in a food product. A post-production microcapsule consists of an interior core of material surrounded by a wall or coating material. Most microcapsules range in size of a few micrometers to millimeters in diameter.[2] They also may range in shape from perfectly spherical to irregular and may have one or more areas of core material.[2]

Microencapsulation in food is achieved through a variety of different techniques including spray drying, spray chilling, fluidized bed drying, electrostatic deposition, pressure extrusion, thermal or ionic gelation, polymer/polymer incompatibility and others.[3] Spray drying, as a microencapsulation method, is reported as the most common and least expensive way to encapsulate food ingredients.[2] During spray drying, a liquid is atomized into a hot gas current to form droplets, allowing the aqueous material to evaporate quickly forming a dry powder. The powder separates from the gas in a cyclone chamber and falls into a collection vessel.

Spray drying is currently being studied as a process to prevent oxidation of fish oil and create a more stable ingredient in dry powder form. Kolanowski et al studied the protection of fish oil from oxidation using spray dried microencapsulation of oil with modified cellulose.[4] Based on peroxide values, the microencapsulated fish oil experienced significantly less oxidation than bulk fish oil during storage. The maximum oil load retained using cellulose and spray dried encapsulation was 400.0 g oil/kg of microcapsules (approximately 40% wt oil).[4] Shaw et al gave

evidence that spray drying using encapsulation of a multilayer emulsion of chitosan, lecithin, and menhaden oil (10% wt oil) with corn syrup solids prevented oxidation of the oil even when reconstituted (200 mg ω3 FA/240 mL).[5] This research suggests using spray dried encapsulation of fish oil with carbohydrate macromolecules is effective in protecting the ω3 FA from oxidation and increasing shelf stability for use in functional foods.[5] Klinkesorn et al also concluded that spray drying fish oil (15% wt fish oil) provided powder with higher oxidative stability for use in foods.[6] Spray drying tuna oil in a multilayer lecithin-chitosan coating resulted in a more stable product than bulk oil. They also found at higher water activities this spray dried product was more stable to oxidation, possibly because Maillard reaction products acted as antioxidants. The addition of EDTA or mixed tocopherols to the emulsions also increased oxidative stability.[6]

Chitosan has been investigated as a wall material for encapsulation of fish oil because of its antioxidant properties, polymer structure, and ability to form complexes with lipids.[4-5, 7] The use of chitosan-based microcapsules in food is only experimental because currently chitosan has not received approved for food use in the United States as it has in other countries. Although several studies have investigated the use of chitosan or chitosan-cellulose combinations for microencapsulation, a unique study of chitosan combined with starch to encapsulate the fish oil was explored in our laboratory.[8] Blending the chitosan with other polymers improved efficiency of the spray-drying process and increased oxidative induction time suggesting an increase in protection against oxidation compared to 100% chitosan wall-material. Chitosan-starch (60:40 wt/wt with 50% wt/wt fish oil to wall material) microcapsules demonstrated an encapsulation efficiency of 62% and 37% surface fat. Particle sizes of chitosan-starch microcapsules were reported as 5.5 μm for 50th percentile and 39.5 μm for the 90th percentile using a 0.5 mm nozzle when spray drying.[8]

Reconstitution of microcapsules is important for use as food ingredients. When added to foods the microcapsules must blend into the food matrix for appropriate desired mouth feel or the products will be deemed undesirable by consumers. The current study investigated how microcapsules of fish oil spray dried with chitosan and starch were affected by pH and temperature during reconstitution and how the wall material behaved when added to a yogurt base. A chitosan-starch wall material (60/40 wt/wt) was used in this research to encapsulate fish oil using spray drying. The long-range goals include developing a microcapsule method for

preventing oxidation, allowing for more uniform distribution of lipids into an aqueous product, and masking fishy flavor and odor associated with fish oil.

Materials and Methods

Microcapsule Manufacture

Microcapsules of fish oil in a chitosan and starch wall blend were manufactured in the laboratory in a manner similar to Hannah et al.[8] This process used spray drying of an emulsion of 40% starch, 60% chitosan with a 1:2 weight fish oil to wall material ratio. Source materials included chitosan (ChitoClear™; donated by Primex, Siglufjordur, Iceland) and high amylose corn starch (Hi-maize™ 260, National Starch, Bridgewater, NJ). Deodorized fish oil was donated by DenOmega (Gamle Fredrikstad, Norway). Starch was solublized in deionized water using a pressure reactor (Parr, Moline, Illinois) at 160°C and 0.4 MPa under nitrogen gas held for 30 min and filtered (Buchner funnel with Whatman No. 1 filter paper) to remove particulate matter. Chitosan and starch solutions with fish oil added were stirred mechanically with a non-aerating overhead stirrer with propeller style blade (Kraft Apparatus, New York, NY) to form the emulsion. The emulsion was homogenized to evenly distribute the lipid using a laboratory homogenizer (model 15MR, APV Gaulin, Inc., Everett, MA) at 20/5 MPa (2950/725 psi) for two passes. The emulsion was spray-dried in a laboratory unit (Buchi 190 mini spray drier, Postfach, Switzerland) with a 0.7 mm two fluid nozzle, air pressure set to 0.55 MPa and inlet and outlet temperatures maintained at 160°C and 90°C, respectively. The emulsion was pumped through the spray drier at a rate of approximately 0.25 L/hour. Each batch yielded approximately 20 g microcapsules and was packaged in an oxygen barrier bag, flushed with nitrogen and frozen at -70°C until a sufficient microcapsule supply for one objective was made. The separate batches were blended into one lot to reduce variability from the spray drying application before study replications were initiated. After blending microcapsules were stored frozen (-15°C) under nitrogen gas to maintain the integrity of the product.

Measurement of Temperature and pH Effect on Microcapsules

Changes in solubility of the microcapsules due to varying temperatures and acidities were compared to determine the best way to reconstitute microcapsules in a dairy matrix. Three acid levels (pH 6.5, 5.5, and 4.5) were tested at four temperatures (30°C, 62°C, 68°C, 85°C). A pH of

4.5 generally corresponds to the acidity of yogurt and a pH of 6.5 represents the acidity of milk; pH 5.5 is an intermediate level. Temperatures were selected to represent vat pasteurization temperatures for milk (62°C), cream (68°C), and yogurt (85°C) and a control at general laboratory temperature (30°C). Approximately 0.25 grams of microcapsules were mixed, with continuous stirring (stir bar on magnetic plate) into 20 grams of magnesium phosphate dairy blank solution, as prepared by dairy standard methods[9]; each solution was adjusted to the proper pH using 1% acetic acid or 0.1 N sodium hydroxide. After pH adjustment, solutions were heated at specified temperatures in a hot water bath with shaking at 125 revolutions per minute for 30 minutes to simulate pasteurization. Solutions were removed and cooled to 27°C when the pH was measured again.

The diameter of dissolved microcapsules was measured using a particle size analyzer (Horiba LA-700, Irvine, CA). Samples were added dropwise (15-25) to the sample cup, containing approximately 200 mL deionized water, until the sample concentration was in an appropriate range for the instrument to read the particle size. Between each sample, the lines (silicone tubing, 9 or 6 mm diameter) and flow cell were washed with ethanol then rinsed with deionized water. The median diameter of the particles indicates the amount of microcapsules dissolved into the media after undergoing the set temperature and pH conditions. The desired oil droplet size of emulsions before spray drying was targeted as less than 2 μm[8]; therefore, a median diameter as read by the particle size analyzer of less than 2 μm also was the criterion for a reconstituted solution of microcapsules. Visible clumps of microcapsules and median diameters >2 μm as read by the particle size analyzer indicated a solution that was not fully reconstituted.

Statistical Analysis

All particle size analysis was conducted in triplicate with four replications. The statistical model for each analysis included the main treatment effects of pH (4.5, 5.5, 6.5) with temperature (30°C, 62°C, 68°C, 85°C) as a repeated variable, replication (n=4) and the interaction (pH x temperature). Statistical evaluation used JMP (SAS, Cary, NC) models, and a preset alpha of 0.05 for determining significant differences. Mean separations were conducted using Tukey's HSD.

Investigation of Microcapsules Added to Yogurt Pre-Pasteurization

This phase of the project investigated how the chitosan-starch wall material affected the textural characteristics of a yogurt base. Microcapsules were prepared as described above. Milk and yogurt were processed in the dairy processing pilot plant. Raw milk (Virginia Tech dairy farm) was heated to 54°C and separated using a pilot scale cream separator (The Creamery Package MF; model # P50STT0, Chicago, IL) into cream and skim milk. Yogurt formulation consisted of skim milk, sugar, non-fat dry milk, and either microcapsules or free oil (Figure 4.1).

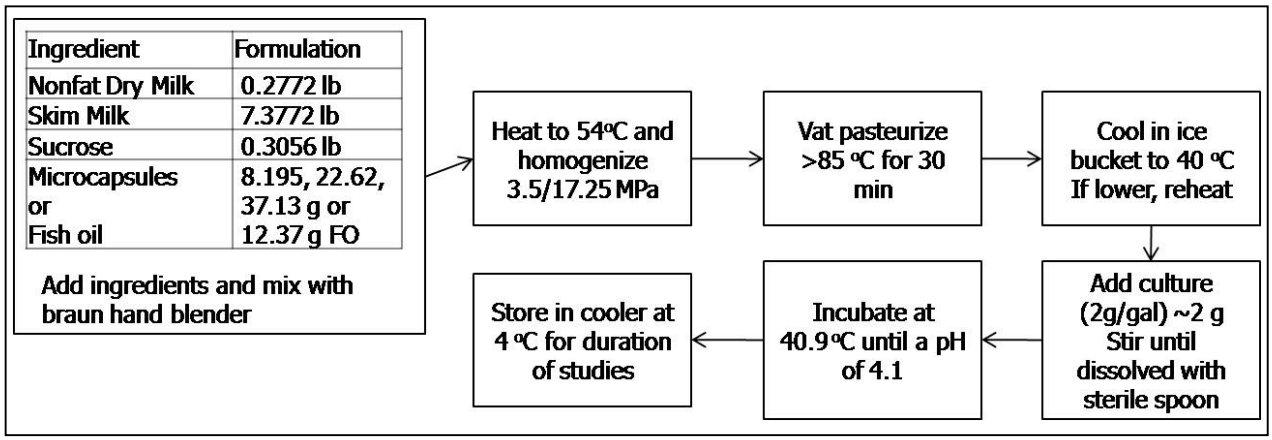

Figure 4.1: Formulation and process flow chart of yogurt mix (8 lbs) with fish oil microcapsules or fish oil (control)

Ingredients were blended together using a hand blender (Kitchen Aid, St. Joseph, MI) and homogenized (model 15MR, APV Gaulin, Inc., Everett, MA) for one pass at 3.5/17.25 MPa (500/1500 psi) for uniform distribution into the yogurt. Yogurt mix was pasteurized at 85°C for 30 minutes, cooled to 40°C and inoculated with *Streptococcus thermophilus and Lactobacillus bulgaricus* cultures (Ultra-gro, Cargill, Minneapolis, MN). The mix then was weighed (170g) into sterile yogurt cups and incubated at 40°C to a pH of 4.3, removed from incubator and stored at 4°C.

Microcapsules were added prior to pasteurization in order to ensure a sterile environment for the added culture bacteria to flourish. No stabilizer was added with the thought that chitosan would interact with the casein proteins to form a firm set-style yogurt. Four levels of chitosan-starch microcapsules (1.74 g, 1.062 g, 384 mg, and 0 mg (control)) per 170 g of yogurt were added to yogurt formulations. Microcapsule weight was based on percent oil/g microcapsule and estimate of 25% total DHA plus EPA in the oil.

The levels of microcapsules represent the recommended daily intake (145 mg) of EPA+DHA (1.740 g), a median value (1.062 g), a current market yogurt enriched with 32 mg DHA (384 mg), and a control with only fish oil and no encapsulation material (0 mg microcapsules, 580 mg fish oil/cup, equivalent to 145 mg DHA and EPA/cup). The varied levels of microcapsules provided different levels of fat in the product but all products had less than 0.5% total fat (nonfat).

Results and Discussion

Effect of pH and Temperature

For this study successful reconstitution is defined by lack of visible clumps of microcapsules and median diameters less than 2 μm as read by the particle size analyzer. Solution pH had a statistically significant ($p<0.001$) effect on the reconstitution of the microcapsules. Reconstituting microcapsules in a dairy blank solution at pH 4.5 was more effective than pH 6.5. Aggregates (clumps) of microcapsules were commonly visible at all temperature treatments at pH 6.5, suggesting complete reconstitution had not occurred. An increase in temperature from 30 to 63°C significantly ($p<0.001$) increased the ability for reconstituting microcapsules. There was a visual difference in turbidity and microcapsule size between samples at the extreme temperatures (85°C and 30°C) for each pH after 30 minutes of heat treatment. The temperature differences had a much smaller effect than the pH differences on microcapsule reconstitution. As the solutions cooled it was visually observed that they became more homogeneous (Appendix I).

The effect of temperature and pH on microcapsule particle size is reported in Table 4.1. The median particle size diameter decreased with decreasing pH and median particle size is higher at 30°C than at other temperatures. The percentage of particles less than 2 μm increased with pH and temperature. At pH 4.5 and temperatures greater than 63°C, over 50% of the particles were less than 2 μm whereas no more than 18% were in that range at pH 6.5. Based on the 90th percentile particle sizes, small amounts of non-dissolved microcapsules, swollen microcapsules and/or aggregates were evident at all conditions, but lower at combinations of higher temperature and low pH. Previous research in our laboratory found that dried microcapsules reported a 90th percentile particle size of 39.5±6.8 μm for a 60:40 chitosan-starch blend made with a 0.5mm spray nozzle.[8] Using this number for comparison, temperatures

greater than 63°C at pH 4.5 and 68°C at pH 5.5 were the only combinations that truly reconstituted the microcapsules. Particle size analysis results suggest that microcapsules would be sufficiently reconstituted under conditions similar to yogurt pasteurization and fermentation.

Table 4.1: Particle size data (averages with standard deviations) for microcapsules (60:40 chitosan:starch wall-material, 1:2 fish oil:wall-material wt/wt) by pH (4.5, 5.5, 6.5) and temperature (30, 63, 68, 85 °C); n=4 replications

median diameter reading[1] (µm)				
		pH		
		4.5	**5.5**	**6.5**
temperature (°C)	**30**	8.39 ±7.53	40.1 ±25.3	112 ±35
	63	1.97 ±0.12	8.95 ±3.43	44.2 ±4.9
	68	1.70 ±0.31	2.56 ±0.67	39.6 ±18.4
	85	1.66 ±0.20	1.98 ±0.31	33.0 ±35.9
90% of particles under[2] (µm)				
		pH		
		4.5	**5.5**	**6.5**
temperature (°C)	**30**	48.8 ±38.4	95.2 ±46.1	172 ±30
	63	18.9 ±19.7	50.0 ±32.2	92.1 ±32.1
	68	12.3 ±18.6	33.2 ±17.6	82.8 ±38.8
	85	2.79 ±0.78	5.71 ±4.79	91.6 ±58.5
% < 2 µm[3]				
		pH		
		4.5	**5.5**	**6.5**
temperature (°C)	**30**	31.7 ±6.1	13.9 ±6.2	2.68 ±1.32
	63	52.6 ±7.0	36.1 ±12.5	13.7 ±5.8
	68	71.1 ±19.9	42.5 ±7.7	14.2 ±5.2
	85	73.6 ±13.1	56.3 ±15.7	17.3 ±11.4

[1]the median diameter measured of particles in solution
[2]the particle size measured on the 90th percentile of particles in solution
[3]the percentage of particles under 2 µm

Effect of Microcapsules on a Yogurt Matrix

Processing problems occurred during manufacture of yogurt containing microcapsules. Microcapsules tended to float in the yogurt mix, were not evenly homogenized into the mix, and distribution was inconsistent. Addition of microcapsules by blending with milk seemed to improve distribution in the mixture, and improved homogenization efficiency but as the yogurts fermented more problems became obvious. During fermentation, the control yogurt containing bulk fish oil reached the final pH endpoint (4.3) in approximately 5.5 hrs but yogurt treatments

with microcapsules never set into a gel matrix characteristic of yogurt even after 12 hours of incubation. Yogurt with the lowest level of microcapsules had significant whey separation. The whey separation was greater than that which could be due to syneresis; this could have resulted from not using a stabilizer in yogurt formulation. Yogurt with higher amounts of microcapsules (1.74 and 1.06 g/170 g cup) had separated clumps of coagulant in the bottom of the cups and significant amounts of free whey on the surface. Alternate ways to add the microcapsules to yogurt were attempted by sonicating the microcapsules in milk, adding a paste of microcapsules and milk, or mixing the microcapsules into milk with a high shear mixer. None of these resulted in a desirable yogurt gel matrix.

There are a few possible explanations for the failure of these microcapsules in a yogurt matrix. The pH of milk (6.5) is around the pK_a of the chitosan,[10] deprotonation of chitosan would lower the pH of the milk solution and the casein proteins could have aggregated in the acidic conditions, becoming insoluble. Chitosan in research has affected the isoelectric point of proteins in solution, this could cause the milk proteins to aggregate and become insoluble.[11] The use of a typical dairy stabilizer could have interacted with the proteins and chitosan to reduce the separation that occurred during fermentation. Another explanation for the failure to create a characteristic gel structure is based on the interaction of chitosan and casein.[12] Microcapsules added prior to yogurt fermentation possibly caused aggregation of casein micelles, preventing fermentation process from occurring. Research on chitosan induced precipitation of casein supports this conclusion. Ausar et al reported that casein precipitation from the addition of chitosan to milk is not dependent on chitosan-lipid interactions, that α-, β-, and κ-casein all precipitate equally in the presence of chitosan, and that phosphate interactions of milk matrices have no effect on the precipitation of casein by chitosan.[12] Hydrophobic and electrostatic interactions were suggested as the primary interactions between casein micelles and chitosan at pH 5.9, even in different molecular weight chitosan molecules. Casein and chitosan interactions at pH 2.3 caused no casein precipitation and researchers determined this was due to casein being protonated below its isoelectric point and unable to interact with positively charged chitosan at this pH. The concentration of chitosan to precipitate casein micelles is temperature dependent; as the temperature increases the concentration of chitosan needed for coagulation decreases. They also documented that UHT milk gave the same coagulation trends as other milks when chitosan was added even though it exhibits reduced coagulation from rennet.[12] With more

inventive formulation our product could overcome these obstacles because research demonstrates chitosan coated alginate beads have been successfully added into yogurts pre-and post-fermentation.[13]

The addition of chitosan microcapsules post-fermentation may be a possible alternative. The acidic nature of yogurt, at pH 4.5 or less, makes it a viable matrix to add chitosan without casein coagulating because the isoelectric point of casein is a pH of 4.6.[14] In preliminary research on this project, chitosan was added to yogurt and no coagulation problems were observed over short storage. The product did have a grainy texture and would not have the desired mouthfeel for a yogurt product. Chitosan-starch microcapsules also were stirred into commercially processed yogurt purchased at a local grocery store, but resulted in a product with microcapsules that did not fully reconstitute in the yogurt matrix (lumpy). This could be investigated further by washing the surface of the microcapsules with hexane to remove any fat left on it before addition to premade yogurt to determine if surface fat was preventing the microcapsules from interacting with the yogurt matrix.

Other researchers have explored the addition of nanopowdered or commercially powdered chitosan post-fermentation to cholesterol-reduced yogurts.[15] They reported increased pH compared to controls and increasing pH trends as increasing levels of chitosan were added. Reduced *Streptococcus thermophilus* and *Lactobacillus bulgaricus* bacteria counts were observed during 20 day storage in chitosan-added yogurts as chitosan concentration increased when compared to the control. Results from a trained sensory panel (n=8) indicated that the chitosan-added yogurts did not have significant ($p<0.05$) grainy texture or whey separation but fishy flavor was significant between high levels of chitosan addition compared to control and rancidity flavor increased over the 20 day storage of chitosan added yogurts.[15]

Other possibilities for food application of chitosan-starch encapsulated microcapsules exist. The microcapsules might be better suited for use in foods when added as dry ingredients, such as cookies or breads. Foods with microcapsules added in a weakly acidic environment (~4.5) would provide a good matrix because chitosan has been shown to be soluble in weak inorganic and some organic acids[16] and this project demonstrated chitosan solubility improved as pH decreased from 6.5 to 4.5. Another market that has not been explored is adding ω3 FA to soy yogurts; this would eliminate the problem of the chitosan causing casein to coagulate because no

casein is present in a soy yogurt matrix. Future research could investigate the microcapsules formulated in this project added to a soy yogurt pre-pasteurization.

Conclusion

Microcapsules of fish oil with chitosan-starch are soluble in weak acidic environments at dairy pasteurization temperatures, suggesting delivery in acidic beverages may be possible. Interactions with casein above its isoelectric point precludes addition to yogurt mix but addition post-fermentation, contingent on appropriate food safety considerations, may be possible. Applications in other food matrices, such as soy yogurt, with no casein, or as a dried ingredient in cookies or breads may prove feasible. Further research in functionality of these microcapsules for protection against oxidation and masking of fishy odor and flavor is needed.

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Appendices

Appendix A: Institutional Review Board Approval Letter

VirginiaTech

Office of Research Compliance
Institutional Review Board
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24061
540/231-4991 Fax 540/231-0959
e-mail moored@vt.edu
www.irb.vt.edu
FWA00000572 (expires 6/13/2011)
IRB # is IRB00000667

DATE: February 18, 2010

MEMORANDUM

TO: Susan E. Duncan
Kim M. Waterman
Marnie Rognlien

FROM: David M. Moore

SUBJECT: **IRB Amendment 4 Approval:** "Sensory Evaluation and Attitudes about Functional Foods: Omega-3 Lipids in Dairy Products" , IRB # 09-726

This memo is regarding the above referenced protocol which was previously granted approval by the IRB on September 9, 2009. You subsequently requested permission to amend your IRB application. Approval has been granted for the requested protocol amendment, effective as of February 18, 2010.

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

1. Report promptly proposed changes in the research protocol. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

cc: File

Appendix B: Study 1, Triangle Test Sensory Documents

Flavor Consent Form

Virginia Polytechnic Institute and State University

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

Title Project: The investigation of new flavors for use in yogurt.

Investigators: Marnie Rognlien, Susan E. Duncan, PhD, RD

I. Purpose of this Research/Project
You are invited to participate in a sensory test to determine if flavor difference can be detected in low fat yogurts that have been created with different flavor profiles.

II. Procedures
There will be one sensory test lasting approximately 20 minutes. You will be presented with four individual sets of three yogurt samples. In each set of samples, you will be asked to compare the three by smell then taste and try to find which of the samples is different. You will then be asked to complete a consumer survey.

III. Risks
There are no more than minimal risks for participating in this study. If you are aware of any allergies to dairy products, please inform the investigator.

IV. Benefits
Your participation in this study will provide valuable information about the notability of different flavors in yogurt. Results from this sensory evaluation will be used to determine if proposed yogurt products are pleasing to consumers, at which levels and what levels should be used to formulate a later product. If you would like a summary of the research results, please contact the researcher at a later time.

V. Extent of Anonymity and Confidentiality
The results of your performance as a panelist will be kept strictly confidential except to the investigator. Individual panelists will be referred to by a code number for data analyses and for any publication of the results. You may be contacted for future participation on a trained sensory panel.

VI. Compensation
You will not be compensated for participating in this study.

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

VII. Subject's Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

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

IX. Subject's Permission

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

_____ Date _____
Subject Signature

_____Email_____
Subject Printed Name

☐ Please check here if you would be willing to participate in another yogurt sensory on Thursday, October 8, 2009.

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

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

Marnie Rognlien, Graduate Research Assistant,
Investigator (540) 272-5763; mxrogn@vt.edu

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

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

Oil Sensory Consent Form

Virginia Polytechnic Institute and State University

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

Title Project: The investigation of an omega-3 fatty acid enriched yogurt product.

Investigators: Marnie Rognlien, Susan E. Duncan, PhD, RD

I. Purpose of this Research/Project

You are invited to participate in this sensory test to determine if an aroma or taste difference can be detected in low fat yogurts that have been fortified with omega-3 fatty acids.

II. Procedures

There will be one sensory test lasting approximately 15 minutes. You will be presented with three individual sets of three yogurt samples. In each set of samples, you will be asked to eat a cracker to cleanse your palate, compare the three by smell then taste, then expectorate and identify which of the samples is different. You then will be asked to complete a consumer survey.

III. Risks

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

IV. Benefits

Your participation in this study will provide valuable information about the detection of different oils in yogurt. Results from this sensory evaluation will be used to prepare subsequent yogurt products fortified with omega-3 fatty acids. If you would like a summary of the research results, please contact the researcher at a later time.

V. Extent of Anonymity and Confidentiality

The results of your performance as a panelist will be kept strictly confidential except to the investigator. Individual panelists will be referred to by a code number for data analyses and for any publication of the results. You may be contacted for future participation on a trained sensory panel.

VI. Compensation

You will not be compensated for participating in this study.

VII. Freedom to Withdraw

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

VII. Subject's Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

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

IX. Subject's Permission

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

_____ Date _____
Subject Signature

_____Email_____
Subject Printed Name

☐ Please check here if you would be willing to participate in a trained sensory panel that would require 5-10 hours of training throughout the semester.

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

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

Marnie Rognlien, Graduate Research Assistant,
Investigator (540) 272-5763;
mxrogn@vt.edu

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

David Moore
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Board for the Protection of Human Subjects
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Triangle Sensory Score Card

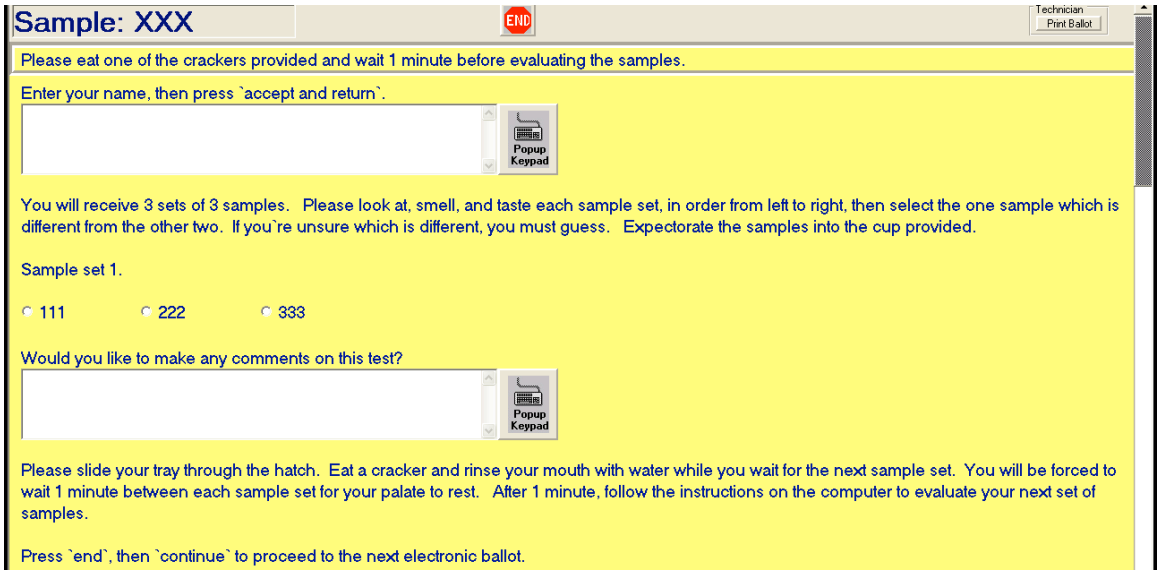

Figure B.1: Sample SIMS sensory score card from triangle sensory tests

Flavor Sensory Demographic Questions

Panelist # _____ (match with consent form.) Date: October 1st, 2009

Demographic Questionnaire

1) Indicate your age group:
   - ☐ 18-25
   - ☐ 26-35
   - ☐ 36-45
   - ☐ 46-55
   - ☐ 56-65
   - ☐ over 65

2) Indicate your gender:
   - ☐ Male
   - ☐ Female

3) How often (# times per week) do you consume milk or dairy products?
   - ☐ Never or up to several times per month
   - ☐ 1-3 servings per week
   - ☐ 4-6 servings per week
   - ☐ 7-9 servings per week
   - ☐ 10-12 servings per week
   - ☐ more than 12 servings per week

4) How often (# times per week) do you consume yogurt products?
   - ☐ Never or up to several times per month
   - ☐ 1-3 servings per week
   - ☐ 4-6 servings per week
   - ☐ 7-9 servings per week
   - ☐ 10-12 servings per week
   - ☐ more than 12 servings per week

5) Do you consume milk and/or yogurt at least once per week?
   - ☐ YES, If YES, continue to Q6.
   - ☐ NO, If No, continue with Q7.

6) If yes, what percent fat content do you normally consume?
   - ☐ Non-fat or skim, < 0.5% fat.
   - ☐ Low-fat, 1% fat.
   - ☐ Reduced-fat, 2% fat.
   - ☐ Whole fat, 3.25% fat.

7) Identify the statement that best describes your view of the potential health benefits associated with regular consumption of milk or dairy products.
   - ☐ I am not aware.
   - ☐ I am vaguely aware.
   - ☐ I am generally aware.
   - ☐ I am very aware.

8) Would you eat a full serving (6 oz cup) of a savory (defined as full-flavored, not sweet) yogurt product?
   - ☐ Highly likely
   - ☐ Likely
   - ☐ Unlikely
   - ☐ Highly unlikely
   - ☐ Don't know

9) Would you consume any of the products you just tasted on a regular basis?
   - ☐ Highly likely
   - ☐ Likely
   - ☐ Unlikely
   - ☐ Highly unlikely
   - ☐ Don't know

10) Do you cook your own food at least one time per week?

□ YES, If YES, continue to Q11.

□ NO, If NO, continue to Q12.

11) How often do you experiment with food flavors or complementary sauces?
   - ☐ Never or up to several times per month
   - ☐ 1-4 times per week
   - ☐ 5-7 times per week
   - ☐ 8-12 times per week
   - ☐ more than 12 times per week

12) What, if anything, would be the best food complement to go with the product you just tasted, please list a specific food in the category? (list one or more foods)
   - ☐ Nothing, eat it alone
   - ☐ Beverage: _____
   - ☐ Meat: _____
   - ☐ Fruit: _____
   - ☐ Vegetable: _____
   - ☐ Grain: _____
   - ☐ Other: _____

13) What other flavors might compliment the product you just tasted?

_____

14) How might you use this product as an ingredient in a recipe or meal?

_____

Thank you for completing this sensory training. Please come to the kitchen to receive a treat for your time.

Oil Sensory Demographic Questions

Panelist # _____ (match with consent form.) Date: October 8th, 2009

Demographic Questionnaire

1) Indicate your age group:
   - ☐ 18-25
   - ☐ 26-35
   - ☐ 36-45
   - ☐ 46-55
   - ☐ 56-65
   - ☐ over 65

2) Indicate your gender:
   - ☐ Male
   - ☐ Female

3) How often (# times per week) do you consume fatty fish (such as salmon, tuna, mackerel) with a meal?
   - ☐ Never or up to several times per month
   - ☐ 1-3 meals per week
   - ☐ 4-6 meals per week
   - ☐ 7-9 meals per week
   - ☐ 10-12 meals per week
   - ☐ more than 12 meals per week

4) Identify the statement that best describes your view of the potential health benefits associated with regular consumption of fatty fish.
   - ☐ I am not aware.
   - ☐ I am vaguely aware.
   - ☐ I am generally aware.
   - ☐ I am very aware.

5) Identify the statement that best describes your view of the potential health benefits associated with regular consumption of omega-3 fatty acids.
   - ☐ I am not aware.
   - ☐ I am vaguely aware.
   - ☐ I am generally aware.
   - ☐ I am very aware.

6) Do you make any attempt to supplement your diet or increase your dietary intake of omega-3 fatty acids? Check all that apply.
   - ☐ No
   - ☐ Yes, by eating more fish
   - ☐ Yes, by consuming omega-3 fortified foods

☐ Yes, by taking omega-3 fatty acid supplements, list _____
☐ Yes, by consuming other foods naturally rich in omega-3 fatty acids; list foods
_____

7) Omega-3 fatty acids have potential health benefits which include improved cardiovascular health and brain functions. If you could consume one serving daily of an omega-3 fortified dairy product to achieve the recommended level of omega-3 fatty acids for potential health benefits how often would you choose to consume this product.

☐ Would not consume this product.
☐ 1 up to several times a month.
☐ 1-3 days a week.
☐ 4-5 days a week.
☐ Every day of the week.

Thank you for completing this sensory training. Please come to the kitchen to receive a treat for your time.

Appendix C: Study 2, Experienced Panel Sensory Documents

Experienced Panel Consent Form

Virginia Polytechnic Institute and State University

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

Title Project: The investigation of a flavored, omega-3 fatty acid enriched yogurt product.

Investigators: Marnie Rognlien, Susan E. Duncan, PhD, RD

I. Purpose of this Research/Project
You are invited to participate in this sensory test to determine if a taste difference can be detected in low fat yogurts that have been flavored and fortified with omega-3 fatty acids.

II. Procedures
There will be six sensory training sessions lasting approximately 1 hour each, two validation sensory sessions lasting approximately 30 minutes each, and three test sessions lasting approximately 30 minutes each. At the test sessions you will be presented with six individual yogurt samples and be asked to mark six attributes on each sample as discussed in training. Samples will be tested as taught in training by eating a cracker to cleanse your palate, rinsing your mouth, tasting the sample, then expectorate and identify each attribute.

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

IV. Benefits
Your participation in this study will provide valuable information about the attributes of this omega-3 enriched yogurt. Results from this sensory evaluation will be used to prepare subsequent yogurt products fortified with omega-3 fatty acids. If you would like a summary of the research results, please contact the researcher at a later time.

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

VI. Compensation
You will not be compensated for participating in this study.

VII. Freedom to Withdraw
If you agree to participate in this study, you are free to withdraw from the study at any time without penalty. There may be reasons under which the investigator may determine you should

not participate in this study. If you have allergies to dairy products, fish, wheat, or flavors, or are under the age of 18, you are asked to refrain from participating.

VII. Subject's Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

3) Participate in all six hours of training sessions, Nov 2, 4, 6, 9, 11, and 13, 10:30-11:30 am, if you need to make one up the researcher will accommodate you.
4) Participate in the two validation training sessions, Nov 16 and 17 10-11:30 am.
5) Participate in the three test sensory sessions, Dec 4, 11, and 17 10-11:30 am.
6) Taste and expectorate the yogurt products and rate each described attribute.

IX. Subject's Permission

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

_____ Date _____
Subject Signature

_____Email_____
Subject Printed Name

----------For human subject to keep----------
Schedule:

1) **Training: Nov 2, 4, 6, 9, 11, and 13, 10:30-11:30 am**
2) **validation training sessions, Nov 16 and 17 10-11:30 am**
3) **test sensory sessions, Dec 4, 11, and 17 10-11:30 am**

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

Marnie Rognlien, Graduate Research Assistant,
Primary Investigator (540) 272-5763; mxrogn@vt.edu

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

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Ingredients for Training Standards

Table C.1: Ingredients used for sensory attribute training standards

attribute or base	standard used	source
fishy	Fish Oil	Chunk light Tuna, Kroger, DenOmega, Gamle Fredrikstad, Norway
heat	Natural Chile Flavor Concentrate	Gold Coast Ingredients, Inc, Commerce, California
lime	Organic Lime Flavor	Gold Coast Ingredients, Inc, Commerce, California
sweet	sucrose	
acid	Citric Acid	New England Cheesemaking Supply Co (C13), Ashfield, MA
oxidized	oxidized canola oil from laboratory fridge	
very oxidized	very oxidized vegetable oil from laboratory fridge	
water	filtered water	Brita Filtered
oil	Pure vegetable oil	Kroger brand, Cincinnati, OH
milk	(1%) low-fat milk	Kroger brand, Cincinnati, OH
yogurt	low-fat plain blended yogurt	Kroger brand, Cincinnati, OH

Outline of Training for Study 2

Table C.2: Training goals and results

Session	Goals	Training Standards and Samples	Comments
1	Orientation, introduction, basic tastes (sweet, acidic)	sucrose (4 levels, 0.77-2.44%) or citric acid (4 levels, 0.012-0.033%) in filtered water, low-fat milk, plain low-fat yogurt	sucrose levels 1 & 4 were obviously different; samples 1& 2 were not differentiated
		tasted and discussed basic tastes in water, then milk; ranked sweet and acidity in yogurts	acid ranking more difficult for panel because of natural acidity of yogurt; acidity levels too low, had to be repeated
	Established evaluation protocol	bring sample to mouth without smelling; swish sample while exploring flavors and then expectorate sample; filtered water and low-sodium crackers provided for cleansing palate between samples	
	Discussed expectations for panelist participation and how to prevent expectation bias	discussion in group setting	
2	Introduced oxidized and fishy flavors characteristics	oxidized fish oil (4 levels, 0.41-1.02%) or tuna oil (4 levels, 0.41-0.81%) in vegetable oil, low-fat milk, plain low-fat yogurt	oxidized levels 3 & 4 easily recognized; milk most intense base for oxidized flavor
		tasted and discussed water, then milk, then low and high only of yogurt, then ranked 4 yogurts	fishy level 4 similar to canned tuna; fishy flavor exaggerated in yogurt base
		provide education of fish oil fortification in yogurt and oxidation problems that can occur and how to prevent	panelists suggested pairing flavors of dill, lemon, soy or as ingredient in spicy Indian dishes
3	Introduced lime flavor; discussed chile-lime and reasoning for flavoring selection	lime flavoring (4 levels, 0.05-0.12%) in water, milk, and yogurt	panelists generally positive about lime in milk
		tasted and discussed lime flavor in water, then milk; ranked lime flavor in yogurts	ranking lime yogurts was difficult because of natural acidity and unsweetened yogurt
	Compared fishy and oxidized flavors	panelists evaluated coded samples of fish oil, oxidized fish oil, oxidized vegetable oil samples and recorded responses of "fishy", "oxidized", or "both"	50% of panelists got correct; fishy flavor more intense in oxidized fish oil than fresh fish oil

	Reviewed acid	"A"-"Not A" test of 8 samples (5-citric acid at 0.77% in yogurt, 3-plain low-fat yogurt)	2/3 of panel got 7/8 or more correct
4	Introduced scaling technique on 15 cm unstructured line scale	4 samples (each of fishy, oxidized, sweet, acidic) tow of known (low/high from training) and two of unknown (intermediate and one repeated) attributes and intensities were provided; panelists marked attribute and intensity on scales	
	Revised evaluation protocol	cleanse palate with one bite of cracker; rinse mouth with water; stir sample with spoon; take half a teaspoon of sample; bring to mouth without smelling; swish sample in mouth; expectorate	
5	Introduced chile flavor	chile (4 levels, 0.40-0.11%) in milk and yogurt	identifying chile easier than other attributes; hard to rank because of fatigue from burn
		tasted and discussed milk taste and mouth sensation, then ranked yogurts	called 'heat' because did not taste much flavor
			range of heat: prefer salsa hotter but higher than most Indian foods and chili
	Reviewed oxidized tastes	Rated intensity of 4 samples of increasing oxidized vegetable oil on line scale; instructed not to retaste	overall correct order was identified, not all panelists used scale in same manner
	Practiced using line scales with multiple attributes in one product	2 samples evaluated for attributes and intensities on line scale. Samples: 1 known (told the flavors were chile and lime), one unknown (sweet, fishy)	heat of chile+lime sample difficult to place; all panelists identified 'sweet' in unknown sample; fishy was confused as oxidized or lime; some panelists suffered from heat carryover
6	Modified protocol to reduce 'heat' carryover	no salt-topped saltines or low sodium oyster crackers	oyster crackers best because have more of bland taste to cleanse chile flavor from palate
	Reviewed 'fishy'	fish oil at high and low levels; marked intensity 1-4	difficult for panelists; high level had more aftertaste
		received 2 more samples (low and high) with 1 extra attribute; marked 'fishy' level (1-4) and identified attribute	lime and oxidized masked 'fishy' taste
	Decided order of attributes on palate	evaluated 4 samples with >1 attribute; panelists identified attributes and order	decided order was lime, sweet, heat, acid, fishy, oxidized

	Practiced rating of multiple attributes in yogurt using unstructured line scales	evaluated 4 samples with multiple attributes asked to mark six line scales for each attributed in defined order	panelists found that many of the attributes interacted, making detection more difficult; examples of interactions were acid and heat, lime and acid, and fishy and oxidized
week before testing	Practiced oxidized attribute	"A"-"Not A" test comparing no, low, and high levels of oxidized oil in low-fat plain yogurt, told the three options and looking at oxidized attribute	all found slightly difficult
before 1st replication	Retested oxidation validation	four triangle tests comparing oxidation (none vs high, low vs none, low vs medium, low vs high)	panelists continued to find this difficult
weekly during testing	Practiced oxidized attribute	three paired comparison tests (4 levels; none, low, med, high), required to get 3 or more correct to not have to repeat	panelists improved with practice using paired comparison tests

Samples used for Validation of Training for Study 2

Table C.3: Samples prepared for validation of experienced panel and levels of attributes formulated

sample	heat	lime	acid	sweet	fishy	oxidized	very oxidized	yogurt	% heat	% lime	% acid	% sweet	% fishy	% oxidized	% very oxidized
A	0.31			2.65	1.41	2.34		299.83	0.10			0.88	0.47	0.78	
B		0.16	0.12			2.02	0.04	300.60		0.053	0.040			0.67	0.013
C	0.13			7.36		2.03	0.04	299.93	0.043			2.50		0.68	0.013
D			0.28		1.52	1.60		299.89			0.093		0.51	0.53	
E	0.29	0.15		7.30	2.13			300.16	0.097	0.050		2.40	0.71		
F		0.37	0.12		2.12			300.02		0.12	0.040		0.71		

Experienced Panel Score Card

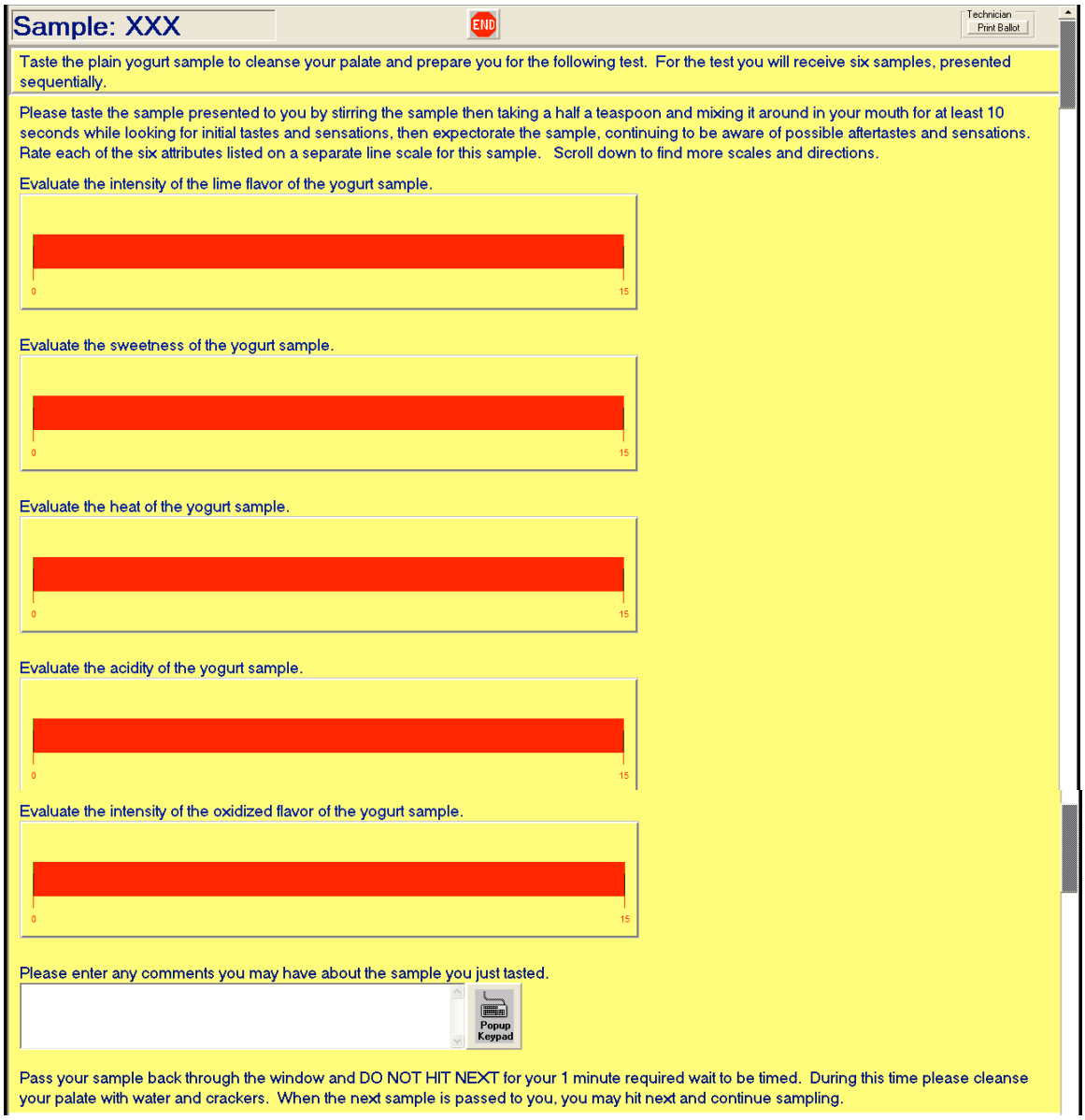

Figure C.1: Sample SIMS sensory score card for experienced panel using rating scales for 5 attributes of chile-lime yogurt

Appendix D: Study 3, Consumer Panel Sensory Documents

Consumer Panel Consent Form

Virginia Polytechnic Institute and State University

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

Title Project: Consumer acceptability of an innovative healthy yogurt product.

Investigators: Marnie Rognlien, Susan E. Duncan, PhD, RD

I. Purpose of this Research/Project
You are invited to participate in a sensory test to determine consumer acceptance of a new flavor of low fat yogurt enriched with healthy fats.

II. Procedures
There will be one sensory test lasting approximately 15 minutes. You will be presented with four individual yogurt samples. For each sample, you will be asked to rate the level of acceptance you feel for the product and record any specific likes or dislikes. You will then be asked to answer a few additional questions about the flavoring.

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

IV. Benefits
Your participation in this study will provide valuable information about the acceptability of this new yogurt product. Results from this sensory evaluation will be used to determine if proposed yogurt products are pleasing to consumers. If you would like a summary of the research results, please contact the researcher at a later time.

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

VI. Compensation
You will be compensated with a snack for participating in this study.

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

VII. Subject's Responsibilities
I voluntarily agree to participate in this study. I have the following responsibilities:

1) Smell and taste the yogurt products and rate my acceptability based on aroma and taste.
2) Answer a few questions about flavoring of the product.

IX. Subject's Permission

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

_____ Date _____
Subject Signature

_____
Subject Printed Name

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

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

Marnie Rognlien, Graduate Research Assistant,
Investigator (540) 272-5763; mxrogn@vt.edu

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

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

Consumer Sensory Score Card

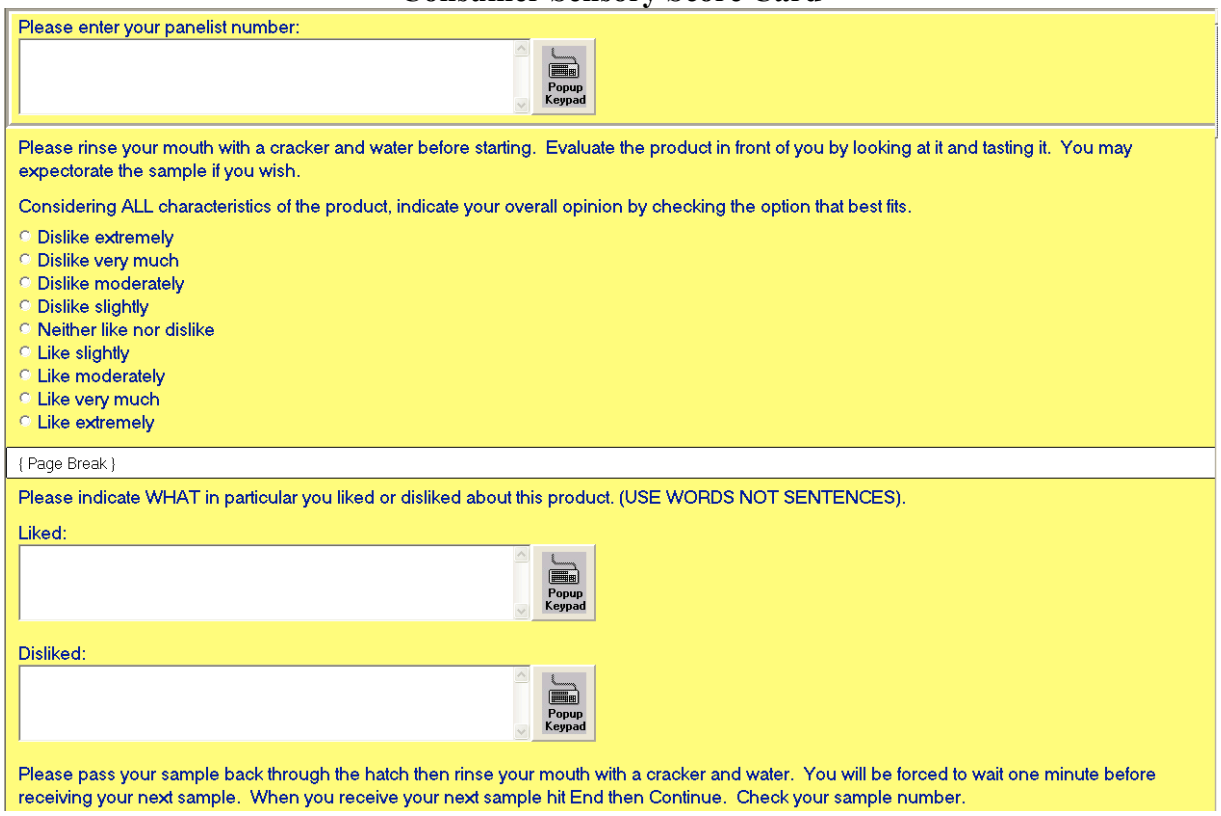

Figure D.1: Sample SIMS sensory score card for hedonic scale consumer sensory panel

Consumer Sensory Screening Questions

Survey on omega-3 fatty acids and yogurt products

By completing and returning this survey your consent is implied that the data you provide may be used for determining if you fall into the target population for our sensory study. The data you provide of consumer knowledge and interest of omega-3 fatty acids and yogurt products in the diet may be used for research purposes. The results of your performance as a panelist will be kept strictly confidential except to the investigator. There are no more than minimal risks for participating in this survey; completion of this survey is voluntary and you are free to withdraw from this study at any time without penalty. If you are eligible to participate in the sensory panel, you will be contacted within a week.

Please fill in the following:
Full Name: _____

Phone contact: _____

Email: _____

Which is the best means of contacting you? _____

Overall demographics

1. Mark one: Male Female

2. Age:
   Under 18 36-45
   18-25 46-55
   26-35 Over 55

3. How would you classify yourself?
   - ☐ American Indian or Alaska Native
   - ☐ Asian
   - ☐ Black or African American
   - ☐ Caucasian/White
   - ☐ Hispanics of any race
   - ☐ Native Hawaiian or Pacific Islander
   - ☐ Multiracial
   - ☐ Would rather not say
   - ☐ Other: _____

4. What country were you born in? _____

5. Describe your knowledgeable of foods or nutrition:
    a. I have very limited knowledge
    b. I have a general knowledge (ie. high school level food and nutrition class or HNFE 1004)
    c. I am knowledgeable (ie. 2-3 collegiate level classes in food or nutrition)
    d. I am extremely knowledgeable (ie. undergraduate or graduate training in FST or HNFE)

Product usage questions

6. How often do you consume yogurt products?
    ☐ Never
    ☐ 1-3 servings per **month**
    ☐ 1-3 servings per week
    ☐ 4-7 servings per week
    ☐ more than 7 servings per week

7. Identify the statement that best describes your view of the potential health benefits associated with regular consumption of milk or dairy products.
    ☐ I am not aware.
    ☐ I am vaguely aware.
    ☐ I am generally aware.
    ☐ I am very aware.

8. Identify the statement that best describes your view of the potential health benefits associated with regular consumption of omega-3 fatty acids.
    ☐ I am not aware.
    ☐ I am vaguely aware.
    ☐ I am generally aware.
    ☐ I am very aware.

9. Do you make any attempt to supplement your diet or increase your dietary intake of omega-3 fatty acids? Check all that apply.
    ☐ No
    ☐ Yes, by eating more fish
    ☐ Yes, by consuming other foods (other than fish) naturally rich in omega-3 fatty acids
    ☐ Yes, by consuming omega-3 fortified foods
    ☐ Yes, by taking omega-3 fatty acid supplements, list _____

Interest in participating in a sensory panel

10. Are you interested in participating in a sensory panel to taste a new flavor of low fat yogurt enriched with healthy fats? The panel will last 15 minutes on February 10, 11, or 12 in the Food Science Building at Virginia Tech?

    Yes No

11. Do you have any of the following conditions or use any of the following products?

	Yes	No
a. Lactose intolerance		
b. Food allergies to dairy or fish		
c. Diabetes		
d. Hypoglycemia		
e. Oral or gum disease		
f. Tobacco products		
g. Take medication that could affect your taste and/or smell		

12. Have you previously participated in sensory panels in the FST department before?

Yes, continue below No, continue with next question

If yes to above: Have you participated in dairy product sensory panels in the FST department before?

Yes No

13. Are you interested in exploring new flavors in traditional foods?

Yes No

14. During February 10^{th}-12^{th} please check any of your preferred times to come to the Food Science Building for a 15 min sensory test?

Wednesday	11 am-12 pm	12-1 pm	1-1:40 pm
Thursday	2-3 pm	3-4 pm	
Friday	11 am-12 pm	12-1 pm	

Thank you for completing the survey. We will contact you very soon about participating on our panel!

Consumer Sensory post-tasting Questions

Demographic Questionnaire "to be asked after completion of the sensory panel"

1) How often do you enjoy spicy foods?
   - ☐ Never or up to several times per month
   - ☐ 1-3 servings per week
   - ☐ 4-6 servings per week
   - ☐ 7-9 servings per week
   - ☐ 10-12 servings per week
   - ☐ more than 12 servings per week

2) Would you consume any of the products you just tasted on a regular basis?
   - ☐ Highly likely
   - ☐ Likely
   - ☐ Unlikely
   - ☐ Highly unlikely
   - ☐ Don't know

3) What, if anything, would be the best food complement to go with the product you just tasted, please list a specific food in the category? (list one or more foods)
   - ☐ Nothing, eat it alone
   - ☐ Beverage
   - ☐ Meat
   - ☐ Fruit
   - ☐ Vegetable
   - ☐ Grain
   - ☐ Other: _____

   Please list examples from the categories you checked above. _____

4) What other flavors might complement the product you just tasted?

   _____

5) How might you use this product as an ingredient in a recipe or meal?

   _____

Thank you for completing this sensory training. Please come to the kitchen to receive a treat for your time.

Appendix E: Analytical Results of Yogurt Samples

Table E.1: Results of yogurt analyses, based on standard methods, for all yogurts produced

	Total Solids (%)	Moisture (%)	Soluble Solids (%)	Protein (g/g)	Fat (%)	pH	Titratable acidity (%)	Coliform (count at 10^{-3})
trained panel (3 reps)								
butter oil-low	14.91±0.23bc	85.09±0.23ab	11.0±0.6a	0.0477±0.0044a	1.1±0.0c	4.20±0.05b	0.019±0.000a	0±0
butter oil-high	15.31±0.09ab	84.69±0.09bc	10.9±0.4a	0.0491±0.0064a	1.6±0.1a	4.20±0.04b	0.019±0.000a	0±0
fish oil-low	15.13±0.28abc	84.87±0.28abc	11.0±0.3a	0.0504±0.0078a	1.2±0.0c	4.18±0.04b	0.019±0.001a	0±0
fish oil-high	15.42±0.17ab	84.58±0.17bc	11.3±0.3a	0.0471±0.0097a	1.6±0.1a	4.17±0.06b	0.020±0.000a	0±0
oxidized fish oil-low	15.13±0.31abc	84.87±0.31abc	11.2±0.1a	0.0491±0.0048a	1.2±0.0c	4.17±0.05b	0.019±0.000a	0±0
oxidized fish oil-high	15.64±0.38a	84.36±0.38c	10.8±0.2a	0.0540±0.0034a	1.6±0.1a	4.17±0.04b	0.020±0.000a	0±0
consumer panel (1 rep)								
butter oil	14.60±0.08c	85.40±0.08a	10.8±0.4a	0.0493±0.0032a	1.2±0.1bc	4.12±0.03b	0.020±0.001a	0±0
fish oil	15.19±0.09abc	84.81±0.09abc	10.7±0.0a	0.0579±0.0048a	1.5±0.0ab	4.13±0.01b	0.020±0.000a	0±0
flavor sensory (avg n=4)	15.04±0.17bc	84.96±0.17ab	10.9±0.4a	0.0527±0.0070a	1.3±0.2bc	4.56±0.18a	0.019±0.001a	0±0
oil sensory (avg n=3)	14.91±0.12bc	85.09±0.12ab	10.8±0.4a	0.0523±0.0006a	1.1±0.0c	4.21±0.03b	0.019±0.000a	0±0

a-c - means within columns with different super script are significantly different ($p<0.05$)

Appendix F: Complete Fatty Acid Profile and Chromatograms

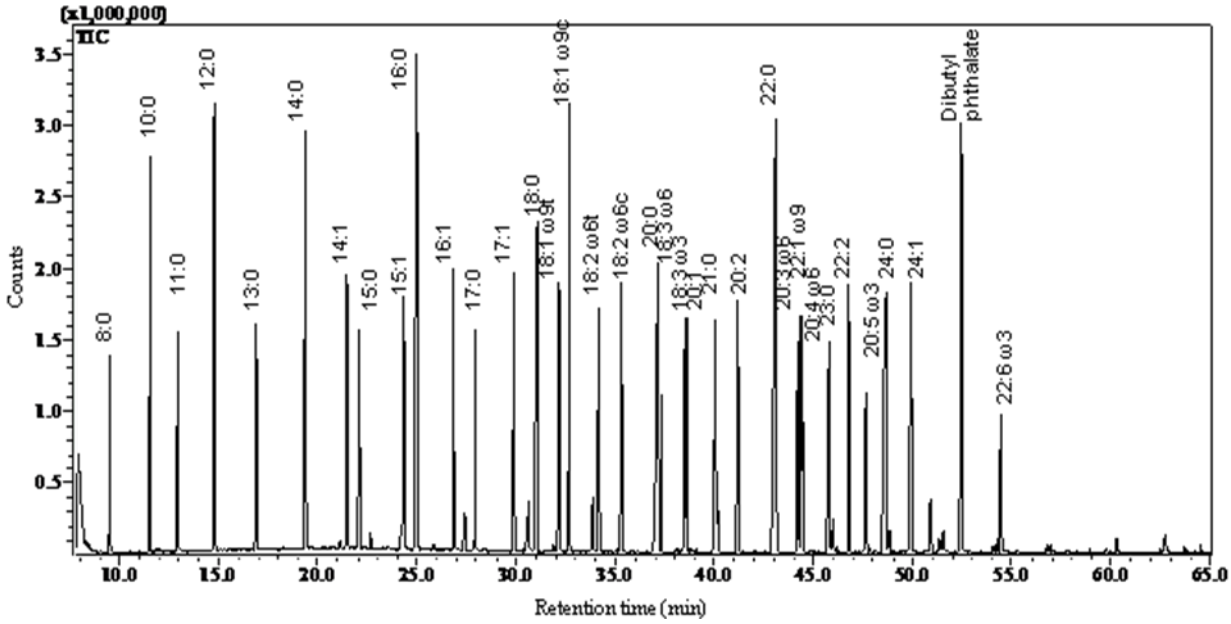

Figure F.1: Labeled FAME chromatogram of external fatty acid methyl ester standard (SupelCo, Bellefonte, PA) used for identification of peaks

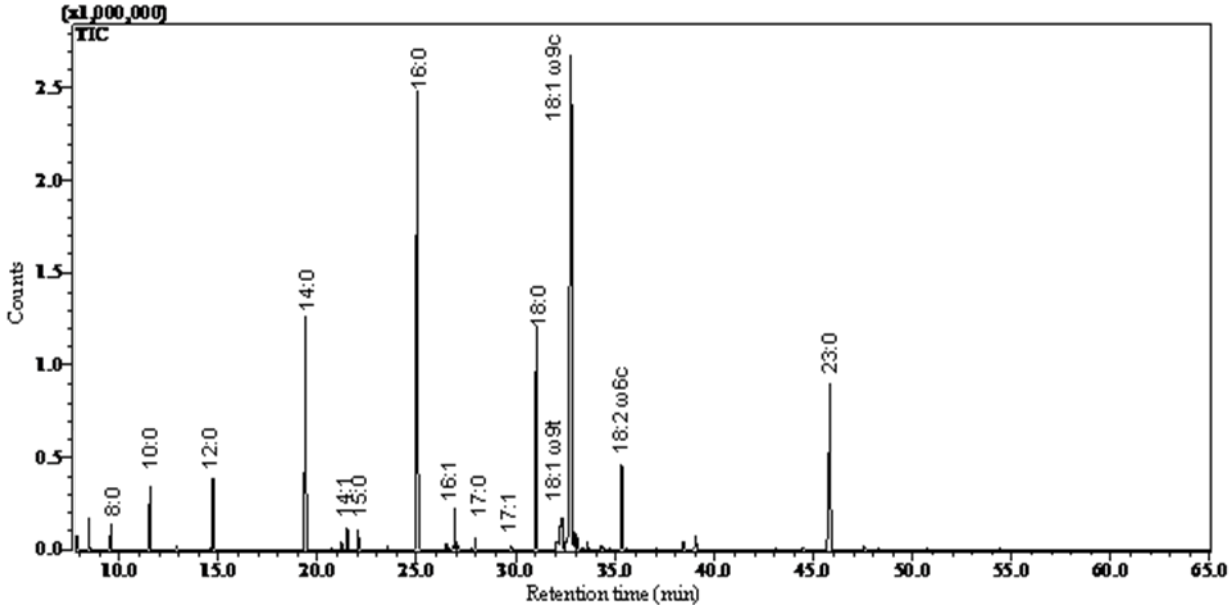

Figure F.2: Labeled FAME chromatogram of butter oil

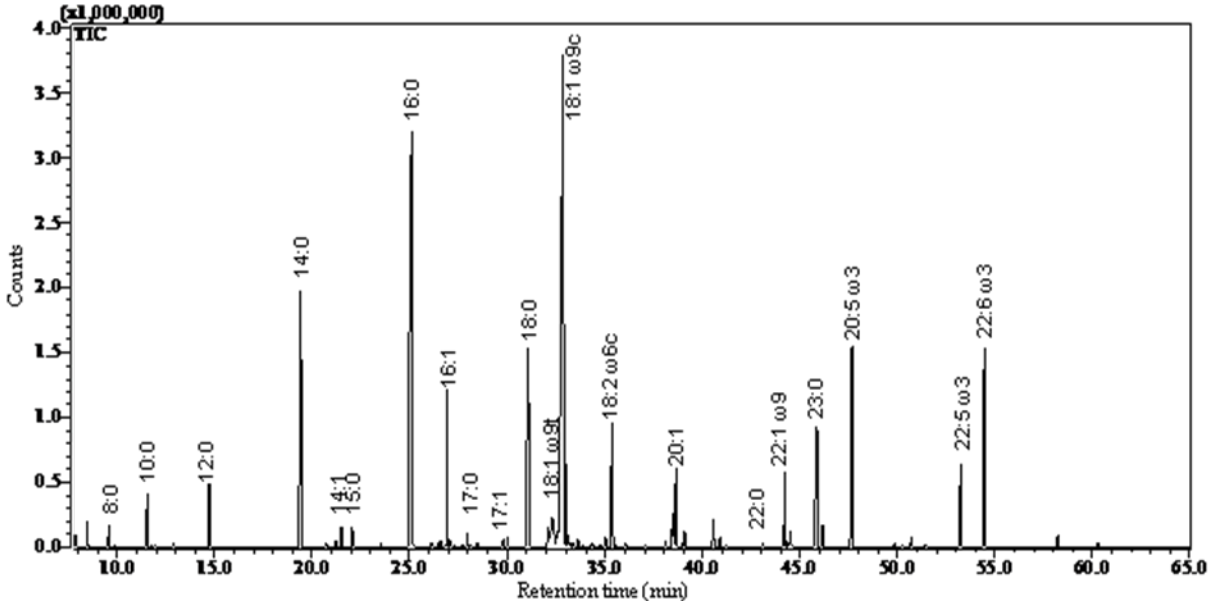

Figure F.3: Labeled FAME chromatogram of fish oil (DenOmega, Gamle Fredrikstad, Norway)

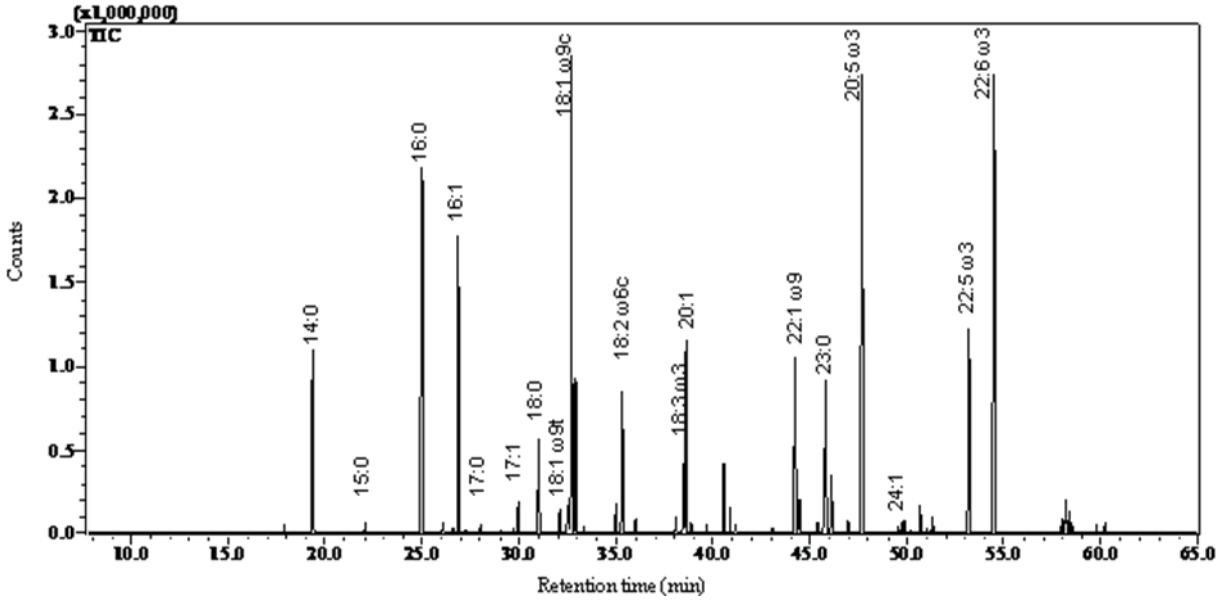

Figure F.4: Labeled FAME chromatogram of oxidized fish oil

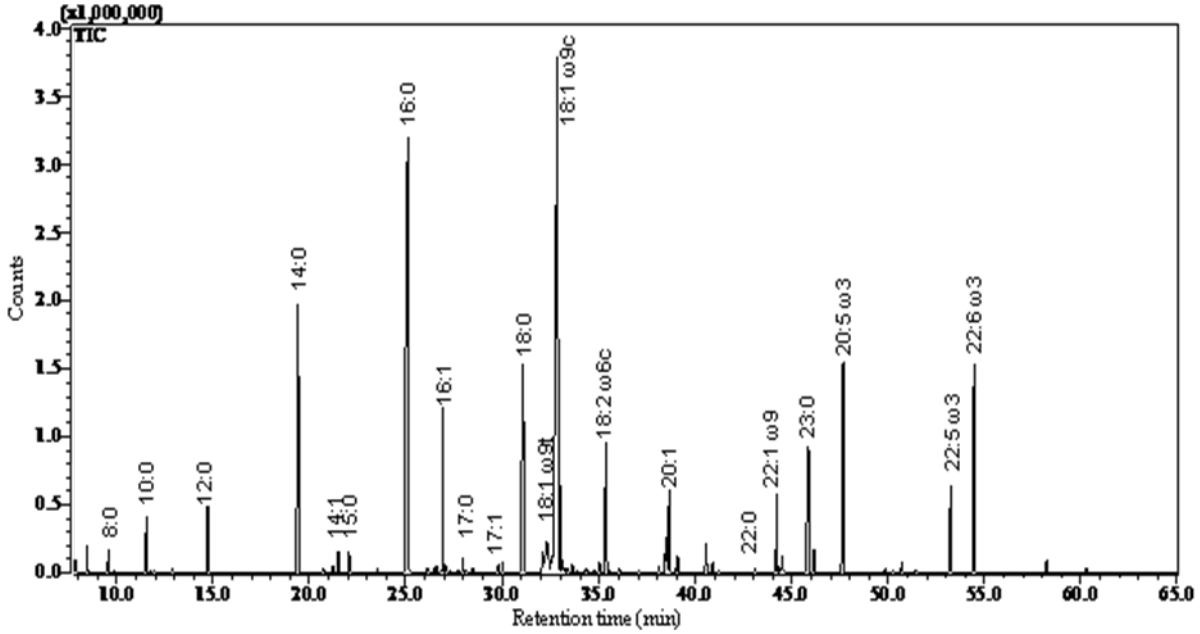

Figure F.5: Labeled FAME chromatogram of chile-lime yogurt with 0.43% wt/wt fish oil (DenOmega, Gamle Fredrikstad, Norway) total fat 1%

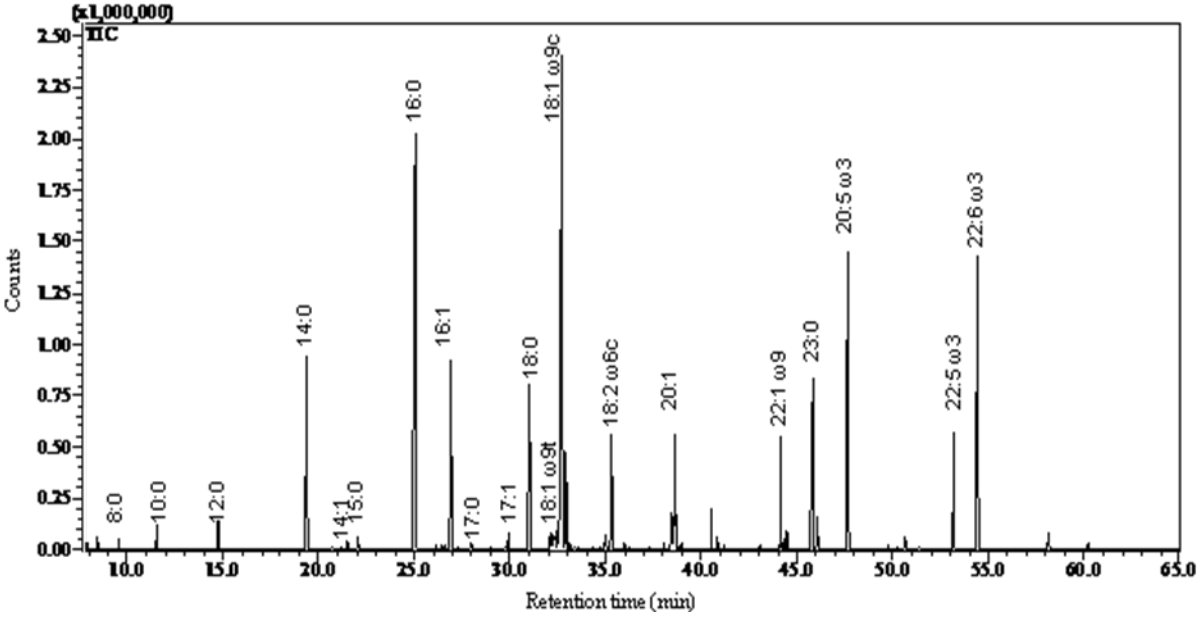

Figure F.6: Labeled FAME chromatogram of chile-lime yogurt with 1% wt/wt fish oil (DenOmega, Gamle Fredrikstad, Norway) total fat 1.5%

Table F.1: Average concentrations (mg/g oil) with standard deviations of major fatty acids in chile-lime yogurt formulations (low or high levels, butter, fish or oxidized fish oils) and source oils (butter, fish, oxidized fish), replications n=3

Fatty acid	Butter oil-low	Butter oil-high	Fish oil-low	Fish oil-high	Oxidized Fish oil-low	Oxidized Fish oil-high	Butter oil	Fish oil	Oxidized fish oil
4:0	1.82±0.10	1.65±0.09	1.08±0.12	0.69±0.18	0.84±0.08	0.67±0.07	1.72±0.55		
6:0	4.10±0.19	3.93±0.31	2.44±0.19	1.38±0.10	1.88±0.06	1.32±0.10	3.82±1.12		
8:0	3.53±0.12	3.61±0.18	2.13±0.25	1.19±0.11	1.63±0.11	1.09±0.10	3.49±1.00		
10:0	10.26±0.55	11.16±0.25	6.18±0.75	3.47±0.08	4.61±0.39	3.20±0.44	10.30±2.77		
11:0				0.35±0.08	0.11±0.03				
12:0	14.22±0.76	15.40±0.51	8.45±0.89	4.74±0.17	6.39±0.56	4.45±0.60	13.98±3.60		
13:0	0.14±0.05	0.23±0.01					0.22±0.08		
14:0	55.12±2.68	58.38±1.80	44.87±4.30	38.45±1.13	40.65±1.94	38.98±3.89	53.95±14.23	29.52±1.72	32.33±1.18
14:1ω5	5.17±0.36	5.63±0.22	2.95±0.24	1.44±0.04	2.22±0.30	1.43±0.26	4.99±1.54		
15:0	4.41±0.31	4.71±0.23	3.23±0.20	2.44±0.03	2.87±0.20	2.54±0.29	4.37±1.26	1.63±0.08	1.83±0.26
16:0	178.2±10.90	181.8±7.90	136.39±10.53	115.38±5.17	124.30±3.77	113.77±9.63	165.45±42.44	82.38±4.44	87.54±1.91
17:0 iso	1.57±0.01	1.61±0.04	1.11±0.06	0.70±0.14	0.88±0.18	0.82±0.09	1.34±0.50	1.11±0.06	1.18±0.20
16:1ω7	10.82±0.70	11.17±0.78	28.78±2.22	41.55±1.57	33.63±0.52	42.96±3.70	9.73±2.89	57.49±3.01	60.88±2.63
17:0 anti iso	2.05±0.37	2.30±0.09	1.41±0.09	0.92±0.02	1.12±0.15	0.82±0.11	1.95±0.62	0.75±0.07	0.78±0.16
17.0	3.03±0.17	3.11±0.13	2.24±0.13	1.60±0.11	2.01±0.21	1.71±0.11	2.66±0.87	0.95±0.07	0.99±0.04
16:2				1.67±0.15	3.26±0.29			5.71±0.15	6.16±0.50
18:0	74.57±4.21	72.92±4.23	51.24±1.72	37.86±2.46	45.04±0.73	37.74±3.84	66.59±18.33	17.96±0.81	19.16±0.64
16:3								4.80±0.16	3.62±2.47
18:1ω9t	15.95±5.93	14.24±5.47	12.92±0.56	2.96±0.22	5.06±0.92	4.94±0.92	12.93±7.04	4.80±0.16	5.22±0.43
18:1ω11				4.24±0.73				6.07±0.20	6.49±0.39
18:1ω9c	192.65±7.74	193.45±15.51	161.62±2.89	146.74±10.57	151.82±5.32	147.29±16.88	172.63±48.72	122.89±5.83	127.97±8.07
18:1ω7			13.32±0.73	19.55±0.55	15.95±0.40	20.45±1.53		27.93±1.21	29.06±1.56
18:2ω6c	22.56±0.55	23.00±1.45	23.08±0.62	24.42±1.00	23.32±0.65	24.89±1.73	21.10±6.08	26.67±1.34	27.53±1.18
18:3ω3	2.38±0.10	2.41±0.10	6.63±0.27	9.62±0.46	8.15±0.41	10.16±0.57	2.13±0.76	2.32±1.08	14.58±0.72
20:1ω9				13.55±1.08	23.67±0.51	18.22±0.81	25.01±1.00	37.84±1.89	38.95±1.72
20:2					0.66±0.07	0.93±0.06		1.60±0.06	1.56±0.09
22:1ω9			13.12±0.86	23.82±0.71	18.25±0.92	25.36±0.99		38.84±2.11	39.50±1.53

unknown				0.92±0.12			6.88±9.34	2.12±0.02	3.80±0.27
20:4ω6			2.44±0.13	3.84±0.27	3.14±0.24	4.18±0.11		6.14±0.28	6.41±0.32
23:0	41.65±6.09	40.30±4.65	38.40±8.54	42.26±10.18	39.14±6.22	38.52±5.51	43.72±18.28	29.69±16.53	39.66±9.66
20:5ω3			38.15±2.95	65.92±0.87	50.68±2.61	69.18±4.01	0.15±0.25	99.55±6.25	104.26±8.38
24:0							0.02±0.04		
24:1				1.10±0.14	0.83±0.08	1.23±0.07		2.28±0.17	2.46±0.14
22:5ω3			14.15±0.89	25.16±0.69	19.26±0.92	26.77±1.24		38.72±2.25	40.83±2.37
22:6ω3			37.32±2.66	65.26±0.54	50.17±2.47	68.67±3.77		99.74±4.88	104.32±7.84
omega-3	2.38±0.10	2.41±0.10	96.27±6.77	165.96±2.57	128.26±6.41	174.79±9.59	2.28±1.01	240.33±14.46	263.99±19.31
DHA+EPA	ND	ND	75.48±5.61	131.18±1.42	100.85±5.08	137.85±7.78	0.15±0.25	199.29±11.13	208.57±16.22

Appendix G: SPME Chromatograms

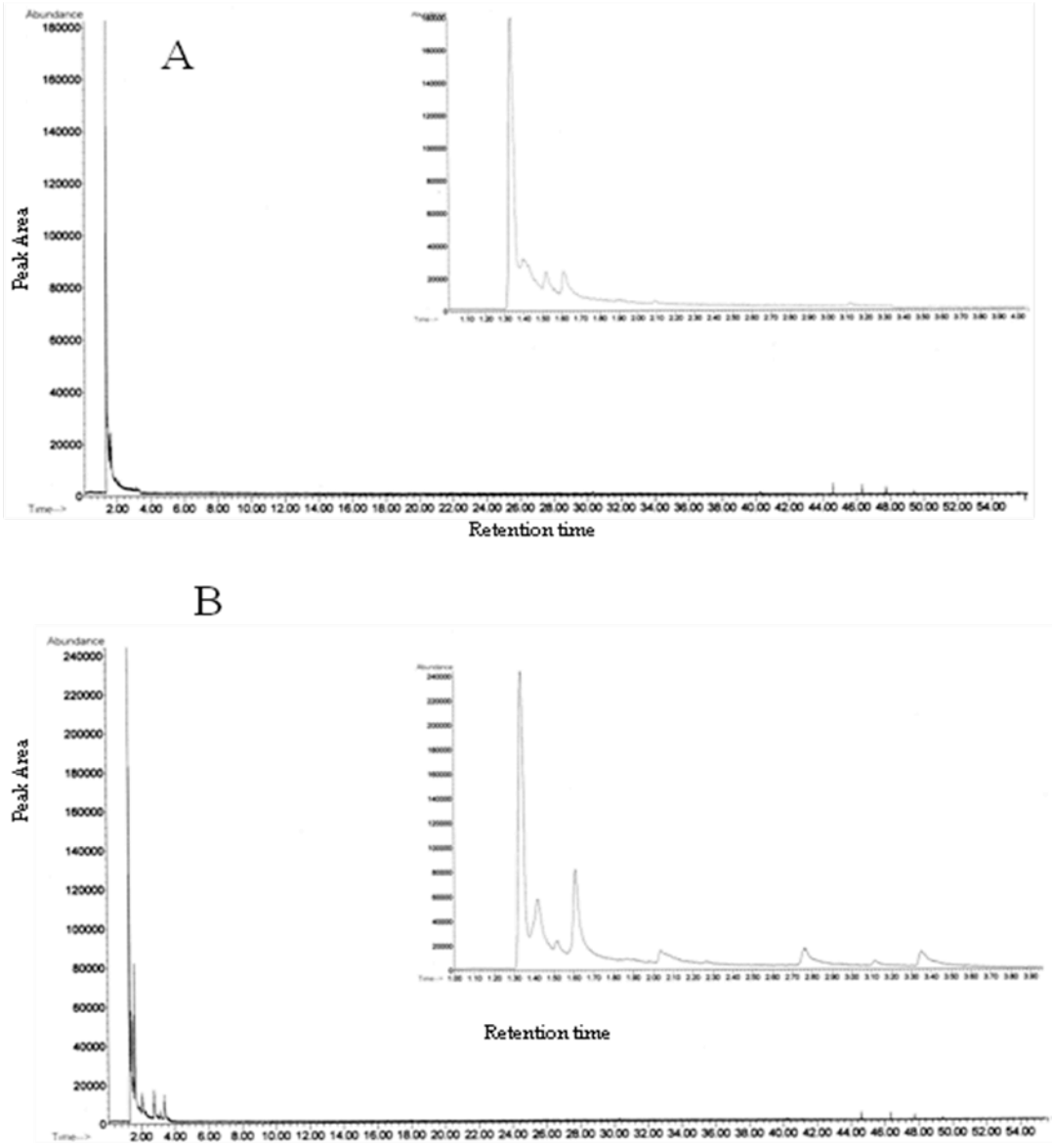

Figure G.1: SPME chromatograms of source oils A) fresh fish oil (DenOmega, Gamle Fredrikstad, Norway), B) oxidized fish oil

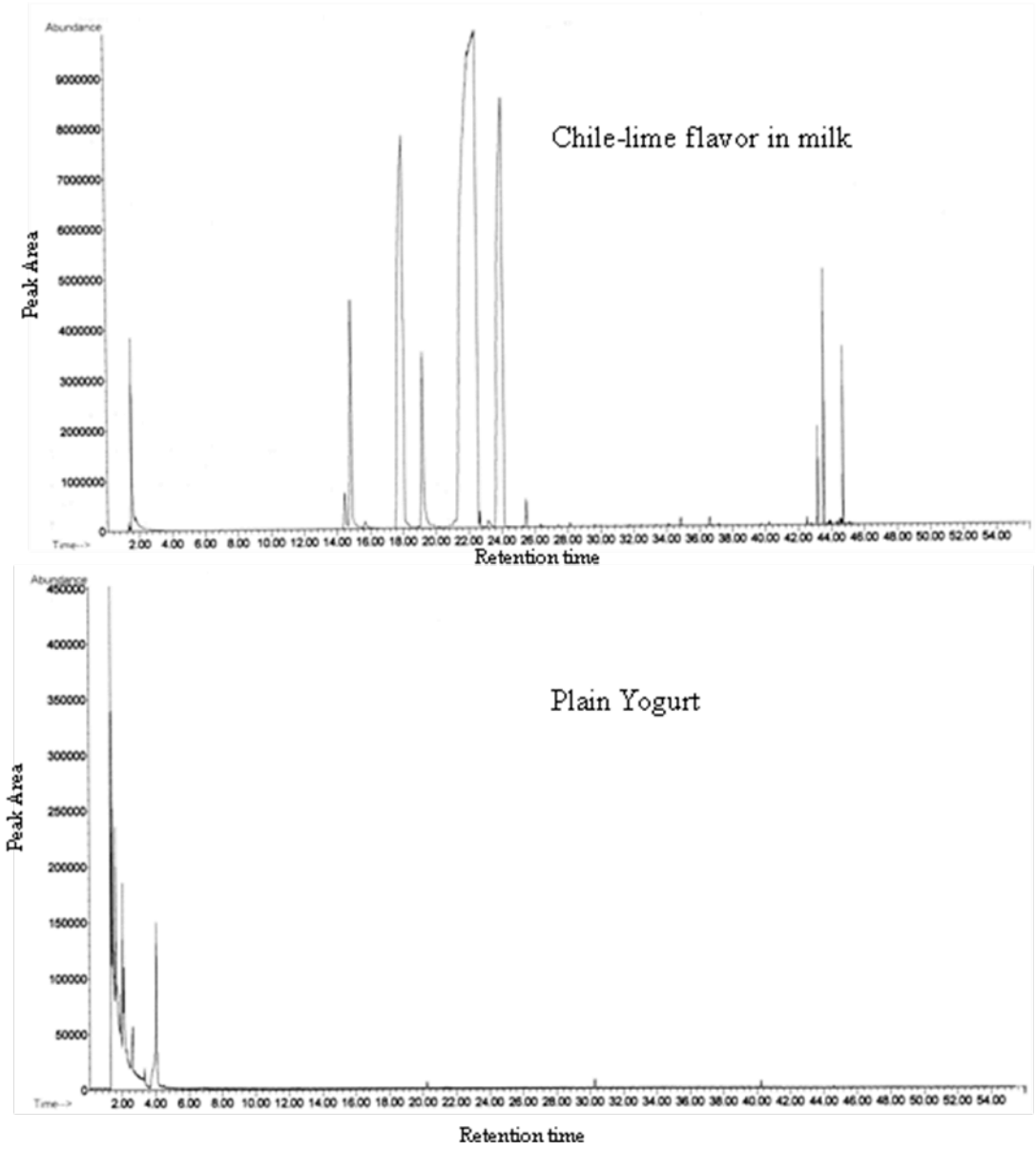

Figure G.2: SPME chromatograms of plain yogurt (Kroger, Cincinnati, OH) and chile-lime flavor (Gold Coast Ingredients, Inc, Commerce, California) in reconstituted milk

Appendix H: Demographic Results

Table H.1: Demographic survey results from all sensory panels

	overall survey (n=186)	consumer panel			flavor panel	oil panel
		panelists (n=100)	male (n=34)	female (n=66)	panelists (n=32)	panelists (n=32)
		%	%	%	%	%
Gender						
male	34%	34%	100%		25%	35%
female	66%	66%		100%	75%	65%
Age						
18-25	69%	66%	85%	56%	50%	52%
26-35	16%	16%	12%	18%	34%	32%
36-45	5%	6%	0%	9%	6%	10%
46-55	6%	6%	0%	9%	6%	6%
over 55	4%	6%	3%	8%	3%	0%
Race						
American Indian or Alaska Native	1%	1%		2%		
Asian	7%	6%	6%	6%		
Black or African American	2%	2%	6%			
Caucasian/White	81%	84%	79%	86%		
Hispanic of any race	4%	3%	3%	3%		
Would rather not say	2%	2%		3%		
Other	3%	2%	6%			
Country of birth						
USA		90%	85%	92%		
Canada		1%		2%		
Chile		1%	3%			
China		1%	3%			
India		1%		2%		
Iran		1%		2%		
Italy		1%	3%			
Peru		1%		2%		
Puerto Rico		1%				
United Arab Emirates		1%	3%			
Yugloslavia		1%	3%			
Knowledge of foods or nutrition						
limited	12%	9%	15%	6%		
general knowledge	47%	49%	59%	44%		
knowledgeable	16%	15%	9%	18%		
extremely knowledgeable	24%	27%	18%	32%		

Dairy product consumption						
never up to several times per month					3%	
1-3 servings/week					19%	
4-6 servings/week					16%	
7-9 servings/week					0%	
10-12 servings/week					47%	
>12 servings/week					16%	
Consumption of yogurt						
never	6%	0%	0%	0%		
1-3 servings/month	28%	31%	41%	26%	19%	
1-3 servings/week	42%	43%	41%	44%	34%	
4-7 servings/week	18%	22%	15%	26%	38%	
> 7 servings/week	5%	4%	3%	5%	9%	
Fat content of yogurt consumed						
do not consume yogurt once per week					16%	
<0.5%					22%	
0.01					38%	
0.02					6%	
0.0325					19%	
View of potential health benefits of milk and dairy products						
not aware	1%	1%	0%	2%	6%	
vaguely aware	11%	12%	24%	6%	9%	
generally aware	56%	53%	65%	47%	53%	
very aware	31%	34%	12%	45%	31%	
Consumption of fatty fish						
never up to several times per month						81%
1-3 meals/week						19%
View of potential health benefits of fatty fish consumption						
not aware						0%
vaguely aware						23%
generally aware						32%
very aware						45%
View of potential health benefits of omega-3 fatty acids						
not aware	9%	9%	12%	8%		3%
vaguely aware	27%	26%	35%	21%		13%
generally aware	37%	35%	26%	39%		42%
very aware	27%	30%	26%	32%		42%
Supplementation of diet with omega-3 fatty acids						
none	35%	26%				26%
eat more fish	49%	34%				24%

other foods naturally rich in omega-3's	30%	19%			13%
omega-3 fortified foods	16%	10%			8%
omega-3 supplements	17%	11%			29%
Interest in exploring new flavors in traditional foods					
yes	84%	100%	100%	100%	
no	15%	0%			
Likelihood to consume this product again					
highly likely		2%		3%	
likely		38%		34%	
unlikely		29%		34%	
highly unlikely		15%		25%	
don't know		6%		3%	
Spicy food consumption					
never-several times per month		29%			
1-3 servings/week		44%			
4-6 servings/week		16%			
7-9 servings/week		7%			
10-12 servings/week		3%			
> 12 servings/week		1%			
Best food compliment for this product					
nothing, eat alone		5%		7%	
beverage		4%		4%	
meat		12%		16%	
fruit		35%		40%	
vegetable		9%		9%	
grain		28%		18%	
other		7%		7%	
Would you consume a savory yogurt					
highly likely				13%	
likely				38%	
unlikely				31%	
highly unlikely				13%	
don't know				6%	
Willingness to consume fortified dairy product					
would not consume					6%
1 up to several times per month					23%
1-3 days/week					39%
4-6 days/week					16%
every day of week					16%

Appendix I: Visual Observations of Microcapsule Reconstitution

Table I.1: Visual observations recorded at room temperature after reconstitution of microcapsules (60:40 chitosan:starch wall-material, 1:2 fish oil:wall-material wt/wt) in a dairy blank solution; n=4 replications

replication	temp	ph	final ph	reconstituted	particles	clumps
4	30	4.51	4.45	partially	small	small
3	30	4.52	4.38	almost	visible	
1	30	4.53	4.58	mostly	small	
2	30	4.55	4.21	almost		very few
4	30	5.50	5.59	not		yes
2	30	5.52	5.26	not		many
3	30	5.52	5.54	not		visible
1	30	5.54	5.72	somewhat		large
3	30	6.51	6.39	not		lots
2	30	6.53	6.30	not		large
4	30	6.53	6.46	not		many
1	30	6.61	6.61	not		
4	63	4.53	4.60	mostly	few	1 large
1	63	4.54	4.21	yes		1 large
3	63	4.54	4.66	yes	some	none
2	63	4.57	4.10	yes		none
1	63	5.54	5.06	mostly	few	
3	63	5.54	5.56	somewhat		few
4	63	5.54	5.78	somewhat	some	some
2	63	5.56	4.95			few
1	63	6.52	5.89	not very		few
3	63	6.52	6.31	not		still visible
4	63	6.52	6.52	not		large
2	63	6.54	5.78			still
1	68	4.54	4.38	yes		
3	68	4.54	4.58	yes	few	none
4	68	4.54	4.49	mostly	on sides	
2	68	4.56	4.42	yes		none
3	68	5.51	5.56	mostly		very few
4	68	5.51	5.46	mostly		very few
2	68	5.53	5.33			few
1	68	5.55	5.30	mostly	fine	
2	68	6.53	6.02			still
4	68	6.53	6.31	not		visible
3	68	6.54	6.42	not		visible
1	68	6.59	6.01	not		many
3	85	4.53	4.57	yes	none	

1	85	4.55	4.50	yes		1 large
4	85	4.55	4.55	fully	none	none
2	85	4.56	4.36	yes	none	none
4	85	5.51	5.49	partially		few small
1	85	5.54	5.47	yes	small	
2	85	5.54	5.30	mostly	some	none
3	85	5.54	5.72			
1	85	6.51	6.20	somewhat	small	
3	85	6.52	6.42	almost		v few
2	85	6.53	6.11	not		some
4	85	6.58	6.34	not	yes	small