February 2016

Fish Oil Nanoemulsions: Optimization of Physical and Chemical Stability for Food System Applications

Rebecca M. Walker
University of Massachusetts Amherst

Fish Oil Nanoemulsions: Optimization of Physical and Chemical Stability for Food System Applications

A Thesis Presented

By

REBECCA M. WALKER

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2015

Department of Food Science

Fish Oil Nanoemulsions: Optimization of Physical and Chemical Stability for Food System Applications

A Thesis Presented

By

REBECCA M. WALKER

Approved as to style and content by:

_____
David Julian McClements, Chair

_____
Eric Decker, Member

_____
Guodong Zhang, Member

_____
Eric Decker, Department Head
Department of Food Science

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. McClements, for this wonderful opportunity and his guidance throughout my research. I would also like to thank Dr. Decker for his support and assistance as my research expanded into lipid oxidation. Additionally, I would like to thank Dr. Zhang for being on my committee and for his input and encouragement.

I am very appreciative of the assistance and support from my lab mates, especially Jennifer Komaiko, Cansu Gumus, Gabriel Davidov Prado, and Laura Salvia-Trujillo. A very special thank you to lab manager, Jean Alamad, who was constantly patient and always helpful throughout my research. Lastly, I would like to thank my family and friend for their unwavering love and support throughout my academic career.

ABSTRACT

FISH OIL NANOEMULSIONS: OPTIMIZATION OF PHYSICAL AND CHEMICAL STABILITY FOR FOOD SYSTEM APPLICATIONS

SEPTEMBER 2015

REBECCA M. WALKER, B.S., OREGON STATE UNIVERSITY

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor David Julian McClements

Emulsion-based delivery systems offer many potential benefits for incorporating omega-3 oils into foods and beverages. Nanoemulsions are emulsion-based delivery systems that are gaining popularity because of their ease of preparation, small particle size, relatively high stability, high bioavailability, and production of optically transparent emulsions. Fish oil (FO) nanoemulsions are potentially more susceptible to lipid oxidation because of their high degree of lipid unsaturation, high surface area of exposed lipids, and greater light penetration. In the first study, spontaneous emulsification, a low-energy method, was used to fabricate FO nanoemulsions. The influence of surfactant-to-oil-ratio (SOR) on particle size, turbidity, and physical stability was evaluated. Furthermore, the oxidative stability of these nanoemulsions was compared to emulsions produced by microfluidizer, a high-energy method. The effect of particle size and SOR on oxidation was monitored by measuring lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS). Optically transparent nanoemulsions were formed and

maintained physical stability after being held at 37 °C for 14 days. FO nanoemulsions produced by high- and low-energy methods had similar oxidative stabilities at 55 °C for 14 days. These results demonstrate that spontaneous emulsification can produce fish oil nanoemulsion that are physically stable and oxidize at similar rates as traditionally prepared nanoemulsions, and are therefore potentially suitable for fortification of clear food systems. Additionally, carrier oils can also impact the physical and oxidative stability of FO nanoemulsions. Medium chain triglycerides, lemon oil, and thyme oil were chosen as carrier oils and added to the oil phase at different ratios of FO to carrier oil for emulsions produced by the microfluidizer. Medium chain triglycerides and lemon oil produced stable FO nanoemulsions but the thyme oil only produced stable FO nanoemulsions at lower concentrations of carrier oil. On the other hand, at FO to carrier oil ratios of 75/25, lemon oil and thyme oil nanoemulsions had high oxidative stability because of natural of their antioxidants. These findings suggest that lemon oil and thyme oil can produce FO nanoemulsions that are physically and chemically stable and can be used for food system fortification.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Fish oil (FO) is high in omega-3 fatty acids (FAs), micronutrients that are essential for all humans and provide multiple health benefits (1-5). Unfortunately, under consumption of omega-3 FA occurs in many Western cultures (2-5). Foods with increased or added omega-3 FAs have been expanding on the food market as a way to increase their consumption. There are many approaches to adding omega-3 FAs into foods, one of which is emulsion systems. Emulsion systems are a useful platform for food fortification because they can encapsulate lipids and lipophilic bioactives for incorporation into aqueous foods. Nanoemulsions in particular can expand the range of food products that are fortified with omega-3 FAs because of their unique characteristics. Nanoemulsions have small particle sizes, can be optically transparent, and have high kinetic stability (6-8).

High- or low-energy methods are used to produce nanoemulsion. This paper will focus on microfluidizer (MF) as a high-energy method and spontaneous emulsification (SE) as a low-energy method. Besides the amount of energy used during production, MF and SE methods have other differences. MF can make nanoemulsion using lower concentrations of surfactant while SE commonly needs higher concentrations to form nanoemulsions. Additionally, MF can expose the emulsion to heat during production while the SE method can expose it to air. Both heat and air exposure can be undesirable when trying to control pro-oxidant exposure but can be difficult to avoid during emulsion fabrication.

Carrier oils can be used to further decrease the particle size of emulsions. Carrier oils are mixed with the FO to make up the total oil phase. A variety of oils can be used including citrus oils, herb oils, and medium chain triglyceride (MCT). Carrier oils can mask the FO flavor and odor, making them extremely beneficial for consumer acceptance of the fortified food product. Additionally, depending on the characteristics of the carrier oil, they can affect the physical and oxidative stability of the emulsion. Carrier oils can act as ripening retarders when they have low water solubility, which increases the emulsion's resistance to Ostwald ripening (9-12). On the other hand, carrier oils with natural antioxidants can inhibit oxidation while other constituents such as monoterpenes can themselves oxidize and increase the rate of oxidation (13, 14).

The physical stability of emulsions describes their ability to stay homogenous. Emulsions can become unstable due to many forces including gravitational separation (creaming and sedimentation), coalescence, flocculation, and Ostwald ripening. Nanoemulsions are more stable against gravitational separation, coalescence, and flocculation than conventional emulsions however they are more susceptible to Ostwald ripening (12). Physically unstable emulsions are undesirable for incorporation into food products because they can negatively affect the appearance, texture, and shelf life of the fortified food product.

Chemical stability of the emulsion refers to its ability to retain its properties and in this case, resist oxidation, under environmental conditions including air, heat, and light exposure. Delaying lipid oxidation is important in order to extend the shelf life of a food and to maintain a palatable product that is safe and has positive health benefits (15, 16). FO's high degree of unsaturation along with the larger surface area and higher degree of

light penetration in nanoemulsions may make FO nanoemulsion more susceptible to oxidation (17).

In our investigation of FO nanoemulsions, we began by using lemon oil (LO) as the carrier oil for the FO and evaluated the physical stability of emulsions produce by the low-energy method of SE using various concentrations of surfactant. Next we compared FO nanoemulsions fabricated by low-energy (SE) and high-energy (MF) methods while controlling the particle size and surfactant concentration in order to evaluate their effects on the oxidation rate of the nanoemulsions. Lastly, we used medium chain triglycerides (MCT), LO, and thyme oil (TO) as carrier oils and compared their ability to form FO nanoemulsions and inhibit lipid oxidation.

CHAPTER 2

DEVELOPMENT OF FOOD-GRADE NANOEMULSIONS AND EMULSIONS FOR DELIVERY OF OMEGA-3 FATTY ACIDS: OPPORTUNITIES AND OBSTACLES IN THE FOOD INDUSTRY

2.1. Introduction

Ecosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the long chain polyunsaturated fatty acids (LC-PUFAs) most commonly found in FO and are linked to brain development, cardiovascular health, and inflammation (2-5). Western diets have been reported to be severely lacking in the amount of omega-3 FAs consumed (2-5). Consumption of sufficient levels of omega-3 FAs have been identified as a way to reduce mortality risks, especially for cardiovascular disease (18). It is estimated that the mortality risk of low omega-3 intake was responsible for 84,000 deaths in the US in 2005. Low consumption of EPA and DHA is due to numerous factors such as the high cost of fish, dislike of seafood by many consumers, presence of methyl mercury, and low availability in many geographical locations (19-21). The low consumption of EPA and DHA mean that fortification of foods may be one of the most effective ways to increase omega-3 intake and improve health.

Much of the early research on omega-3 FAs focused on enrichment of foods using alpha-linolenic acid (ALA), however more attention is now being paid to EPA and DHA. This rise in interest may be a result of the specific recommendation for EPA and DHA

intake by the *National Academies* and the *Dietary Guidelines for Americans* in 2010 or the Food and Drug Administration's (FDA) approval of a qualified health claim for foods or supplements that contain EPA and DHA in 2004 (3, 22-25).

The food industry is now taking measures to help consumers increase their omega-3 FA consumption by introduction of various kinds of functional foods. Functional foods provide health benefits over and above their basic nutritional aspects (26). Omega-3 enriched foods are quite popular, especially beverages, and there are large areas of growth for omega-3 products in countries with both small and large existing omega-3 markets (27). At present, there are a number of functional foods enriched with omega-3 FAs that are on the market, such as milk, eggs, yogurts, breads, and spreads. Some of these products have been naturally enriched through the diet of the chicken or cow they were obtained from, while others have been enriched through the addition of omega-3 FAs as bulk oils, emulsions, or powders (27-29). Nevertheless, there are considerable challenges to incorporating omega-3 FAs into many types of functional food products due to their low water-solubility, poor chemical stability, and variable bioavailability. Consequently, there has been growing interest in the development of appropriate delivery systems to encapsulate, protect, and release omega-3 fatty acids.

Nanoemulsions have great potential for overcoming the challenges associated with developing omega-3 enriched food and beverage products. They can be used to encapsulate oils and increase their water-dispersibility (11). They can be designed to have good kinetic stability and high optical clarity, which is important for application in many food and beverage products (6). They can also be designed to increase the oral bioavailability of encapsulated lipophilic components (30, 31). Despite these advantages,

nanoemulsion-based delivery systems must still be carefully designed to ensure good physical and chemical stability, and high bioavailability. The purpose of this article is to highlight the potential of nanoemulsions for the encapsulation, protection and release of omega-3 fatty acids. These delivery systems could be used in the food industry to fortify foods and beverages with these bioactive lipids, or they could be used in the supplement or pharmaceutical industry to increase the bioactivity of therapeutic omega-3 fatty acid formulations.

2.2. Omega-3 fatty acids

2.2.1. Chemistry and health benefits

Fat consumption is necessary for human development, health, and longevity (1). There are two fatty acids that have been identified as being essential in the human diet: linoleic acid (LA) (18:2 n-6) and alpha-linolenic acid (ALA) (18:3 n-3), which are also known as omega-6 and omega-3 FAs, respectively. These substances are part of a lipid group collectively known as long chain polyunsaturated fatty acids. These fatty acids are considered essential because they cannot be synthesized by the human body as a result of the lack of enzymes that can form double bonds beyond the Δ^9 carbon (1). After consumption, the essential fatty acids can then be converted in the human body by desaturation and elongation into longer chained and more unsaturated fatty acids, which are more bioactive than their precursors (32). The most common derivative of LA is arachidonic acid (20:4 n-6) (1, 32). ALA is converted to EPA (20:5 n-3), which is further elongated to DHA (22:6 n-3) (32).

The conversion of ingested ALA to EPA and DHA within the body is not usually considered to be a reliable source of LC-PUFAs in the human diet. The elongation and

desaturation conversions are highly inefficient as most of the fatty acid precursors are utilized for energy (32). Furthermore, the conversion yield of ALA to EPA and DHA in men is only 0.3-8% and < 4%, respectively (33). In women, the conversion yield of ALA to EPA and DHA is 21 % and 9%, respectively. This poor production of LC-PUFA in the body makes it more beneficial to consume omega-3 FAs as preformed EPA and DHA, rather than as ALA.

2.2.2. Food sources

There are many dietary sources of omega-3 FAs including fish, krill, algae, and land plants (19). The type and amount of omega-3 FAs varies between sources. Fish is the most common source of omega-3 FAs and the amount of EPA and DHA varies between fish species, time of year, the fish's diet, and geography. Cold water, pelagic fish usually have the highest levels of EPA and DHA. Overall, in marine fish the most important factor is their total fat content, with high fat fish having the highest amount of omega-3s per serving. Sardine, mackerel, herring, and halibut have some of the highest omega-3 PUFA levels but are uncommon in many diets (19). In the United States, salmon, anchovies, herring, sardines, Pacific oysters, trout, and Atlantic and Pacific mackerel are the most commonly consumed low mercury seafood varieties (24).

The frequent consumption of fish does raise some safety and environmental concerns. Fish is susceptible to bioaccumulation of toxins and pollutants, one of the most common being mercury (19). An advantage of using FOs (rather than consuming whole fish) is that oil refining removes the majority of these toxins. Another concern is overfishing of the supply that could strain the sustainability of the market (21).

Alternative marine sources are available as a source of omega-3 FA, without facing some of the challenges associated with using fish. Krill oil can achieve higher levels of EPA and DHA than FO but the product has a higher cost so it is usually used in supplements (19). In addition, there are marine plant sources of omega-3 FAs that can be used commercially in food products. Algae are primary producers of omega-3 FAs, which can be cultivated to produce a continuous supply of omega-3 FAs. While algae produce high amounts of DHA, the EPA levels are often lower than those found in FO (32, 34). Until recently, relatively high production and purification costs limited the large scale manufacturing of algae oils, however, considerable advances have been made in recent years that have led to their increased commercial use (32, 34-36).

The Dietary Guidelines and American Dietetic Association encourage nutrient consumption from food rather than supplements however, people may choose to consume supplements or fortified foods for many reasons including cost, their dislike of seafood, allergies, a vegan diet, convenience, and the inability to meet recommended EPA and DHA levels from their normal diet (4, 21, 24). Consumers seeking alternative sources of omega-3 FAs should be aware if the products contain ALA, EPA or DHA in order to receive maximum health benefits.

Land plant sources of omega-3 FAs include canola, soy, flaxseed, and walnuts mainly in the form of ALA (24, 32, 37). An increased consumption of omega-3 FAs from these sources may have a limited effect in decreasing cardiovascular disease or a stroke because of the inefficient conversion of ALA to EPA and DHA (32).

Supplements may contain EPA and DHA in different forms than the common triglyceride form typically found in FO (19). Ethyl esters of omega-3 FAs are commonly

used in dietary supplements and pharmaceuticals because of the ability to distill the ethyl esters and produce highly concentrated oils (19). The ethyl esters of EPA and DHA have a different absorption route in the human body than triglyceride forms, but plasma lipid levels appear to be equivalent, however the triglyceride form can be better utilized in the body (19, 38).

2.2.3. Fish oil

In the remainder of this section, we will primarily focus on FO as it is considered to be the most common, least expensive, and best source of both EPA and DHA in the human diet (37). However, other sources of omega-3 fatty acids are becoming more economically viable, such as genetically engineered oil seeds (39, 40).

2.2.4. Health aspects

FOs have been reported to have many mechanisms by which they can reduce cardiovascular disease (CVD) risk factors for morbidity and sudden death. The combined effects of decreased blood pressure, positive shifts in blood lipid profiles (a decrease in low density lipoprotein (LDL) cholesterol and increase in high density lipoprotein (HDL) cholesterol), lowering of plasma triacylglycerols, improved cell membrane stability (especially in the heart muscles that control heart rate), decreased platelet aggregation, and reduced inflammation contribute to these health benefits (37, 41). Bread rolls, cereal bars and crackers were fortified with DHA and participants consumed the products in order to achieve 2 g of DHA/day. The consistent consumption of DHA increased HDL cholesterol in middle aged men and women and these fortified foods present a convenient way to incorporate omega-3 FAs into the diet (42).

The EPA and DHA found in FO is also associated with the prevention and possible treatment of inflammatory disease like asthma, cystic fibrosis, and rheumatoid arthritis (37, 43). The anti-inflammatory properties of omega-3 fatty acids may also help patients recover after surgery. Omega-3 FAs administered through a parenteral route to patients after undergoing a liver transplant had positive effects including decreasing the duration of the post-transplant hospital care, reducing infectious morbidities, and protecting the liver from injury partially as a result of the anti-inflammatory effects of the PUFA (44).

DHA has been associated with brain development because of the large amounts of DHA in the human nervous system (43). The cell membranes of the brain and retina of the eye experience a surge of DHA inclusion between the third trimester and the first year after birth (45). Omega-3 FAs are essential for proper brain functioning and development and studies have found connections between maternal consumption of fish and the visual acuity, higher developmental scores at 18 months, and higher IQ of infants (43, 45-47). These preliminary studies highlight the potential importance of DHA consumption for pregnant women.

Besides brain development, omega-3 FAs have also been investigated for connections with mental health conditions including attention deficit hyperactivity disorder (ADHD), dyslexia, depression, and adult cognitive decline including dementia and Alzheimer's disease (43). All of these areas require further investigation for various reasons including small sample sizes, inconsistencies in regimes, drug interactions, or conflicting conclusions.

2.2.5. Dietary recommendations for LC-PUFA

Many organizations at the national and international levels have published recommendations for omega-3 FA. These recommendations vary in the specificity of omega-3 FA forms taken, such as fish, ALA, EPA, and DHA, and if subsets of the general population require different recommendations. In the United States, the 2010 Dietary Guidelines for Americans suggests consuming 250 mg of EPA and DHA per day through the means of 8 ounces (227 g) of a variety of seafood a week (24). It is recommended that pregnant women consume 8 to 12 ounces (227 to 340 g) of low mercury seafood per week (24).

The *National Academies* (USA) has made its omega-3 FA recommendations using adequate intake values. An adequate intake value is used if a recommended daily allowance cannot be established and is determined based on the intake of healthy people (4). For males and females 14 years old and above, the adequate intake value of ALA, EPA and DHA are 1.6 and 1.1 g/day, respectively with most of the recommendation coming from ALA (48). Pregnant and lactating women have an adequate intake value of 1.4 and 1.3 g omega-3s/day, respectively.

The American Dietetic Association and Dieticians of Canada recommend 2 servings of fatty fish per week; 8 oz of cooked fish should provide 500 mg of EPA and DHA per day (49). The American Diabetes Association suggest at least 2 servings of fish per week for adequate omega-3 FA consumption (50). Commercially fried fish filets are excluded from this recommendation. The American Heart Association recommends 2 servings of fatty fish per week, a total of 8 oz in order to obtain beneficial amounts of EPA and DHA (51).

The European Food Safety Agency proposes the dietary intake of 250-500 mg of EPA and DHA/day for adults (37, 52). They also acknowledge that supplementing up to 1 g of DHA per day is safe. The Scientific Advisory Committee on Nutrition of Great Britain recommends at least 2 servings of fish (140 g) per week with at least one of the servings from oily fish (3). In France, the French National Nutrition and Health Program (PNNS) recommends eating fish two times a week (53). The French Food Safety Agency (AFFSA) recommends that individuals over the age of 10, including pregnant and lactating women, should consume 500 mg of EPA and DHA/day and a minimum of 250 mg of DHA/day.

The World Health Organization recommends 2 servings of fish per week in order for the consumer to intake about 200 to 500 mg of EPA and DHA per day (54). The Australian and New Zealand National Health and Medical Research Council recommends 430 and 610 mg/day of DHA/EPA/DPA (docosapentaenoic acid) for women and men between the ages of 19 and 69 years (55). For pregnant and lactating women from 19-50 years old, 115 and 145 mg/day of DHA/EPA/DPA is recommended.

Western diets in general do not provide satisfactory omega-3 FA intakes. American's current consumption of EPA and DHA is lower than the recommended values (2, 5). On average, Americans are currently consuming 3.5 ounces (99 g) of seafood per week and much of it is low in omega-3 FAs (4, 24). The National Health and Nutrition Examination Survey (NHANES) determined the mean intake of EPA and DHA through food sources by people over 19 years is 23 and 63 mg/day, respectively (2, 5). For individuals over the age of 19 consuming EPA and DHA through both food and supplement sources, they are consuming 41 and 72 mg/day, respectively. As of the

2008/2009 and 2010/2011 surveys, the actual consumption of oily fish by the population of Great Britain was not meeting the recommendation (3, 56). On average, only 54 g of oily fish were consumed per week across the age range of 19-64 years. Adults over 65 consumed an average of 90 g of oily fish per week. In contrast, Japanese diets easily provide sufficient omega-3 FA. The Japanese population achieves the recommended intake values of DHA and EPA through their diet high in seafood, and their use of dietary fats high in ALA (57). Japanese adults consume about 80 g of fish and shellfish per day, resulting in around 1-2 g of omega-3 FA per day.

Although many of the dietary recommendations for omega-3 encourage consumption of fish, this is not always convenient: some people do not like fish; some people cannot afford fish; fresh fish spoils rapidly; fish may contain undesirable contaminants (such as heavy metals); overfishing may reduce the supply of fish available; the growing global population puts a higher demand on the available fish (19-21). Consequently, there is great interest in the development of alternative means of incorporating omega-3 fatty acids into the human diet (21).

2.3. Nanoemulsions

Emulsion-based delivery systems offer a number of potential benefits for introducing omega-3 oils into foods and beverages (17, 58, 59). Nanoemulsions are a class of emulsion-based delivery systems that are becoming increasingly popular because of their ease of preparation, small particle size, relatively high stability, and high bioavailability.

2.3.1. Characteristics of nanoemulsions

Oil-in-water nanoemulsions, which are the most suitable for encapsulating omega-3 oils, consist of emulsifier-coated lipid droplets dispersed within an aqueous continuous phase. Nanoemulsions have been defined as emulsions that have mean particle radii below 100 nm (6, 7). Unlike microemulsions, which also contain small lipid droplets dispersed in water, nanoemulsions are thermodynamically unstable systems (60, 61). Nanoemulsions have been utilized in the food and pharmaceutical industries as delivery systems to encapsulate, protect, and control the release of a variety of bioactives (17, 58, 62). The small particle size provides both benefits and challenges for nanoemulsions.

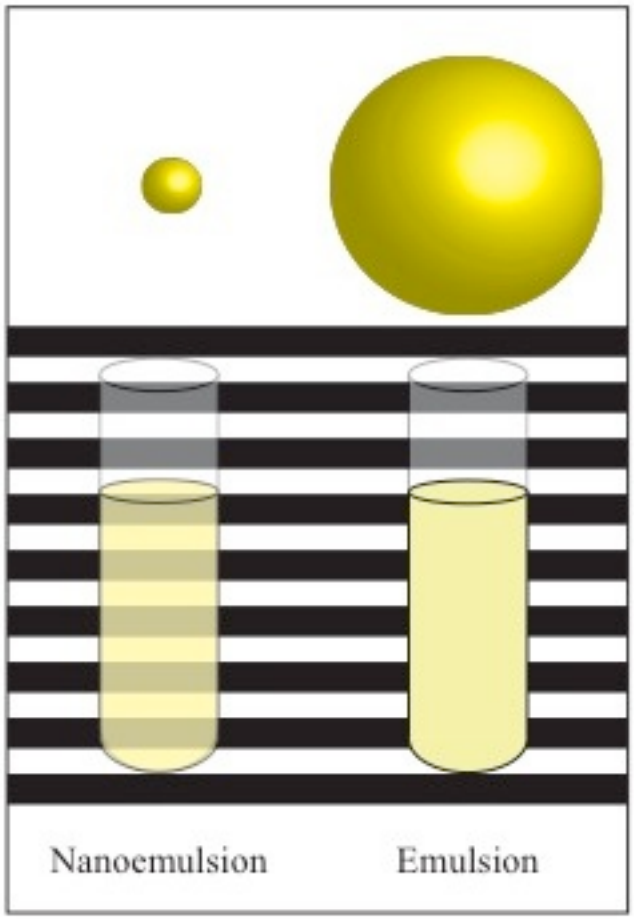
The bioavailability of lipophilic bioactive components encapsulated in small particles is usually greater than those in larger particles, which may be due to various mechanisms (30, 62). Smaller particles have a larger specific surface area allowing for increased enzyme activity at the oil-water interface and therefore faster lipid digestion (11, 62). Smaller particles can also penetrate into the mucus layer coating the epithelium cells of the small intestine, thereby increasing the time for lipid digestion and absorption. In addition, smaller particles may be able to pass through the mucus layer and be absorbed by epithelium cells. Lastly, smaller particle sizes increase the solubility of encapsulated lipophilic components in the aqueous phase close to the particle surfaces due to a curvature effect, thereby increasing the driving force for absorption (11, 62).

Nanoemulsions are not thermodynamically stable since the separate oil and water phases have a lower free energy than the emulsified system (7, 11). Nevertheless, they can be designed to have high kinetic stability (8). For example, nanoemulsions typically

are more resistant to gravitational separation, flocculation, and coalescence than conventional emulsions (11). Their high stability to gravitational separation can be attributed to two reasons: (i) the creaming or sedimentation velocity is proportional to the square of the particle size; (ii) Brownian motion dominates gravitational forces for small droplets (7). The high stability of nanoemulsions to droplet aggregation is due to the fact that the attractive forces that normally promote flocculation or coalescence weaken with decreasing droplet size (7). On the other hand, nanoemulsions are often more susceptible to Ostwald ripening than conventional emulsions. Ostwald ripening in O/W nanoemulsions involves the diffusion of the oil phase from small droplets to larger ones resulting in an increase in the mean droplet size (7, 11, 63). Droplet growth due to this mechanism can be inhibited by careful selection of the oil phase or by addition of ripening inhibitors (17).

Another potential advantage of nanoemulsions for certain applications is that they can appear transparent or only slightly turbid when they are fabricated to have particle sizes much smaller than the wavelength of light (**Figure 1**) (6). Typically, the mean droplet radius should be less than about 20-25 nm to ensure high optical clarity of a nanoemulsion, which requires careful control of fabrication conditions and product formulation.

Figure 1. A schematic diagram comparing the appearance and particle size of emulsions and nanoemulsions. Nanoemulsions appear transparent because the particle size is smaller than the wavelength of light and so they only scatter light weakly.



2.3.2. Fabrication methods

Typically, nanoemulsions require the use of high mechanical energy, high surfactant levels, or both in order to be produced (64). In general, nanoemulsion production can be divided into high-energy and low-energy methods (65). High-energy methods rely on the application of mechanical energy to disrupt the separate oil and water phases, mix the two phases together, and form tiny oil droplets (7, 17). High-energy methods based on this principle include high pressure valve homogenizers, MF, and sonicators (11). Droplet size is dependent on many variables including the production

method, operation settings, and system components (11). Typically, the droplet size decreases with increasing energy input and duration, provided there is sufficient surfactant present and the oil, water, and surfactant type are carefully selected.

In contrast, low-energy methods rely on changes in the environment or solution conditions to promote the spontaneous formation of tiny oil droplets (17). The ability of low-energy methods to produce nanoemulsions are closely related to the physicochemical properties of the surfactant, and depend on the type and amount of surfactant, oil and water present (8). Low-energy methods for nanoemulsion fabrication are becoming more popular because they can better create smaller particles sizes compared to high energy methods, they have lower manufacturing costs, and they have simple production methods (17).

A number of low-energy emulsification methods are available, including the SE, phase inversion temperature (PIT), phase inversion composition (PIC), and emulsion inversion point (EIP) methods (11). SE uses simple mixing as one phase is slowly added to another to spontaneously form an emulsion, *e.g.,* an organic phase containing surfactant and oil is added to an aqueous phase containing water (66). The final emulsion can be manipulated by controlling many variables including which phase is added into the other, the composition of the phases, environmental factors (*i.e.* temperature and pH), and mixing conditions (*i.e.* stir speed and rate of addition) (17). The PIT method utilizes alterations in temperature to change the solubility or optimum curvature (molecular geometry) of non-ionic surfactants, which results in the conversion of an oil-in-water to a water-in-oil emulsion or *vice versa* (11, 66, 67). Typically, a surfactant-oil-water mixture of appropriate composition is heated above the PIT, and then rapidly cooled with

continuous stirring to form a nanoemulsion. The PIC method is similar to the PIT method as it relies again on a change in the solubility or optimum curvature of the surfactant, however instead of changing the temperature of the system, the formulation of the system is altered, *e.g.,* salt concentration (66). Both the PIT and PIC methods rely on a transitional-phase inversion which utilizes the change in surfactant's functional characteristics (17). The EIP method however relies on catastrophic-phase inversion instead of transitional-phase inversion methods. Catastrophic-phase inversion changes the ratio between the oil and water phases while maintaining the surfactant's properties (17). This may occur by preparing a water-in-oil emulsion and then adding water while stirring. The water will initially form more droplets in the oil however when excess water is added, the water becomes the continuous phase and the oil becomes droplets leading to the formation of an oil-in-water emulsion.

2.3.3. Formulating safe nanoemulsions

When formulating nanoemulsions for food systems, food safety is one of the greatest concerns (30), followed by the consumer's desire for clean labels on their foods (68). Reducing the particle size into the nano-range ($r < 100$ nm) may substantially change the gastrointestinal fate of ingested foods, which has led to some concern about the presence of engineered nanoparticles in foods (30). As mentioned earlier, there may be a considerable increase in the oral bioavailability of encapsulated bioactive agents when they are incorporated into nanoemulsions. In many cases, this increase may be desirable, but in some cases it may be undesirable. For example, a bioactive agent may have an optimum blood level concentration for efficacy, but may become toxic at higher levels. If a nanoemulsion greatly increased the concentration of this type of bioactive

agent, it may lead to high blood levels that increase toxicity. However, this should not be a problem with omega-3 oils because they can usually be consumed at high levels without causing health problems. Nevertheless, if the oil is highly oxidized then it may contain toxic reaction products that would cause a problem, although consumers usually reject this type of product due to poor sensory characteristics.

The presence of certain components in nanoemulsions may also cause concern, particularly high levels of surfactants or solvents. Surfactants are commonly used to stabilize nanoemulsions by adsorbing to droplet surface and protecting them from aggregation (58). Large amounts of surfactant are typically needed to fabricate nanoemulsions using low-energy methods (such as the spontaneous emulsification or phase inversion temperature methods), but this is less of a problem with the high-energy methods commonly used in the food industry (such as high pressure homogenization or sonication) (69, 70). In addition, the surfactants used to form nanoemulsions are typically small molecule synthetic surfactants (such as Tweens), although some progress has been achieved forming nanoemulsions using natural surfactants such as phospholipids or saponins (71, 72). There are some health concerns associated with using high amounts of certain types of synthetic surfactants in foods, and so their use is limited by government regulations (73). Natural biopolymer-based emulsifiers, such as polysaccharides and proteins, cannot currently be used to form nanoemulsions by low-energy fabrication methods (17), although they can be used to form nanoemulsions by high-energy methods (74). Toxicity may also arise from the utilization of organic solvents in certain solvent displacement or evaporation methods used to prepare nanoemulsions (17). Small traces of these solvents may remain in the emulsion and must

be monitored. However, most of the fabrication methods currently used to create food emulsions do not require the utilization of organic solvents. Another important issue affecting the potential toxicity of nanoemulsions is the fact that lipid nanoparticles may behave differently in the human body than the larger particles conventionally used in foods, *e.g.,* the location, rate, and extent of absorption (30).

2.3.4. Formulating label-friendly nanoemulsions

Consumers are increasingly demanding products that are perceived to have "clean labels" (71, 75). Changing to natural surfactants may be one way to meet these demands. One natural surfactant that has been investigated is extracted from the bark of the *Quillaja saponin* Molina tree and is marketed commercially as Q-Naturale® (Ingredion, New Jersey). This surfactant has been compared to Tween 80, a common nonionic surfactant used in the food industry, to form nanoemulsions by a high-energy method (microfluidization) using medium chain triglycerides as the oil phase (71). Q-Naturale exhibited effective surfactant properties, as it was able to form stable nanoemulsions under certain circumstances at relatively low surfactant-to-oil ratios (1:10). The use of clean label ingredients also extends to any cosolvents or antioxidants that are added to the emulsion formulation to increase physical and chemical stability, which may narrow the formulation possibilities for omega-3 nanoemulsions.

2.4. Applications of nanoemulsions in foods and beverages

The most widely used delivery systems for incorporating omega-3 oils into foods and beverages are bulk oils, emulsions, and powders (76). These powders are typically formed by spray drying emulsions. Microencapsulation has proved to be a popular way of creating powdered omega-3 that can be incorporated into a variety of food products

including baked goods, spreads, and fruit beverages (77). However, this technology typically only delivers relatively small levels of bioactive lipids since powders usually only contain around 1 to 30% omega-3 FAs (29). Microencapsulated emulsions for food applications have previously been discussed in detail elsewhere and will therefore not be reviewed further here (29, 77).

Nanoemulsions offer a convenient means of fortifying many aqueous-based food and beverage products with omega-3 oils. Fortified nanoemulsions could be introduced into food systems such as beverages, salad dressings, sauces, dips, and desserts (78, 79). Current liquid or semisolid food products that have been enriched with omega-3 FAs using emulsion-based delivery systems include table spreads, yogurts, and milk (80-83). None of these products requires the delivery system to be optically transparent, and therefore emulsions or nanoemulsions could be used, although there may be some advantages in terms of long-term stability and bioavailability from using nanoemulsions (84). The optical transparency that can be achieved with nanoemulsions allows their application within clear food and beverage products, which would expand the functional food market for lipophilic bioactives. Low-energy fabrication methods are also becoming a larger area of interest because of their beneficial characteristics mentioned previously, *e.g.,* simplicity, low cost, and gentle processing conditions (66, 79). That being said, nanoemulsions must be carefully formulated to create physically and chemically stable systems suitable for food applications.

2.5. Obstacles to incorporating Omega-3 nanoemulsions in foods

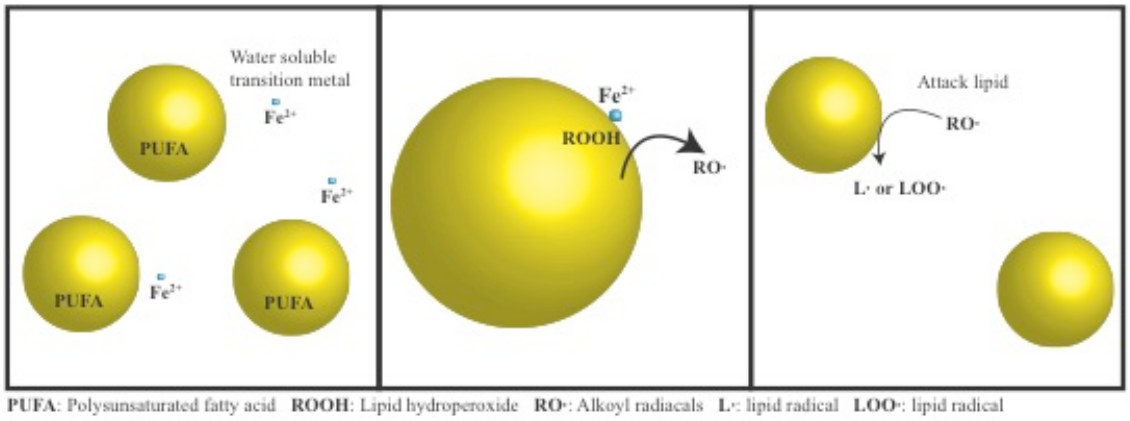
A number of obstacles must be overcome before omega-3 fortified nanoemulsions can be successfully incorporated into commercial food products (58), such as their

susceptibility to lipid oxidation, ensuring the physical stability of the system, delivering a nutritionally beneficial quantity of bioactive in a bioavailable form, and providing a palatable product that is acceptable to consumers. A number of these challenges are discussed in more detail in the remainder of this section.

2.5.1. Oxidation

Lipid oxidation in food products causes multiple problems that impact shelf-life, safety, nutritional value, functionality, and flavor (15, 16). Oxidation is readily noticed by consumers because the products of the reaction cause undesirable sensory attributes in food products at very low levels (58). Oxidation is the reaction of unsaturated FAs free radicals and oxygen (**Figure 2**) and occurs in three stages: initiation/induction, propagation, and termination (15, 85, 86). The most common mechanism for oxidation in emulsions is the reaction of free radicals with unsaturated lipids leading to the formation of lipid radicals. These lipid radicals react with oxygen and other lipids, thus beginning the chain reaction (propagation) stage of lipid oxidation (85). Before oxidation occurs, there is a lag phase, which is the phase that food processors attempt to extend through means of storage in cooler temperatures, decreased oxygen exposure, and addition of antioxidants (86). Once the initiation phase has begun, the rate of oxidation increases exponentially and the food is spoiled.

Figure 2. Proposed mechanism of lipid oxidation in an oil-in-water emulsion or nanoemulsion. Key: PUFA, polyunsaturated fatty acid; ROOH, lipid hydroperoxide; RO, alkyl radicals; L·, lipid radical; LOO-, lipid radical.

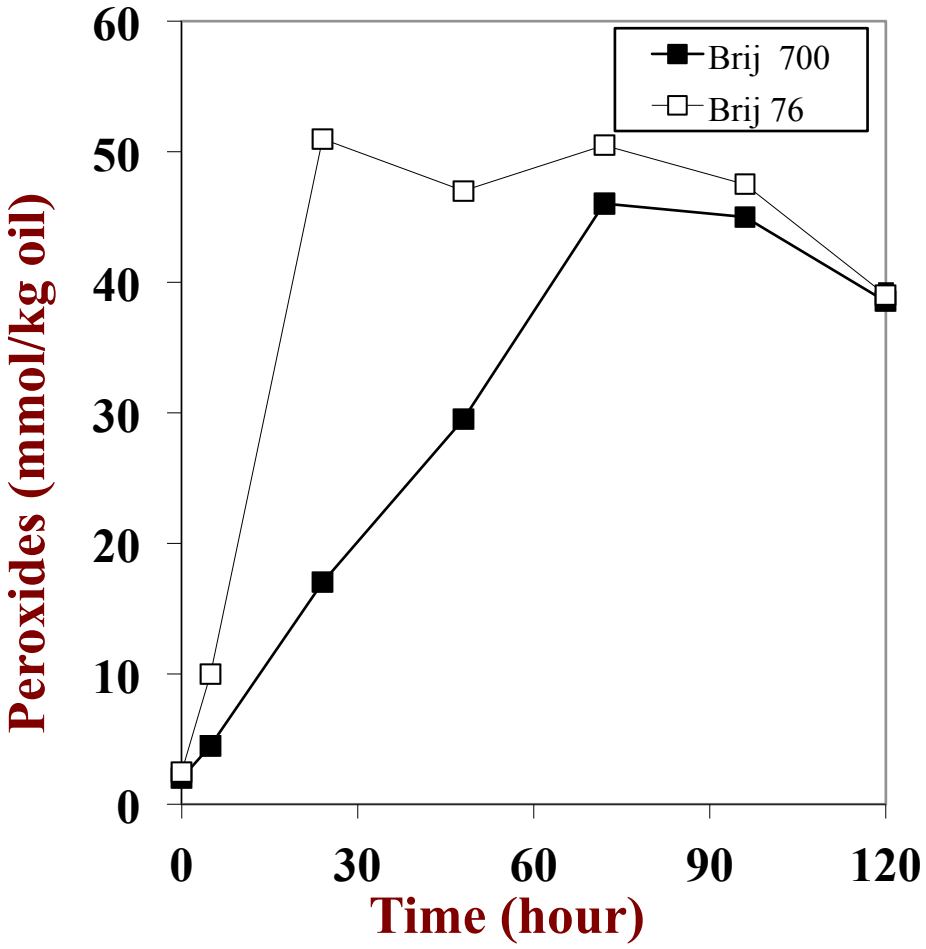


Lipid oxidation is promoted by exposure of unsaturated lipids to air, light, heat, and irradiation (15). Many factors contribute to an emulsion-based delivery system's susceptibility to oxidation including the composition, structure and organization of the oil, water and interfacial phases, as well as the type, amount, and location of any antioxidants present (17). FO nanoemulsions are particularly susceptible to lipid oxidation for a number of reasons: high degree of lipid unsaturation; high surface area of exposed lipids; and greater light penetration (17). Indeed, experimental studies have shown that lipid oxidation is faster in protein-stabilized nanoemulsions than in conventional emulsions with similar compositions, which was attributed to the higher lipid surface area (87). Consequently, it may be necessary to take additional steps to stabilize omega-3 oils encapsulated within nanoemulsions when compared to conventional emulsions.

Oxidation in nanoemulsions can be partially managed by controlling their physicochemical characteristics. Surfactants can influence the droplet charge, thickness, and permeability, all of which control the ability of pro-oxidants, free radicals, and

oxygen to interact with the lipids in the droplets (17, 88). Several studies have shown that anionic surfactants attract cationic transition metals while cationic surfactants repulse them thereby decreasing the rate of oxidation (17, 89-92). In addition, the interfacial layer of an emulsion can form a physical (steric) barrier against the aqueous phase of a system that contains pro-oxidants (17, 92). Thicker interfacial layers offer more protection, which depends on the dimensions and composition of the surfactant's head and tail group. A surfactant with a larger head group (Brij 700) was found to be better at slowing lipid oxidation in salmon oil-in-water emulsions than one with a smaller head group (Brij 76) (**Figure 3**) (92). Conversely, surfactant tail length has been shown to have only a minor impact on oxidative stability (93).

Figure 3. Comparison of lipid peroxide formation in salmon oil nanoemulsions (mean diameter = 200 nm) stabilized by Brij 76 and Brij 700. Samples were stored at pH 7.0 and 32 °C. Graph replotted from Silvestre, Chaiyasit, Brannan, McClements and Decker (92).



To prevent oxidation in food systems, radical scavenging and metal chelation are the main antioxidant strategies (15, 17, 94). Free radical scavengers react with free radicals before they can react with unsaturated FAs, and their effectiveness depends on their ability to donate a hydrogen atom to the free radical (95). Flavonoids tend to be effective free radical scavengers by donating a hydrogen from their hydroxyl groups, however their ability to act as an antioxidant depends on their volatility, pH sensitivity, and polarity. Metal chelation is a mechanism by which an antioxidant reduces the

reactivity of the transition metal or physically blocks it from interacting with the lipid (17). Metal chelators in oil-in-water emulsions have been shown to promote the movement of iron out of the lipid phase and to remove it from the surface of oil droplets, thereby inhibiting lipid oxidation (90, 96, 97). Studies of the chemical degradation of β-carotene in nanoemulsions (another polyunsaturated bioactive lipid) have shown that the rate of oxidation depends on system conditions (such as pH, ionic strength, temperature, droplet size, and emulsifier type) and can be inhibited by adding appropriate antioxidants (98-101). The addition of antioxidants has also been found to improve the stability of citral oil in nanoemulsions (102). Similar factors are likely to affect the rate of omega-3 oxidation in nanoemulsions.

Flavonoids can act as antioxidants through the means of radical and oxygen scavenging and have been found to be successful in inhibiting oxidation in FO emulsions (103). Two Flavonoids from apples (phloretin and phloridzin) have been tested for their ability to inhibit oxidation of PUFA methyl esters in oil-in-water emulsions (104). Both of these natural components had a significant effect in preventing lipid oxidation, with phloretin having a higher antioxidant activity than phloridzin, which was attributed to the fact that it was more lipophilic and therefore tended to accumulate within the lipid droplets where oxidation occurs. Certain flavanols (quarcetine glucosides) have also been evaluated for their antioxidant activity in bulk FO and in fish oil-in-water emulsions, and compared with butylated hydroxytoluen (BHT) and alpha-tocopherol (105). The emulsions were formed with methyl linolenate or DHA as the lipid phase. In oil-in-water emulsions, the flavanols were less effective than BHT but more effective

than alpha-tocopherol in preventing oxidation. In addition, the flavanols were more effective than both BHT and alpha-tocopherol in the bulk oil oxidation prevention.

2.5.2. Physical stability

The physical stability of nanoemulsions impacts their shelf life, appearance, functionality, and acceptability to consumers. As previously mentioned, nanoemulsions are most susceptible to Ostwald ripening, which is driven by the degree of water-solubility of the oil phase in the aqueous phase (9, 17). Oils with a higher water-solubility are more susceptible to Ostwald ripening because it is easier for them to migrate through the aqueous continuous phase. Oils with a lower degree of water-solubility, like long chain triglycerides, rarely experience Ostwald ripening. FOs contain long chain triglycerides, which makes them resistant to droplet growth due to Ostwald ripening (106). If nanoemulsion-based delivery systems are formulated using more water-soluble oils (such as flavor oils to mask off flavors), then it may be necessary to carefully design them to avoid Ostwald ripening. For example it may be necessary to mix a certain amount of water-insoluble oil (such as fish, flaxseed, or algae oil) with a flavor oil to prevent droplet growth (17). In this case, the water-insoluble oil acts as a ripening inhibitor.

The surfactant type and concentration used to create a nanoemulsion or emulsions impacts its susceptibility to flocculation and coalescence (6, 17, 107). Non-ionic surfactant-coated and polysaccharide-coated droplets tend to be stable across a wide range of salt and pH conditions because they are mainly stabilized by steric repulsion. On the other hand, phospholipid-coated and protein-coated droplets tend to be highly susceptible to changes in pH and ionic strength because they are mainly stabilized by

electrostatic interactions. Non-ionic surfactant stabilized nanoemulsions are influenced by other factors, such as surfactant characteristics and temperature. For example, nanoemulsions formed by spontaneous emulsification experienced coalescence during one month storage when using surfactants with intermediate hydrophilic/lipophilic balance (HLB) numbers (5-9). These surfactants tend to be soluble in both oil and water and form lamellar structures instead of micelles due to their optimum curvature, which do not stabilize nanoemulsions very effectively (79). Non-ionic surfactant stabilized nanoemulsions may also coalesce upon heating due to changes in the optimum curvature of the surfactant monolayer at elevated temperatures, *i.e.,* dehydration of the head group (69, 108).

Protein-coated lipid droplets are highly susceptible to flocculation at high salt levels or at pH values close to their isoelectric point (pI) due to a reduction in electrostatic repulsion between the droplets (109, 110). Protein-stabilized nanoemulsions should therefore only be used under conditions that favor a strong electrostatic repulsion between the droplets, *i.e.,* low ionic strength and/or pH far from pI. Alternatively, they should be incorporated into products that are highly viscous or gel-like, since then even if aggregation does occur the nanoparticles will not separate from the product due to gravitational separation.

2.5.3. Reaching the RDA

For a product to be considered to be a functional food, it must provide health benefits exceeding those of basic nutrition (26). The incorporation of FO in foods and beverages meets this definition based on the potential health benefits previously mentioned. However, it is important that the amount of omega-3 FAs present in a

functional food is large enough to demonstrate a beneficial health effect (111). Thus products should be fortified with an amount of FO that is a substantial amount of the recommended intake value if not the total amount. The total amount of omega-3 FAs in a functional food product ($m_{w\text{-}3}$) depends on the fraction of omega-3 FAs in the oil phase ($\Phi_{w\text{-}3}$), the fraction of oil phase in a nanoemulsion-based delivery system (Φ_{nE}), the amount of nanoemulsion added to the food product (Φ_P), and the serving size of the product (m_P):

$$m_{w\text{-}3} = m_P \times \Phi_{w\text{-}3} \times \Phi_{nE} \times \Phi_P$$

For example, for a FO containing 50% omega-3 fatty acids ($\Phi_{w\text{-}3} = 0.5$), that is converted into a 20 wt% oil-in-water nanoemulsion ($\Phi_{nE} = 0.2$), that is added to a food product that has a serving size of 280 g at a level of 10 wt% ($\Phi_P = 0.1$), then the final amount of omega-3 oil present is 2.8 g (2,800 mg). As mentioned earlier, the recommended intake values of omega-3 fatty acids are around 250 to 1000 mg per day, and therefore this amount should be achievable. The amount of nanoemulsion added to a food product may be limited by changes in optical properties if the nanoemulsion is not completely transparent. Typically, the smaller the droplet size, the more transparent the nanoemulsion and therefore the more that can be incorporated before the system becomes turbid. It is also important to ensure that the droplets do not grow after the food product has been manufactured, or this could result in an increase in turbidity during storage.

2.5.4. Bioavailability

With the growing use of emulsion-based delivery systems for human consumption, it is important to evaluate the gastrointestinal fate of the systems to ensure that there are no adverse health effects, and that the bioactive being delivered is indeed being absorbed into the body (30, 112). *In vitro* and *in vivo* digestion models have become instrumental in undertaking this kind of evaluation (113-115). Bioaccessibility is an important marker used in these studies that describes the fraction of an ingested compound (the bioactive) that is transferred into a mixed micelle after lipid digestion (116).

An ingested nanoemulsion will pass through the mouth and stomach before reaching the small intestine where lipid absorption normally occurs (117, 118). The size, composition, and surface characteristics of the lipid droplets within a nanoemulsion may change appreciably when they are exposed to gastrointestinal conditions (30). Upon entering the small intestine, lipase adsorbs to the surfaces of emulsified fats and coverts triacylglycerols into monoacyglycerols and free fatty acids (FFA) (1). These FAs are then incorporated into mixed micelles, travel through the mucus layer, and are absorbed by epithelium cells. The bioavailability of encapsulated fatty acids may be inhibited if the ability of the lipase to adsorb to the surface of lipid droplets and hydrolyze the triglycerides is prevented. The type and amounts of surfactants in a nanoemulsion may therefore impact the rate and extent of lipid digestion and FFA release. For example, corn oil nanoemulsions made using high-energy methods experienced a lag period before FFA release that ranged from 5 to 20 minutes as the mean droplet radius increased (119). This was a result of the lipase not being able to adsorb to the surface of the droplets due

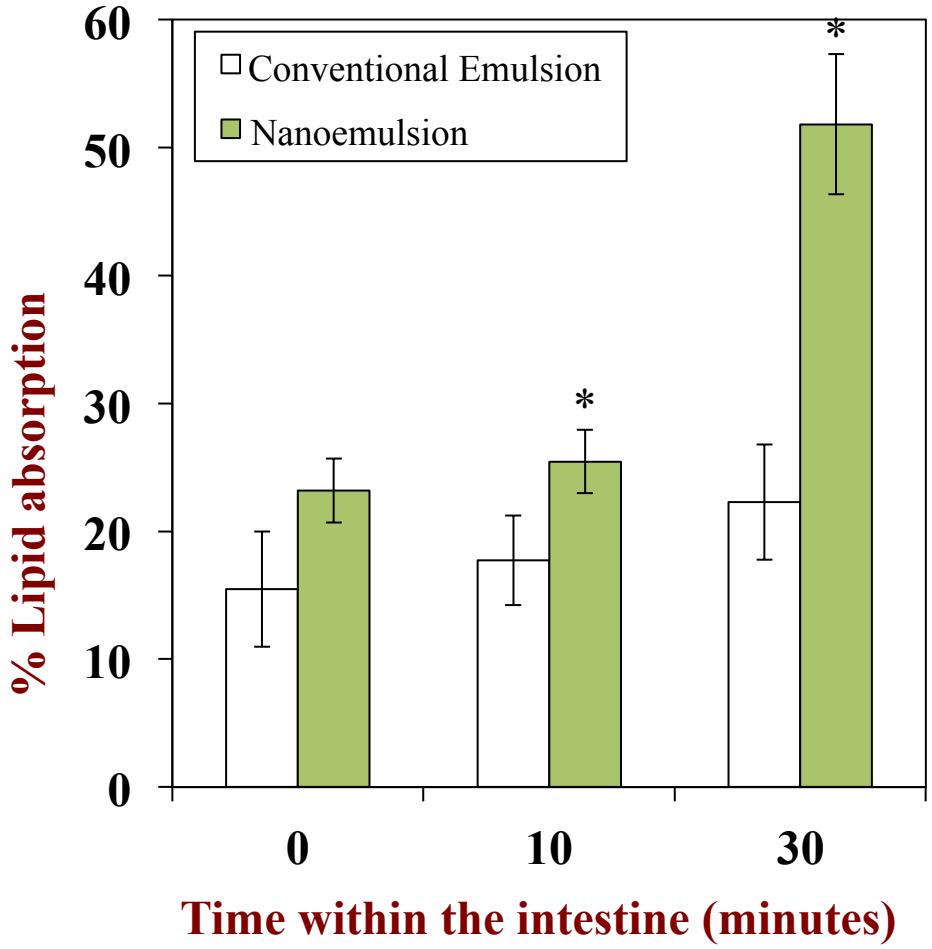
to the presence of excess surfactant that competed for the droplet surfaces. In these emulsion, 61-71% of the FFAs were released with higher amounts of FFA being release as the particle radius decreased. The obstruction of lipase as a result of high surfactant concentrations was also seen in medium chain triglyceride nanoemulsions containing vitamin E acetate made from both high and low energy methods (120). In this study, both the high and low energy emulsions had comparable particle sizes and similar behaviors throughout the *in vitro* digestion and both released similar amounts of FFA.

Surfactants can also impact the rate of lipid digestion based on their molecular and physicochemical characteristics. A study by Speranza et al. evaluated the effect of nonionic and anionic surfactants with a range of HLB numbers on the bioaccessibility of lipids (trioctanoyl glycerol) in emulsions using an *in vitro* digestion model (121). The results showed that an increasing HLB number increased the lag time in the jejunum and decreased the rate of lipolysis. In contrast, increasing the length of the aliphatic chain decreased the lag time in the jejunum, but increased the rate of lipolysis in the small intestine.

After FFA and bioactives are liberated from the lipid droplets, they form mixed micelles that travel through the mucus layer, and are then absorbed by the intestinal epithelial cells. When conventional FO emulsions were compared with FO nanoemulsions, the nanoemulsions had a significantly higher percentage of lipid absorbed compared to the conventional emulsions, which was attributed to their smaller particle size (**Figure 4**) (122). A recent study showed that the bioaccessibility of an oil-soluble bioactive component (vitamin E acetate) was higher in nanoemulsions prepared using a low-energy method (emulsion phase inversion) than in those prepared using a

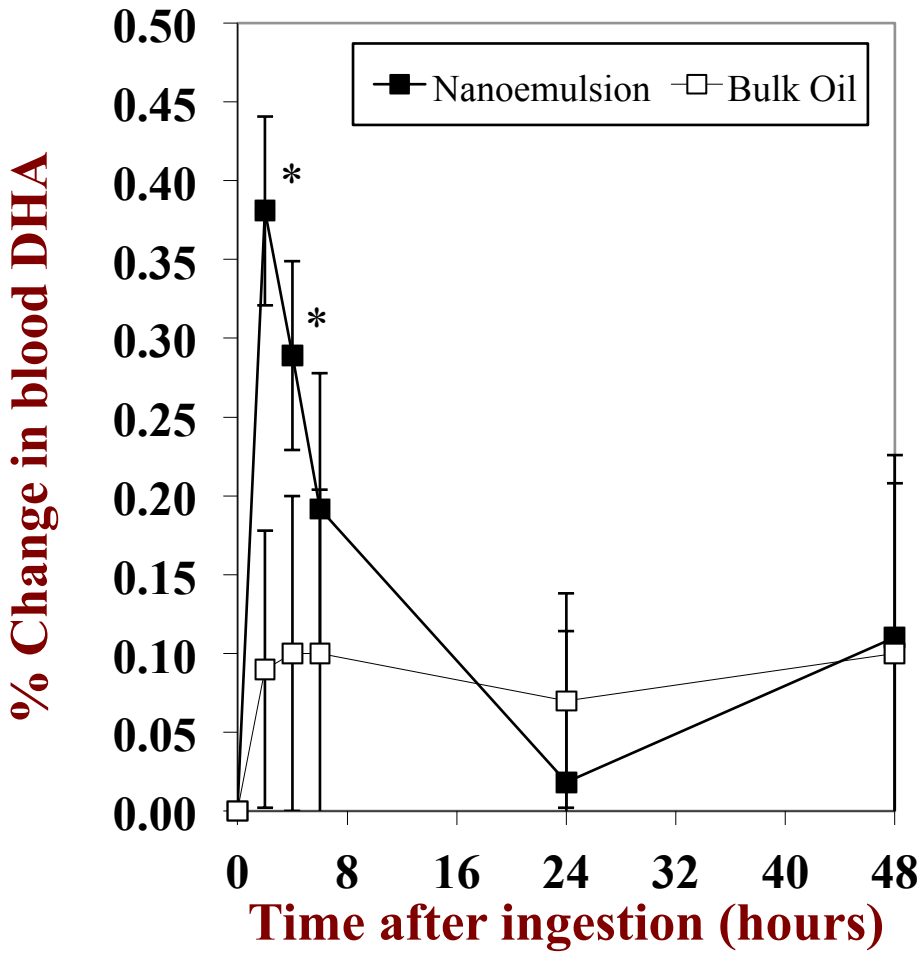
high-energy method (microfluidization) (120). It was suggested that the high levels of surfactant used in the low-energy method may have increased the amount of bioactive incorporated into the mixed micelles. The surfactant characteristics can also impact FFA absorption (121). An increasing surfactant HLB has been reported to increase the bioaccessibility of FFAs in the small intestine.

Figure 4. Comparison of EPA and DHA absorption in the intestinal tract of rats when delivered as nanoemulsions (mean diameter = 82 nm) or conventional emulsions (mean diameter = 1580 nm). Volume percentage of the emulsion absorbed was measured at three time intervals. *Mean values were significantly different (P<0.05). Graph replotted from Dey, Ghosh, Ghosh, Koley and Dhar (122).



Lastly, the absorption of FOs from ingested foods is important when developing functional food systems. Researchers investigated the absorption of FO in capsules *versus* microencapsulated FO incorporated into a milk shake (123). Both treatments resulted in similar increases of EPA and DHA in blood plasma. Another study looked at yogurt as a carrier product for algal oil nanoemulsions (mean droplet size 258 nm) *versus* bulk oil (81). In this study, both the nanoemulsion and bulk oil increased DHA levels in blood lipids however; the DHA from the nanoemulsion was more bioavailable than the bulk oil during the first four hours of digestion (**Figure 5**). Both of these studies support the use of microencapsulated or emulsified FO in food products and provide an alternative way for consumers to supplement their EPA and DHA intake without swallowing a large pill. The properties of a food system that accompanies the FO also has importance. When supplements were consumed with a higher fat meal compared to a lower fat meal, more long chain omega-3 PUFA were available, possibly due to the higher fat content stimulating more digestive enzymes and more mixed micelles (38). This again supports the use of functional foods to incorporate omega-3 FA and increase the absorption of the fats as an alternative to supplements.

Figure 5. Comparison of the mean baseline adjusted percentage blood fatty acid levels for DHA after subjects consumed algal oil delivered in either nanoemulsions or bulk oil incorporated into yogurt. *Mean values were significantly different (P<0.05). Graph replotted from Lane, Li, Smith and Derbyshire (81).



2.5.5. Flavor

As previously mentioned, some consumers must find alternative sources of omega-3 FAs because they do not like the flavor or texture of fish or seafood. High quality refined FOs have little to no flavor. This is unlike oils used in some dietary supplement that are low quality and have strong fishy flavors. Some consumers avoid soft gel capsules of FO supplements because of the reflux of FO resulting in "fish burps" (124). This is caused by the formation of a layer of the FO on top of the stomach

contents because the oils have a lower density than the gastric juices. By using a FO nanoemulsions incorporated into food products, consumers can receive the benefits of EPA and DHA in a form other than seafood. In addition, nanoemulsions can be designed to be resistant to coalescence and creaming within gastric environments by selecting appropriate emulsifiers so that the oil will not form a layer of oil on the top of the stomach contents and cause reflux issues (125-127). When functional foods are concerned, consumers will not sacrifice the taste of a product, even if the consumer is aware of the potential health benefits of the functional food (128).

2.5.6. Consumer acceptance

Studies disagree about which types of food a bioactive component, such as omega-3 oils, should be added for maximum consumer interest. In a study by Ares and Gámbaro consumers were more accepting of a functional food when the carrier food was perceived as being healthy (129). In a separate study by Bech-Larsen and Grunert, it was concluded that functional foods with a healthier base food were perceived as healthier compared to functional foods with an unhealthy base food, however this study also stated that consumers rationalized the enrichment of less healthy foods better than that of already healthy foods (130). Some consumers have concerns about unhealthy foods that have been fortified because they may now be perceived as a health food by others when in fact they are not (131).

Regardless of the carrier product, it is important to the consumer that the bioactive ingredient and base food are compatible; this is a stronger driving force for the purchasing of functional food products compared to health benefits and attitude towards functional foods (132). For example, products where FO appears to be a more natural fit

such as fish balls, rye bread, and tuna salad were expected to receive more positive attention by consumers (131). Another characteristic of FO enriched foods that should be considered when choosing an appropriate food carrier and in the product formulation is the sweetness profile. Participants in a study evaluating the acceptance of FO fortified foods were put off by sweet products such as yogurt drinks and sports bars having the addition of FO (131). In a separate study, women between the ages of 40 and 60 years did not accept the addition of sweeteners into a functional food and would rather consume a more natural product (128).

It is suggested that the use of health claims on functional food labels will have a positive impact on the consumer's view of the healthfulness of that food (130). The source of omega-3 fatty acids used in the fortification of foods can affect the cost of the products but also their health benefits. ALA omega-3s may give a cleaner label because they are from plant sources along with a lower price for consumers however, the conversion of ALA to LC-PUFA is quite low, decreasing its actual health benefits (32). The FDA health claim for EPA and DHA containing foods can aid in the marketing and advertising for qualifying products while differentiating them from products that only provide ALA.

Finally, sensory aspects also play a key role in consumer acceptance of foods. Few studies have researched the effect of nanoemulsions on the sensory properties of enriched foods. Dairy products have been the main focus of these studies. One study evaluated the fishy off flavor intensity of strawberry yogurt containing emulsified omega-3 oils after 14 days storage (80). This study found no significant difference between the control and fortified yogurt samples amongst an untrained consumer panel. Another

study evaluated a strawberry drinking yogurt fortified with bulk algae oil and algae oil nanoemulsion for smell, appearance, flavor, texture, consistency, aftertaste, and overall acceptability (133). Consumers were able to identify a sensory difference between yogurts fortified with either bulk oil or nanoemulsions in a triangular test. However, no statistically significant differences were found between the nanoemulsion-fortified, bulk oil-fortified, and unfortified yogurts in terms of their consistency and appearance. The sensory properties of cheese fortified with bulk FO or FO nanoemulsion have also been evaluated (134). Fishy off flavor was dependent on the concentration of FO in the sample and was more easily detected in the bulk oil-fortified samples compared to the nanoemulsion-fortified samples. Clearly, more research should be conducted to evaluate the sensory aspects of foods fortified with nanoemulsions to better understand their effect on consumer acceptance.

2.6. Conclusions

The low consumption of omega-3 FAs in Western diets clearly shows the need for alternative food sources on the market that provide these essential fatty acids. FO is an effective functional food ingredient because it is a good source of both EPA and DHA. Consumers will be more likely to buy functional foods with FO if the carrier food is compatible with the fat and if the foods are more savory instead of sweet. Whether the FO should be added to healthy or unhealthy foods is debated and should be evaluated on a product-by-product basis. Nanoemulsions are a promising way to deliver FOs into liquid food systems with the capabilities to protect the oil from oxidation, mask undesirable off-flavors, and increase oral bioavailability. Most importantly, the ability of nanoemulsions to be added to clear products increases the range of products that omega-3

FA enrichment can be applied to. That being said, there is still a need to expand omega-3 nanoemulsion research in order to optimize the fabrication method and formulation as a way to increase palatability, shelf life, and other physical characteristics of the food product.

CHAPTER 3

PHYSICAL AND OXIDATIVE STABILITY OF FISH OIL NANOMEULSIONS PRODUCED BY SPONTANEOUS EMULSIFICATION

3.1. Introduction

ALA (18:3 n-3), which is a type of omega-3 FA, cannot be synthesized in the human body and therefore it is an essential fat that must be consumed from the diet (1). ALA is a relatively short chain polyunsaturated fatty acid (SC-PUFA) that is converted to LC-PUFA after undergoing desaturation and elongation in the body (32). These LC-PUFA products include EPA (20:5 n-3) and its derivative, DHA (22:6 n-3), both of which are more bioactive than the precursor ALA (32). The conversion of ALA to EPA and DHA is not very efficient in the human body as many of the FAs are utilized for energy rather than converted to PUFAs (32). For this reason, it is usually recommended to consume EPA and DHA directly, rather than ALA, to obtain the beneficial health effects of omega-3 FAs.

Omega-3 FAs can be found in plants and seafood, however the amount and form of omega-3 FAs varies between sources (19). Land plant sources such as canola, soy, flaxseed, and walnuts provide omega-3 FAs mainly in the form of ALA (24, 32, 37). As for aquatic plants, algae can provide high amounts of DHA but it contains lower levels of EPA than FO (32, 34). FO is a more highly regarded as an excellent dietary source of omega-3 FAs because it contains high amounts of preformed EPA and DHA and does not

need to rely on the inefficient conversion of ALA to EPA and DHA in the human body (34).

FO is known to provide health benefits associated with brain development, inflammation, and cardiovascular disease (4). In the United States, the 2010 Dietary Guidelines for Americans recommends the consumption of 250 mg of EPA and DHA per day through the means of 8 ounces (227 g) of a variety of seafood a week (24). Unfortunately, Americans are falling short of this recommendation with a current consumption of 3.5 ounces (99 g) of seafood per week, mostly from sources low in omega-3 FAs (4, 24). This under-consumption of seafood may be attributed to taste, price, contamination concerns (such as heavy metals), and availability (19-21). As a result, there is a need to develop alternative sources for omega-3 FAs in consumer's diets. Emulsion-based delivery systems are particularly suitable for incorporating FOs into functional food products.

Nanoemulsions, a class of emulsion-based delivery systems, have been of particular interest lately because of their simple fabrication, high physical stability, and high bioavailability (11). By definition, nanoemulsions have a mean droplet radii below 100 nm, and may become optically transparent at sufficiently small particle sizes (6, 7). In contrast to microemulsions, which are thermodynamically stable systems, nanoemulsions are thermodynamically unstable systems but can be designed to be kinetically stable (8, 60, 61). Nanoemulsions can be fabricated by high- or low-energy methods. High-energy methods use specialized mechanical devices to breakdown the droplets into very fine particles, such as MF, high pressure valve homogenizers, or sonicators (7, 11, 12). In contrast, low-energy methods are able to spontaneously form

very fine droplets as a result of controlled changes in the environment or solution conditions (12). The interest in low-energy methods for certain applications is increasing because of their lower manufacturing costs, simple production methods, and ability to create smaller particle sizes than high-energy methods (12). SE is one of the simplest low-energy methods to implement since it only involves the addition of one phase into another phase with continuous stirring to spontaneously form a nanoemulsion (66). Typically in SE, the organic phase consisting of oil and surfactant is added to the aqueous phase.

This study will focus on the potential of SE to fabricate FO nanoemulsions that are suitable for application in clear beverages. As part of this study, the nanoemulsions created using this low-energy method will be compared to those produced using a high-energy method (MF) to highlight the advantages and limitations of these different approaches. The physical stability of the nanoemulsions and their susceptibility to lipid oxidation are obstacles that must be addressed when producing foods fortified with omega-3 FAs (58), and so these issues will also be evaluated in this research.

3.2. Materials and Methods

3.2.1. Materials

FO (Ropufa 30 *n*-3 food oil) was provided by DSM Nutritional Products Ltd. (Basal, Switzerland). The oil was composed of 101 mg of EPA/g of oil, 148 mg of DHA/g oil, and 312 mg of total n-3 PUFA/g of oil. LO was kindly donated by Citrus & Allied Essences (Lake Success, NY, USA). The supplier reported the chemical composition as determined by gas chromatography (**Table 1**). Non-ionic surfactant, polysorbate 80 (Tween 80), sodium benzoate, thiobarbituric acid (TBA), butylated

hydroxytoluene, 1,1,3,3-tetraethoxypropane (TEP), barium chloride, iron (II) sulfate heptahydrate, hydrochloric acid, and cumene hydroperoxide were purchased from Sigma-Aldrich Co. (St. Lois, MO, USA). Citric acid, isooctane, 1,2-propanol, methanol, and butanol were purchased from Fisher Scientific (Waltham, MA, USA). Trichloroacetic acid (TCA) and ammonium thiocyanate were purchased from Acros Organics (Geel, Belgium). Ethanol was purchased from Pharmco-AAPER (Brookfield, CT, USA). All solvents and reagents were of analytical grade or higher. Double distilled water was used to prepare all solutions.

Table 1. Concentration of constituents in threefold (3x) lemon oil, provided by Citrus & Allied Essences (Lake Success, NY).

Constituent	Concentration in lemon oil (%)
α-Thujene	0.00
α-Pinene	0.70
Camphene	0.00
Sabinene	0.30
β-Pinene	4.90
Myrcene	0.80
Octanal	0.01
Limonene	63.00
α-Phellandrene	0.02
γ-Terpinene	14.00
Terpinolene	1.80
Linalool	0.60
Citronellal	0.30
α-Terpineol	0.30
Neral (citral B)	2.38
Geranial (citral A)	3.84
Neryl acetate	0.97
Geranyl acetate	0.60
(E)-caryophyllene	0.90
Trans-α-bergamotene	0.90
β-bisabolene	1.40
Total (%)	**97.72**

3.2.2. Emulsion preparation

3.2.2.1. Low-energy method: Spontaneous emulsification

Nanoemulsions were prepared by SE for physical and oxidation stability evaluation. The organic phase consisted of a mixture of FO (5 wt %) and LO (5 wt %), which were stirred at 750 rpm for 15 min, and then stirred for an additional 30 min after adding non-ionic surfactant (2.5-20 wt% Tween 80). The aqueous phase was buffer (70.0 - 87.5 wt%) consisting of 0.8 wt% citric acid and 0.08 wt% sodium benzoate at pH 3.0, in order to simulate the aqueous phase of a beverage system. In this method, the organic phase was added to an aqueous phase using an automatic pipette (Ranin 10 mL E4 XLS, Mettler-Toledo International Inc., Columbus, OH, USA) while stirring at 500 rpm for 15 min.

3.2.2.2. High-energy method: Microfluidizer

Nanoemulsions were also prepared using a MF for oxidation stability evaluation. FO (10 wt%) and LO (10 wt%) were mixed for 15 min at 750 rpm. Buffer (78 wt%) was mixed with Tween 80 (2 wt%) for 30 min at 750 rpm. The two phases were added together and mixed for 2 min with a hand mixer (Bamix ESGE Ltd, Swtizerland) to form a course emulsion. Samples were passed through a MF (M-110L, Microfluidics, Newton, MA) 3 times at 12,000 PSI.

3.2.2.3. Post-production alterations of emulsions

All emulsions were diluted to 1 wt% oil (0.5 wt% FO and 0.5 wt% LO) with buffer solution and then stirred for 5 minutes at 300 rpm. Finished emulsions were held

in 50 mL disposable centrifuge polypropylene tubes (Fisher Scientific, Pittsburg, PA, USA). Each formulation was made in duplicate.

For the emulsions used in oxidation studies, additional surfactant was added during the dilution stage to evaluate the effect of surfactant and particle size on oxidation. For these emulsions, Tween 80 was mixed with the volume of buffer used for dilution at 750 rpm for 30 min. This solution was added to the emulsion to dilute to 1 wt% oil (0.5 wt% FO and 0.5 wt% LO) and stirred at 300 rpm for 5 min. Iron (100 μM) as $FeSO_4$ was also added to all emulsions used in the oxidation studies to accelerate the lipid oxidation reaction. Emulsions were observed using optical microscopy on day 0 and 14 of the oxidation experiments (Nikon Eclipse 80i, Nikon Instrument Inc., Melville, NY). Each formulation was made in duplicate.

3.2.3. Surfactant concentration

The effect of surfactant concentration in the SE nanoemulsions was evaluated by varying the surfactant-to-oil ratio (SOR) while keeping the amount of oil (FO and LO) constant (10 wt %).

$$\%SOR = 100 \times m_s/m_o$$

where m_s is the mass of the surfactant and m_o is the total mass of the oil phase.

3.2.4. Particle size measurements

The particle size distribution (PSD) of all emulsions was measured by either dynamic (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) or static light scattering instruments (Masterszier 2000, Malvern Instruments, Malvern, UK). Static

light scattering was used to measure the size of the droplets in emulsions containing relatively large droplets ($d > 1$ μm), and in this case the particle size was reported as d_{32}. On the other hand, dynamic light scattering was used to analyze emulsions containing relatively small droplets ($d < 1$ μm), and the particle size was reported as the Z-average diameter and polydispersity index (PDI). PDI is a measure of the narrowness of the size distribution; values ≤ 0.1 designate a very narrow size distribution (135). All samples were measured in duplicate.

3.2.5. Turbidity measurement

The turbidity of samples was determined using a Thermo Scientific Evolution Array UV-Visible Spectrophotometer (Waltham, MA, USA). The turbidity was measured at 600 nm at the temperature at which the samples were being held. All samples were measured in duplicate.

3.2.6. Oxidation measurements

3.2.6.1. Peroxide value

Emulsions were held in 50 mL disposable centrifuge polypropylene tubes (Fisher Scientific, Pittsburg, PA, USA) and incubated in the dark at 55 °C for 14 days, with measurements being taken every 2 days. Lipid hydroperoxides were measured using a method adapted from Shantha and Decker (136). Lipids from the emulsion were extracted by adding the emulsion (0.3 mL) to a 3:1 v/v mixture of isooctane/2-propanol (1.5 mL) and vortexing the mixture for 10 s, 3 times followed by centrifugation (1000 *g*) for 2 min. The top layer (0.2 mL) was mixed with 2:1 v/v methanol/1-butanol (2.8 mL), followed by 30 μL of 1:1 v/v 3.94 M ammonium thiocyanate/ferrous iron solution

(solution prepared by mixing equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min, absorbance was measured at 510 nm using an UV- visible spectrophotometer (Ultraspec 3000 *pro*, Biochrom Ltd, Cambridge, UK). Hydroperoxides were calculated as mM cumene hydroperoxide using a cumene hydroperoxide standard curve at concentrations of 0-0.5 mM. All samples were measured in triplicate.

3.2.6.2. Thiobarbituric acid-reactive substances (TBARS)

TBARS samples were held in 50 mL disposable centrifuge polypropylene tubes (Fisher Scientific, Pittsburg, PA, USA) at 55 °C in the dark and were measured every 2 days for 14 days using the method of McDonald and Hultin (137). Emulsion (1.0 mL) and TBA reagent (2.0 mL) (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, and 0.25 M HCl with 2% BHT in ethanol solution) were vortexed in glass test tubes with screw caps. The tubes were placed in a water bath (90 °C) for 15 minutes, and then moved to a room temperature water bath to cool for 10 minutes. The tubes were centrifuged (1000 *g*) for 15 minutes, then sat for 10 more minutes. The absorbance of the supernatant was measured at 532 nm. Concentrations of TBARS were calculated as μM using a standard curve of TEP at concentrations between 0-20 μM. All samples were measured in triplicate.

3.3. Experimental design and data analysis

All measurements were done in duplicate or triplicate and results are reported as mean ± standard deviation. Statistical analysis was carried out by analysis of variance (ANOVA) and Tukey test with confidence interval of 95% using Minitab 16 software (Minitab Inc., State College, PA, USA).

3.4. Results and discussion

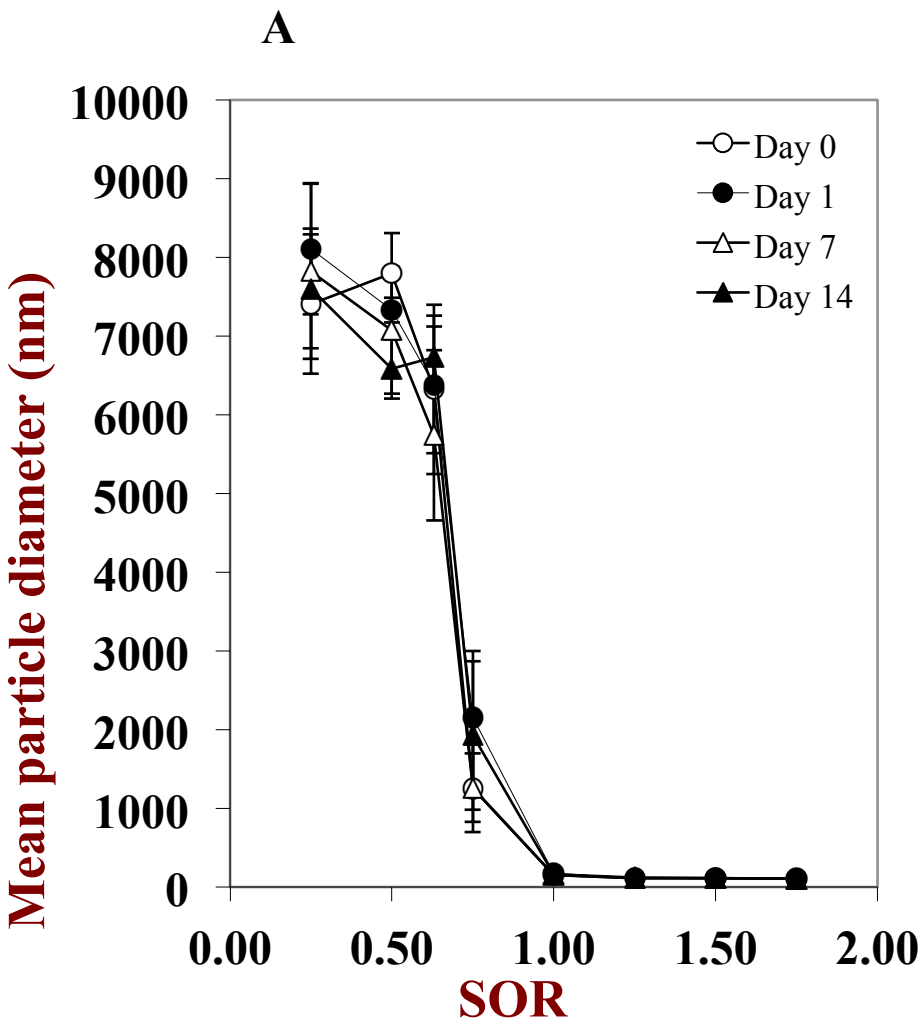
The term "emulsions" is used to refer to both nanoemulsions (d < 200 nm) and conventional emulsions (d > 200 nm) in the remainder of this study, since both types of systems could be formed depending on the initial composition.

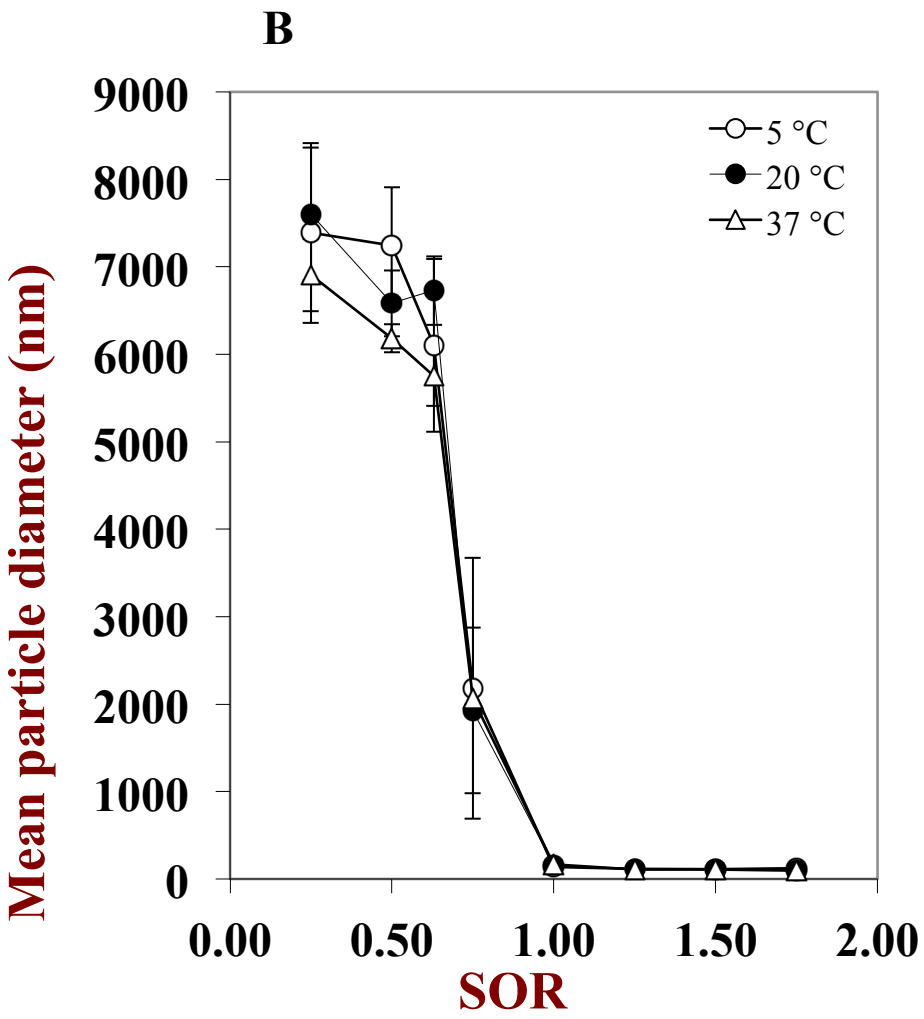
3.4.1. Effect of surfactant concentration on physical stability

Emulsions were prepared using SE with 10 wt% total oil phase (50 wt% FO and 50 wt% LO) and different SOR values. These emulsions were then diluted to 1 wt% total oil phase and held at 5, 20, and 37 °C for 14 days to evaluate their physical stability. SOR had a large impact on the mean particle diameter of the emulsions: as the SOR increased, the mean particle diameter decreased (**Figure 6**). The dependence of the mean particle size on SOR did not depend strongly on storage time (**Figure 6a**) or holding temperature (**Figure 6b**). Previous studies have also reported similar trends when using low-energy methods such as SE and emulsion phase inversion to prepare emulsions (70, 79, 106, 135, 138).

After fabrication of the emulsions, the mean particle diameters ranged from 106 to 7,800 nm for SORs 1.75 and 0.5, respectively (**Figure 6**). Emulsions with SORs of 0.63 and below were significantly larger than those with SORs of 0.75 and above ($p < 0.05$). However, true nanoemulsions (d < 200 nm) were only formed with SORs from 1.00 to 1.75 (6, 7). These particle size results agree with those of a recent study that also prepared FO nanoemulsions using SE (106). The fact that there was no significant change in the mean particle size of the emulsions after they were held at 5, 20, and 37 °C for 14 days, suggests that they were stable against droplet growth from flocculation, coalescence, and Ostwald ripening (12).

Figure 6. Influence of surfactant-to-oil ratio (SOR) on the mean particle diameter of oil-in-water emulsions produced by spontaneous emulsification (SE) (A) after being held at 14 °C for 14 days and (B) on day 14 at all temperatures. All systems were made with a total oil phase content of 10% wt (50% fish oil (FO) and 50% lemon oil (LO)) and were diluted to 1% wt total oil phase before measurement. Particle sizes on day 0 were measured immediately after producing the emulsion.

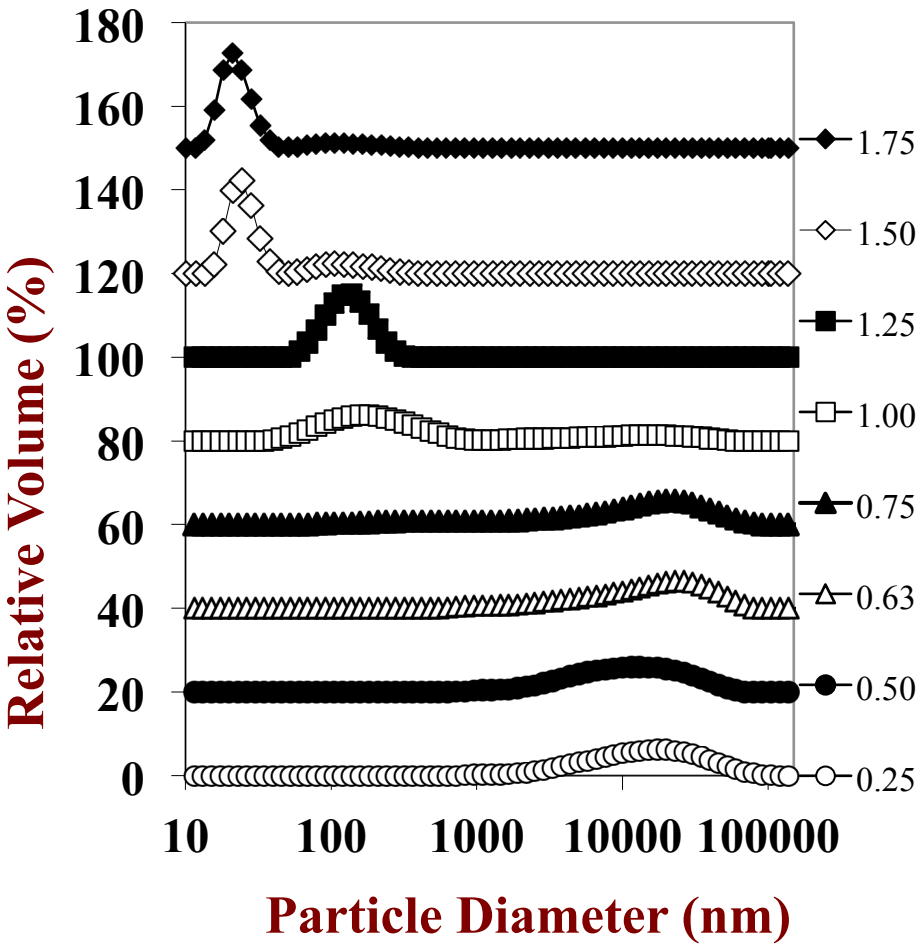




Only the particle size distributions of the emulsions held at 20 °C for 14 days are shown since these were representative of the other holding temperatures and storage times (**Figure 7**). At SORs from 1.25-1.75, emulsions containing small droplets with a narrow particle size distribution were formed. At higher SORs the emulsions still had a monomodal particle size distribution, but the majority of droplets were much larger (> 10 μm). It is important to note that during the fabrication of emulsions using high SORs (≥ 1.75) gel-like globules were observed by eye and under an optical microscope (data not shown). At SOR values of 2.00 and above, these gel-like structures did not dissolve during the 15 min period after preparing the emulsions, and so these samples were not

analyzed further. Gel-like structures also formed at SOR 1.75, but fewer were observed and they disappeared after the emulsions were diluted to 1% wt oil. We hypothesize that these were liquid-crystalline regions formed from surfactant, oil, and water at relatively high surfactant levels. Further research is needed to evaluate the precise range of conditions where these globules form, and to develop effective strategies to disrupt them, if this is possible. While emulsions with such high surfactant concentrations may not be of commercial interest to the food industry because of legal and cost concerns, they may be suitable for application in the pharmacological industry, especially for topical treatments.

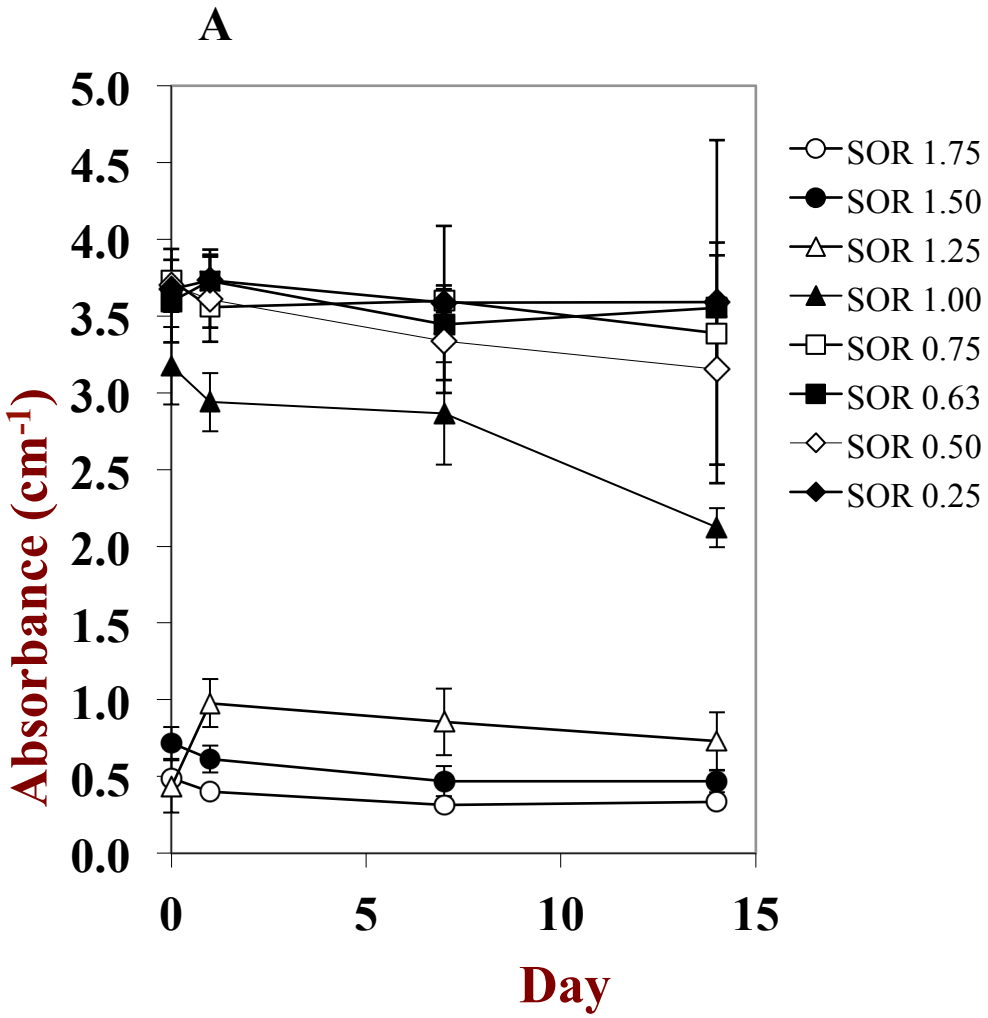
Figure 7. Influence of SOR on the particle size distribution of oil-in-water emulsions produced by SE held at 20 °C on day 14. All systems were made with a total oil phase content of 10% wt (50% FO and 50% LO) and were diluted to 1% wt total oil phase before measurement.

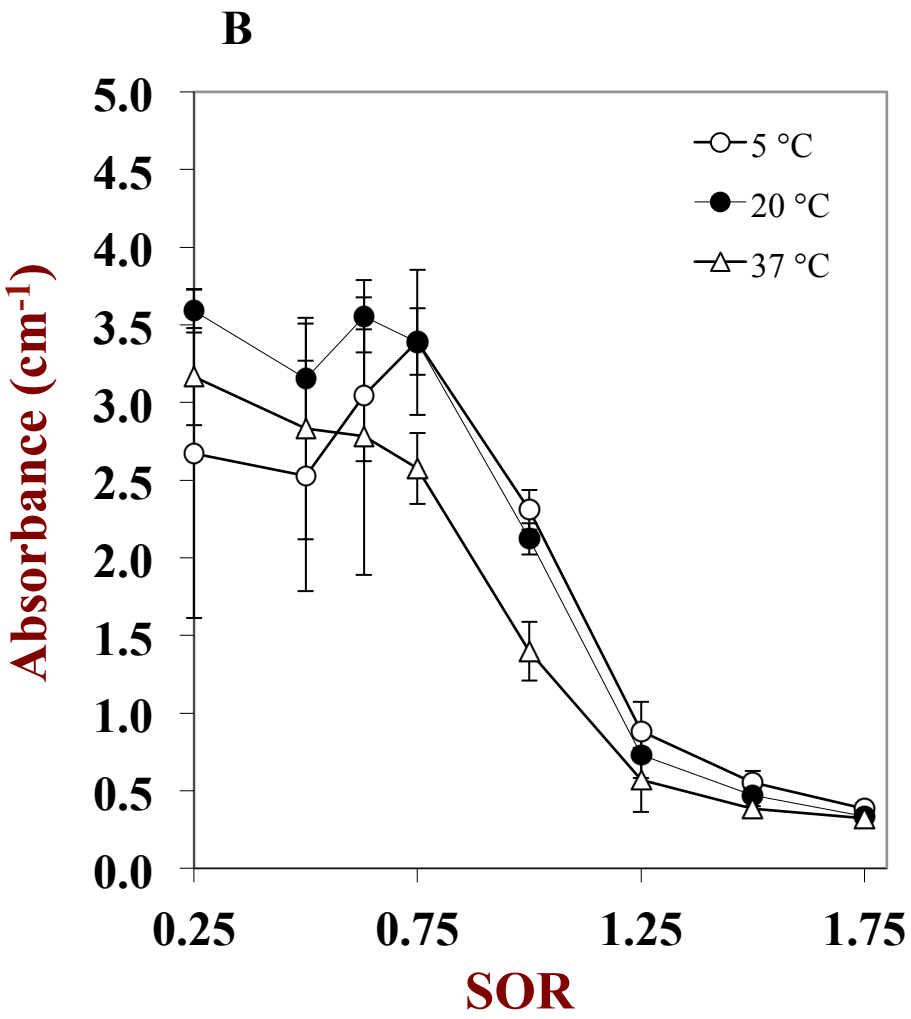


The turbidity of the emulsions was monitored throughout their storage to evaluate changes in their opacity. The turbidity of the nanoemulsions decreased as the SOR increased for all holding temperatures and storage times (**Figure 8**). This was expected, as the emulsions should become less turbid and more transparent as the mean particle size decreased. Emulsions appear opaque when their particle size is comparable to the wavelength of light and the light is scattered strongly (139). In contrast, they appear only

slightly turbid or transparent when their particle sizes are smaller than the wavelength of light (6).

Figure 8. Influence of SOR on the turbidity of oil-in-water emulsions produced by SE (A) after being held at held at 20 °C for 14 days and (B) on day 14 at all temperatures. All systems were made with a total oil phase content of 10% wt (50% FO and 50% LO) and were diluted to 1% wt total oil phase before measurement. Turbidity on day 0 was measured immediately after producing the emulsion.





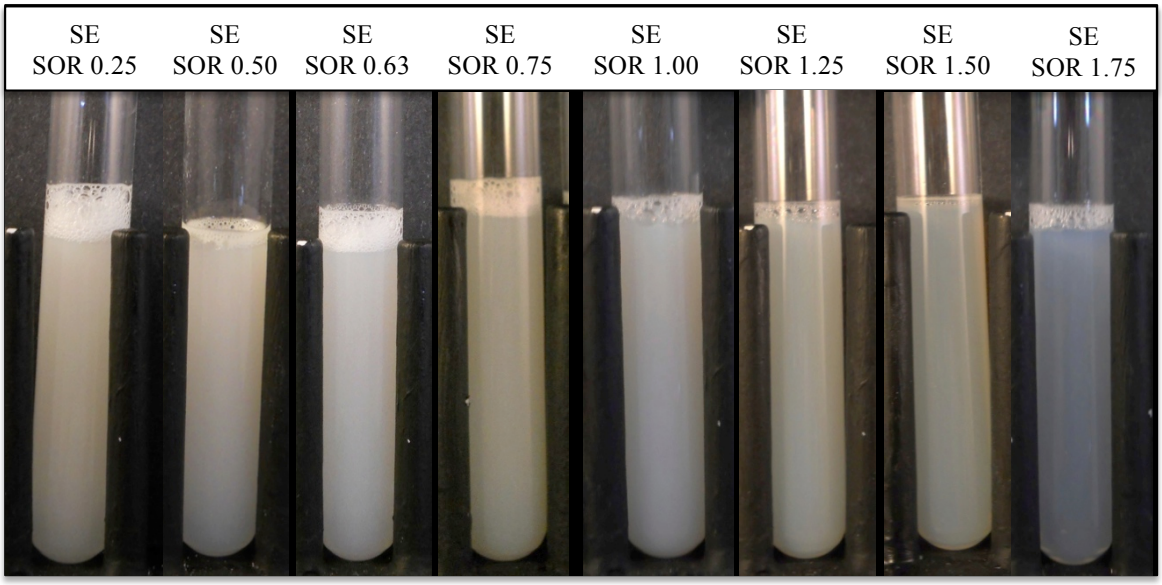
The overall dependence of turbidity on SOR for emulsions prepared using the SE method did not depend strongly on storage time (**Figure 8a**) or holding temperature (**Figure 8b**). Emulsions with SORs of 0.75 and below were significantly more turbid than those with SORs of 1.00 and above ($p < 0.05$). The origin of this effect can be attributed to the observed differences in droplet size (**Figure 6**). The droplets formed at lower SORs (0.25-0.75) are much larger than those formed at higher SORs (1.00-1.75), and therefore they scatter light more strongly leading to a higher turbidity. The turbidity of many of the emulsions was lower after 14 days storage than immediately after preparation (**Figure 8a**), which suggests that there may have been a decrease in droplet

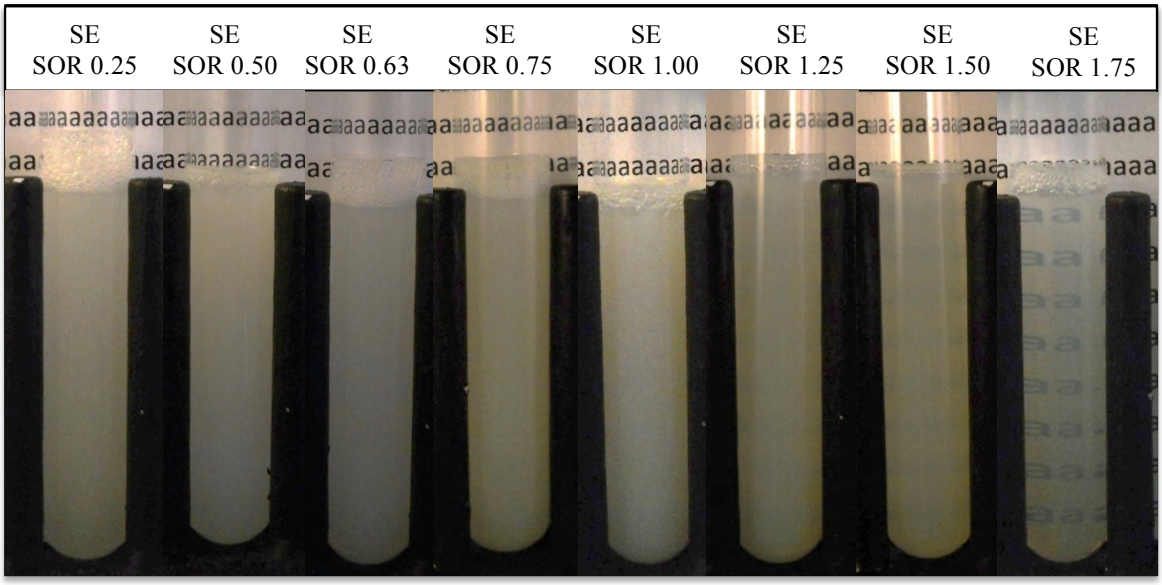
number or size. A possible explanation for this phenomenon is solubilization of some of the oil phase in surfactant micelles during storage.

Additional information about the influence of SOR on the storage stability of the emulsions was obtained from visual observations of their appearance (**Figure 9)**. For the sake of brevity, only images from day 0 and 14 for emulsions stored at 37 °C are shown since they gave similar trends as the other samples, but exhibited the largest overall change in appearance. Immediately after fabrication (day 0), only the emulsions with SORs from 1.25 to 1.75 were optically transparent, whereas emulsions with lower surfactant concentrations were opaque. After 14 days storage, the range of SOR levels where emulsions appeared transparent at higher surfactant values increased, which again suggested that there was a reduction in the number or size of the oil droplets during storage (possibly due to solubilization). In addition, there was evidence of an opaque layer on the top of the emulsions with the lower SOR levels, which can be attributed to rapid creaming of the large fat droplets.

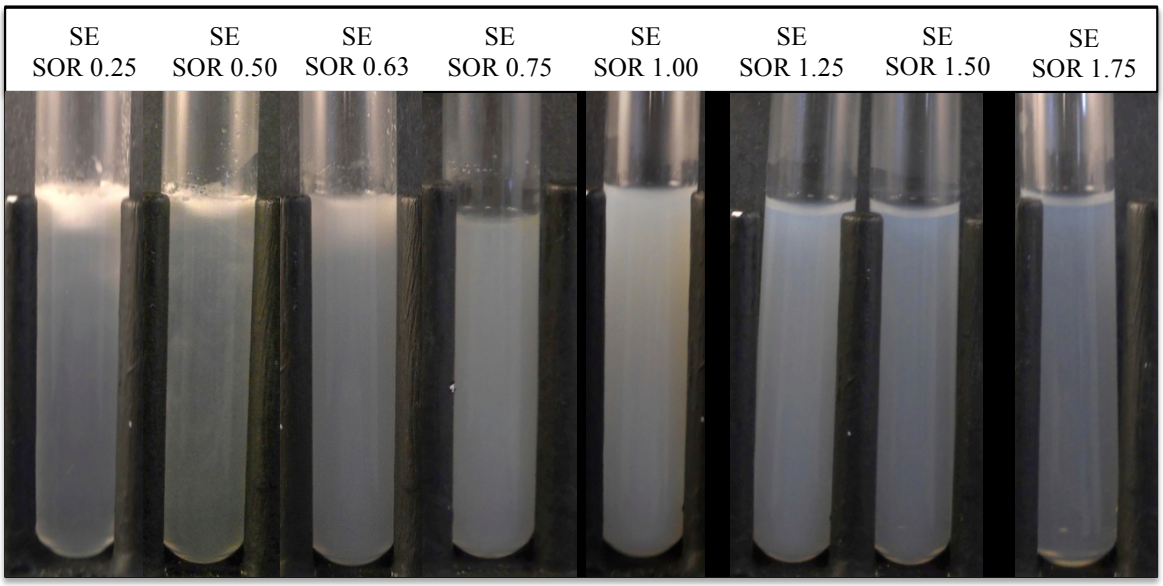
Figure 9. Influence of SOR on the appearance of oil-in-water emulsions produced by SE (A) on day 0 at 20 °C and (B) on day 14 held at 37 °C. All systems were made with a total oil phase content of 10% wt (50% FO and 50% LO) and were diluted to 1% wt total oil phase before measurement. Images on day 0 were taken immediately after producing the emulsion.

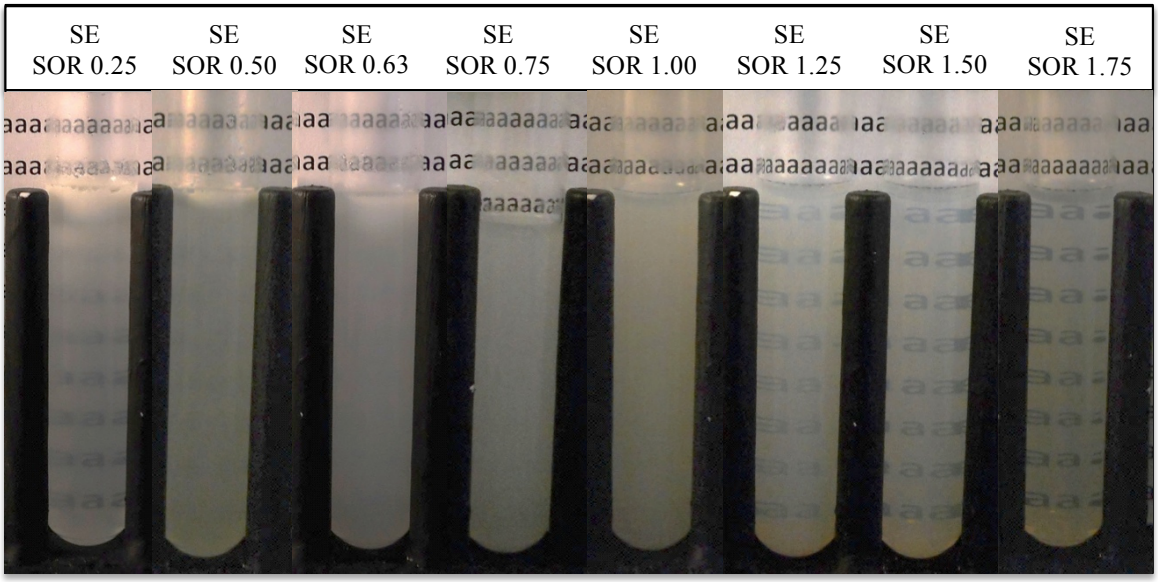
A





B





3.4.2. Effect of particle size and surfactant concentration on lipid oxidation

In this series of experiments, the influence of droplet size and surfactant concentration on the oxidative stability of fish oil-in-water emulsions prepared by low-energy (SE) and high-energy (MF) methods was examined. Emulsions with different

droplet sizes were prepared using the low-energy method by varying the SOR (**Figure 6**). However, the fact that these emulsions had different surfactant levels may also have influenced their oxidative stability. For this reason, we added extra surfactant to some of the emulsions after they were prepared so that we could produce emulsions that had different particle sizes, but similar surfactant concentrations. A similar procedure was also carried out for the emulsions prepared using the high-energy method. These samples could then be used to disentangle the effects of droplet size from those of surfactant concentration on lipid oxidation. A summary of the different samples prepared for this study is given in **Table 2**.

Table 2. Comparison of the composition and structure of different nanoemulsions used in the oxidation studies. All of the emulsions contained the same final level of oil (1 wt%). **Key**: SE = spontaneous emulsification (Low-energy); MF = Microfluidization (High-energy).

Sample Name	Preparation Method	SOR	Initial Droplet Diameter (nm)
SE D 1.00	SE – all surfactant added initially	1.00	132.7 ± 6.4
SE D 1.75	SE – all surfactant added initially	1.75	96.5 ± 2.0
SE S 1.75	SE – some surfactant added to SE D 1.00 after formation	1.75	136.0 ± 3.8
MF D 0.10	MF – all surfactant added initially	0.10	151.8 ± 2.1
MF S 1.00	MF – some surfactant added to MF D 0.10 after formation	1.00	161.1 ± 1.8
MF S 1.75	MF – some surfactant added to MF D 0.10 after formation	1.75	166.5 ± 1.8

Initially, low-energy emulsions were prepared using SORs of 1.00 (“SE D 1.00”) and 1.75 (“SE D 1.75”) because they gave relatively stable emulsions containing large and small droplet sizes, respectively (**Table 2**). Additional surfactant was then added to a

portion of the SE D 1.00 emulsion to produce an emulsion that contained relatively large droplets and a high surfactant concentration ("SE S 1.75"). Here, the middle letter designations that either the diameter ("D") or surfactant level ("S") was being controlled. The diameter was controlled by adding all the surfactant prior to emulsion formation, whereas the surfactant level was controlled by adding some of the surfactant after emulsion formation. For the sake of comparison, a high-energy emulsion was also prepared with an SOR of 0.10 ("MF D 0.10") using a MF. Additional surfactant was also added to some of this emulsion to prepare final samples that had SOR values similar to those found in the low-energy emulsions: "MF S 1.00" and "MF S 1.75" (**Table 2**).

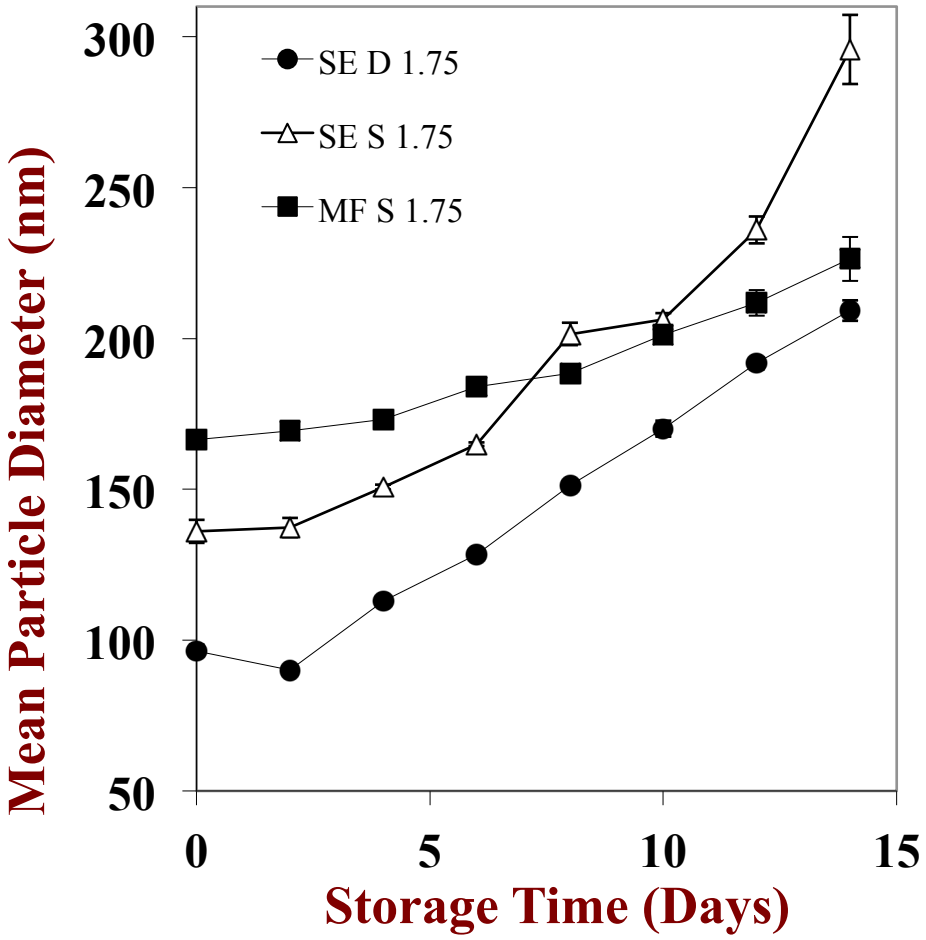
The prepared emulsions were found to be relatively stable to lipid oxidation when stored under ambient conditions. We therefore accelerated the lipid oxidation reaction by adding a pro-oxidant (100 μM iron) and holding them at an elevated temperature (55 °C) during storage.

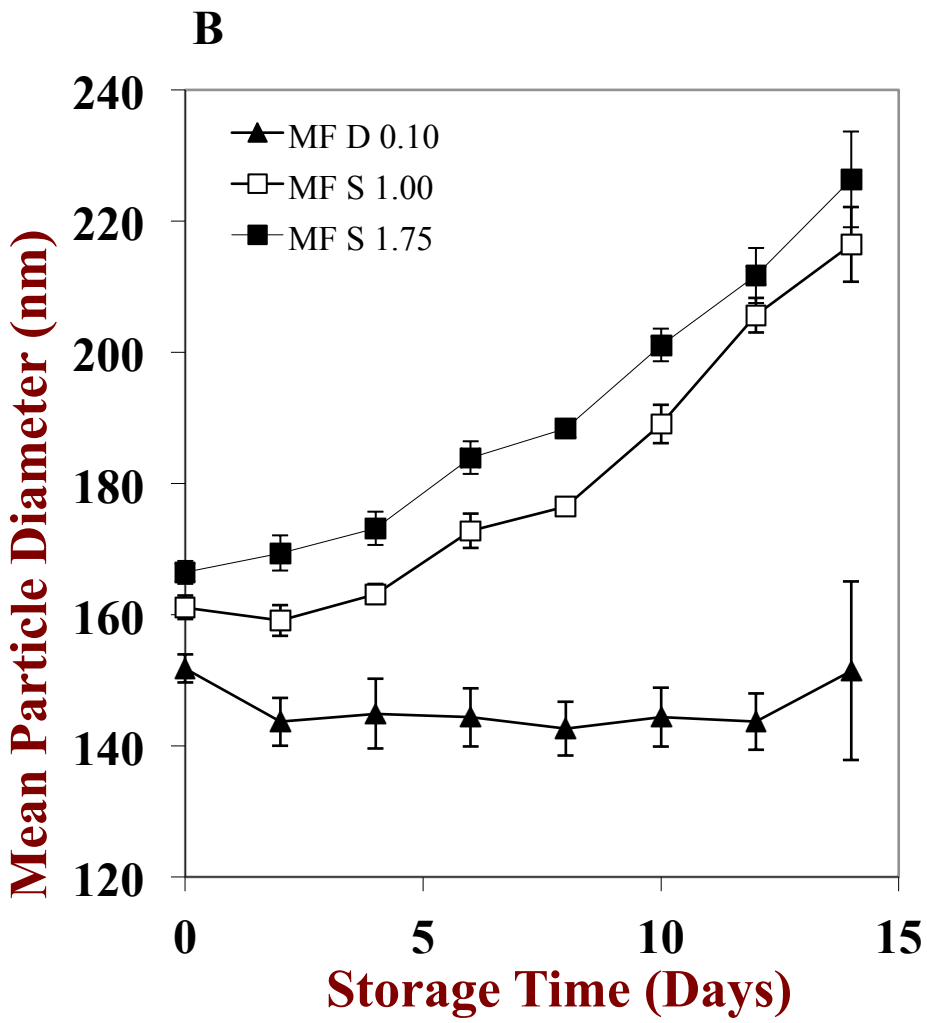
3.4.2.1. Physical stability of emulsions during oxidation

Influence of initial particle size: We examined the influence of the initial particle size on the stability of the emulsions to droplet growth during storage at the same surfactant level, *i.e.* SOR = 1.75 (**Figure 10a**). Interestingly, the droplet growth rate depended on both the initial particle size and the homogenization approach used. Droplet growth was fastest for the low-energy emulsion to which surfactant was added after emulsion formation, which initially had an intermediate particle size. On the other hand, droplet growth was slowest for the high-energy emulsion, which initially had the largest particle size. This result suggests that the initial structural organization of the oil and

surfactant molecules within the emulsions played an important role in determining their stability to droplet growth.

Figure 10. Effect of (A) SOR and (B) added surfactant on mean particle diameter for emulsions made by MF and SE and held at 55 °C with added iron. All emulsions were diluted to 1% wt total oil phase.





Influence of surfactant concentration: We also examined the influence of the SOR on the stability of emulsions that initially had similar particle sizes (**Figure 10b**). These emulsions were all fabricated using the MF, but different amounts of surfactant were added after production. There was little change in the mean particle diameter during storage of the emulsion containing no surfactant added after homogenization (MF D 0.1), which suggests that it was relatively stable to droplet growth. On the other hand, there was an appreciable increase in the mean droplet diameter for the two emulsions containing additional surfactant (MF S 1.00 and MF S 1.75) during storage, with the growth rate increasing with increasing surfactant concentration. A similar result was

obtained for emulsions produced by SE: the droplet diameter increased by 60% after 14 days storage for the SE S 1.00 emulsion, but by 117% for the SE D 1.75 emulsion. These results suggest that the presence of free surfactant within the aqueous phase of the emulsions increased the droplet growth rate. There are a number of potential reasons for this phenomenon: (i) micelles formed by non-ionic surfactants are known to generate an osmotic attraction to oil droplets due to a depletion effect, which can promote coalescence (140); (ii) micelles can solubilize and transport oil molecules between emulsion droplets, which can enhance Ostwald ripening (141).

In summary, the stability of the emulsion droplets to growth during storage appears to depend on at least three factors: initial particle size; total surfactant concentration; and preparation method (low-energy versus high-energy). The frequency of droplet collisions is known to decrease with increasing droplet size, whereas the coalescence efficiency is known to increase (142). The rate of Ostwald ripening is known to increase with decreasing particle size, increasing polydispersity, and increasing free surfactant concentration (63, 143). Consequently, the dependence of droplet growth on these factors may be quite complex.

In all the emulsions produced using the low-energy method, there was a considerable increase in the mean particle diameter during storage (**Figure 10a**). This increase was not observed in the experiments on the influence of SOR on emulsion stability reported earlier, and can be attributed to the elevated storage temperature and the presence of iron. Elevated temperatures are known to promote droplet coalescence in emulsions stabilized by non-ionic surfactants due to dehydration of their head groups. For example, Gulotta et al. (106) conducted a thermal stability analysis on nanoemulsions

that contained 10% oil (50% FO and 50% LO), 10% Tween 80, and 80% aqueous phase. This experiment showed that rapid droplet growth occurred between 50 and 68 °C, which was attributed to coalescence caused by surfactant head group dehydration. Absorbance measurements and visual observations were also made on the same samples during storage, which showed that there was an increase in turbidity in the emulsions that underwent droplet growth (data not shown), as would be expected due to the increase in the light scattering ability of the larger droplets.

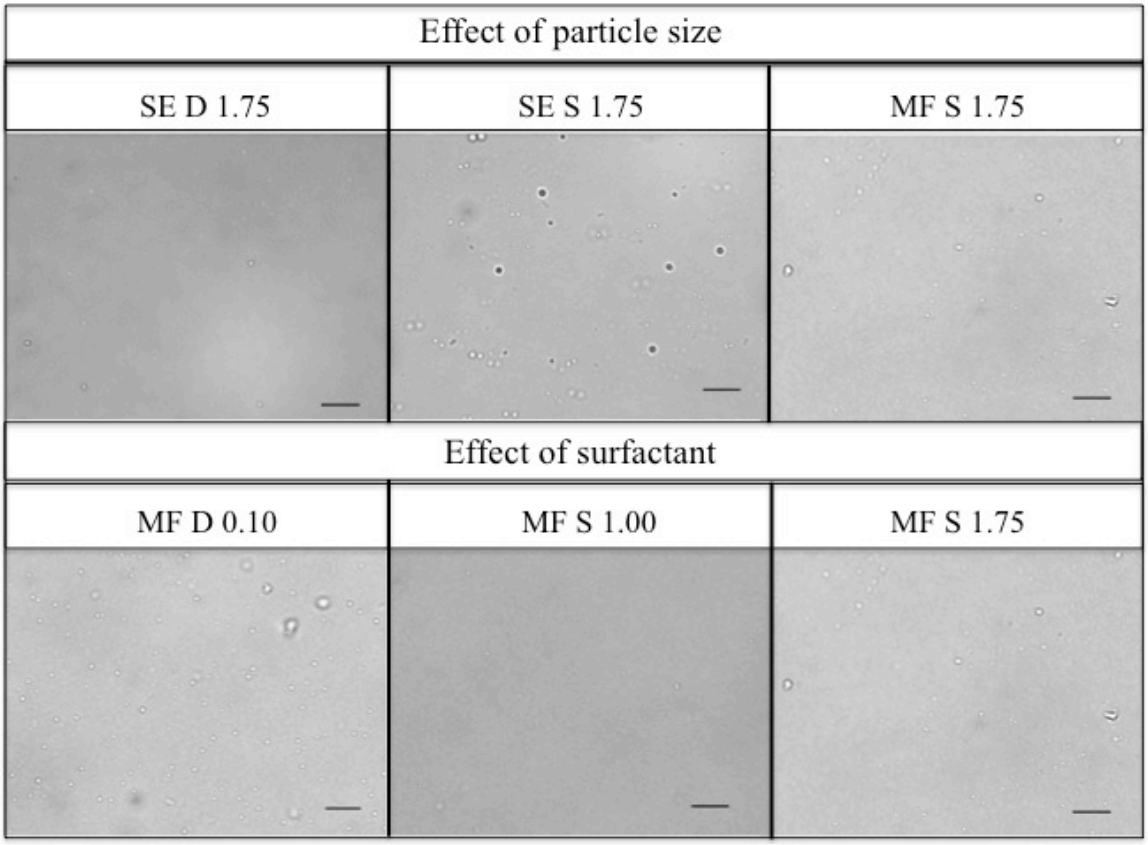
The ζ-potential was measured throughout emulsion storage to provide some indirect information about changes in interfacial properties (data not shown). The charges of the emulsions were initially between around -1 and -1.5 mV, which can be attributed to the fact that the emulsions were stabilized by a non-ionic surfactant, and that any free fatty acid impurities will be protonated under acidic conditions. Haahr et al. (144) reported similar values for emulsions prepared from medium chain triglycerides (MCT), FO, Tween 80, and buffer solution (10 mM acetate-imidazole buffer, pH 3). The droplet charge of the emulsions prepared in our study became slightly more negative after 14 days storage, which may have occurred due to the generation of some free fatty acids caused by hydrolysis of the triglyceride molecules during storage. Emulsion MF S 0.10 had the lowest concentration of free surfactant and a slightly more negative charge (-1.5 and -2.0 mV on days 0 and 14, respectively) than the other emulsions (-0.9 and -1.2 mV), which may have been because the surfactant micelles in these emulsions contributed to the signal used to calculate the ζ-potential.

Microscopic images of the emulsions on days 0 and 14 were used to provide further information about their structural properties (**Figure 11**). Initially, there was no

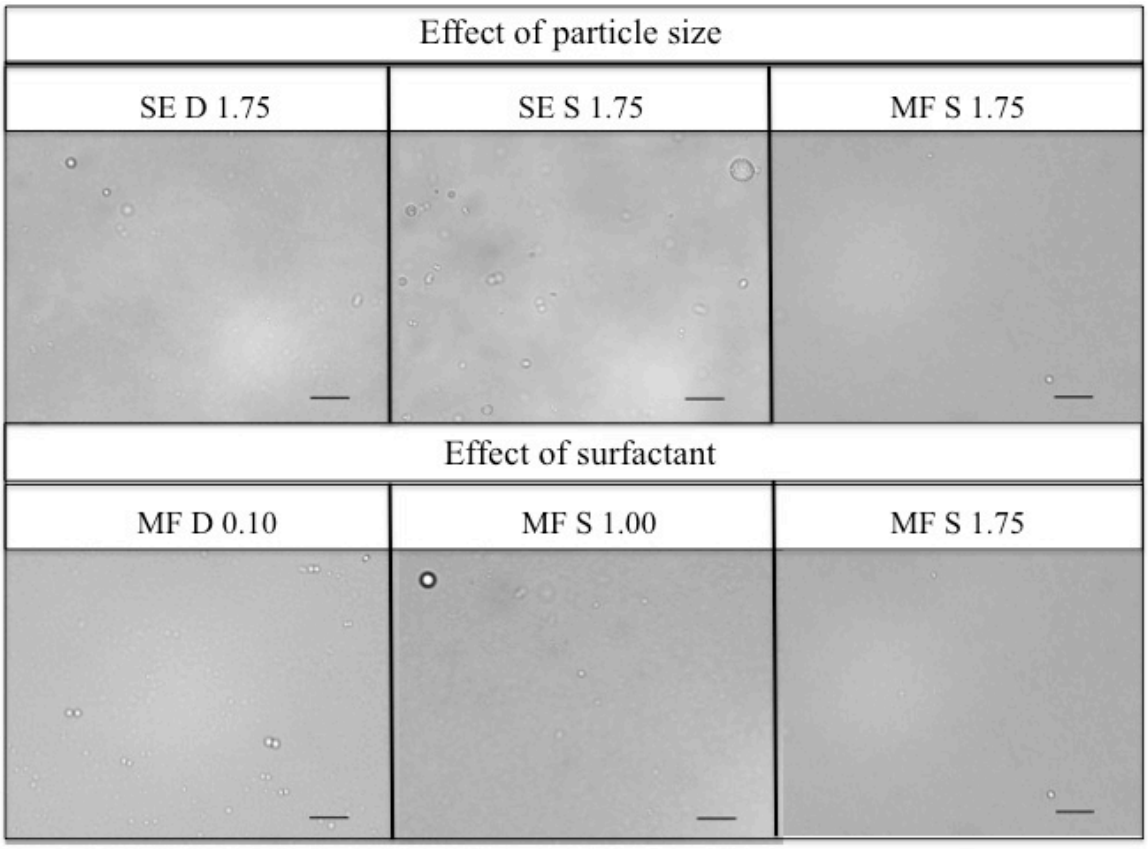
evidence of any visible particles in any of the samples, which is to be expected given the small particle sizes of the emulsions. Nevertheless, there was evidence of some multiple emulsions within the SE S 1.75 emulsion on day 14. These results suggest that some complex, non-equilibrium structures were formed in the emulsions when excess surfactant was added to the SE D 1.00 emulsion. In addition, transparent fragments were observed in the SE S 1.75 and SE D 1.75 emulsions (**Figure 11c**). We also attempted to study these fragments using cross-polarized optical microscopy but they did not polarize light and remained dark against the black background (data not shown), meaning that they are isotropic and not anisotropic liquid crystalline structures (79, 145). At this time, it is unclear what these fragments may be and further investigation is needed. Nevertheless, the microscopy images clearly show that different structures are present in emulsions that have similar overall compositions (SOR = 1.75), which highlights the importance of the preparation method used on their structural and physicochemical properties.

Figure 11. Microscopic images of emulsions made by MF and SE methods and held at 55 °C with added iron at (A) day 0 and (B) day 14. (C) shows enlarged sections of abnormalities found in the emulsions on day 14. All emulsions were diluted to 1% wt total oil phase. Images were taken at 60x magnification. The black bars at the bottom of each picture are the scales for 10 μm.

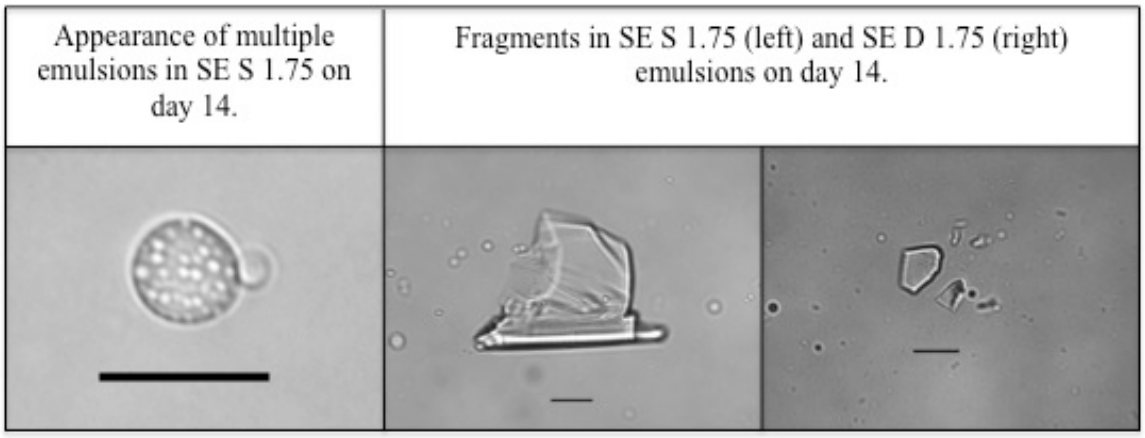
A



B


Effect of particle size
SE D 1.75
SE S 1.75
MF S 1.75
Effect of surfactant
MF D 0.10
MF S 1.00
MF S 1.75

C


Appearance of multiple emulsions in SE S 1.75 on day 14.
Fragments in SE S 1.75 (left) and SE D 1.75 (right) emulsions on day 14.

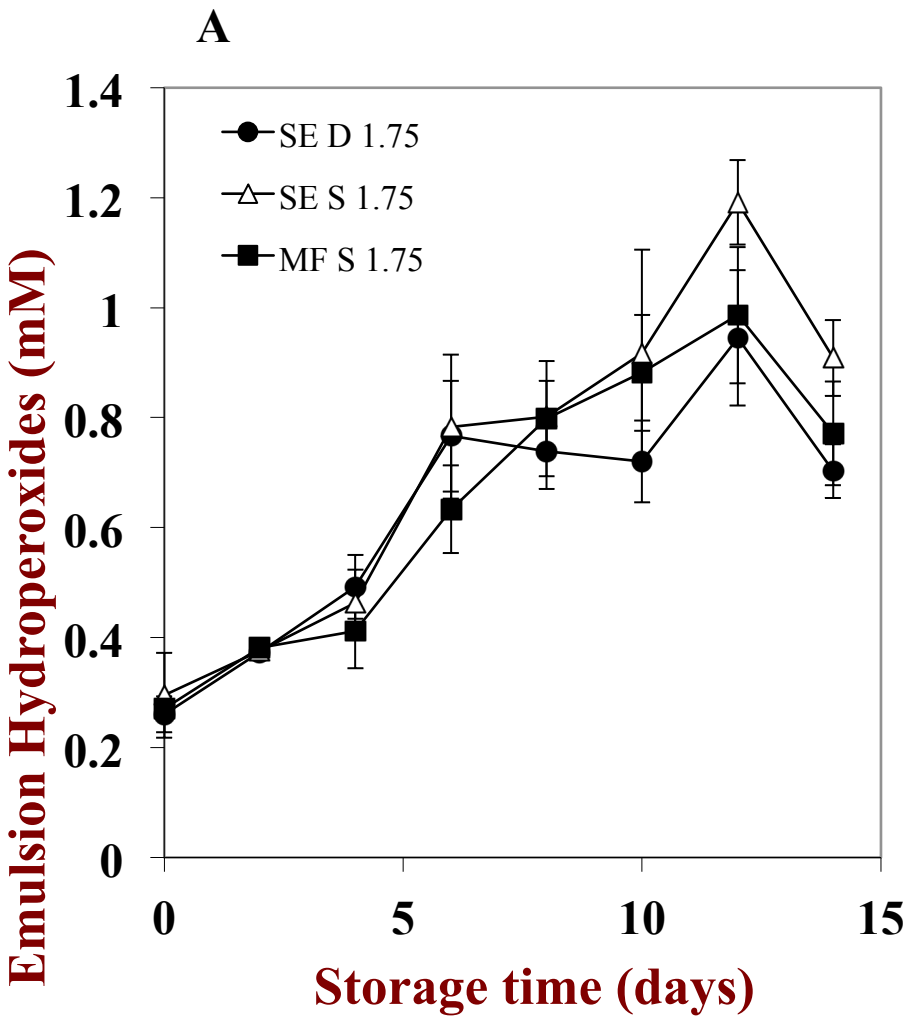
3.4.2.2. Oxidative stability of emulsions

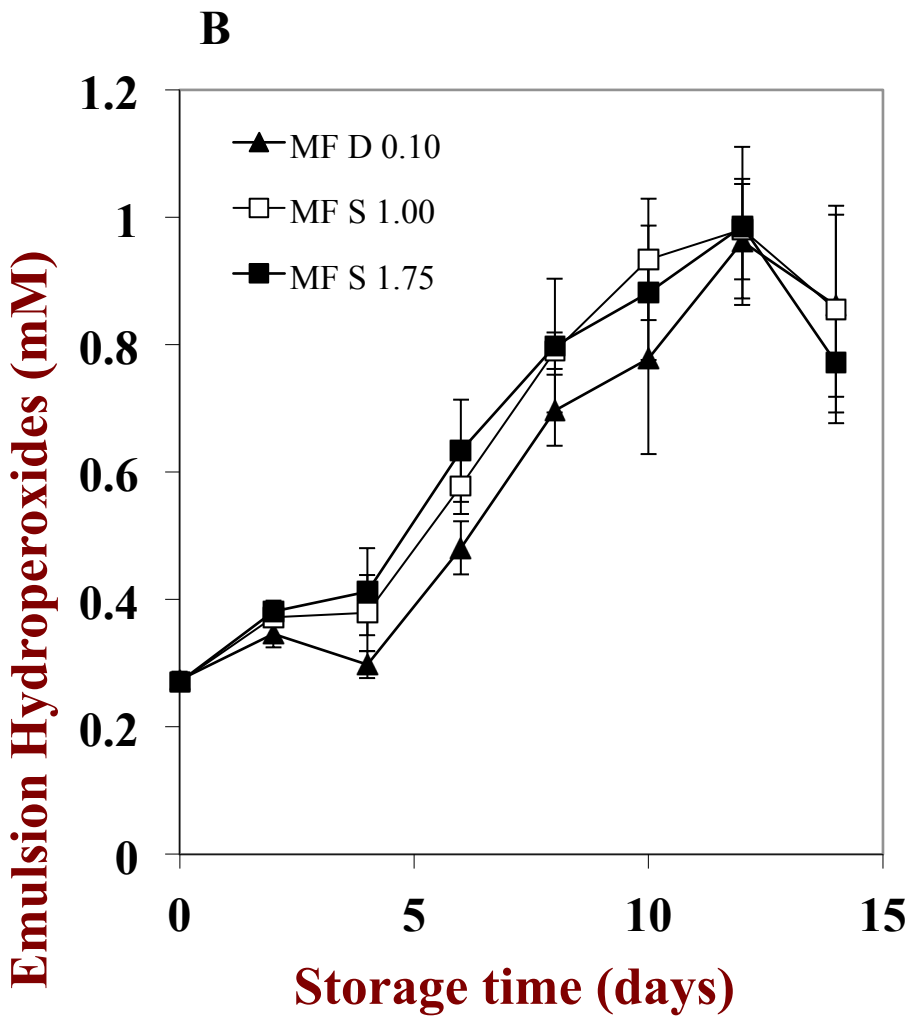
Lipid oxidation in emulsions typically occurs at the oil-water interface due to the interaction of free radicals with unsaturated lipids within the droplets (15, 85). In these systems, lipid oxidation is usually catalyzed by transition metals, such as iron, which are also responsible for accelerating lipid oxidation by decomposing hydroperoxides into free radicals (85, 146). The rate of lipid oxidation in emulsions is therefore dependent on the relative location of lipid substrates (polyunsaturated fatty acids) and pro-oxidants (transition metals and hydroperoxides) in the system (85). In the current study, lipid hydroperoxide levels and TBARS were measured to monitor the primary and secondary oxidation products of the emulsions throughout a 14-day oxidation study.

Influence of initial particle size: In this series of experiments, we compared the oxidation in emulsions that had similar surfactant levels (SOR = 1.75), but different initial particle sizes and preparation methods. Overall, the time-dependence of lipid oxidation in all of the emulsions was fairly similar, irrespective of the preparation method, with all of the emulsions reaching a peak in hydroperoxide levels after 12 days storage (**Figure 12a**). However, the low-energy emulsions with added surfactant (SE S 1.75) did have a slightly higher hydroperoxide value than the other emulsions towards the end of the incubation period. Interestingly, the SE S 1.75 emulsions also had a greater particle size (236 nm) than the SE D 1.75 (192 nm) and MF S 1.75 (212 nm) emulsions at these storage times. We had expected a higher rate of oxidation in emulsions with smaller particle sizes because of the greater surface area of the oil droplets, which would allow for more oxidation reactions to occur at the droplet surface. However, it is also possible for participants in the lipid oxidation reaction to partition into the surfactant

micelles, which would be expected to alter the lipid oxidation rate. In addition, there may have been only a limited number of reactants in the system and all of them may already have been at the droplet surface (85, 147).

Figure 12. Effect of (A) particle size and (B) surfactant on hydroperoxide values for emulsions made by MF and SE methods and held at 55 °C with added iron for 14 days. All emulsions were diluted to 1% wt total oil phase.



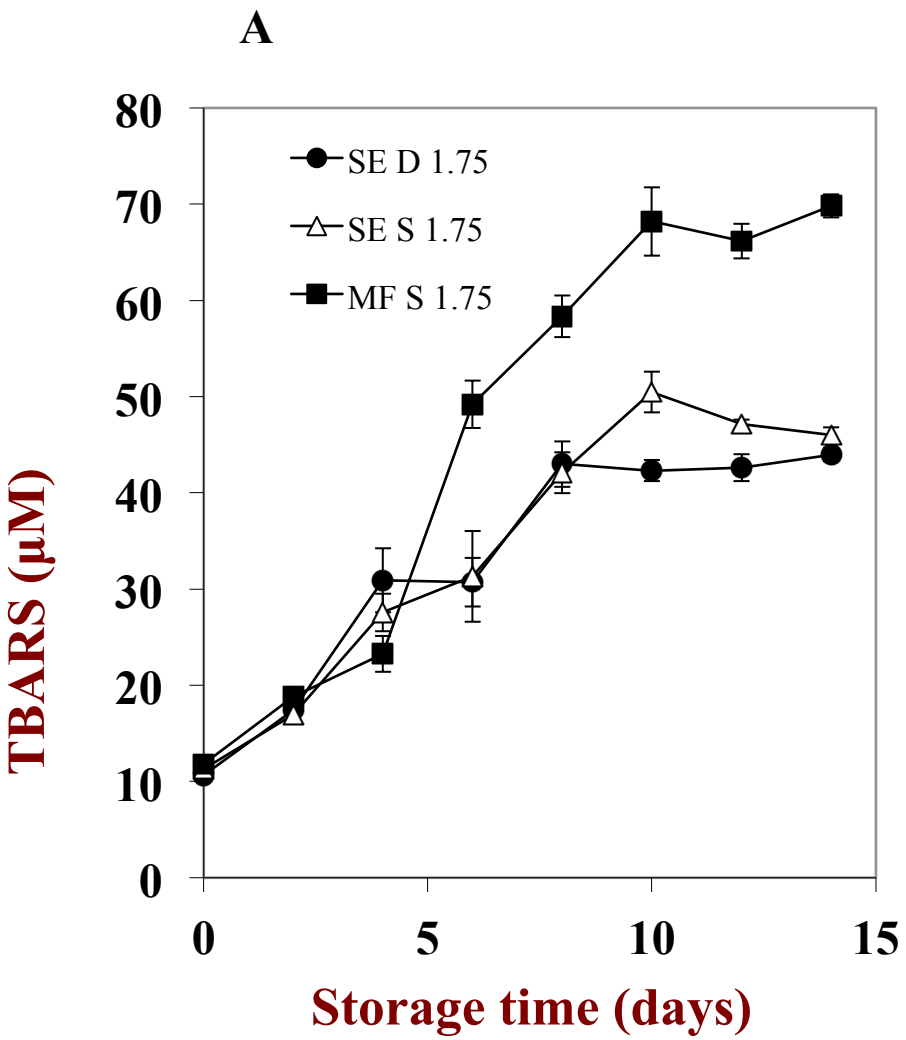


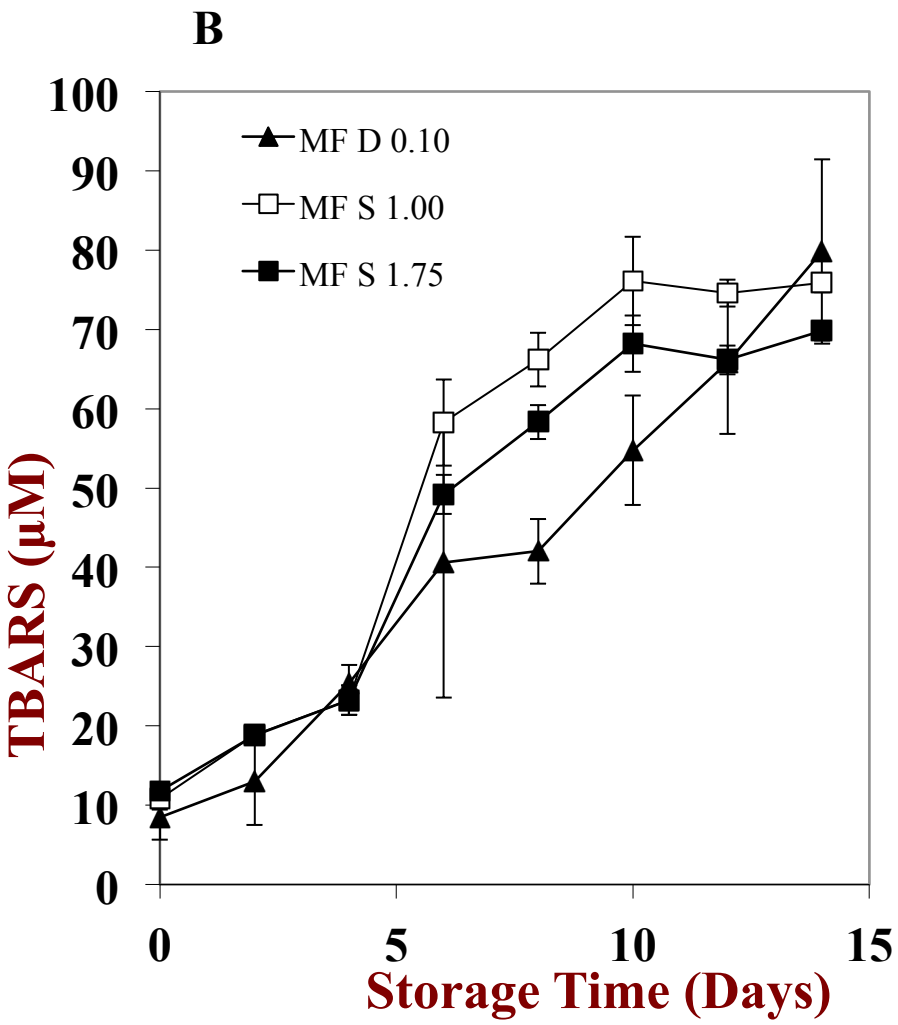
Previous researchers have also reported different dependences of lipid oxidation on particle size. Studies of oxidation in protein-stabilized corn oil-in-water emulsions found that particle size did not have a major impact on their oxidative stability, but emulsifier type did (148). Studies with fish oil-in-water emulsions reported that oxidation occurred more quickly in smaller droplets, but this effect could also have been due to the fact that different emulsifier types were used (144). Another study using fish oil-in-water emulsions also reported that particle size was not a major factor influencing the rate of oxidation, when compared to other factors such as emulsifier type (149).

Overall, these findings suggest that droplet surface area is not a major factor impacting the chemical stability of encapsulated polyunsaturated lipids.

As for secondary oxidation products, emulsion MF S 1.75, which had the largest initial particle size (166 nm) but ended with an intermediate particle size (226 nm), reached the highest TBARS value within the 14-day study compared to the SE emulsions (SE D 1.75 and SE S 1.75) (**Figure 13a)**. Based on these results, the fabrication method may have a larger impact on the secondary oxidation products than the emulsion particle size. The lower concentration of secondary oxidation products in the low-energy emulsions suggests a slower lipid oxidation rate or inhibition of hydroperoxide degradation.

Figure 13. Effect of (A) particle size and (B) surfactant on TBARS for emulsions made by MF and SE methods and held at 55 °C with added iron for 14 days. All emulsions were diluted to 1% wt total oil phase.





Influence of surfactant concentration: All low-energy emulsions (SE D 1.00 and SE S 1.75; data not shown) and high-energy emulsions (MF D 0.10, MF S 1.00 and MF S 1.75) reached similar peak hydroperoxide values on day 12 (**Figure 12b)**. The SE emulsions followed a similar TBARS trend but SE D 1.00 had higher TBARS values than SE S 1.75 starting on day 2. In addition the MF D 0.10 emulsion reached a peak TBARS value on day 14 while the emulsions with added surfactant (MF S 1.00 and MF S 1.75) had high TBARS values on day 10, which plateaued through day 14 (**Figure 13b**).

It was expected that the higher concentration of surfactant would have slowed down the rate of oxidation in the emulsions. Surfactants affect many characteristics of an emulsion including droplet charge, interfacial layer thickness, and permeability, which in turn influence accessibility of pro-oxidants, free radicals, and oxygen to the droplet lipids (17, 88). Surfactant can be found surrounding the oil droplets in the emulsion and as surfactant micelles in the aqueous phase when excess surfactant is used (85). In this case, the non-ionic surfactant produced minimal particle charge and because of that, it is not expected to have impacted the lipid oxidation greatly. Surfactant at the interfacial layer can also form a physical (steric) barrier between pro-oxidants and the lipid phase (17, 92). The effectiveness of the interfacial layer relies on its thickness, which is dependent on surfactant's head group. Brij 700 has a larger head group than Brij 76 and was found to be more effective at decreasing the rate of oxidation than Brij 76 (92).

Tween 80 has a low critical micelle concentration (CMC; <0.1 mM), a point where the surface of the lipid particles are saturated with surfactant and the excess surfactant forms surfactant micelles (150). Surfactant micelles have been shown to decrease lipid oxidation through multiple mechanisms (151). One such way is by solubilizing iron or hydroperoxides as a means to decrease pro-oxidants in the emulsion (152, 153). Secondly, surfactants, including Tween 20, have been shown to increase the solubilization of antioxidants into the aqueous phase of the emulsion (154). Lastly, it has also been hypothesized that tocopherols in FO can form surfactant co-micelles, which act as a reservoir for the antioxidants and they can be released as they were needed to extend the lag period of the oxidation process (154).

Despite all of the potentially positive impacts that surfactant can have on lipid oxidation in emulsions, surfactant itself can form hydroperoxides (146). The polyethers found in the hydrophilic head group of Tweens are easily oxidized and form hydroperoxides and their degradation products (146, 155, 156). Nuchi et al. showed that Tween 20 high in hydroperoxides increased the rate of oxidation in salmon oil emulsions (146). The oxidation of the surfactants themselves may have contributed to the hydroperoxides measured in the FO emulsions in our study instead of slowing down the oxidation. Additionally, the high temperature and addition of iron into the FO emulsions may have increase the oxidation rate making it difficult to find differences in the oxidation rate between the emulsions.

3.5. Conclusions

This study determined that the low-energy method of spontaneous emulsification can produce optically transparent nanoemulsions at higher SORs (≥ 1.25). These nanoemulsions were physically stable when stored at 37 °C for 14 days, but exhibited some droplet growth when stored at a higher temperature, which was attributed to coalescence caused by surfactant head group dehydration. Neither particle size nor surfactant had a major impact on the rate of lipid oxidation in the FO emulsions. These findings on physical and chemical stability suggest that spontaneous emulsification may be used to produce FO emulsions used to fortify transparent food or beverage systems.

CHAPTER 4

INFLUENCE OF CITRUS AND HERB OILS ON FISH OIL NANOEMULSION FORMATION AND OXIDATIVE STABILITY

4.1. Introduction

Sufficient consumption of omega-3 FAs has been linked to reduced mortality risks, especially for cardiovascular disease (18). The mortality risk of insufficient omega-3 consumption was responsible for an estimated 84,000 deaths in the US in 2005. Additionally, EPA and DHA, omega-3 LCPUFAs most commonly found in FO, are linked to other health benefits including brain development and the treatment of inflammatory diseases (2-5). Currently, Western cultures are greatly under consuming omega-3 FAs (2-5). This low consumption may be for many reasons including the high cost of fish, dislike of seafood by many consumers, presence of methyl mercury, and low availability in many geographical locations (19-21). The fortification of foods with fish oil, a good source of EPA and DHA, may be an effective way to increase omega-3 consumption and nanoemulsions offer a versatile platform in which to do this (34).

Nanoemulsions are a type of emulsions-based delivery system characterized by their small particle size (r < 100 nm) and thermodynamic instability (6, 7, 60, 61). They are gaining popularity because of their ease of preparation, small particle size, relatively high stability, and high bioavailability. The food and pharmaceutical industries are currently using these delivery systems to encapsulate, protect, and control the release of bioactives (17, 58, 62).

Despite the many benefits of nanoemulsions, there are still obstacles to overcome for their food fortification including lipid oxidation. Oxidation in emulsion systems usually occurs at the oil-water interface and is commonly attributed to free radicals reacting with unsaturated lipids within the droplet and forming lipid radicals (15, 85). In food products, lipid oxidation causes a variety of problems that negatively affect shelf life, safety, nutritional value, functionally, and flavor (15, 16).

Physical stability of the nanoemulsion can also be a challenge in nanoemulsions. Physically unstable emulsions can cause undesirable changes to the appearance, texture, and shelf life of the food product. Compared to conventional emulsions, nanoemulsions are more stable against gravitational separation, coalescence, and flocculation but are more susceptible to Ostwald ripening (12). Ostwald ripening in oil-in-water nanoemulsions is described as an increase in mean droplet size as a result of the diffusion of the oil phase from small droplets to larger ones (7, 11, 63).

Carrier oils offer some benefits to address these problems of oxidative and physical instability. Carrier oils are mixed with the lipophilic bioactive, in this case FO, to make up the total oil phase. Carrier oils with natural antioxidants can improve oxidative stability. In addition, when carrier oils have low water solubility, they can act as ripening retarders and improve the emulsion's resistance to Ostwald ripening (9-12).

A variety of oils can be used as carrier oils, including essential oils. Essential oils are bioactives utilized by the food and pharmaceutical industries not only for their flavor and aroma but also for their antioxidant, antibacterial, and antifungal activity (157, 158). While the monoterpenes in essential oils can oxidize (13, 14), essential oils have not exhibited pro-oxidant activity at any concentration (Misharina and others 2011).

Additionally, the flavoring capabilities of essential oils are especially beneficial for FO nanoemulsions because they can mask the FO flavor and odor, making the FO nanoemulsion fortified food product more acceptable to consumers. This paper will focus on lemon oil (LO) and thyme oil (TO) as citrus and herb oil carrier oils, respectively.

LO is widely used in the food industry and can be found in soft drinks, dairy products, candies, and baked goods (14). It is largely comprised of limonene followed by α-pinene and γ-terpinene but citral is the major flavor and aroma constituent (14, 158, 159). In emulsions, lemon oil has had positive impacts on the physical and oxidative stability (13, 160).

TO has been studied as a natural antioxidant for meat and meat products (161-163). In emulsions, it has exhibited antimicrobial and antioxidant activity (160, 164, 165). The main phenols in thyme are thymol and carvacrol, which are also largely responsible for its antioxidant activity (166). The antioxidant mechanisms of thyme oil include radical scavenging and inhibition against oxidation induced by Fe^{2+}/ascorbate and Fe^{2+}/H_2O_2 (167).

This study will focus on the potential use of essential oils as carrier oils in FO nanoemulsions produced with the high-energy method of MF. The carrier oils evaluated include MCT as a control, model carrier oil; LO as a citrus oil, which can be used in beverages or sweet food applications; and TO as an herb oil, which can be used in savory food applications. These emulsions will be evaluated based on their physical stability and susceptibility to lipid oxidation as those are common obstacles that must be overcome in omega-3 FA fortified foods (58).

4.2. Materials and Methods

4.2.1. Materials

FO (Omega 30 TG Food Grade Fish Oil (Non-GMO)) was provided by DSM Nutritional Products Ltd. (Basal, Switzerland). The oil was composed of 157 mg of EPA/g oil, 99 mg of DHA/g oil, and 326 mg of total omega-3 as triglycerides/g of oil. Ten fold LO was kindly donated by International Flavors & Fragrances Inc. (New York, NY, USA). TO was purchased from Essential7 (Golden, CO, USA). Polysorbate 80 (Tween 80), polysorbate 20 (Tween 20), sodium benzoate, barium chloride, iron (II) sulfate heptahydrate, hydrochloric acid, cumene hydroperoxide, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), 1,1,3,3-tetraethoxypropane (TEP), sodium carbonate, Folin & Ciaocatleu reagent, and gallic acid were purchased from Sigma-Aldrich Co. (St. Lois, MO, USA). Citric acid, 1,2-propanol, isooctane, butanol, and methanol were purchased from Fisher Scientific (Waltham, MA, USA). Trichloroacetic acid (TCA) and ammonium thiocyanate were purchased from Acros Organics (Geel, Belgium). Ethanol was purchased from Phamco-AAPER (Brookfield, CT, USA). All solvents and reagents were of analytical grade or higher. Double distilled water was used to prepare all solutions.

4.2.2. Emulsion preparation

All emulsions were prepared by microfluidizer (MF) with a total oil phase of 5 wt%. For emulsions with mixtures of FO and carrier oil, FO (1.25-3.25 wt%) and carrier oil (1.25-3.25 wt%) were mixed for 15 min at 750 rpm. Buffer (0.8% citric acid, 0.08% sodium benzoate, pH 3.0) simulating an acidic beverage system was mixed with Tween 80 (1.5 wt%) for 30 min at 750 rpm. The oil and aqueous phases were combined and

mixed with a hand mixer (Bamix ESGE Ltd, Swtizerland) for 2 min to form a course emulsion. Samples were passed through a MF (Microfluidics M-110P, Westwood, MA, USA) 5 times at 20,000 PSI. Each formulation was produced in duplicate. All samples were stored in amber glass bottles at 20 °C in the dark for 28 days for physical stability evaluation.

4.2.3. Particle size measurements

The particle size (Z-average), particle size distribution (PSD), and polydispersity index (PDI) of all emulsions, except the 100% TO emulsion, was measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) because of their smaller droplet size (d < 500 nm). The mean particle diameter (d_{32}) and PSD of the 100% TO emulsion was measured by static light scattering (Masterszier 2000, Malvern Instruments, Malvern, UK) because of its larger droplet size (d > 500 nm). All samples were measured in duplicate.

4.2.4. Visual observations

The influence of carrier oil on the macroscopic appearance of the emulsions was documented using a digital camera (Nikon CoolPix L4, Melville, NY, USA) for both the physical and oxidative stability studies. During the oxidation experiments, emulsions were observed using optical microscopy on the first and last day of measurements (Nikon Eclipse 80i, Nikon Instrument Inc., Melville, NY).

4.2.5. Oxidation measurements

2.5.1. Lipid hydroperoxides

Emulsions were held in amber glass bottles and incubated in the dark at 20 °C for 42 days. Measurements were taken every 3 days. Lipid hydroperoxides were measured using a method adapted from Shantha and Decker (136). An extraction of the emulsion lipids was performed by adding the emulsion (0.3 mL) to a mixture of isooctane/2-propanol (3:1 v/v; 1.5 mL) and vortexing it 3 times for 10 s, followed by centrifugation (1000 *g*) for 2 min. The top layer (0.2 mL) was mixed with methanol/1-butanol (2:1 v/v; 2.8 mL), followed by the addition of 3.94 M ammonium thiocyanate/ferrous iron solution (1:1 v/v; 30 μL). The ferrous iron solution was prepared by mixing equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$. The absorbance was measured at 510 nm after 20 min using an UV- visible spectrophotometer (Ultraspec 3000 *pro*, Biochrom Ltd, Cambridge, UK). Hydroperoxides were calculated as mM cumene hydroperoxide using a cumene hydroperoxide standard curve (0-0.5 mM) and all data was normalized based on the concentration of day 0. All samples were measured in triplicate.

4.2.5.2. Thiobarbituric acid-reactive substances (TBARS)

TBARS samples were held in amber glass bottles at 20 °C in the dark for 42 days. Samples were measured every 3 days using the method of McDonald and Hultin (137). TBA reagent (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, and 0.25 M HCl with 2% BHT in ethanol solution; 2.0 mL) and emulsion (1.0 mL) were combined and vortexed in glass test tubes with screw caps. The tubes were placed in a water bath (90 °C) for 15 min, and then cooled in a water bath (room temperature) for 10 min. The tubes were centrifuged (1000 *g*) for 15 min, and left to sit for 10 min. The absorbance of

the supernatant was measured at 532 nm (Ultraspec 3000 *pro*, Biochrom Ltd, Cambridge, UK). Concentrations of TBARS were calculated as μM using a standard curve of TEP (0-20 μM) and all data was normalized based on concentrations of day 0. All samples were measured in triplicate.

4.2.6. Total phenolic content

4.2.6.1. Extraction of phenolic compounds

Phenolic compounds were extracted from the LO and TO using an adapted method from Rombaut et al. (168). Tween 20 (0.2 g) and oil (5g) were mixed with methanol/water (4:1 v/v; 10 mL) for 5 min at 400 rpm. The mixture was sonicated in an ultrasonic bath for 15 min, and then mixed for another 5 min. The mixture was centrifuged (2,000 *g*) for 20 min. The supernatant was removed and the remaining oil phase was re-extracted using the described method above. The supernatants from both extractions were combined and stored at 4 °C in the dark for further analysis. Each oil was extracted in duplicate.

4.2.6.2 Folin-Ciocatleu assay

Total phenolic content of the oil extracts was determined (168, 169). Folin-Ciocatleu reagent/water (9/1 v/v; 1 mL) was added to 0.2 mL extract. After 5 min, sodium carbonate solution (7.5% in water m/v; 0.8 mL) was added and the mixture was vortexed. The samples were placed in a water bath (50 °C) for 10 min then cooled for 5 min. The absorbance was measured at 750 nm (Ultraspec 3000 *pro*, Biochrom Ltd, Cambridge, UK). Gallic acid (0- 80 mg/L) in methanol/water (4:1 v/v) was used to make

a standard curve. Results are expressed as g gallic acid equivalents (GAE) per kg oil. All samples were measured in triplicate.

4.3. Experimental design and data analysis

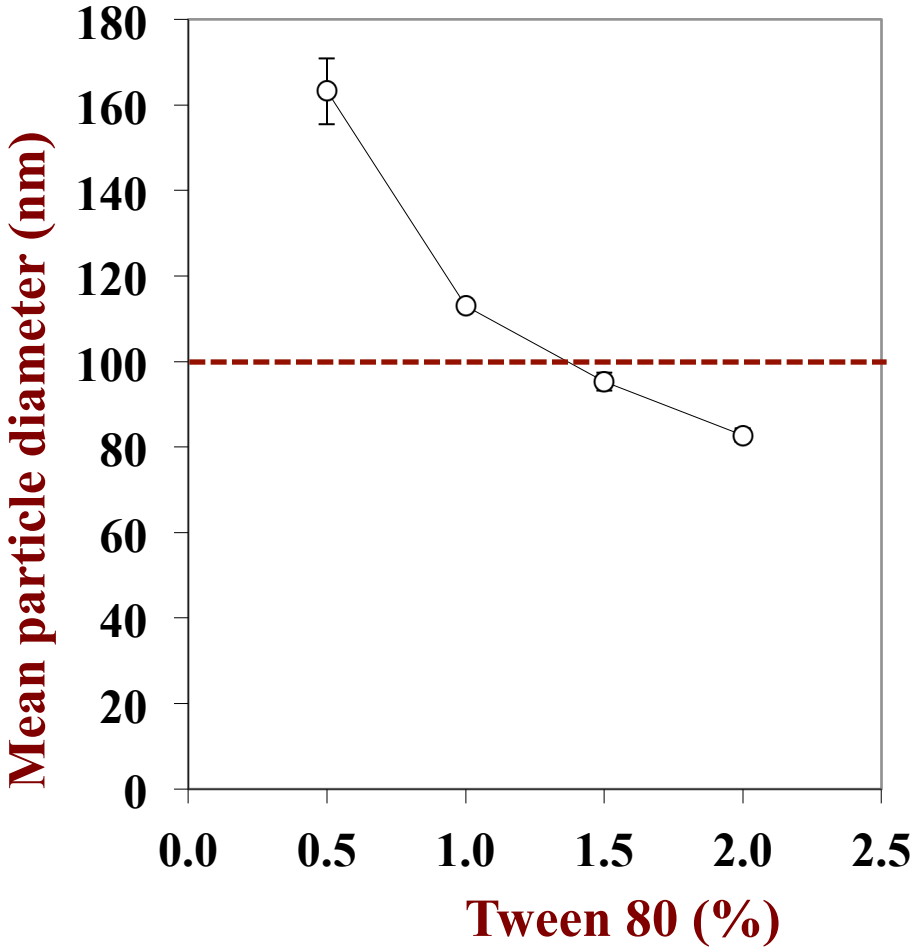
All measurements were made in duplicate or triplicate and results are stated as mean ± standard deviation. Statistical analysis was carried out with Minitab 17 software (Minitab Inc., State College, PA, USA) by analysis of variance (ANOVA) and Tukey test with a confidence interval of 95%.

4.4. Results and discussion

4.4.1. Determination of surfactant concentration

The effect of surfactant concentration on the mean particle diameter of FO nanoemulsions was evaluated in order to determine the optimal Tween 80 concentration to use throughout the subsequent studies. Surfactant concentrations of 0.5, 1.0, 1.5, and 2.0 wt% were evaluated in FO nanoemulsions produced with 5 wt% total oil phase (50 wt% FO and 50 wt% MCT). As the surfactant concentration increased, the mean particle diameter of the nanoemulsions decreased significantly ($p < 0.05$) (**Figure 14**). Nanoemulsions with surfactant concentrations of 1.5 and 2.0 wt% had mean particle diameters below 100 nm (95 and 82 nm, respectively). Based on these results, we chose to produce all remaining emulsions for this study with 1.5 wt% surfactant since it was the lowest concentration at which the mean particle diameter was below 100 nm.

Figure 14. Effect of surfactant concentration on mean particle diameter of fish oil nanoemulsions with 5% wt total oil phase (50% wt FO and 50% wt medium chain triglyceride).



4.4.2. Physical stability

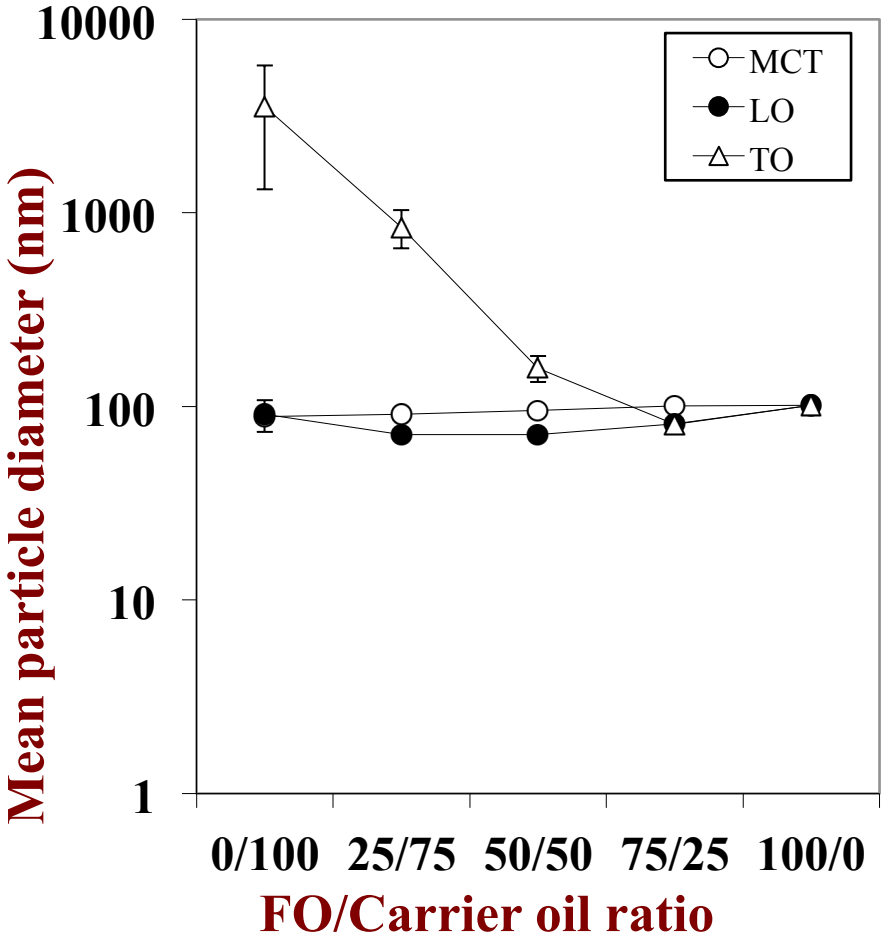
The effect of carrier oil type and ratio of FO to carrier oil on the physical stability of FO nanoemulsions was evaluated. FO nanoemulsions were producing using a MF with 5 wt% total oil phase and 1.5 wt % Tween 80 as the surfactant. FO/carrier oil ratios evaluated were 25/75, 50/50, 75/25. The carrier oils used were MCT as a control, LO as an essential oil for beverage or sweet food application, and TO as an essential oil for savory food application. Emulsions were also made with the FO and each of the carrier oils as the total oil phase (referred to as 100% FO, 100% MCT, 100% LO, or 100% TO).

These emulsions were then held at 20 °C in the dark for 28 days to evaluate their physical stability.

4.4.2.1. Mean particle diameter

Carrier oil type had a large impact on the particle size of these emulsions (**Figure 15**). For the emulsions made with MCT as the carrier oil, the 100% MCT nanoemulsion had the smallest mean particle diameter (89 nm) compared to the 100% FO nanoemulsion (101 nm) and FO/MCT nanoemulsions (91-101 nm). For these emulsions, as the amount of FO in the emulsion increased, the mean particle diameter increased. In contrast, the 100% LO nanoemulsion had a larger mean particle diameter (91 nm) than all of the FO/LO nanoemulsions (72-82 nm) and the 100% FO nanoemulsion had the largest mean particle diameter (101 nm). Again, as the amount of FO increased, the mean particle diameter increased. The TO emulsions saw the largest difference in mean particle diameters amongst themselves. The 100% TO emulsion was significantly larger than all other emulsions ($p < 0.05$). The 100% TO emulsion and 25/75 FO/TO emulsion did not form nanoemulsions (d < 200 nm) with mean particle diameters of 3,549 and 844 nm, respectively. The 75/25 FO/TO nanoemulsion had the smallest mean particle diameter (81 nm) out of all of the TO emulsion.

Figure 15. Effect of fish oil to carrier oil ratio on mean particle diameter after production (day 0). X/Y indicates the % Fish oil (FO)/% Carrier oil that made up the total oil phase. Key: MCT: medium chain triglyceride; LO: lemon oil; TO: thyme oil.



A previous study also reported a large particle size (d > 7,000 nm) for a 100% TO emulsion, which they attributed to rapid Ostwald ripening in the system (164). The researchers were able to decrease the particle diameter by the addition of oils (MCT and corn oil) that acted as ripening inhibitors. At MCT and corn oil concentrations up to 60 and 70 wt% of the total oil phase, respectively, the mean particle diameter of the emulsions decreased, compared to the 100% TO emulsion. In the case of our study, the FO acted as the ripening inhibitor since the mean particle diameter of the FO/TO emulsions decreased as the concentration of FO increased. The increase in particle size

between the 75/25 FO/carrier oil nanoemulsions and the 100% FO emulsion may be due to the effect of oil viscosity and interfacial tension on droplet disruption during high-energy fabrication of emulsions. As the oil viscosity and interfacial tension decrease, the droplet size usually decreases. In this study, the essential oils have the lowest viscosities and interfacial tensions followed by MCT and then FO (164).

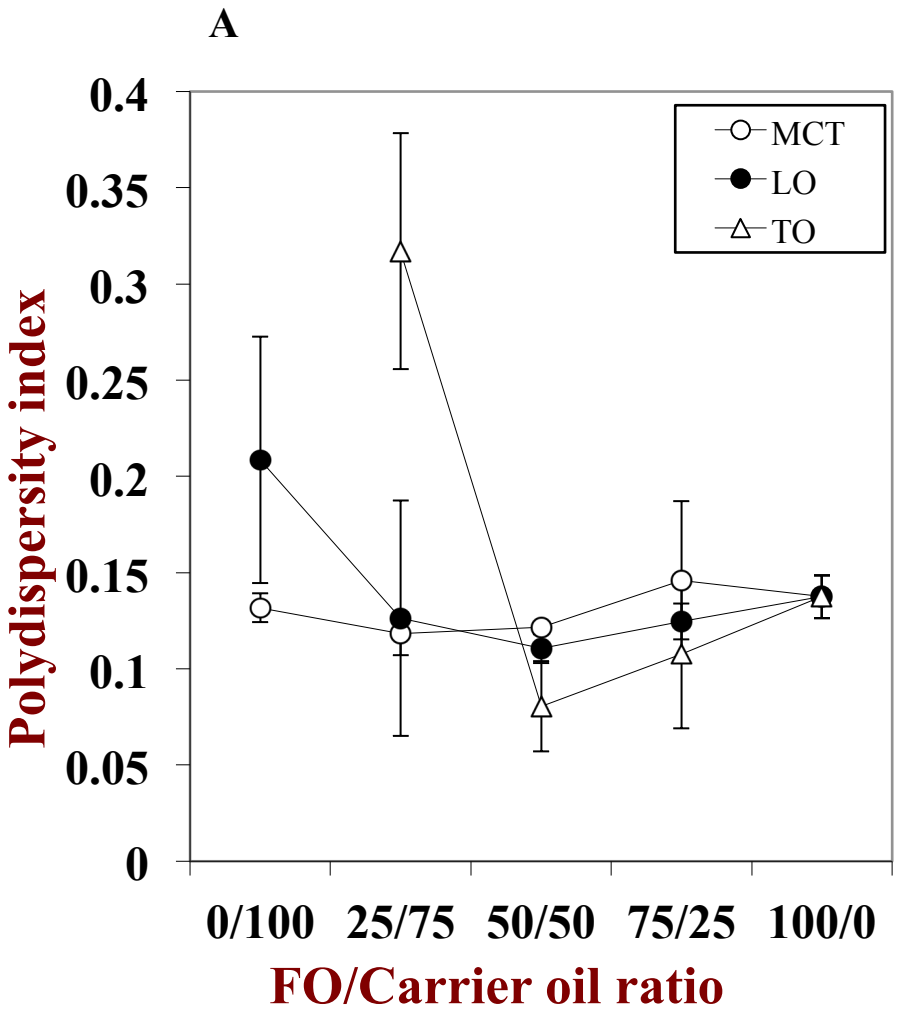
As for the stability of the emulsions over the 28-day period, the 100% FO nanoemulsion had no significant change in mean particle diameter. The nanoemulsions made with MCT were relatively stable. The 100% MCT and 25/75 FO/MCT nanoemulsions had significant increases in their mean particle diameters increasing from 89 and 91 nm to 95 and 95 nm, respectively. The nanoemulsions made with LO as the carrier oil were stable throughout the 28-day period, with minor, insignificant changes in their mean particle diameters. On the other hand, the emulsion made with 100% TO experienced significant droplet growth ($p < 0.05$), ending with a mean particle diameter of 119,295 nm, a 33 fold increase from the initial particle diameter of 3,549 nm. The 25/75 FO/TO emulsion also had increases and decreases in the mean particle diameter over time but ended with the same mean particle diameter (844 nm). The instability of the TO emulsions can be attributed to the essential oil's susceptibility to Ostwald ripening since it has a high water solubility (≈ 1 g L^{-1} for thymol at 25 °C) (164).

4.4.2.2. Polydispersity index (PDI) and particle size distribution (PSD)

The PDI (**Figure 16**) is a measure of the narrowness of the particle size distribution (**Figure 17**) with PDI values ≤ 0.1 indicating a very narrow distribution. After fabrication (day 0), only the 50/50 FO/TO emulsion had a PDI below 0.1 (**Figure 16 A**). The 100% LO nanoemulsion and the 25/75 FO/TO emulsion had the largest PDI

values of 0.21 and 0.32, respectively, due to their bimodal distributions (**Figure 17 B and C**). All other emulsions had PDI values below 0.15 nm, still indicating quite narrow distributions. The PDI values for all emulsions stayed relatively constant throughout the 28-day study (**Figure 16 B**). PSD graphs for day 28 are not shown as they are similar to those of day 0.

Figure 16. Effect of fish oil to carrier oil ratio on polydispersity index (PDI) after production (day 0; A) and on day 28 (B). No PDI data available for 0/100 TO as it was measured using static light scattering. X/Y indicates the % FO/% Carrier oil that made up the total oil phase. Key: MCT: medium chain triglyceride; LO: lemon oil; TO: thyme oil.



B

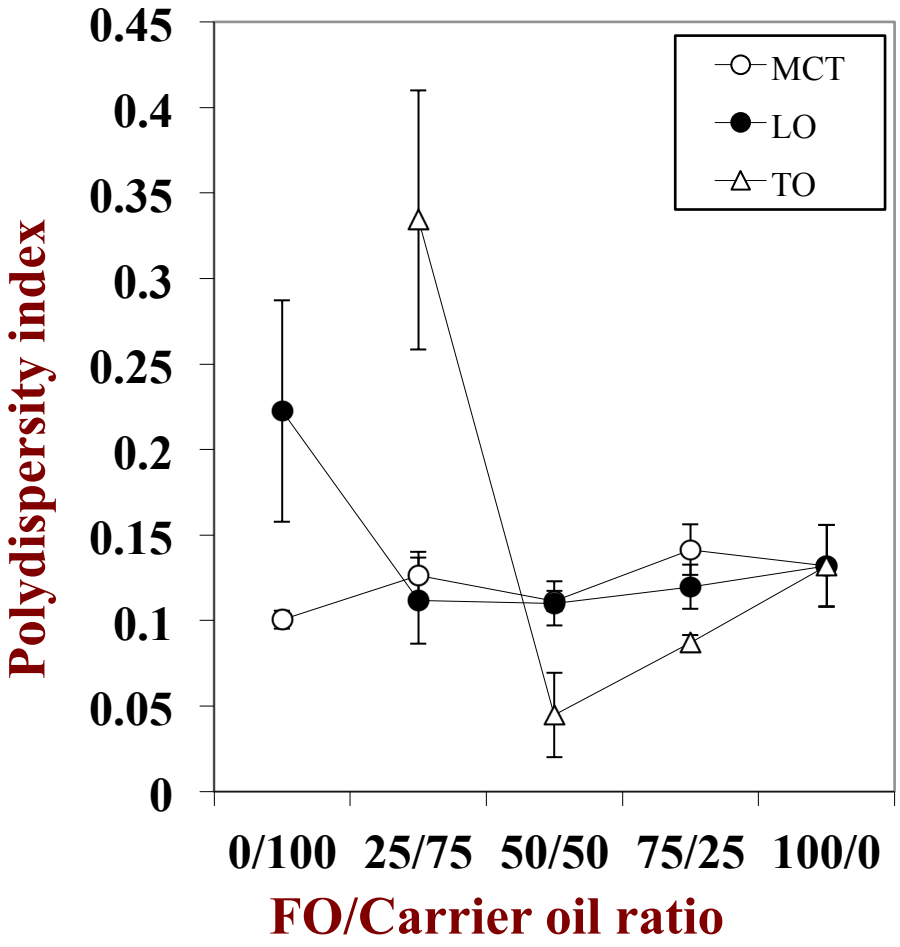
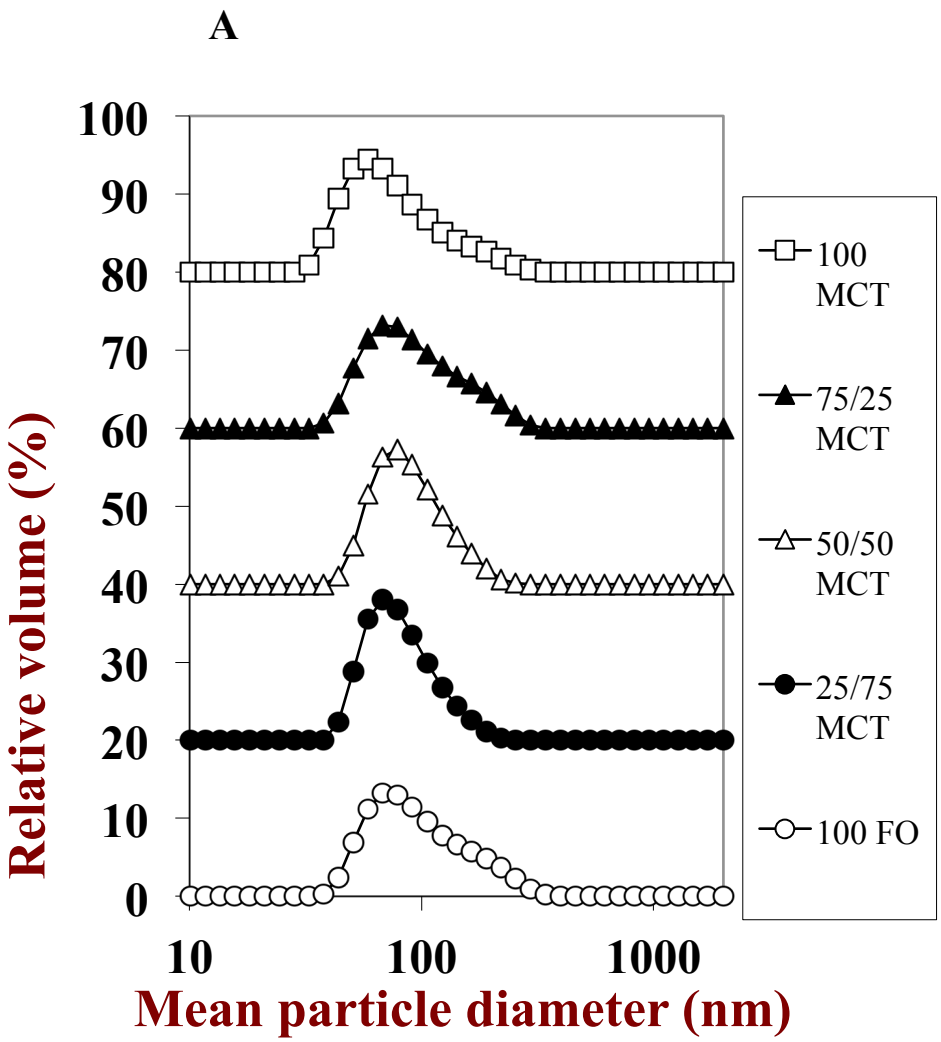
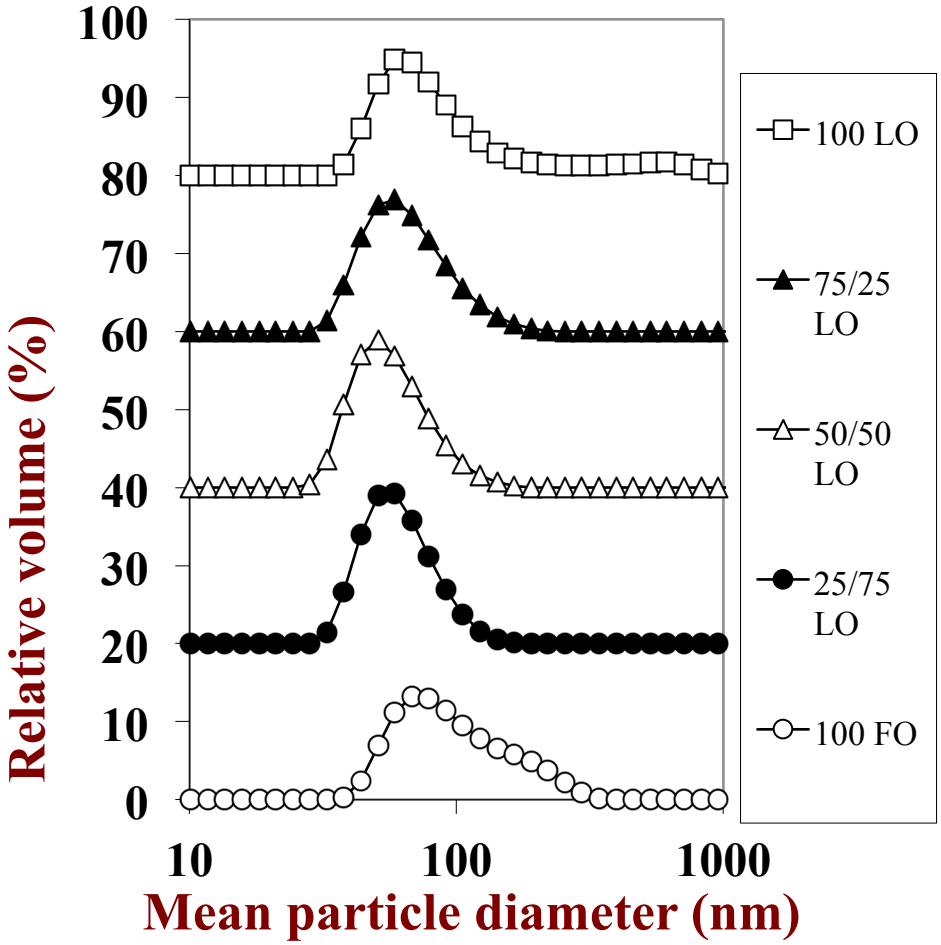
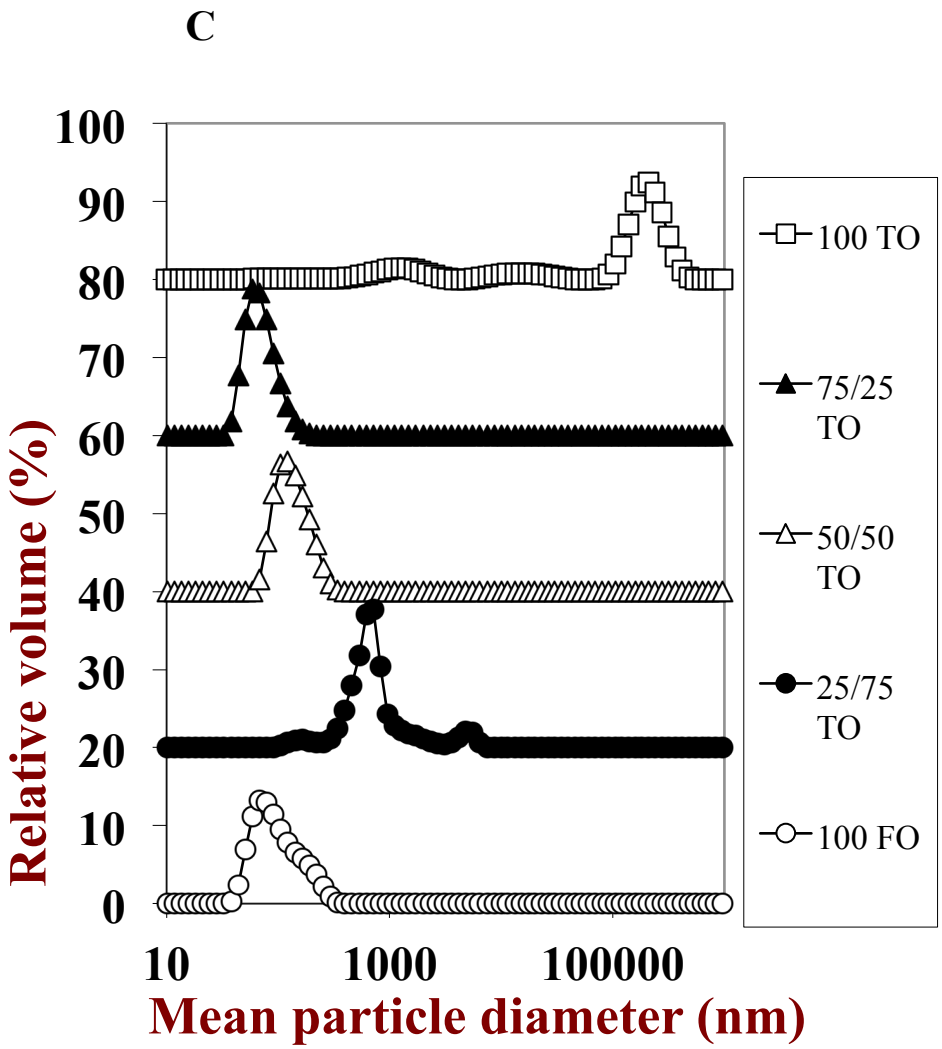

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Polydispersity index
MCT
LO
TO
0/100
25/75
50/50
75/25
100/0
FO/Carrier oil ratio

Figure 17. Effect of fish oil to carrier oil ratio on particle size distribution after production (day 0) for MCT (A), LO (B), and TO (C). X/Y indicates the % fish oil/% carrier oil that made up the total oil phase. Key: MCT: medium chain triglyceride; LO: lemon oil; TO: thyme oil.



B


100
90
80
70
60
50
40
30
20
10
0
Relative volume (%)
10
100
1000
Mean particle diameter (nm)
100 LO
75/25 LO
50/50 LO
25/75 LO
100 FO



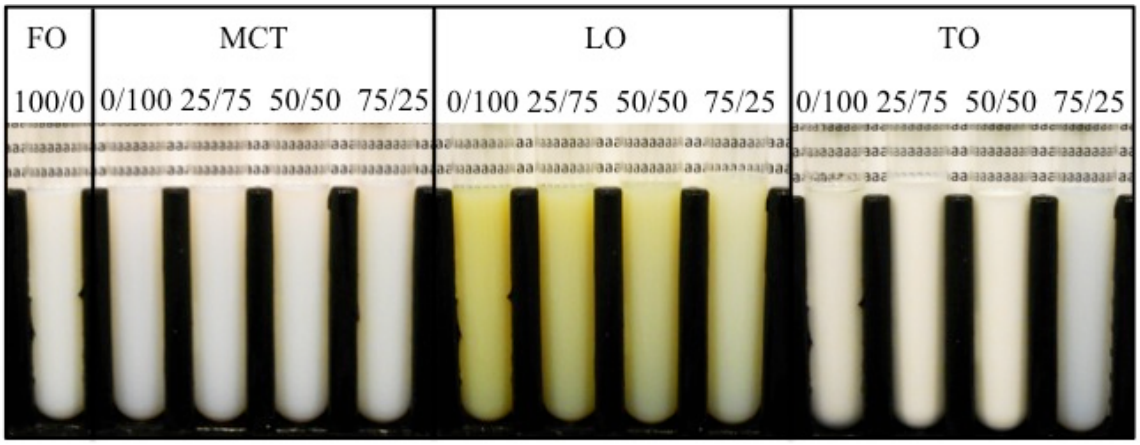
4.4.2.3. Appearance

The visual appearance of the emulsions was evaluated with images of the emulsion samples. After fabrication (day 0), all emulsions were opaque (**Figure 18**). The nanoemulsions produced with LO had a yellow tint due to its natural coloring, which increased as the percent of LO in the nanoemulsion increased, otherwise all other emulsions appeared milky white. By day 1, the 100% TO emulsion had oiling off (data not shown), which developed into a clear separation of phases, resulting in a layer of TO on top of a clear layer of buffer by day 28. This creaming and oiling off was supported by the increase in mean particle diameter. The 25/75 FO/TO emulsion also appeared to

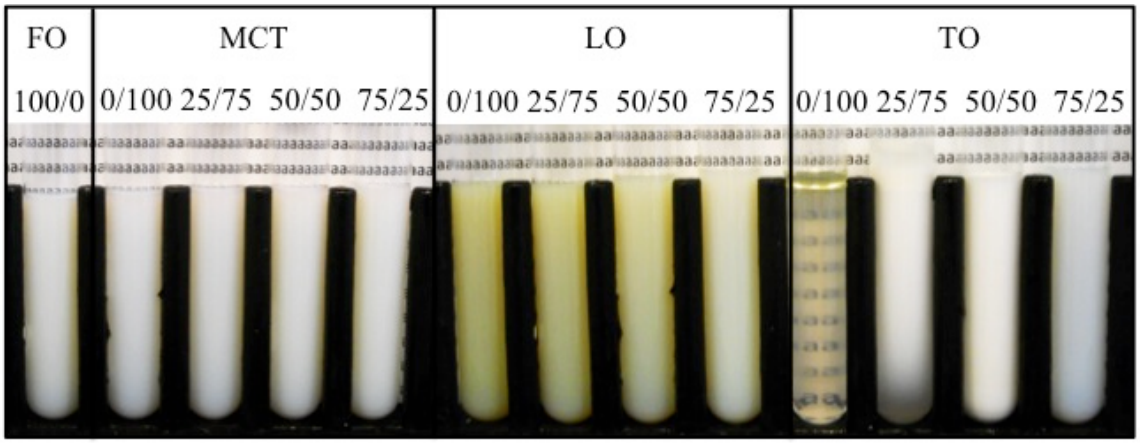
be unstable toward the end of the study since it became more transparent at the bottom of the test tube on day 28. Again, this instability is due to the TO's high water solubility and susceptibility to Ostwald ripening (164).

Figure 18. Images of emulsions made by microfluidization with different fish oil to carrier oil ratios on (A) day 0 (after production) and (B) day 28. X/Y indicates the % fish oil/% carrier oil that made up the total oil phase. Key: MCT: medium chain triglyceride; LO: lemon oil; TO: thyme oil.

A



B



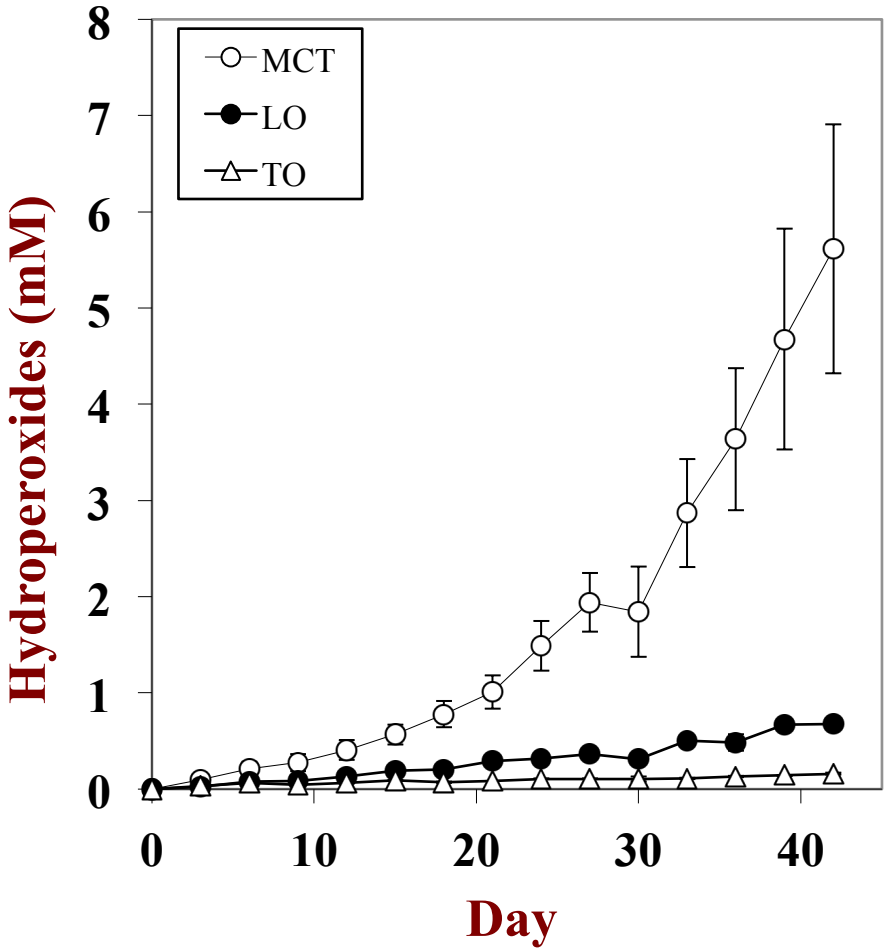
4.4.3. Oxidative stability

The effect of different carrier oils (MCT, LO, and TO) on the oxidative stability of FO nanoemulsions held at 20 °C was evaluated. FO nanoemulsions were produced with 5 wt% total oil consisting of a FO/carrier oil ratio of 75/25. This ratio was chosen based on the physical stability results in section 4.4.2 as it produced stable nanoemulsions with similar particle sizes amongst all the carrier oils while maintaining a high load of FO in the nanoemulsion. Lipid hydroperoxides and TBARS were measured to monitor the primary and secondary oxidation products in this 42-day study. The total phenolic contents of the essential oils were determined by the Folin-Ciocatleu assay. LO and TO had a total phenolic contents of 28.43 and 123.20 g GAE/kg oil, respectively.

4.4.3.1. Oxidative stability

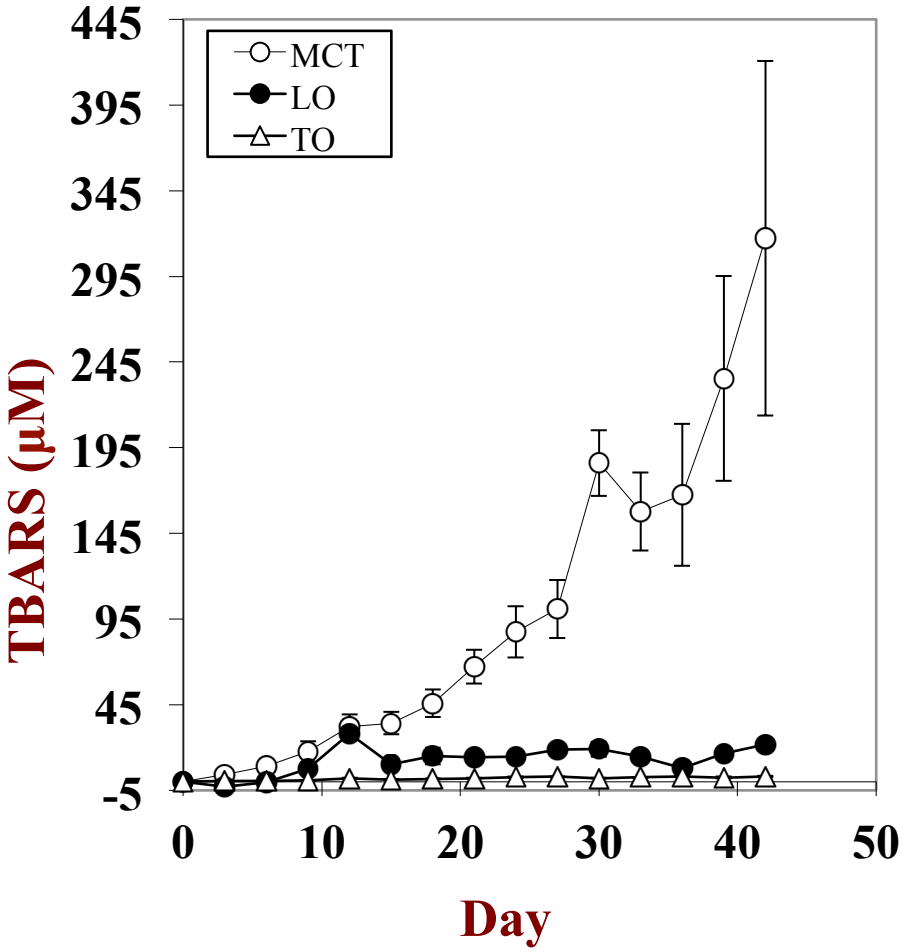
Overall, the lipid hydroperoxides were consistently higher for the FO nanoemulsion with MCT as a carrier oil compared to those made with LO and TO (**Figure 19**). On day 15, the lipid hydroperoxides for the MCT nanoemulsion were 3 and 6 fold higher than those of the LO and TO nanoemulsions, respectively. By day 42, lipid hydroperoxides of the MCT nanoemulsion were 8 and 35 fold higher than those of the LO and TO nanoemulsions, respectively. The LO began to have slightly higher lipid peroxides levels than the TO around day 12.

Figure 19. Lipid hydroperoxides of fish oil nanoemulsions made with different carrier oils at a ratio of 75/25 fish oil to carrier oil after being held at 20 °C for 42 days. Key: MCT: medium chain triglyceride; LO: lemon oil; TO: thyme oil.



This same trend was seen with the TBARS concentrations. By day 15, the TBARS for the MCT emulsion were 3 and 24 fold higher than the LO and TO nanoemulsions, respectively (**Figure 20**). At the end of the study, on day 42, the TBARS of the MCT emulsion were 15 and 99 fold higher than the LO and TO nanoemulsions, respectively.

Figure 20. TBARS of fish oil nanoemulsions made with different carrier oils at a ratio of 75/25 fish oil to carrier oil after being held at 20 °C for 42 days. Key: MCT: medium chain triglyceride; LO: lemon oil; TO: thyme oil.



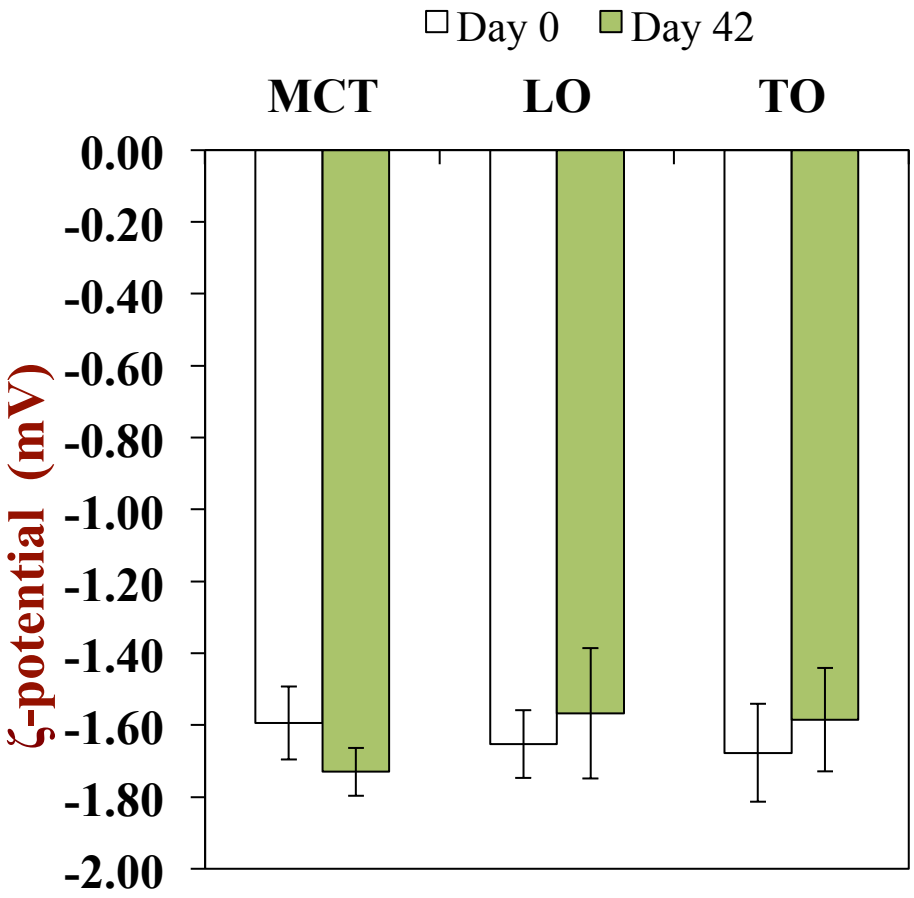
Overall, the LO and TO exhibited antioxidant behavior against lipid oxidation in the nanoemulsion systems. This may be attributed to the natural antioxidants in the essential oils. Previous studies have also demonstrated the inhibition of lipid oxidation in sunflower oil emulsions by both LO and TO and in corn oil emulsions by TO (160, 165, 166). The total phenolic content of the TO was 4 fold higher than that of the LO, which may explain why the LO nanoemulsion began experiencing an increase in lipid peroxides much earlier than the TO nanoemulsion.

4.4.3.2. Physical stability of emulsions during oxidation

The physical stability of the FO/carrier oil nanoemulsions was monitored throughout the oxidation study. The MCT emulsion had significantly larger mean particle diameters than the LO and TO nanoemulsions on day 0 and 42 ($p < 0.05$). However, the mean particle diameter of each FO/carrier oil nanoemulsion was stable for the 42 days with no significant change in size. Nanoemulsions produced with MCT, LO, and TO carrier oils had initial mean particle diameters of 101, 82, and 80 nm, respectively, and their final mean particle diameters were 102, 81, and 79 nm, respectively.

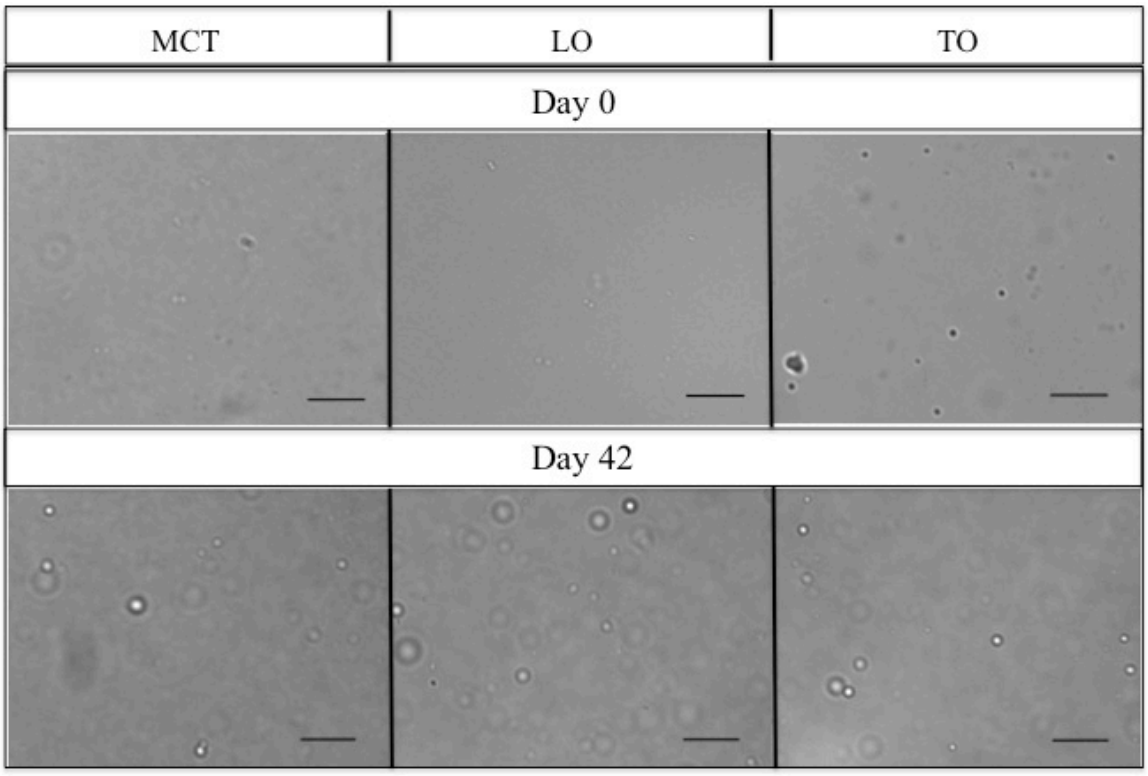
The ζ-potential for all nanoemulsions was measured on the first and last day of the oxidation experiment (day 0 and 42) in order to evaluate any changes in interfacial properties. The ζ-potentials for all nanoemulsions were only slightly negative with no significant difference in charge between them on day 0 or 42 ($p < 0.05$). The charge was also stable throughout the 42-day study (**Figure 21)**. This minimal charge was expected since Tween 80 is a nonionic surfactant. The nanoemulsion produced with MCT started with an initial ζ-potential of -1.60 mV and became slightly more negative over time, ending at -1.73 mV. In contrast, the nanoemulsions produced with LO and TO became slight more positive over time. The small charges on the emulsion particles were unlikely to have any affect on the oxidation of emulsions.

Figure 21. ζ-potential of fish oil nanoemulsions made with different carrier oils at a ratio of 75/25 fish oil to carrier oil after fabrication (day 0) and after being held at 20 °C for 42 days. Key: MCT: medium chain triglyceride; LO: lemon oil; TO: thyme oil.



Microscopic images of the nanoemulsions were taken on day 0 and 42 to further analyze their structural properties (**Figure 22**). Given the small particle size of the emulsions, there were few visible particles in the images for both days. This supports the stable mean particle size measurements previously discussed.

Figure 22. Microscope images of the fish oil nanoemulsions made with different carrier oils at a ratio of 75/25 fish oil to carrier oil held at 42 °C for 42 days. Images were taken at 60x magnification. The black bars at the bottom of each picture are the scales for 10 μm. Key: MCT: medium chain triglyceride; LO: lemon oil; TO: thyme oil.



4.5. Conclusions

This study showed that physically stable FO nanoemulsions were produced by using MCT, LO, and TO as carrier oils at different FO/carrier oil ratios. All nanoemulsions made with LO as the carrier oil were physically stable for 28 days at 20 °C. MCT and TO nanoemulsions were physically stable at ratios of 50/50 and 75/25 FO/carrier oil. At the ratio of 75/25 FO/carrier oil, LO and TO increased the oxidative stability of FO nanoemulsions as a result of their natural antioxidants. These findings suggest that LO and TO are suitable carrier oils to produce FO nanoemulsions with high physical and oxidative stability for food fortification.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Nanoemulsion food systems are a versatile platform that can be used for the incorporation of FO into aqueous food products in order to increase omega-3 FA consumption in Western cultures. Their small particle sizes can produce transparent emulsions, which can be added to aqueous beverages without minimal impact their visual appearance. Our study demonstrated that FO nanoemulsions were produced using the low-energy method of SE and high concentrations of surfactant. In addition, the rate of oxidation for FO nanoemulsions produced by low- and high-energy methods was not affected by particle size or surfactant concentration. These findings suggest that SE may be a suitable method for producing FO nanoemulsion for food fortification.

Our study also demonstrated that MCT, LO, and TO can be used as carrier oils to produce stable FO nanoemulsions at certain ratios of FO to carrier oil. FO nanoemulsions made with a 75/25 FO to carrier oil ratio had increased oxidative stability when LO and TO were used as the carrier oil. Based on these results, physically and chemically stable FO nanoemulsions can be produced with LO and TO carrier oils. The acceptability of both a citrus and herb oil may lead to the fortification of a wider variety of food products including both sweet and savory foods.

Lastly, the effect of surfactant and concentration of FO in the total oil phase on the chemical stability of FO nanoemulsions can be quite complex and should be studied further. Surfactants have been shown to negatively affect oxidation, making it important to evaluate the oxidation rate of the emulsion as well as their components. Additionally, the effect of FO/carrier oil ratios on physical stability was evaluated but its effect on

oxidative stability was not. Different concentrations of FO and types of carrier oil may have positive or negative impacts on an emulsion's susceptibility to lipid oxidation. Further studies should be conducted to try and optimize the FO to carrier oil ratio for increased oxidative stability while maintaining physical stability.

BIBLOGRAPHY

1. Gropper SAS, Smith JL, Groff JL. Advanced nutrition and human metabolism. 5 ed: Wadsworth Cengage Learning; 2009.

2. Center for Disease Control and Protections. National health and nutrition examination survey. National Center for Health Statistics; 2003.

3. Great Britain Scientific Advisory Committee on Nutrition. The nutritional wellbeing of the British population. Norwich: The Stationary Office; 2008.

4. Kris-Etherton PM, Grieger JA, Etherton TD. Dietary reference intakes for DHA and EPA. Prostaglandins, Leukotrienes and Essential Fatty Acids. 2009;81(2–3):99-104. doi: 10.1016/j.plefa.2009.05.011.

5. Papanikolaou Y, Brooks J, Reider C, Fulgoni VL. U.S. adults are not meeting recommended levels for fish and omega-3 fatty acid intake: Results of an analysis using observational data from NHANES 2003–2008. Nutrition Journal. 2014;13(1). doi: 10.1186/1475-2891-13-31.

6. Mason TG, Wilking JN, Meleson K, Chang CB, Graves SM. Nanoemulsions: formation, structure, and physical properties. Journal of Physics: Condensed Matter. 2006;18(41). doi: 10.1088/0953-8984/18/41/R01.

7. Tadros T, Izquierdo P, Esquena J, Solans C. Formation and stability of nano-emulsions. Advances in Colloid and Interface Science. 2004;108–109:303-18. doi: 10.1016/j.cis.2003.10.023.

8. Anton N, Benoit J-P, Saulnier P. Design and production of nanoparticles formulated from nano-emulsion templates—A review. Journal of Controlled Release. 2008;128(3):185-99. doi: 10.1016/j.jconrel.2008.02.007.

9. Wooster TJ, Golding M, Sanguansri P. Impact of oil type on nanoemulsion formation and Ostwald ripening stability. Langmuir. 2008;24(22):12758-65. doi: 10.1021/la801685v.

10. Li Y, Le Maux S, Xiao H, McClements DJ. Emulsion-based delivery systems for tributyrin, a potential colon cancer preventative agent. Journal of Agricultural and Food Chemistry. 2009;57(19):9243-9. doi: 10.1021/jf901836f.

11. McClements DJ. Edible nanoemulsions: Fabrication, properties, and functional performance. Soft Matter. 2011;7(6):2297-316. doi: 10.1039/C0SM00549E.

12. McClements DJ, Rao J. Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. Critical Reviews in Food Science and Nutrition. 2011;51(4):285-330. doi: 10.1080/10408398.2011.559558.

13. Rao J, McClements DJ. Impact of lemon oil composition on formation and stability of model food and beverage emulsions. Food Chemistry. 2012;134(2):749-57. doi: 10.1016/j.foodchem.2012.02.174.

14. Nguyen H, Campi EM, Roy Jackson W, Patti AF. Effect of oxidative deterioration on flavour and aroma components of lemon oil. Food Chemistry. 2009;112(2):388-93. doi: 10.1016/j.foodchem.2008.05.090.

15. Arab-Tehrany E, Jacquot M, Gaiani C, Imran M, Desobry S, Linder M. Beneficial effects and oxidative stability of omega-3 long-chain polyunsaturated fatty acids. Trends in Food Science & Technology. 2012;25(1):24-33. doi: 10.1016/j.tifs.2011.12.002.

16. Dacaranhe CD, Terao J. Effect of phosphatidic acid and phosphatidylserine on lipid oxidation in beef homogenate during storage and in emulsified sardine oil. Journal of Food Science. 2001;66(3):422-7. doi: 10.1111/j.1365-2621.2001.tb16121.x.

17. Waraho T, McClements DJ, Decker EA. Mechanisms of lipid oxidation in food dispersions. Trends in Food Science & Technology. 2011;22(1):3-13. doi: 10.1016/j.tifs.2010.11.003.

18. Danaei G, Ding EL, Mozaffarian D, Taylor B, Rehm J, Murray CJL, et al. The preventable causes of death in the United States: Comparative risk assessment of dietary, lifestyle, and metabolic risk factors. PLoS Medicine. 2009;6(4):1-23. doi: 10.1371/journal.pmed.1000058.

19. Racine RA, Deckelbaum RJ. Sources of the very-long-chain unsaturated omega-3 fatty acids: eicosapentaenoic acid and docosahexaenoic acid. Current Opinion in Clinical Nutrition & Metabolic Care March 2007. 2007;10(2):123-8. doi: 10.1097/MCO.0b013e3280129652.

20. Glanz K, Basil M, Maibach E, Goldberg J, Snyder DAN. Why Americans eat what they do: Taste, nutrition, cost, convenience, and weight control concerns as influences on food consumption. Journal of the American Dietetic Association. 1998;98(10):1118-26. doi: 10.1016/S0002-8223(98)00260-0.

21. Kennedy ET, Luo H, Ausman LM. Cost implications of alternative sources of (n-3) fatty acid consumption in the United States. The Journal of Nutrition. 2012;142(3):605S-9S. doi: 10.3945/jn.111.152736.

22. Gillies PJ, Harris WS, Kris-Etherton PM. Omega-3 fatty acids in food and pharma: The enabling role of biotechnology. Current Atherosclerosis Reports. 2011;13(6):467-73. doi: 10.1007/s11883-011-0206-z.

23. Center for Food Safety and Applied Nutrition. Labeling & nutrition - guidance for industry: A food labeling guide (12. Appendix d: Qualified health claims). 2013.

24. U. S. Department of Agriculture, U. S. Department of Health and Human Services. Dietary guidelines for Americans 2010. 7th ed. Washington DC: U.S. Government Printing Office; 2010.

25. U. S. Department of Agriculture, U. S. Department of Health and Human Services. Report of the dietary guidelines advisory committee on the dietary guidelines for Americans, 2010. USDA; 2010.

26. Hasler C, Bloch A, Thomson C. Position of the American Dietetic Association: Functional foods. Journal of the American Dietetic Association. 2004;104(5):814-26. doi: 10.1016/j.jada.2004.03.015.

27. Hudson E. Omega-3 fatty acids: The whole package. Nutraceutical Business & Technology. 2010;6(2):30-2.

28. Fraeye I, Bruneel C, Lemahieu C, Buyse J, Muylaert K, Foubert I. Dietary enrichment of eggs with omega-3 fatty acids: A review. Food Research International. 2012;48(2):961-9. doi: 10.1016/j.foodres.2012.03.014.

29. Garg ML, Wood LG, Singh H, Moughan PJ. Means of delivering recommended levels of long chain n-3 polyunsaturated fatty acids in human diets. Journal of Food Science. 2006;71(5):R66-R71. doi: 10.1111/j.1750-3841.2006.00033.x.

30. McClements DJ. Edible lipid nanoparticles: Digestion, absorption, and potential toxicity. Progress in Lipid Research. 2013;52(4):409-23. doi: 10.1016/j.plipres.2013.04.008. PubMed PMID: WOS:000328810400005.

31. Yao MF, Xiao H, McClements DJ. Delivery of lipophilic bioactives: Assembly, disassembly, and reassembly of lipid nanoparticles. Annual Review of Food Science and Technology, Vol 5. 2014;5:53-81. doi: 10.1146/annurev-food-072913-100350. PubMed PMID: WOS:000336429000004.

32. Deckelbaum RJ, Torrejon C. The omega-3 fatty acid nutritional landscape: Health benefits and sources. The Journal of Nutrition. 2012;142(3):587S-91S. doi: 10.3945/jn.111.148080.

33. Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n−3 fatty acids in humans. The American Journal of Clinical Nutrition. 2006;83(6):S1467-76S.

34. Maki KC, Yurko-Mauro K, Dicklin MR, Schild AL, Geohas JG. A new, microalgal DHA- and EPA-containing oil lowers triacylglycerols in adults with mild-to-moderate hypertriglyceridemia. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA). 2014;91(4):141-8. doi: 10.1016/j.plefa.2014.07.012.

35. Adarme-Vega TC, Lim DKY, Timmins M, Vernen F, Li Y, Schenk PM. Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. Microbial Cell Factories. 2012;11(1).

36. Lenihan-Geels G, Bishop K, Ferguson L. Alternative sources of omega-3 fats: Can we find a sustainable substitute for fish? Nutrients. 2013;5(4):1301-15. PubMed PMID: doi:10.3390/nu5041301.

37. Tur JA, Bibiloni MM, Sureda A, Pons A. Dietary sources of omega 3 fatty acids: Public health risks and benefits. The British journal of nutrition. 2012;107 Suppl 2.

38. Schuchardt JP, Hahn A. Bioavailability of long-chain omega-3 fatty acids. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA). 2013;89(1):1-8. doi: 10.1016/j.plefa.2013.03.010.

39. Kitessa SM, Abeywardena M, Wijesundera C, Nichols PD. DHA-containing oilseed: A timely solution for the sustainability issues surrounding fish oil sources of the health-benefitting long-chain omega-3 oils. Nutrients. 2014;6(5):2035-58. doi: 10.3390/nu6052035. PubMed PMID: WOS:000337160100018.

40. Pottel L, Lycke M, Boterberg T, Foubert I, Pottel H, Duprez F, et al. Omega-3 fatty acids: physiology, biological sources and potential applications in supportive cancer care. Phytochemistry Reviews. 2014;13(1):223-44. doi: 10.1007/s11101-013-9309-1. PubMed PMID: WOS:000331818000015.

41. Din JN, Harding SA, Valerio CJ, Sarma J, Lyall K, Riemersma RA, et al. Dietary intervention with oil rich fish reduces platelet-monocyte aggregation in man. Atherosclerosis. 2008;197(1):290-6. doi: 10.1016/j.atherosclerosis.2007.04.047.

42. Harrison RA, Sagara M, Rajpura A, Armitage L, Birt N, Birt CA, et al. Can foods with added soya-protein or fish-oil reduce risk factors for coronary disease? A factorial randomised controlled trial. Nutrition, Metabolism and Cardiovascular Diseases. 2004;14(6):344-50. doi: 10.1016/S0939-4753(04)80024-3.

43. Ruxton CHS, Reed SC, Simpson MJA, Millington KJ. The health benefits of omega-3 polyunsaturated fatty acids: A review of the evidence. Journal of Human Nutrition and Dietetics. 2004;17(5):449-59. doi: 10.1111/j.1365-277X.2004.00552.x.

44. Zhu X-H. Liver-protecting effects of omega-3 fish oil lipid emulsion in liver transplantation. World Journal of Gastroenterology. 2012;18(42). doi: 10.3748/wjg.v18.i42.6141.

45. Jorgensen MH, Hernell O, Hughes EL, Michaelsen KF. Is there a relation between docosahexaenoic acid concentration in mothers' milk and visual development in term infants? . Journal of Pediatric Gastroenterology. 2001;32(3):293-6.

46. Daniels JL, Longnecker MP, Rowland AS, Golding J. Fish intake during pregnancy and early cognitive development of offspring. Epidemiology. 2004;15(4):394-402. doi: 10.1097/01.ede.0000129514.46451.ce.

47. Helland IB, Smith L, Saarem K, Saugstad OD, Drevon CA. Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. Pediatrics. 2003;111(1):e39-e44.

48. National Research Council. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients). Washington DC: National Academies Press; 2005.

49. Kris-Etherton PM, Innis S. Position of the American Dietetic Association and Dietitians of Canada: Dietary fatty acids. Journal of the American Dietetic Association. 2007;107(9):1599-161.

50. American Diabetes Association. Nutrition Recommendations and Interventions for Diabetes: A position statement of the American Diabetes Association. Diabetes Care. 2008;31(Supplement_1):S61-S78. doi: 10.2337/dc08-S061.

51. Lichtenstein AH, Appel LJ, Brands M, Carnethon M, Daniels S, Franch HA, et al. Summary of American Heart Association diet and lifestyle recommendations revision 2006. Arteriosclerosis, Thrombosis, and Vascular Biology. 2006;26(10):2186-91. doi: 10.1161/01.ATV.0000238352.25222.5e.

52. EFSA Panel on Dietetic Products, Nutrition and Allergies. Scientific opinion on the tolerable upper intake level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). EFSA Journal. 2012;10(7):2815-63. doi: 10.2903/j.efsa.2012.2815.

53. French Food Safety Agency (AFFSA). Opinion of the French Food Safety Agency regarding the benefits/risks of fish consumption. The Director General; 2010.

54. World Health Organisation, Food and Agriculture Organization. Diet, nutrition and the prevention of chronic diseases: Report of a joint WHO/FAO expert consultation. Geneva, CHE: World Health Organization (WHO); 2003 2003.

55. Autralia and New Zealand National Health and Medical Research Council. Nutrient reference values for Australia and New Zealand2006 2006.

56. Bates B, Lennox A, Prentice A, Bates C, Page P, Nicholson S, et al. National diet and nutrition survey: Results from years 1, 2, 3, and 4 (combined) of the rolling programme (2008/2009 - 2011/2012). In: Public Health England, Food Standards Agency, editors. London, UK: Public Health England; 2014.

57. Sugano M, Hirahara F. Polyunsaturated fatty acids in the food chain in Japan. The American Journal of Clinical Nutrition. 2000;71(1):189S-96S.

58. McClements DJ, Decker EA, Weiss J. Emulsion-based delivery systems for lipophilic bioactive components. Journal of Food Science. 2007;72(8):R109-R24. doi: 10.1111/j.1750-3841.2007.00507.x.

59. Li X, Anton N, Vandamme T. Nano-emulsions: Overview and applications. Liang XJ, editor2013. 21-48 p.

60. McClements DJ. Nanoemulsions versus microemulsions: Terminology, differences, and similarities. Soft Matter. 2012;8(6):1719-29. doi: 10.1039/c2sm06903b. PubMed PMID: WOS:000299291900001.

61. Anton N, Vandamme TF. Nano-emulsions and micro-emulsions: Clarifications of the critical differences. Pharmaceutical Research. 2011;28(5):978-85. doi: 10.1007/s11095-010-0309-1. PubMed PMID: WOS:000289302000004.

62. Acosta E. Bioavailability of nanoparticles in nutrient and nutraceutical delivery. Current Opinion in Colloid & Interface Science. 2009;14(1):3-15. doi: 10.1016/j.cocis.2008.01.002.

63. Kabalnov A. Ostwald ripening and related phenomena. Journal of Dispersion Science & Technology. 2001;22(1).

64. Jafari SM, He Y, Bhandari B. Optimization of nano-emulsions production by microfluidization. European Food Research and Technology = Zeitschrift für Lebensmittel-Untersuchung und -Forschung A. 2007;225(5-6):733-41. doi: 10.1007/s00217-006-0476-9.

65. Forgiarini A, Esquena J, González C, Solans C. Formation of nano-emulsions by low-energy emulsification methods at constant temperature. Langmuir. 2001;17(7):2076-83. doi: 10.1021/la001362n.

66. Anton N, Vandamme TF. The universality of low-energy nano-emulsification. International Journal of Pharmaceutics. 2009;377(1-2):142-7. doi: 10.1016/j.ijpharm.2009.05.014.

67. Gutiérrez JM, González C, Maestro A, Solè I, Pey CM, Nolla J. Nano-emulsions: New applications and optimization of their preparation. Current Opinion in Colloid & Interface Science. 2008;13(4):245-51. doi: 10.1016/j.cocis.2008.01.005.

68. Sloan AE. Top 10 functional food trends. Food Technology. 2012;66(4):1-5.

69. Saberi AH, Fang Y, McClements DJ. Fabrication of vitamin E-enriched nanoemulsions by spontaneous emulsification: Effect of propylene glycol and ethanol on formation, stability, and properties. Food Research International. 2013;54(1):812-20. doi: 10.1016/j.foodres.2013.08.028. PubMed PMID: WOS:000329377700100.

70. Ostertag F, Weiss J, McClements DJ. Low-energy formation of edible nanoemulsions: Factors influencing droplet size produced by emulsion phase inversion. Journal of Colloid and Interface Science. 2012;388:95-102. doi: 10.1016/j.jcis.2012.07.089. PubMed PMID: WOS:000310495600014.

71. Yang Y, Leser ME, Sher AA, McClements DJ. Formation and stability of emulsions using a natural small molecule surfactant: Quillaja saponin (Q-Naturale®). Food Hydrocolloids. 2013;30(2):589-96. doi: 10.1016/j.foodhyd.2012.08.008.

72. Ozturk B, Argin S, Ozilgen M, McClements DJ. Formation and stabilization of nanoemulsion-based vitamin E delivery systems using natural surfactants: Quillaja saponin and lecithin. Journal of Food Engineering. 2014;142:57-63. doi: 10.1016/j.jfoodeng.2014.06.015. PubMed PMID: WOS:000340305600009.

73. Kralova I, Sjöblom J. Surfactants used in food industry: A review. Journal of Dispersion Science & Technology. 2009;30(9):1363-83. doi: 10.1080/01932690902735561.

74. Qian C, McClements DJ. Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: Factors affecting particle size. Food Hydrocolloids. 2011;25(5):1000-8. doi: 10.1016/j.foodhyd.2010.09.017. PubMed PMID: WOS:000288247300020.

75. Güçlü-Üstündağ Ö, Mazza G. Saponins: Properties, applications and processing. Critical Reviews in Food Science and Nutrition. 2007;47(3):231-58. doi: 10.1080/10408390600698197.

76. Jacobsen C. Enrichment of foods with omega-3 fatty acids: a multidisciplinary challenge: Omega-3-enriched foods. Annals of the New York Academy of Sciences. 2010;1190(1):141-50. doi: 10.1111/j.1749-6632.2009.05263.x.

77. Drusch S. An industry perspective on the advantages and disadvantages of different fish oil delivery systems. In: Garti N, McClements DJ, editors. Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals2012. p. 488-504.

78. Velikov K, Pelan E. Colloidal delivery systems for micronutrients and nutraceuticals. Soft Matter. 2008;4:1964-80.

79. Yang Y, Marshall-Breton C, Leser ME, Sher AA, McClements DJ. Fabrication of ultrafine edible emulsions: Comparison of high-energy and low-energy homogenization methods. Food Hydrocolloids. 2012;29(2):398-406. doi: 10.1016/j.foodhyd.2012.04.009.

80. Chee CP, Gallaher JJ, Djordjevic D, Faraji H, McClements DJ, Decker EA, et al. Chemical and sensory analysis of strawberry flavoured yogurt supplemented with an algae oil emulsion. Journal of Dairy Research. 2005;72(3):311-6. doi: 10.1017/S0022029905001068.

81. Lane KE, Li W, Smith C, Derbyshire E. The bioavailability of an omega-3-rich algal oil is improved by nanoemulsion technology using yogurt as a food vehicle. International Journal of Food Science & Technology. 2014;49(5):1264-71. doi: 10.1111/ijfs.12455.

82. O' Dwyer SP, O' Beirne D, Ni Eidhin D, Hennessy AA, O' Kennedy BT. Formation, rheology and susceptibility to lipid oxidation of multiple emulsions (O/W/O) in table spreads containing omega-3 rich oils. LWT - Food Science and Technology. 2013;51(2):484-91.

83. Sharma R. Market trends and opportunities for functional dairy beverages. Australian Journal of Dairy Technology. 2005;60(2):195-8.

84. McClements DJ, Xiao H. Potential biological fate of ingested nanoemulsions: Influence of particle characteristics. Food & Function. 2012;3(3):202-20. doi: 10.1039/c1fo10193e. PubMed PMID: WOS:000300991300002.

85. McClements DJ, Decker EA. Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. Journal of Food Science. 2000;65(8):1270-82. doi: 10.1111/j.1365-2621.2000.tb10596.x.

86. McClements DJ, Decker EA. Lipids. In: Damodaran S, Parkin KL, O.R. F, editors. Food Chemistry, 4th Edition. Boca Raton: CRC Press; 2008. p. 155-216.

87. Lee SJ, Choi SJ, Li Y, Decker EA, McClements DJ. Protein-stabilized nanoemulsions and emulsions: Comparison of physicochemical stability, lipid oxidation, and lipase digestibility. Journal of Agricultural and Food Chemistry. 2011;59(1):415-27. doi: 10.1021/jf103511v. PubMed PMID: WOS:000285735200052.

88. Villiere A, Viau M, Bronnec I, Moreau N, Genot C. Oxidative stability of bovine serum albumin- and sodium caseinate-stabilized emulsions depends on metal availability. Journal of Agricultural and Food Chemistry. 2005;53(5):1514-20. doi: 10.1021/jf0486951.

89. Boon CS, Xu Z, Yue X, McClements DJ, Weiss J, Decker EA. Factors affecting lycopene oxidation in oil-in-water emulsions. Journal of Agricultural and Food Chemistry. 2008;56(4):1408-14. doi: 10.1021/jf072929+.

90. Mancuso JR, McClements DJ, Decker EA. The effects of surfactant type, pH, and chelators on the oxidation of salmon oil-in-water emulsions. Journal of Agricultural and Food Chemistry. 1999;47(10):4112-6. doi: 10.1021/jf990203a.

91. Mancuso JR, McClements DJ, Decker EA. Iron-accelerated cumene hydroperoxide decomposition in hexadecane and trilaurin emulsions. Journal of Agricultural and Food Chemistry. 2000;48(2):213-9. doi: 10.1021/jf990757d.

92. Silvestre MPC, Chaiyasit W, Brannan RG, McClements DJ, Decker EA. Ability of surfactant headgroup size to alter lipid and antioxidant oxidation in oil-in-water emulsions. Journal of Agricultural and Food Chemistry. 2000;48(6):2057-61. doi: 10.1021/jf991162l.

93. Chaiyasit W, Silvestre MPC, McClements DJ, Decker EA. Ability of surfactant hydrophobic tail group size to alter lipid oxidation in oil-in-water emulsions. Journal of Agricultural and Food Chemistry. 2000;48(8):3077-80. doi: 10.1021/jf000323e.

94. Belhaj N, Arab-Tehrany E, Linder M. Oxidative kinetics of salmon oil in bulk and in nanoemulsion stabilized by marine lecithin. Process Biochemistry. 2010;45(2):187-95. doi: 10.1016/j.procbio.2009.09.005. PubMed PMID: WOS:000273897400007.

95. Damodaran S, Parkin KL, Fennema OR. Fennema's food chemistry, fourth edition. 4 ed. Damodaran S, Parkin KL, Fennema OR, editors: CRC Press; 2008 2007/09/13/. 1160 p.

96. Cho YJ, Alamed J, McClements DJ, Decker EA. Ability of chelators to alter the physical location and prooxidant activity of iron in oil-in-water emulsions. Journal of Food Science. 2003;68(6):1952-7. doi: 10.1111/j.1365-2621.2003.tb07000.x.

97. Mei L, McClements DJ, Wu J, Decker EA. Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH and NaCl. Food Chemistry. 1998;61(3):307-12. doi: 10.1016/S0308-8146(97)00058-7.

98. Qian C, Decker EA, Xiao H, McClements DJ. Inhibition of beta-carotene degradation in oil-in-water nanoemulsions: Influence of oil-soluble and water-soluble antioxidants. Food Chemistry. 2012;135(3):1036-43. doi: 10.1016/j.foodchem.2012.05.085. PubMed PMID: WOS:000310396700025.

99. Qian C, Decker EA, Xiao H, McClements DJ. Physical and chemical stability of beta-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. Food Chemistry. 2012;132(3):1221-9. doi: 10.1016/j.foodchem.2011.11.091. PubMed PMID: WOS:000301022400015.

100. Yi J, Li Y, Zhong F, Yokoyama W. The physicochemical stability and in vitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions. Food Hydrocolloids. 2014;35:19-27. doi: 10.1016/j.foodhyd.2013.07.025. PubMed PMID: WOS:000326270000003.

101. Xu DX, Wang XY, Jiang JP, Yuan F, Decker EA, Gao YX. Influence of pH, EDTA, alpha-tocopherol, and WPI oxidation on the degradation of beta-carotene in WPI-stabilized oil-in-water emulsions. Lwt-Food Science and Technology. 2013;54(1):236-41. doi: 10.1016/j.lwt.2013.05.029. PubMed PMID: WOS:000321991200035.

102. Zhao Q, Ho CT, Huang QR. Effect of ubiquinol-10 on citral stability and off-flavor formation in oil-in-water (O/W) nanoemulsions. Journal of Agricultural and Food Chemistry. 2013;61(31):7462-9. doi: 10.1021/jf4017527. PubMed PMID: WOS:000323020500006.

103. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine. 1996;20(7):933-56. doi: 10.1016/0891-5849(95)02227-9.

104. Rupasinghe HPV, Yasmin A. Inhibition of oxidation of aqueous emulsions of omega-3 fatty acids and fish oil by phloretin and phloridzin. Molecules. 2010;15(1):251-7. doi: 10.3390/molecules15010251.

105. Huber GM, Vasantha Rupasinghe HP, Shahidi F. Inhibition of oxidation of omega-3 polyunsaturated fatty acids and fish oil by quercetin glycosides. Food Chemistry. 2009;117(2):290-5. doi: 10.1016/j.foodchem.2009.04.007.

106. Gulotta A, Saberi AH, Nicoli MC, McClements DJ. Nanoemulsion-based delivery systems for polyunsaturated (ω-3) oils: Formation using a spontaneous emulsification method. Journal of Agricultural and Food Chemistry. 2014;62(7):1720-5. doi: 10.1021/jf4054808.

107. Qian C, Decker EA, Xiao H, McClements DJ. Nanoemulsion delivery systems: Influence of carrier oil on beta-carotene bioaccessibility. Food Chemistry. 2012;135(3):1440-7. doi: 10.1016/j.foodchem.2012.06.047. PubMed PMID: WOS:000310396700083.

108. Saberi AH, Fang Y, McClements DJ. Effect of glycerol on formation, stability, and properties of vitamin-E enriched nanoemulsions produced using spontaneous emulsification. Journal of Colloid and Interface Science. 2013;411:105-13. doi: 10.1016/j.jcis.2013.08.041. PubMed PMID: WOS:000326135600015.

109. Lee SJ, McClements DJ. Fabrication of protein-stabilized nanoemulsions using a combined homogenization and amphiphilic solvent dissolution/evaporation approach. Food Hydrocolloids. 2010;24(6-7):560-9. doi: 10.1016/j.foodhyd.2010.02.002. PubMed PMID: WOS:000277717200002.

110. Troncoso E, Aguilera JM, McClements DJ. Influence of particle size on the in vitro digestibility of protein-coated lipid nanoparticles. Journal of Colloid and Interface Science. 2012;382:110-6. doi: 10.1016/j.jcis.2012.05.054. PubMed PMID: WOS:000307027600016.

111. Patch CS, Tapsell LC, Williams PG. Attitudes and intentions toward purchasing novel foods enriched with omega-3 fatty acids. Journal of Nutrition Education & Behavior. 2005;37(5):235-41.

112. Bouwmeester H, Dekkers S, Noordam MY, Hagens WI, Bulder AS, de Heer C, et al. Review of health safety aspects of nanotechnologies in food production. Regulatory Toxicology and Pharmacology. 2009;53(1):52-62. doi: 10.1016/j.yrtph.2008.10.008. PubMed PMID: ISI:000263460800008.

113. McClements DJ, Li Y. Review of in vitro digestion models for rapid screening of emulsion-based systems. Food & Function. 2010;1(1):32-59. doi: 10.1039/C0FO00111B.

114. Porter CJH, Charman WN. In vitro assessment of oral lipid based formulations. Advanced Drug Delivery Reviews. 2001;50:S127-S47. doi: 10.1016/s0169-409x(01)00182-x. PubMed PMID: WOS:000171793100009.

115. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, et al. A standardised static in vitro digestion method suitable for food - an international consensus. Food & Function. 2014;5(6):1113-24. doi: 10.1039/c3fo60702j. PubMed PMID: MEDLINE:24803111.

116. Sy C, Gleize B, Dangles O, Landrier J-F, Veyrat CC, Borel P. Effects of physicochemical properties of carotenoids on their bioaccessibility, intestinal cell uptake, and blood and tissue concentrations. Molecular Nutrition & Food Research. 2012;56(9):1385-97. doi: 10.1002/mnfr.201200041.

117. McClements DJ, Decker EA, Park Y. Controlling lipid bioavailability through physicochemical and structural approaches. Critical Reviews in Food Science and Nutrition. 2009;49(1):48-67. doi: 10.1080/10408390701764245. PubMed PMID: ISI:000261802900003.

118. Singh H, Ye A, Horne D. Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. Progress in Lipid Research. 2008.

119. Troncoso E, Aguilera JM, McClements DJ. Fabrication, characterization and lipase digestibility of food-grade nanoemulsions. Food Hydrocolloids. 2012;27(2):355-63. doi: 10.1016/j.foodhyd.2011.10.014.

120. Mayer S, Weiss J, McClements DJ. Behavior of vitamin E acetate delivery systems under simulated gastrointestinal conditions: Lipid digestion and bioaccessibility of low-energy nanoemulsions. Journal of Colloid and Interface Science. 2013;404:215-22. doi: 10.1016/j.jcis.2013.04.048.

121. Speranza A, Corradini MG, Hartman TG, Ribnicky D, Oren A, Rogers MA. Influence of emulsifier structure on lipid bioaccessibility in oil–water nanoemulsions. Journal of Agricultural and Food Chemistry. 2013;61(26):6505-15. doi: 10.1021/jf401548r.

122. Dey Tk, Ghosh S, Ghosh M, Koley H, Dhar P. Comparative study of gastrointestinal absorption of EPA & DHA rich fish oil from nano and conventional emulsion formulation in rats. Food Research International. 2012;49(1):72-9. doi: 10.1016/j.foodres.2012.07.056.

123. Morrissey PA, Higgins S, Carroll YL, O'Brien NM. Use of microencapsulated fish oil as a means of increasing n-3 polyunsaturated fatty acid intake. Journal of Human Nutrition & Dietetics. 1999;12(4):265-71.

124. Haug IJ, Sagmo LB, Zeiss D, Olsen IC, Draget KI, Seternes T. Bioavailability of EPA and DHA delivered by gelled emulsions and soft gel capsules. European Journal of Lipid Science and Technology. 2011;113(2):137-45. doi: 10.1002/ejlt.201000450.

125. Wilde PJ, Chu BS. Interfacial & colloidal aspects of lipid digestion. Advances in Colloid and Interface Science. 2011;165(1):14-22. doi: 10.1016/j.cis.2011.02.004. PubMed PMID: WOS:000291337600004.

126. Marciani L, Wickham M, Singh G, Bush D, Pick B, Cox E, et al. Delaying gastric emptying and enhancing cholecystokinin release and satiety by using acid stable fat emulsions. Gastroenterology. 2006;130(4):A227-A. PubMed PMID: WOS:000236961701443.

127. Marciani L, Wickham M, Singh G, Bush D, Pick B, Cox E, et al. Enhancement of intragastric acid stability of a fat emulsion meal delays gastric emptying and increases cholecystokinin release and gallbladder contraction. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2007;292(6):G1607-G13. doi: 10.1152/ajpgi.00452.2006. PubMed PMID: WOS:000247935900018.

128. Cox DN, Koster A, Russell CG. Predicting intentions to consume functional foods and supplements to offset memory loss using an adaptation of protection motivation theory. Appetite. 2004;43(1):55-64. doi: 10.1016/j.appet.2004.02.003.

129. Ares G, Gámbaro A. Influence of gender, age and motives underlying food choice on perceived healthiness and willingness to try functional foods. Appetite. 2007;49(1):148-58. doi: 10.1016/j.appet.2007.01.006.

130. Bech-Larsen T, Grunert KG. The perceived healthiness of functional foods. Appetite. 2003;40(1):9-14. doi: 10.1016/S0195-6663(02)00171-X.

131. Krutulyte R, Grunert KG, Scholderer J, Hagemann KS, Elgaard P, Nielsen B, et al. Motivational factors for consuming omega-3 PUFAs: An exploratory study with Danish consumers. Appetite. 2008;51(1):137-47. doi: 10.1016/j.appet.2008.01.005.

132. Krutulyte R, Grunert KG, Scholderer J, Lähteenmäki L, Hagemann KS, Elgaard P, et al. Perceived fit of different combinations of carriers and functional ingredients and its effect on purchase intention. Food Quality and Preference. 2011;22(1):11-6. doi: 10.1016/j.foodqual.2010.06.001.

133. Lane KE, Derbyshire EJ, Smith CJ, Mahadevan K, Li W. Sensory evaluation of a yogurt drink containing an omega-3 nanoemulsion with enhanced bioavailability. Proceedings of the Nutrition Society. 2013;72(OCE2):E99-E. doi: 10.1017/S0029665113001109.

134. Ye A, Cui J, Taneja A, Zhu X, Singh H. Evaluation of processed cheese fortified with fish oil emulsion. Food Research International. 2009;42(8):1093-8. doi: 10.1016/j.foodres.2009.05.006.

135. Saberi AH, Fang Y, McClements DJ. Fabrication of vitamin E-enriched nanoemulsions: Factors affecting particle size using spontaneous emulsification. Journal of Colloid and Interface Science. 2013;391:95-102. doi: 10.1016/j.jcis.2012.08.069.

136. Shantha NC, Decker EA. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. Journal of AOAC International. 1994;77(2):421-4. PubMed PMID: 8199478.

137. McDonald RE, Hultin HO. Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. Journal of Food Science. 1987;52(1):15-21. doi: 10.1111/j.1365-2621.1987.tb13964.x.

138. Mayer S, Weiss J, McClements DJ. Vitamin E-enriched nanoemulsions formed by emulsion phase inversion: Factors influencing droplet size and stability. Journal of Colloid and Interface Science. 2013;402(0):122-30. doi: 10.1016/j.jcis.2013.04.016.

139. McClements DJ. Theoretical prediction of emulsion color. Advances in Colloid and Interface Science. 2002;97(1–3):63-89. doi: 10.1016/S0001-8686(01)00047-1.

140. McClements DJ. Ultrasonic determination of depletion flocculation in oil-in-water emulsions containing a nonionic surfactant. Colloids and Surfaces a-Physicochemical and Engineering Aspects. 1994;90(1):25-35. doi: 10.1016/0927-7757(94)02881-8. PubMed PMID: WOS:A1994PN34000004.

141. Binks BP, Clint JH, Fletcher PDI, Rippon S, Lubetkin SD, Mulqueen PJ. Kinetics of swelling of oil-in-water emulsions stabilized by different surfactants. Langmuir. 1999;15(13):4495-501. doi: 10.1021/la990054q. PubMed PMID: WOS:000081119900031.

142. Capek I. Degradation of kinetically-stable o/w emulsions. Advances in Colloid and Interface Science. 2004;107(2-3):125-55. doi: 10.1016/s0001-8686(03)00115-5. PubMed PMID: WOS:000220721200004.

143. Kabalnov AS, Shchukin ED. Ostwald ripening theory: Applications to fluorocarbon emulsion stability. Advances in Colloid and Interface Science. 1992;38:69-97. doi: 10.1016/0001-8686(92)80043-W.

144. Haahr A-M, Jacobsen C. Emulsifier type, metal chelation and pH affect oxidative stability of n-3-enriched emulsions. European Journal of Lipid Science and Technology. 2008;110(10):949-61. doi: 10.1002/ejlt.200800035.

145. Fanun M. Microemulsions: Properties and applications. Boca Raton, FL: CRC Press; 2008. 560 p.

146. Nuchi CD, McClements DJ, Decker EA. Impact of Tween 20 hydroperoxides and iron on the oxidation of methyl linoleate and salmon oil dispersions. Journal of Agricultural and Food Chemistry. 2001;49(10):4912-6. doi: 10.1021/jf010370m.

147. Roozen JP, Frankel EN, Kinsella JE. Enzymic and autoxidation of lipids in low fat foods: Model of linoleic acid in emulsified hexadecane. Food Chemistry. 1994;50(1):33-8. doi: 10.1016/0308-8146(94)90089-2.

148. Hu M, McClements DJ, Decker EA. Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. Journal of Agricultural and Food Chemistry. 2003;51(6):1696-700. doi: 10.1021/jf020952j.

149. Sørensen A-DM, Haahr A-M, Becker EM, Skibsted LH, Bergenståhl B, Nilsson L, et al. Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3-enriched oil-in-water emulsions. Journal of Agricultural and Food Chemistry. 2008;56(5):1740-50. doi: 10.1021/jf072946z.

150. Patist A, Bhagwat SS, Penfield KW, Aikens P, Shah DO. On the measurement of critical micelle concentrations of pure and technical-grade nonionic surfactants. Journal of Surfactants and Detergents. 2000;3(1):53-8. doi: 10.1007/s11743-000-0113-4.

151. Richards MP, Chaiyasit W, McClements DJ, Decker EA. Ability of surfactant micelles to alter the partitioning of phenolic antioxidants in oil-in-water emulsions. Journal of Agricultural and Food Chemistry. 2002;50(5):1254-9. doi: 10.1021/jf011324p.

152. Nuchi CD, Hernandez P, McClements DJ, Decker EA. Ability of lipid hydroperoxides to partition into surfactant micelles and alter lipid oxidation rates in emulsions. Journal of Agricultural and Food Chemistry. 2002;50(19):5445-9. doi: 10.1021/jf020095j.

153. Cho Y-J, McClements DJ, Decker EA. Ability of surfactant micelles to alter the physical location and reactivity of iron in oil-in-water emulsion. Journal of Agricultural and Food Chemistry. 2002;50(20):5704-10. doi: 10.1021/jf020433g.

154. Kiralan SS, Doğu-Baykut E, Kittipongpittaya K, McClements DJ, Decker EA. Increased antioxidant efficacy of tocopherols by surfactant solubilization in oil-in-water emulsions. Journal of Agricultural and Food Chemistry. 2014;62(43):10561-6. doi: 10.1021/jf503115j.

155. Ray WJJ, Puvathingal JM. A simple procedure for removing contaminating aldehydes and peroxides from aqueous solutions of polyethylene glycols and of nonionic detergents that are based on the polyoxyethylene linkage. Analytical Biochemistry. 1985;146(2):307-12. doi: 10.1016/0003-2697(85)90544-5.

156. Lever M. Peroxides in detergents as interfering factors in biochemical analysis. Analytical Biochemistry. 1977;83(1):274-84. doi: 10.1016/0003-2697(77)90536-X.

157. Shaw PE. Review of quantitative analyses of citrus essential oils. Journal of Agricultural and Food Chemistry. 1979;27(2):246-57. doi: 10.1021/jf60222a032.

158. Di Vaio C, Graziani G, Gaspari A, Scaglione G, Nocerino S, Ritieni A. Essential oils content and antioxidant properties of peel ethanol extract in 18 lemon cultivars. Scientia Horticulturae. 2010;126(1):50-5. doi: 10.1016/j.scienta.2010.06.010.

159. Misharina TA, Terenina MB, Krikunova NI, Kalinichenko MA. The influence of the composition of essential lemon oils on their antioxidant properties and the stability of the components. Russian Journal of Bioorganic Chemistry. 2011;37(7):883-7. doi: 10.1134/S1068162011070168.

160. Abdalla AE, Roozen JP. Effect of plant extracts on the oxidative stability of sunflower oil and emulsion. Food Chemistry. 1999;64(3):323-9. doi: 10.1016/S0308-8146(98)00112-5.

161. Rimini S, Petracci M, Smith DP. The use of thyme and orange essential oils blend to improve quality traits of marinated chicken meat. Poultry Science. 2014;93(8):2096-102. doi: 10.3382/ps.2013-03601.

162. Loizzo MR, Tundis R, Menichini F, Duthie G. Anti-rancidity effect of essential oils, application in the lipid stability of cooked turkey meat patties and potential implications for health. International Journal of Food Sciences and Nutrition. 2015;66(1):50-7. doi: doi:10.3109/09637486.2014.953454. PubMed PMID: 25265207.

163. Sarıçoban C, Yilmaz MT. Effect of thyme/cumin essential oils and butylated hydroxyl anisole/butylated hydroxyl toluene on physicochemical properties and oxidative/microbial stability of chicken patties. Poultry Science. 2014;93(2):456-63. doi: 10.3382/ps.2013-03477.

164. Chang Y, McLandsborough L, McClements DJ. Physical properties and antimicrobial efficacy of thyme oil nanoemulsions: Influence of ripening inhibitors. Journal of Agricultural and Food Chemistry. 2012;60(48):12056-63. doi: 10.1021/jf304045a.

165. Gallego MG, Gordon M, Segovia F, Skowyra M, Almajano M. Antioxidant properties of three aromatic herbs (rosemary, thyme and lavender) in oil-in-water emulsions. Journal of the American Oil Chemists' Society. 2013;90(10):1559-68. doi: 10.1007/s11746-013-2303-3.

166. Dogu-Baykut E, Gunes G, Decker EA. Impact of shortwave ultraviolet (UV-C) radiation on the antioxidant activity of thyme (Thymus vulgaris L.). Food Chemistry. 2014;157(0):167-73. doi: 10.1016/j.foodchem.2014.02.027.

167. Bozin B, Mimica-Dukic N, Simin N, Anackov G. Characterization of the volatile composition of essential oils of some lamiaceae spices and the antimicrobial and antioxidant activities of the entire oils. Journal of Agricultural and Food Chemistry. 2006;54(5):1822-8. doi: 10.1021/jf051922u.

168. Rombaut N, Savoire R, Thomasset B, Castello J, Van Hecke E, Lanoisellé J-L. Optimization of oil yield and oil total phenolic content during grape seed cold screw pressing. Industrial Crops and Products. 2015;63(0):26-33. doi: 10.1016/j.indcrop.2014.10.001.

169. Singleton VL, Orthofer R, Lamuela-Raventós RM. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In: Lester P, editor. Methods in Enzymology: Academic Press; 1999. p. 152-78.