A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in food science
Department of Food Science
University of Iceland

Nano-laminated Fish Oil Droplets:

Influence of Chitosan Charge Density on Emulsion Stability

Þóra Ýr Árnadóttir
Maí 2013

Supervisors:

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Abstract

The benefits of wholesome consumption, are for example decreased risk of type 2 diabetes, hypertension, and certain cancer, decreased risk of overweigh and obesity and decreased risk of micronutrient deficiencies. Omega-3 fatty acids provide essential fatty acids that our body needs. Fish oil is probably the most beneficial source of omega-3 fatty acids, and there for there could be a substantial marked for ω-3 fatty acid incorporated food, function foods. However, there are some obstacles that need to be overcome before this will be commercially viable.

Fish oil oxidation is very rapid so that could be a problem when it's incorporated in food. Another problem is that fish oil has a rather strong odor as well as taste. With a technique that encapsulates the oil by creating layer or layer´s around the lipid droplets it's possible control the absorption in the body and also to conceal the odor and the taste.

A series of emulsions containing the same oil [1% (w/w) fish oil] and protein [0.1% (w/w) sodium caseinate] contents but different deacetylated chitosan/chitin (50 DDA, 70 DDA and 92% DDA) were prepared by mixing different ratios of stock emulsion [10% (w/w) fish oil, 1% wt% sodium caseinate], chitosan solution [0.04% (w/w) chitosan] and buffer solution. The 1°emulsion contained sodium caseinate layer around the fish oil droplets, the 2°emulsion contained chitosan layer around the sodium caseinate layer and the 3°emulsion contained a pectin layer around the chitosan layer. We compared the influence of repeated freeze-thaw cycles and different pH on the mean particle diameter, microstructure, zeta-potential and creaming stability of primary, secondary and tertiary emulsions. We also compared the influence of oxidation rate on different layered emulsions.

This study has shown that the freeze-thaw stability of oil-in-water emulsions can be improved by engineering the characteristics of the interfacial layers surrounding the lipid droplets using layer-by-layer electrostatic deposition technique. The study also showed that we were able to improve oxidative stability of the emulsion droplets, stabilized by either caseinate or caseinate-chitosan layers versus emulsion droplets stabilized by tween20.

Acknowledgements

I would like to thank all the people who assisted me during this research. I would like to begin by thanking my professors Kristberg Kristbergsson Ph.D. and David Julian McClements Ph.D. I would also thank my lab members in Chenoweth and the staff there. I would like to mention Tarek Award, who was a great help especially at the beginning of my research as well as Þrándur Helgasson. Last but not least my husband Helgi Jónasson who moved with me to the United States and took care of our home.

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1. Introduction

1. 1 Rationale

Chronic diseases—such as heart disease, cancer, and diabetes—are the leading causes of death and disability worldwide. Chronic diseases account for 7 out of 10 deaths among Americans each year. These diseases also cause major limitations in daily living for one-fourth of people with chronic conditions. Although chronic diseases are among the most common and costly health problems, they are also among the most preventable. Adopting healthy behaviors such as eating nutritious foods, being physically active and avoiding tobacco use can prevent or control the devastating effects of these diseases (CDC Centers for Disease Control and Prevention, 2008).

The benefits of wholesome consumption, are for example decreased risk of type 2 diabetes, hypertension, and certain type of cancer, decreased risk of overweigh and obesity and decreased risk of micronutrient deficiencies. Fats and oils are part of a healthy diet and play many important roles in the body. Fat provides energy and is a carrier of important nutrients such as vitamins A, D, E, and K and carotenoids (CDC Centers for Disease Control and Prevention, 2008).

Fat can impact the health of our hearts and arteries in a positive or negative way, depending on the types of fat we eat. It´s recommended too eat fats from fish, nuts and vegetable oils, there is where monounsaturated- and polyunsaturated fat comes in they are the "good" fats. The "bad" fats are saturated- and trans-fats they can raise bad cholesterol, clog arteries and cause heart disease. However, all types of fat contain an equal number of calories, 9 calories per gram, or twice the calories of either protein or carbohydrates. For some applications we could have the option to use a fat replacer that contains fewer calories.

1.2. Background

In recent year´s people are thinking more about what they can do to stay healthy for longest possible time and overall wellness. ω-3 fatty acids provide an essential fatty acids that our body needs. ω-3 fatty acids are unsaturated fatty acids that have a double bond that is 3 carbon atoms from the methyl end of the molecule. The most common ω-3 fatty acids are α-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6). Research has shown that ω-3 fatty acids may have potential health benefits for the human body, for example, prevention of diseases like Alzheimer's and depression,

reduction high blood pressure and reduces cholesterol. Omega-3 fatty acids have numerous physiological roles such as impacting cell membrane fluidity, cellular signaling, gene expression, and eicosanoid metabolism. (McClements, Decker, Weiss, 2007). Fish oil is probably the most beneficial source of ω-3 fatty acid, and there for could it be a big market for ω-3 fatty acids incorporated food and function foods. But there are a few problems that come up that we need to address. Fish oil oxidation is very rapid, which could be a problem when it is incorporated in to food. Another problem is that fish oil has a rather strong odor as well as taste. With a technique that encapsulates the oil droplet by creating layer or layer´s around the lipid droplets it's possible to control the absorption in the body and also to conceal the odor and taste. It is possible to make layer´s with many different biopolymers.

We used an emulsion to make our delivery system. Emulsion is a mixture of two or more immiscible liquids, emulsions are thermodynamically unstable systems that are prone to destabilization. Emulsions destabilization may occur through a variety of different physicochemical processes, including gravitational separation, flocculation, coalescence, and Oswald ripening (McClements, 1999) (Dickinson, 1992). Most of the important and widely used methods of improving the stability in oil-in-water emulsions involve the utilization of emulsifiers. Emulsifiers are surface-active ingredients that readily adsorb at interfaces and facilitate emulsion formation by lowering the interfacial tension. The caseins are examples of emulsifiers, caseins are phosphoproteins with the electronegative domain preferentially located in small peptidic fragments (Dalgeish, 1998) (Fox and McSweeney, 2006) Chitosan has been proposed as a particularly effective material for encapsulating fish oils because of its unique functional attributes, natural abundance and underutilization. (Klaypradit and Huang, 2008) (Klinkesorn, Sophanodora, Chinachoti, McClements and Decker, 2005) (Peniche, Howland, Carrillo, Zaldivar and Arguelles-Monal, 2004). Chitosan is produced commercially by deacetylation of chitin, which is the structural element in shellfish. Primex/Genis is an Icelandic based company producing chitin derivatives. Primex is exploiting the unique features Iceland has to offer for its chitin and chitosan production. This includes abundant year-around source of fresh shrimp shells, which is received through pipelines directly from the shrimp pealing machines, as well as an abundant and high purity water source. The single source of raw material used for these products is the North Atlantic shrimp, *Pandalus borealis.* Primex continues to emphasize product development, involving various modifications of dissolved chitosan, such as microcrystalline chitosan (MCCh) and chito-oligosacharides. They produce purified and consistent products that are intended to serve the increasing need for higher quality chitosan, exhibiting high level of activities pertinent to

many developing market applications. Icelandic seafood products are traditionally recognized worldwide as products of highest quality. Chitosan, derived from shellfish has been used in a research as an edible, invisible coating on Atlantic cod and herring fillets; it has also been use as an anti-microbial agent for fruits, vegetables, cheeses and meat (Duan, Cherian and Zhao, 2009)(Jumaa, Furkert and Müller, 2002). Chitosan has an GRAS (generally recognized as safe) status in the USA. In the EU Chitosan has this self clamed status, it is somewhere in between, it's not allowed in foods but is allowed in pills. It has for example been sold as a diet pills.

The stability of globular protein emulsion to droplet aggregation around the isoelectric point of the adsorbed protein can be greatly improved by coating droplets with chitosan. (Hong and McClements, 2007) Chitosan is metabolized by certain human enzymes, (e.g. lysozyme), and breaks down slowly to harmless products (amino sugars) that are completely absorbed in the human body (Sashiwa, Saimoto, Shigemasa, Ogawa, Tokura, 1990).

1.2.1 Salatrim

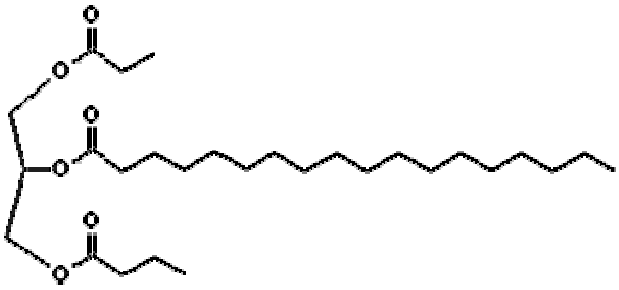


Figure 1 Chemical structure of Salatrim

Salatrim is a fat replacer that is prepared from natural fats that are normally digested by the body. Salatrim is a modified triacylglycerol that is rich in short-chain fatty acids and stearic acid. The melting range is 16-71°C, depending on triglyceride composition. It is used as a lower-calorie fat replacer. The available calories from Salatrim are only 55% of those from normal fats. Short chain organic acids have less energy storage capacity than long chain fatty acids. It does not have any laxative effects, and it does not prevent the absorption of fat-soluble vitamins and carotenoids (Field, 2003)

1.2.2 Solid-Lipid Nanoparticles (SLN).

Solid lipid nanoparticles are a novel carrier system for cosmetic active ingredients, pharmaceutical drugs and bioactive compounds in the food industry. SLN consist of biodegradable physiological lipids or lipid substances and stabilizers (Mozafari, 2006). The particles are stabilized by a

surfactant layer, which may consist of a single surfactant or a binary or ternary mixture of surfactants (Jenning, Mader, Gohla, 2000) (Jenning, Thunemann, Gohla, 2000). Compared to other delivery systems such as liposomes and micro emulsions, SLN possess various advantages. The use of solid lipids instead of liquid lipids has shown to greatly increase control over release and uptake of the bioactive. This is because mobility of the bioactive can be controlled by controlling the physical state of the lipid matrix. In several cases it has been shown that SLN have good encapsulation efficiency and long-term physical stability (Lee. G.S and others 2007). Their mean diameter typically ranges between 80 nm and 400 nm and they have low content of microparticles. The SLN combine advantages of polymeric nanoparticles (solid matrix for controlled release) and o/w fat emulsion for parenteral administration (physiological compounds, production on large industrial scale), but simultaneously avoid the disadvantages of these two systems. Both lipophilic and hydrophilic drugs can be incorporated, which represents considerable advantages. Sterilization by autoclaving is possible (Müller and others 1994)

1.3 Objective

The goals are to make an effective emulsion based delivery system that encapsulates, protects and delivers lipophilic ingredients such as carotenoids or ω-3 fatty acids. In most cases the carrier particles release the active agents within seconds (Westen, 2000). The aim was to make specific double environmental trigger mechanism to encapsulate and release the active agents. By using double environmental triggers the delay and release of the active agents was possible. The environmental trigger could for example be pH, salt concentration or temperature. The goal was to make delivery system that remains associated with the active agents until it has reached the target. Ultimately, this will benefit raw manufacturers e.g. the fishery industry as a source for ω-3 fatty acids as well as food manufacturers and consumers.

2. Literature review

2.1 Emulsion

An emulsion is a mixture of two or more immiscible liquids, often water and oil, where one of the liquids dispersed as small spherical droplets in the other. A system that consists of oil droplets dispersed in an aqueous phase is called an oil-in-water or O/W emulsion. O/W emulsions are common in food, for example, milk, vinaigrette, mayonnaise, beverages, soups and sauces. A system that consists of water droplets dispersed in an oil phase is called water-in-oil or W/O emulsion, for example, margarine and butter. Emulsions contain a dispersed

and a continuous phase. The substance that makes up the droplets in an emulsion is referred to as the dispersed phase, whereas the substance that makes up the surrounding liquid is called the continuous phase. Emulsions tend to have cloudy appearance. The basic color of an emulsion is milky white. Microemulsions and nanoemulsions tend to appear clear due to the small size of the disperse phase.

Emulsions are unstable and therefore do not form spontaneously. Emulsions are formed by applying energy to the two immiscible liquids, this is carried out using mechanical devices known as homogenizers (Figure 1). Homogenizers usually subject the liquids to intense mechanical agitation, for example, high speed blenders, high pressure valve homogenizers, and colloidal mills. It is possible to form an emulsion containing only oil and water, but because emulsions are thermodynamically unstable system the oil phase usually separates rapidly from the water phase. The droplets merge with their neighbors when they come to contact with them, which eventually leads to complete phase separation. All food emulsions are thermodynamically unstable system and will eventually break down. Kinetic stability tells us the rate at which it will proceed if it does occurs.

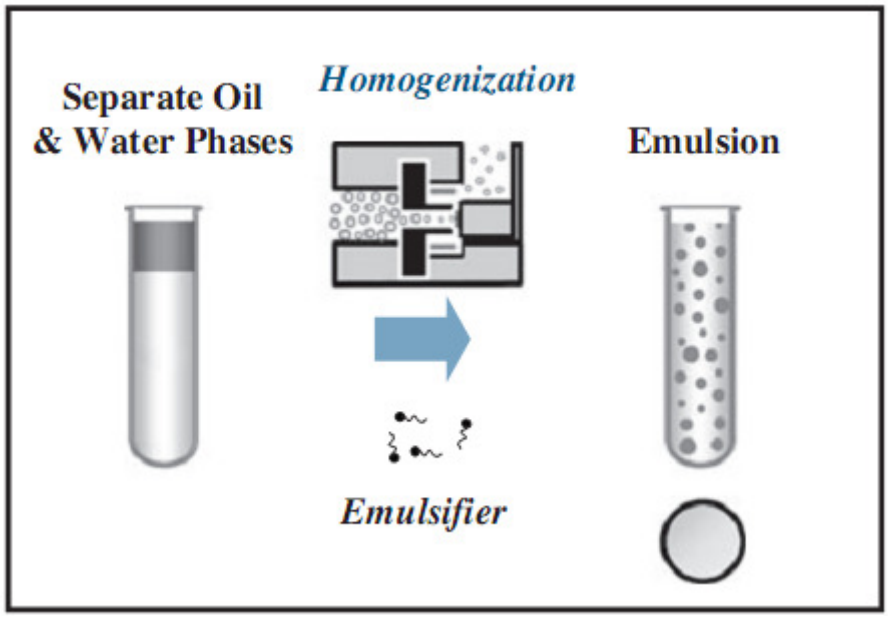


Figure 2. Oil-in-water emulsions are conventionally produced by homogenizing an oil phase and aqueous phase together in the presence of a water-soluble emulsifier.

2.1.1 Emulsion preparation

A variety of different homogenizers are available, including high shear mixers, high-pressure homogenizers, colloid mills, ultrasonic homogenizers and membrane homogenizers

(McClements, 1999; Walstra, 1993, 2003). The droplet size in O/W emulsions can usually be decreased by increasing the homogenizer pressure or number of passes. The breakup of lipid droplets within a homogenizer depends on the disruptive forces being large enough to overcome the interfacial forces holding the droplets together (McClements, 1999)

2.1.2 Structural Design of Emulsions

Emulsion based systems that are dispersible in solutions where the water is the continuous phase are common as food emulsions. The oil droplets in most food emulsions typically have diameters somewhere between 100 nm and 100 µm. The interfacial layer is typically between approximately 1 nm and 10 nm thick for food grade emulsifiers, but may be appreciably thicker if biopolymer multilayers are formed around the droplets (Guzey and McClemments, 2007)

Conventional emulsions: Conventional oil-in-water (O/W) emulsions consist of oil droplets dispersed in an aqueous continuous phase, with the oil droplets being surrounded by a thin interfacial layer consisting of emulsifier molecules (Figure 2) (Dickinson, 1992; Friberg and others, 2004; McClements, 2005a).

Multiple emulsions: Water-in-oil-in-water (W/O/W) emulsions consist of small water droplets contained within larger oil droplets that are dispersed in an aqueous continuous phase (Garti, 1997a, 1997b; Garti and Bisperink, 1998; Garti and Benichou, 2004) (Figure 2).

Multilayer emulsions: Multilayer oil-in-water (M-O/W) emulsions consist of small oil droplets dispersed in an aqueous medium, with each oil droplet being surrounded by a nano-laminated interfacial layer, which usually consists of emulsifier and biopolymer molecules (Figure 2).

Solid lipid particles: In some aspects, solid lipid particle (SLP) emulsions are similar to conventional emulsions consisting of emulsifier coated lipid droplets dispersed in an aqueous continuous phase (Figure 2). However, the lipid phase is either fully or partially solidified, and the morphology and packing of the crystals within the lipid phase are usually controlled to obtain particular functional attributes

Filled hydrogel particles: Filled hydrogel particle emulsions consist of oil droplets contained within hydrogel particles that are dispersed within an aqueous continuous phase (Figure 2). They can therefore be thought of as a type of oil-in-water-in-water (O/W1/W2) emulsion. (McClements, 1999)

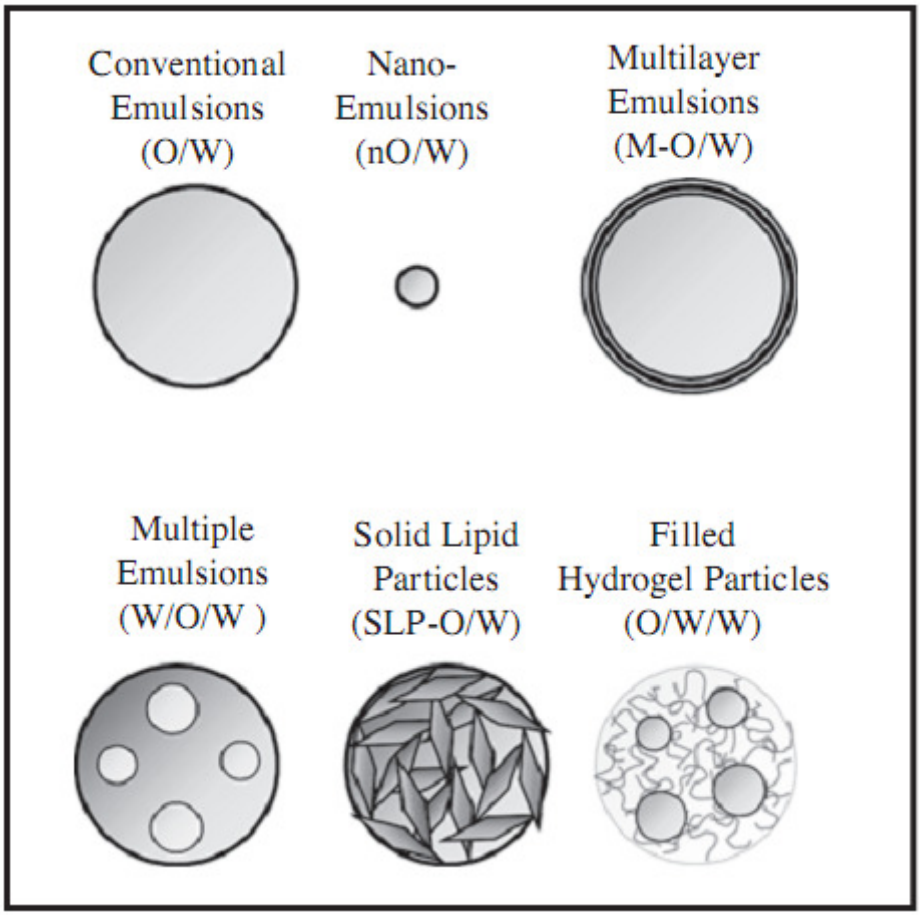


Figure 3. Examples of different kinds of structured emulsion systems that can be designed using food-grade ingredients. These include conventional emulsions, nanoemulsions, multilayer emulsions, multiple emulsions, solid lipid nanoparticles and filled hydrogel particles.

2.1.3 Emulsifiers

To active kinetically stable emulsions which can be stable for reasonable periods of time it is necessary to include substance known as stabilizers. Stabilizer can be classified as emulsifiers, but emulsifiers are surface-active molecules that absorb to the surface of freshly formed droplets during homogenization. An emulsifier is defined as a substance that reduces surface tension between oil-water or air-water, thus enhancing emulsification and increasing emulsion stability. They form a protective membrane that prevents the droplets from merging. Several issues must be evaluated when considering emulsifiers; the concentration, it needs to be sufficient to cover all droplet interfaces formed. The kinetic stability have to be sufficiently to prevent re-coalescence due to collision. It has to be protected against droplet coalescence and the surface pressure has to be sufficiently low to facilitate breakup. An effective emulsifier should have the following general characteristics, it should be capable of rapidly adsorbing to the surface of droplets during homogenization, it should be capable of reducing

the interfacial tension significantly and it should be capable of forming an interfacial membrane (McClements, 1999). The most commonly used emulsifiers in the food industry are an amphiphilic molecules (low-molecular-weight) having both polar and non-polar parts and polysaccharides (high-molecular-weight). Amphiphilic molecules are surfactants that tend to orientate themselves at the oil-water or air-water interfaces so that their hydrophilic head-groups (e.g. carboxyl, hydroxyl) are located in the aqueous phase and their hydrophobic (lipophilic) tail groups are located in the non-aqueous phase. Polysaccharides such as carrageenan or xanthan are stabilizers but not emulsifiers since they cannot be used independently to make an emulsion, only to stabilize one that has already been formed. Proteins on the other hand, act primarily through the properties if their interfacial films, and so in many cases they are able to fulfill both emulsifying and stabilizing roles (Dickinson, 1992). When proteins adsorb to an oil-water interface, the hydrophobic regions of their structure adhere to, or possibly partially dissolve in, the oil phase (Walstra, 2003). Proteins can be used at relatively low levels, but their ability to stabilize emulsions against droplet aggregation is strongly influenced by pH, ionic strength and temperature. Emulsion stabilized by polysaccharides have much better stability to environmental conditions than proteins due to the fact that the predominant stabilizing mechanism is steric rather than electrostatic, but they usually have to be used in much higher levels (McClements, 1999). A mixture of emulsifiers often gives a better stabilization than a single emulsifier at the same total concentration. It is clear that emulsification proceeds more easily if the surface tension is reduced by a combination of surfactants, but when the emulsion is formed it is the mechanical strength of the interfacial layer, rather than the surface tension, which determines the stability with respect to coalescence. In a mixed emulsifier system, extra film strength can arise from, better molecular packing in the layer, the formation of a mixed liquid crystalline mesophase at the droplet surface or the formation of a molecular complex between emulsifier components at the interface (Dickinson, 1992). The manufacture of oil in water food emulsions often involves a homogenization process under turbulent flow conditions. The relative contribution to droplet disruption by energy input (e.g. homogenization pressure) and the effect of emulsifiers on reduction of interfacial tension between oil and water is approximately 100:1. The emulsification process and final particle distribution of the emulsions are thus mainly controlled by the energy input, and the influence of emulsifiers is negligible (Walstra, 2003).

2.1.4 Particle charge

The droplets in emulsions often have an electrical charge, which plays an important role in their functional performance and stability. The electrical properties of a droplet are usually characterized in terms of its surface charge density, surface electrical potential, and/or zeta potential. The surface charge density is the number of unit electrical charges per unit surface area. The surface electrical potential is the free energy required to increase the surface charge density from zero to σ by bringing charges from an infinite distance to the surface through the surrounding medium. Zeta potential depends on the ionic composition of the surrounding medium due to electrostatic screening effects and usually decreases as the ionic strength of the aqueous phase increases (Hunter, 1986). The electrical characteristics of emulsion droplets can be controlled by careful selection of particular emulsifier types. Droplets stabilized by nonionic surfactants tend to only have a small droplet charge (e.g., Tweens and Spans), those stabilized by anionic surfactants have a negative charge (e.g., lecithin, DATEM, CITREM, fatty acids), those stabilized by cationic surfactants have a positive charge (e.g., lauric arginate), those stabilized by polysaccharide emulsifiers tend to have a negative charge (e.g., gum Arabic, modified starch, and beet pectin), and those stabilized by proteins have a positive charge below the isoelectric point (pI) and negative charge above it (e.g., whey protein, casein, soy proteins, egg proteins). The charge on emulsifier-coated droplets may also be altered by adsorption of other charged substances onto their surfaces, such as proteins, polysaccharides, or multivalent ions (McClements, 1999)

2.2 Colloidal Delivery Systems

A colloidal delivery systems consist of a dispersion of small particles that are designed to encapsulate a functional ingredient. The encapsulated ingredient is trapped inside colloidal particles for controlled solubility, to prevent degradation or undesirable chemical reactions. The particles usually have dimensions ranging from a few nanometers to a few hundred micrometers. The functional ingredients may be flavors, colorants, antimicrobials, antioxidants, vitamins, nutraceuticals and drugs just to mention a few. The are many kinds of food grade delivery systems, e.g. nano-droplets, micro-droplets, filled droplets, coated droplets, solid lipid particles, micelles- and microemulsions. Micelles have dimension between 5-20nm. They have non-polar tails clustered together so they can trap hydrophobic particles. Microemulsions have dimension between 10-100 nm. They can be used to encapsulate hydrophobic compounds.

2.3 Multilayer emulsion

A multilayered emulsion consist of a lipid droplets surrounded by nanolaminated biopolymer layers. The best way for formatting a multilayer emulsion is to add one biopolymer at time. It is easier to control the interfacial properties with multilayer emulsions opposed to conventional emulsions. Interfacial refers to; charge sign and density, thickness, packing, selective permeability, rheology and responsiveness. It is also easier to improve the emulsion properties with multilayer emulsions like increased shelf life and controlled-and triggered release. Multilayer technology could be used to improve the stability of many food emulsions to environmental stresses. Emulsions containing lipid droplets coated by nanolaminated layers can be prepared by a simple cost effective method using food ingredients. These emulsions have improved stability to environmental stress, such as heating, freezing, drying, pH extremes, and high mineral contents. Future studies are needed to determine their suitability for use in real foods**.**

2.4 Solid Lipid Nanoparticles (SLN)

Solid lipid nanoparticles emulsions also consist of emulsifier-coated lipid droplets dispersed in an aqueous continuous phase. The lipid phase is either fully or partially solidified and the morphology and packing of the crystals within the lipid phase are usually controlled to obtain some particular functional attributes (Saupe et al. 2005, Souto et al. 2004, Uner et al. 2004, Wissing et al. 2004, Wissing & Muller 2002).

2.4.1 Solid Lipid Nanoparticle preparation

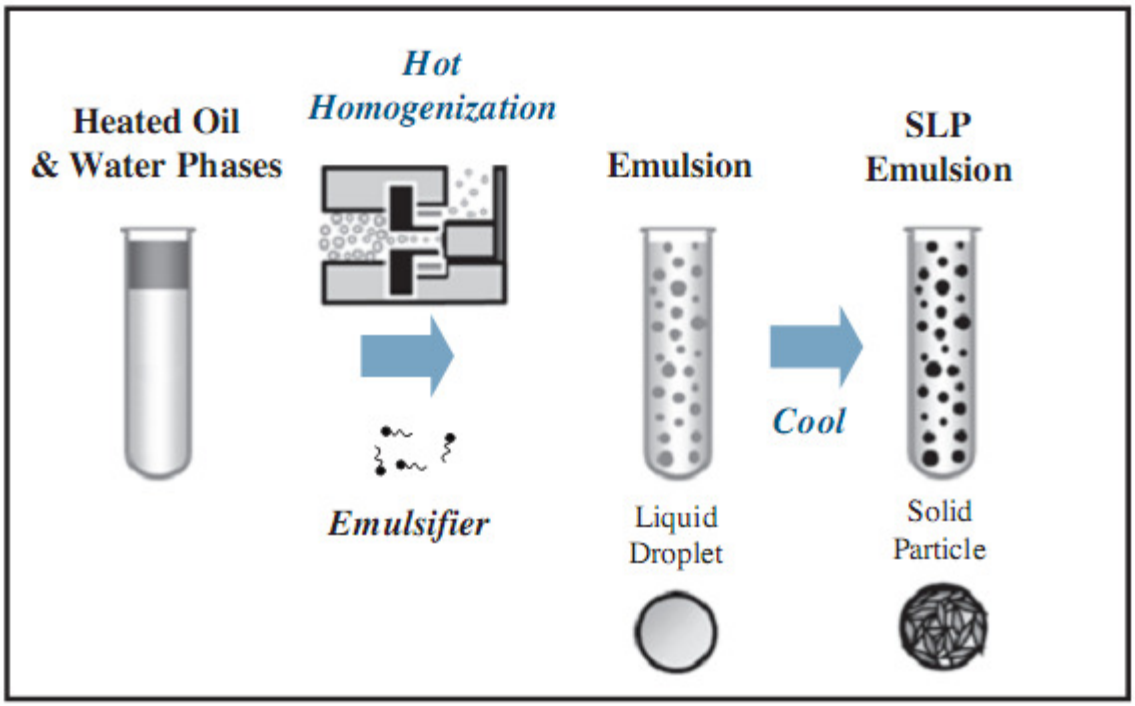


Figure 4. Solid lipid Nanoparticles (SLN) are typically produced by homogenizing an oil phase and aqueous phase together in the presence of a water-soluble emulsifier at a temperature above the melting point of the lipid phase. The emulsion is then cooled to promote fat crystallization

Solid lipid Nanoparticles are usually created by homogenizing an oil phase and aqueous phase together in the presence of a water-soluble emulsifier at a temperature above the melting point of the lipid phase (Figure 3) (Saupe et al. 2005, Souto et al. 2004, Uner et al. 2004, Wissing et al. 2004, Wissing & Muller 2002, Schubert & Muller-Goymann 2005). The emulsion is then cooled so the portion of the lipid phase crystallizes. It is crucial that the emulsion is kept above the crystallization temperature of the lipid phase during homogenization, otherwise the homogenizer may be blocked and damaged. It is possible to create a variety of different internal structures within solid lipid particles, e.g., homogeneous crystal structure, core-shell structures, or crystal dispersions (Figure 4). In addition, it is possible to control the relative location of the different phases within the droplets, e.g., the core could be solid and the shell liquid, or vice versa (McClements, 2010).

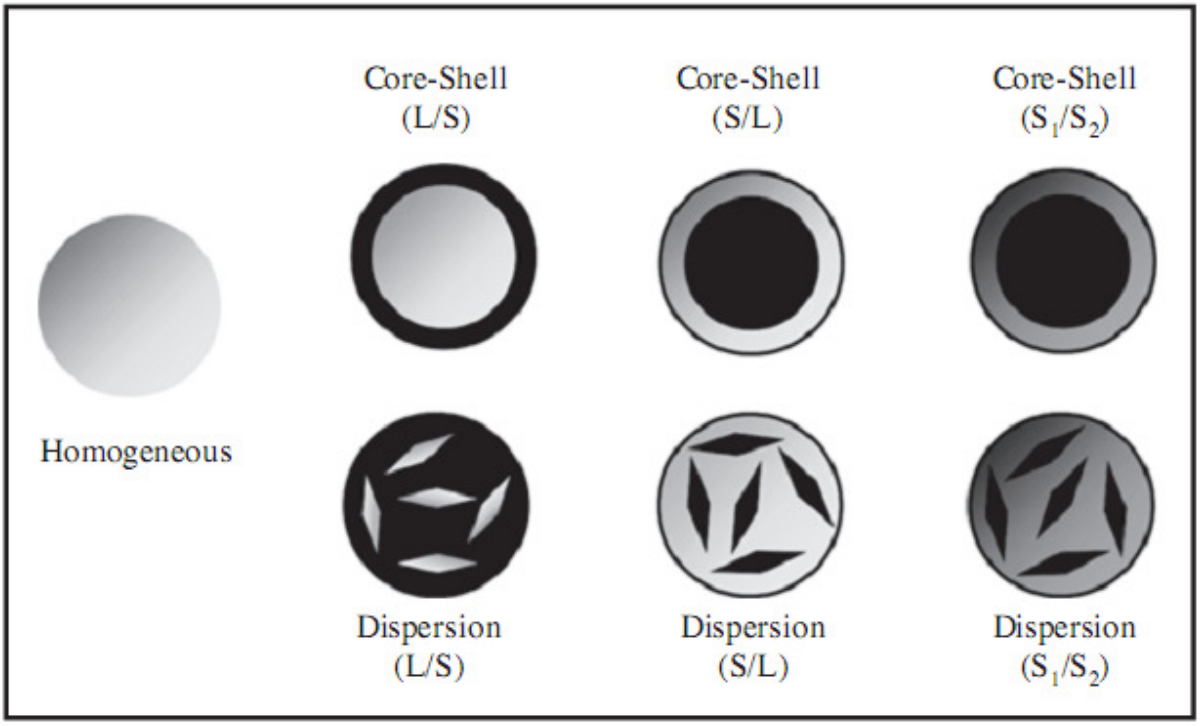


Figure 5. Examples of different kinds of structural organization possible within lipid droplets containing solid fat crystals

2.5 Emulsion stability

Oil and water are liquids which are highly immiscible (e.g. triglycerides + water) and can be vigorously mixed to form a nontransparent, heterogeneous mass; however, on standing the mixture will separate into two clear, homogeneous layers. Thermodynamics predict that spontaneous processes occur in the direction of decreasing Gibbs free energy, therefore we can conclude that the separation of two-phase dispersed systems to form two distinct layers is a change in the direction of decreasing Gibbs free energy. The coarsening process of a thermodynamically unstable dispersion is called coalescence or aggregation.

Coalescence is the irreversible coming together of two or more emulsion droplets to form a larger single particle. When two droplets come close together, their coalescence depends on the stability if the thin liquid film of the continuous phase separating them. Coalescence usually occurs when droplets have been close together for an extended period of time in a cream layer or in some other flocculated state. Only when the intervening film of continuous phase has thinned to a certain critical thickness is there a finite probability of film breakage (Dickinson, 1992).The central feature of coalescence is the fact that total surface area is reduced. Coalescence is extensive, it leads to the formation of a macrophase and the emulsion is said to have been broken. Thermodynamic stability is reached when all the

droplets in an emulsion have coalesced into a single homogeneous region of oil or aqueous solution (Dickinson, 1992). The protective layer of the emulsifier around the droplet in a food emulsion normally inhibits coalescence.

Flocculation is the process by which small particles clump together like a cluster of grapes, but do not fuse into a new particle. In aggregation there is no reduction of surface, although certain surface sites may be blocked at the points at which the smaller particles touch. A colloid that is stable against coalescence or aggregation is called kinetically stable (Hiemenz and Rajagopalan, 1997).

The most obvious visible manifestation of colloid instability is the settling of particles, droplets or aggregates under gravity called sedimentation or creaming depending on whether the dispersed phase moves down or up. Creaming is the movement of oil droplets, under gravity or in a centrifuge, to form a concentrated layer at the top of an oil-in-water emulsion sample, with no accompanying change in the droplet-size distribution. In the early stages there is merely a vertical concentration gradient of droplets, but later on a distinct boundary may appear between an upper cream layer and a lower depleted layer. Creaming is reversible, in the sense that the original uniform distribution of droplet can usually be reestablished by gentle mixing. Stockes' Law is a useful guide to creaming behavior in the absence of flocculation. It tells us that there are three ways to inhibit creaming in a dilute emulsion: reduce the average droplet size, reduce the difference in densities of the phases, or increase the viscosity of the dispersion medium. Flocculation increases the effective size of the particles within the emulsion (which enhances creaming), while decreasing the density contrast between the particles and the surrounding fluid (which retards creaming). The overall influence of flocculation on the creaming velocity can be conveniently characterized by creaming instability ratio: ν flocculation / ν Stokes'law. Liquid edible oils normally have lower densities than liquid water and so creaming is more prevalent in oil-in-water emulsions, whereas sedimentation is more prevalent in water-in-oil emulsions. Thus, droplets in an O/W emulsion tend to cream, whereas those in water-in-oil (W/O) emulsion tend to sediment. Nevertheless, this may not be the case in emulsions that contain fully or partially crystalline lipids because the density of oils usually increases when crystallization occurs. A cream may be flocculated or colloidally stable, but the droplets should not have coalesced to any significant extent (McClements, 2010). In relation to emulsion shelf-life, coalescence is a much more severe form of instability than creaming: when perceived by the consumer in food product, it is almost invariably considered quite unacceptable. On the other hand, the process of coalescence has a positive role in ensuring desirable release of flavor components during

eating. The physical state of the oil phase, especially the degree of solidification, affects the nature of the coalescence process in oil in water emulsions. When the dispersed phase is wholly liquid, the coalescing droplets merge into larger spherical globules, and eventually free oil is seen at the surface. When the oil is partially crystallized, the droplets come together to form irregular clumps composed of a continuous network of flocculated fat crystals held together by necks of liquid fat. Proteins like casein and gelatin are well-known flocculants of colloidal particles. But at higher concentration they act as protective colloids, inhibiting flocculation of emulsion droplets by a steric stabilization mechanism. The other factors affecting the shelf-life of food emulsions are bacterial action and freezing. During freezing, ice crystals nucleate and grow at the expense of liquid water. Stability of emulsions repeated freeze-thaw cycles generally increases with the amount of emulsifier (Buzagh and Rohrsetzer, 1961) (Dickinson, 1992). The more complicated the system, the more sensitive to environmental conditions.

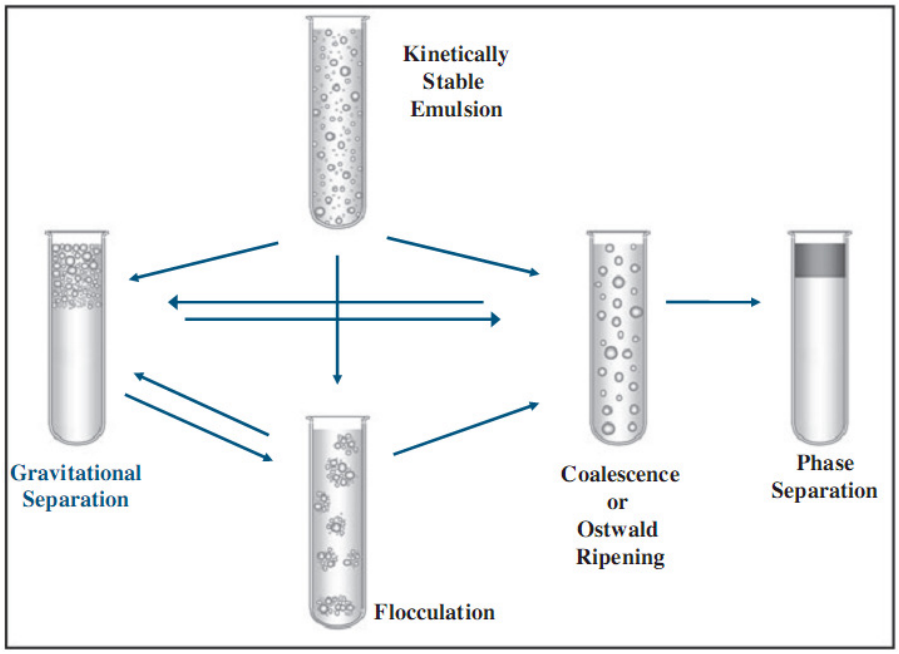


Figure 6. Schematic diagram of most common instability mechanisms that occur in food emulsions: creaming, sedimentation, flocculation, coalescence, Ostwald ripening, and phase separation.

Phase inversion is the process whereby a system changes from an O/W emulsion to a W/O emulsion or vice versa. In some applications phase inversion is desirable, like in the making of butter and margarine. But in other application phase inversion is undesirable, because it has an adverse effect on their appearance, texture, stability, and taste. The physicochemical basis

of phase inversion is believed to be extremely complex, involving aspects of flocculation, coalescence, partial coalescence, and emulsion formation (Brooks, 1998).

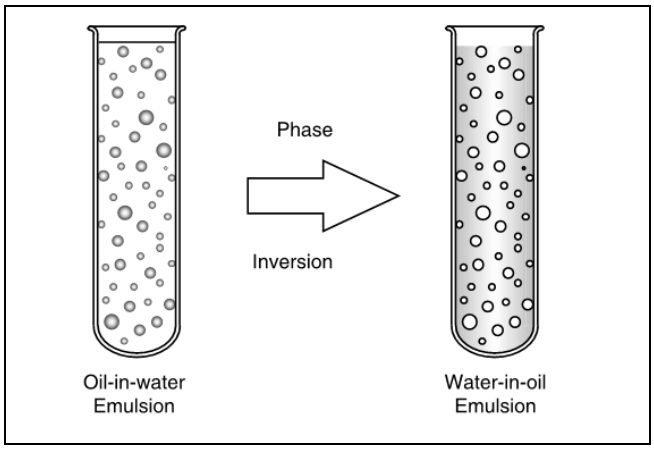


Figure 7. Phase inversion involves the conversion of an oil-in-water emulsion to a water-in-oil emulsion or vice versa.

2.6 Freeze-thaw stability

Freeze/thaw stability is the ability of a product to maintain its composition and integrity after repeated cycles between freezing and ambient temperature levels. Even minor temperature fluctuations can cause slight thawing of liquids within a product. Those ice crystals freeze at a larger size, causing the break-down of a product's structure. When O/W emulsion is frozen only part of the water is initially crystallized and the oil droplets are forced into the remaining liquid region (Sherman, 1968a; Berger, 1997; Dickinson and Stainsby, 1982; Hartel, 2001). The oil droplets may crystallize during a freezing process, which can lead to emulsion instability through partial coalescence. Freezing of the water phase may lead to dehydration of the emulsifier molecules adsorbed to the surface of the droplets, which promotes droplet-droplet interactions. There are many factors that contribute to the instability of emulsions during freezing and thawing (McClements, 1999). Under certain circumstances, freezing can cause cold denaturation of proteins, which may lead to reduction in their functionality (Walstra, 2003).

2.7 Oxidation

The problem of oxidative deterioration is of greatest economic importance in the production of lipid-containing foods. Oxidation of unsaturated lipids not only produces offensive odors and flavors but can also decrease the nutritional quality and safety by the formation of secondary reaction products in foods after cooking and processing. Temperature and water

activity affect lipid oxidation as well as metal ions and light. The effects of lipid oxidation are loss of flavor quality because of hydroperoxide decomposition (e.g. rancid flavor, change of color and texture, consumer acceptance, economic loss). Loss of nutritional quality because of free radicals (e.g. essential fatty acids, vitamins) health risks (e.g. toxic compounds, growth retardation, hearth diseases).

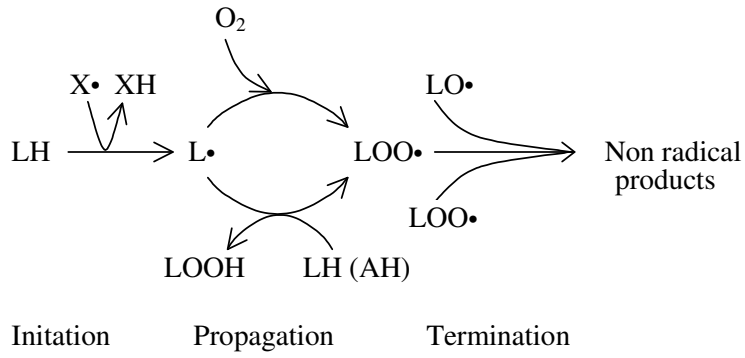


Figure 8 Schematic illustration of the lipid oxidation process. LH = unsaturated lipid, X• = initiator, L• = alkyl radical, LO• = alkoxyl radical, LOO• = peroxy radical, LOOH = hydroperoxide, AH = antioxidant.

Conjugated double bonds are less reactive than non-conjugated double bonds, there can be a lot of double bonds in chemical structure but they are not necessarily conjugated. That is why fish oil is very susceptible (polyunsaturated). As the number of double bonds increases, number and reactivity of radicals increases.

Antioxidants are substances that delay the onset of, or slow down the rate of oxidation. The most common types of lipid soluble antioxidants are mono-or polyhydric phenols with ring substitutes. Antioxidant works either by inhibiting the formation of free radicals in the initiation step or interrupting propagation of the free radical chain. Ideal antioxidants have no harmful physiological effects, no objectionable flavor, odor or color. It has to be effective in low concentration, fat-soluble, carry-through effect, readily-available, economical and not absorbable by the body.

The degree of oxidation that has taken place in a fat or oil can be expressed in terms of Peroxide value. Peroxides are primary reaction products formed during the initial stages of oxidation and give an indication of the progress of lipid oxidation. During oxidation the peroxide value will reach maximum followed by a decrease as the peroxides further react and decompose. Determination of peroxides relies on the fact that peroxides can liberate iodine from potassium iodide. When the double bonds of unsaturated fats become oxidized,

peroxides are among the oxidation production formed (Potter & Hotchkiss, 1998). The lipid to be tested is dissolved in a suitable solvent and excess potassium iodide is added. The amount of iodine liberated is then measured by a relatively simple titration with sodium thiosulphate and starch indicator. The peroxide value (PV) is expressed as milliequivalents of iodine per kg of lipid (meq/kg). The PV may be used as an indicator in its own right to measure the extent of oxidation in oil or it can be followed over time to determine the Induction Period (IP) which is generally regarded as the point of onset of oxidation as antioxidants are exhausted. PV of oil may be significantly reduced by heating in the absence of oxygen. The anisidine value is a measure of the secondary reaction products that occur during lipid oxidation and in many respects is a more robust indicator if the quality of the oil and the extent of oxidation as the secondary reaction products such as aldehydes and ketones are much more stable in the oil.

2.8 Van der Waals

One of the most important forces in surface and colloid chemistry are Van der Waals forces between atoms, molecules, or particles. These forces have their origin in the dipole or induced-dipole interactions at the atomic level and are therefore of extreme importance in almost all aspects of the study of materials. The strength of Van der Waals forces increases in the case of interaction between macroscopic objects such as colloidal particles since typically each particle has a large number of atoms or molecules. Almost all phenomena are influenced to various extents by forces that have their origin in atomic- and molecular-level interactions due to the induced or permanent polarities created in molecules by the electric fields of neighboring molecules or due to the instantaneous dipole caused by the “positions” of the electrons around the nuclei.

Van der Waals forces are always attractive. They are relatively long compared to other atomic- or molecular-level forces and can have an interval of influence ranging from about 0.2 nm to over 10 nm. The dispersion force between two atoms, molecules, or large bodies is influenced by the presence of other nearby particles. Nevertheless, we consistently add pairwise interactions between the atoms in separate bodies as our procedure for scaling up the interactions. This must be viewed as an approximation since perturbations by neighboring atoms limit the additivity of these forces (Hiemenz and Rajagopalan, 1997).

3. Materials and Methods

3.1 Analytical methods

3.1.1 Light scattering

When light is directed at a diluted colloidal system in a glass container, most of it passes through undisturbed, but some is scattered and some is adsorbed. The intensity scattered at a certain angle to an incident monochromatic of the intent beam depends on several factors, the wavelength and polarization of the incident beam, the difference in refractive index between the dispersed phase and the continuous phases, the size and shapes of the particles and the concentration and structure of the dispersion. (Dickinson and Rajagopalan, 1982)

3.1.2 Zeta-potential

Zeta potential measures the direction and velocity of particles, the measurement gives sign and magnitude. The charge is measured by electro-kinetic techniques. The charge strength depends on charge density and ionic strength. The zeta potential can be positive, negative or neutral, which depends on the emulsifier type.

3.1.3 Optical microscopy

The optical microscope is a type of microscope which uses visible light and a system of lenses to magnify images of small samples.

3.2 Chemicals and other materials

Genu Pectin (citrus) type USP/100 was purchased from Sigma-Aldrich (St. Louis, MO)., Chitosan TM1766 and G060724P (Genis, Iceland), Benefat B/D (Danisco. Ardsley, NY), Sodium Caseinate (ALANATE 180) with 1.2% sodium content and 0.1% calcium content was kindly provided from NZMP (Lot # 0034-W5166, Lemoyne, PA). As stated by the supplier, this product contained 92.7% protein, 4.2% moisture, 3.5% ash, 0.8% fat, and 0.1% lactose. High methoxyl pectin (HMP; 59% DE) was obtained from the Sigma Chemical Company (Lot #91K1420, St Louis, MO). Acetic Acid monohydrate (Fisher Scientific. Pittsburgh, PA), Sodium acetate dehydrate (CH_3COONa), (Fisher Scientific), Sucrose (Fisher Scientific. Pittsburgh, PA). Powdered b-lactoglobulin (b-Lg) (pI ≈ 5.2) was kindly supplied by Davisco Foods International (Lot No. JE 001-3-922, LeSueur, MN). The protein content was reported to be 98.3% (dry basis) by the supplier, with b-Lg making up 95.5% of the total protein. The moisture content of the protein powder was reported to be 4.9%. The fat, ash and

lactose contents of this product are reported to be 0.3 ± 0.1, 2.5 ± 0.2 and <0.5 wt%, respectively. Analytical-grade hydrochloride acid (HCI), sodium hydroxide (NaOH) and Sodium Azide (NaN_3) were purchased from Sigma-Aldrich (St. Louis, MO). Water from a water purification system (Nanopure infinity, Barnstead International) was used for preparation of all solutions. Corn oil (Mazola, ACH Food Companies, Inc.) was purchased from a local supermarket and was used without further purification.

3.3 Emulsion ingredients

3.3.1 Fish oil

Fish oil is derived from the tissue of oily fish, fish oil is the best source of omega-3 fatty acids. Our bodies cannot produce carbon-carbon double bonds before the ninth carbon from the methyl end, so we cannot manufacture certain fatty acids such as omega-6 or omega-3, they must come from food and are therefore called essential fatty acids. Omega-3 fatty acids: Linoleic acid, an eighteen carbon essential fatty acid with three double bonds (18:3). It can ultimately be elongated and desaturated to EPA (Eicosapentaenoic acid), with twenty carbons and five double bonds (20:5), and DHA (docosahexaenoic acid), with twenty carbons and six double bonds (20:6). (Insel, Turner and Ross, 2004). In the 1970s a study of the Inuits focused attention on the beneficial effects of EPA and DHA. This group of people had a high intake of fat, saturated fat, and cholesterol from marine mammals and fish, yet they showed little evidence if atherosclerosis. The Inuits were compared to the Danes, among whom atherosclerosis was common and whose diet was similarly high in fat, but from meats and dairy products. It became clear that the high EPA and DHA content of fish in the Inuit diet protects against heart disease, by discouraging blood cells from clotting and sticking to the artery walls, and by reducing inflammation. Many other studies point in the same direction; some show that as few as two or three servings of fish weekly can be protective. (Insel and others 2004).

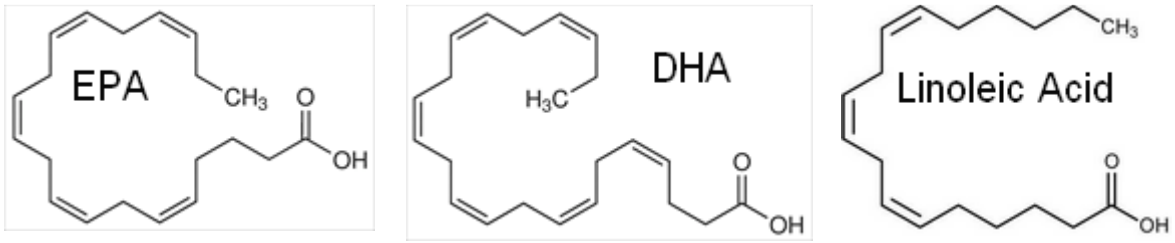


Figure 9. Chemical structure of EPA, DHA and Linoleic Acid

The fish oil (Ropufa 30 *n*-3 food oil) was obtained from DSM Nutritional Products Ltd. (Basel, Switzerland). The oil contained 101 mg of EPA/g of oil, 148 mg of DHA/g oil, and a total omega-3 PUFA of 312 mg/g of oil.

3.3.2 Corn oil

Corn oil, which is extracted from corn germ, has a high polyunsaturated fatty acid content and oxidative stability.

3.3.3 Salatrim

The salatrim we were using came from Danisco sold under the brand name Benefat B/D, Benefat is a Kosher approved triglyceride. Benefat B/D is a mixture of short chain (C3) and long chain (C16-C22) fatty acid esters of glycerol. The application areas are in baked goods, in place of traditional shortening and filling fats. The potential benefits are reduced fat, reduced calories and no compromise in taste. To insure the best creaming properties the Benefat B/D should be applied at a temperature of 23-26°C.

3.3.4 Sodium caseinate

Sodium caseinate is the biochemical name for casein, which is a type of protein found in the milk from all mammals. Casein is a major component of commercial cheese and its principle source of protein. Casein is also used as a food additive and for industrial purposes. Milk contains two proteins in roughly an 80 to 20 ratio, with casein being the primary protein. Sodium caseinate is a stabilized molecule of sodium hydroxide and casein. As a food source, casein supplies amino acids, carbohydrates and two inorganic elements, calcium and phosphorus. Casein is a highly nutritious protein, containing many essential amino acids.

3.3.5 Sodium lauryl sulfate

Sodium lauryl sulfate, in science is referred to as sodium dodecyl sulfate (SDS). SDS is an anionic surfactant used in many cleaning and hygiene products. The salt consists of an anionic

organosulfate consisting of a 12-carbon tail attached to a sulfate group, giving the material the amphiphilic properties required of a detergent. SDS is a highly effective surfactant and is used in a variety of tasks requiring the removal of oily materials and residues. SDS represent a potentially effective topical microbicide, which can also inhibit and possibly prevent infection by various enveloped and non-enveloped viruses such as the Herpes simplex viruses, HIV, and the Semliki Forest Virus. (Piret, Desormeaux, Bergeron, 2002; Piret and others 2000)

In medicine, SDS is used rectally as an laxative in enemas, and as an excipient on some dissolvable aspirins and other fiber therapy caplets.

SDS is probably the most researched anionic surfactant compound available. Like all detergent surfactants (including soaps), SDS removes oils from the skin, and can cause skin and eye irritation.

3.3.6 Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine.

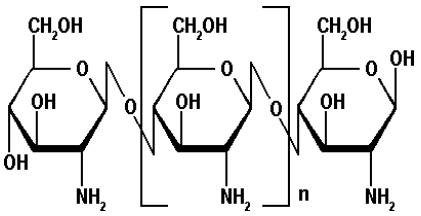


Figure 10. Chemical structure of Chitosan

Chitosan is obtained by alkaline deacetylation of chitin, which is the principal component of the protective cuticles of crustaceans (such as crabs, shrimps, and lobsters) and of the cell walls of some fungi (such as Aspergillus and Mucor) (Muzzarelli, 1973; Roberst, 1992) Chitosan has a high density of ammonium groups and hydroxyl groups in its polymer structure that makes Chitosan cationic polysaccharide (pK_a 6.5), one of few found in the nature. Chitosan possesses high positive charge on $–NH_3$ groups when dissolved in aqueous acidic solution, and therefore it is able to adhere to or aggregate with negatively charged lipids and fats. (Wydro, Krajewska and Hac-Wydro, 2007). Chitosan can be produced as an ingredient with different molecular weights (MW) and degrees of deacetylation (DDA) by varying the chitin extraction and treatment conditions. The DDA can be determined by NMR spectroscopy. Commercial chitosan ranges from 60 to 100%. On average the molecular weight of commercially produced chitosan is between 3800 and 20.000 Daltons. Previous

studies have shown that soluble or insoluble chitosan-caseinate complexes can be formed depending on the pH. The characteristics of the complexes are determined by the biopolymer types and their concentration, as well as by the environmental conditions. At pH 3.0-3.8, where chitosan and sodium caseinate have similar charges, they may dissociate from each other and become solubilized in solution (Anal, Tobiassen, Flanagan and Singh, 2008). Chitosan has been proposed as a particularly effective material for encapsulating fish oils because of its unique functional attributes, natural abundance and underutilization. (Klaypradit and Huang, 2008; Klinkesorn and others 2004; Peniche, Howland, Carrillo, Zaldivar and Arguelles-Monal, 2004).

3.3.7 Pectin

Pectin is a complex mixture of polysaccharides that makes up about one third of the cell wall dry substance of higher plants. Much smaller proportions of these substances are found in the cell walls of lower, plants like grasses. Like most other plant polysaccharides, it is both polydisperse and polymolecular and its composition varies with the source and the conditions applied during isolation. In any samples of pectin, parameters such as molecular weight or content of particular subunits will differ from molecule to molecule. The highest concentrations of pectin are found in the middle lamella of the cell wall, with a gradual decrease as one passes through the primary wall toward the plasma membrane (Kertesz, 1951). At present, commercial pectins are almost exclusively derived from citrus peel or apple pomace, both by-products from juice manufacturing. (May, 1990). In human digestion, pectin goes through the small intestine more or less intact. Pectin is thus a soluble dietary fiber. Consumption of pectin has been shown to reduce blood cholesterol levels. The mechanism appears to be an increase in viscosity in the intestinal tract, leading to a reduced absorption of cholesterol from bile or food. In the large intestine and colon, microorganisms degrade pectin and liberate short-chain fatty acids that have positive influence on health

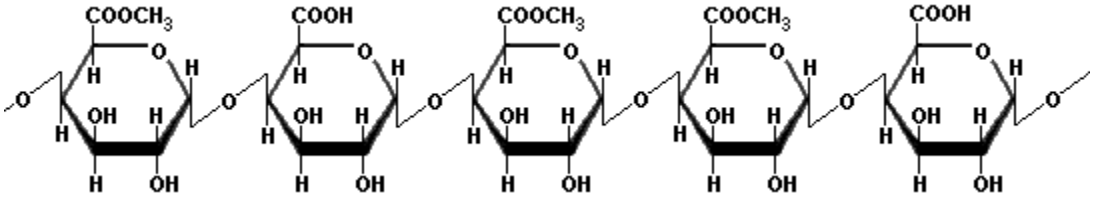


Figure 11. Chemical structure of pectin.

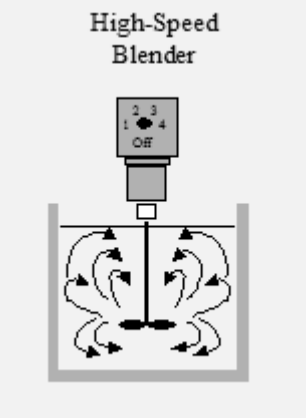
Pectin is a polysaccharide, composed of different sub-structural entities that vary with the extraction methodology, raw material, location, and other environmental factors. The linear backbone of the pectin polymer is called homogalacturonan, and it is built by sequences of α (1→ 4) linked D-galacturonic acid residues. These building blocks of polygalacturonic acid can be esterified and present as the methylesters and the free acid groups. They may be partly or fully neutralized with cations such as sodium, potassium, calcium, or ammonium. The structural properties of the pectin molecule, such as molecular weight, degree of esterification (DE), degree of amidation (DA) and presence of acetyl groups considerably affect the gelation properties of pectin. The DA value indicates the percentage of carboxyl groups that have been converted to the amide form by ammonia processing. Commercial pectin types are, divided into product groups depending on their DE. Due to processing parameters the molecular weight will also decrease as the DE decreases. There can be a wide range of DEs dependent on species, tissue, and maturity. In general, tissue pectins range from 60 to 90% DE. The pectin classes based on the DE are high methoxyl (HM) pectins, and the low methoxyl (LM) pectins which are either the conventionally demethylated or the amidated molecule. DEs values for commercial HM-pectins typically range from 60 to 75% and those for LM-pectins range from 20 to 40%. These two groups of pectin gel by different mechanisms. HM-pectin requires a minimum amount of soluble solids and a pH within a narrow range, around 3.0, in order to form gels. HM-pectin gels are thermally reversible. In general, HM-pectins are hot water soluble and often contain a dispersion agent such as dextrose to prevent lumping. LM-pectins produce gels independent of sugar content. They are also not as sensitive to pH as the HM-pectins are. LM-pectins require the presence of a controlled amount of calcium or other divalent cations for gelation. (CP Kelco, 2008)

3.4 Equipment

3.4.1 Magnetic stirrer

Is a device that employs a rotating magnetic field to cause a stir bar immersed in a liquid to spin, in most cases it is possible to control the speed of the stir bar. The magnetic stirrer is for example used to blend chemicals together and make them homogeneous, or to dissolve powder in liquid. Corning Stirrer/Hot Plate.

3.4.2 High speed mixer



High speed mixers use turbines or propellers to pre-mix emulsions of low-viscosity liquids. They operate by a shearing action in the food at the edges and tips of the blades (Fellows, 2000). It is possible to make a coarse emulsion using a high speed blender. The specific one we used in these experiments was, M133/1281-0, Biospec Products, Inc., ESGC, Switzerland

Figure 12. Handheld high speed mixer

3.4.3 Microfluidizer

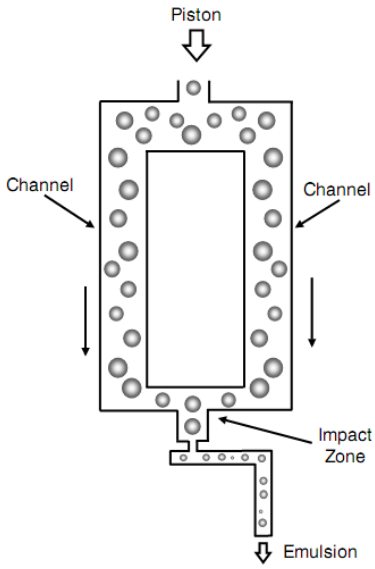


Figure 13. Microfluidizer

Is a high intensity method that makes the smallest droplets. This type of homogenizer usually consists of a fluid inlet, some kind of pumping device, and an interaction chamber containing two channels through which the fluids are made to flow and interact with each other. Fluids are introduced into the homogenizer, accelerated to a high velocity within the channels using the pumping device, and then made to simultaneously impinge with each other on a solid surface. Intense disruptive forces are generated when the fluids collide with each other, which cause the fluids to intermingle, and the droplets to be disrupted. A microfluidizer can be used to reduce the size of the droplets in a preexisting emulsion by directing portions of the coarse emulsion to flow through different channels (Figure 12). (Dickinson and Stainsby, 1988) External pressure-type micro kit, MG-20-5, Kiyomto Iron Works Ltd. Japan.

3.4.4 Sonicator

The ultrasound-driven sonication is performed using a sonicator containing a piezoelectric quartz crystal that can expand and contract in response to alternating electrical voltage. As the tip of a sonicator probe contacts the process liquid, it generates mechanical vibrations and therefor cavitation occurs. (Eisenberg & Tulin, 1961; Krishnan, Vekatasubramainan & Rajagopal, 1959; Fridman, 1972; Higgins & Skauen, 1972). Cavitation is the process of vapor bubble formation in a liquid under a reduced pressure at ambient temperature. These cavitation bubbles grow, migrate and collapse in the fluid. The pressure associated with the collapse of the cavitation bubbles is extremely high and can reach 10.000-20.000 psi (Eisenberg & Tulin, 1961). The con of using a sonicator is that the brake up only happens around the probe, it is important to have homogenous sample and a stir bar stirring while making the emulsion. Continuous application of ultrasound to a sample can cause appreciable heating, and so it is often advantageous to apply the ultrasound in a number of short bursts. In our case the beaker glass containing the emulsion stood in ice water while the sonication took place. The sonicator we used in this experiment was from Fisher Scientific, Sonic Dismembrator Model Pittsburgh, PA

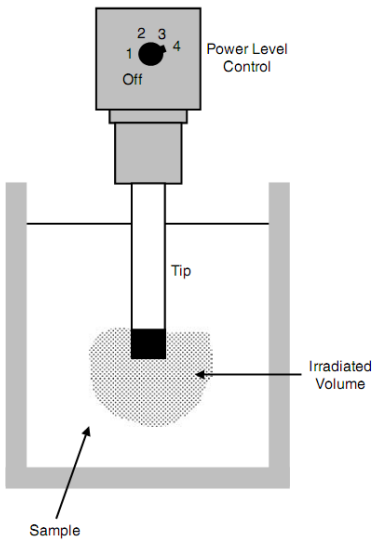


Figure 14. Ultrasonic probe

3.4.5 pH meter

pH meter is an electronic instrument used for measuring the pH of a liquid. A typical pH meter consists of a special measuring probe connected to an electronic meter that measures and displays the pH reading. The pH meter we used in this study was Thermo Orion,
Beverly, MA.

Figure 15. pH meter

3.4.6 Particle size / DLS

Dynamic light scattering measures the time dependent fluctuations in the scattering intensity to determine the translational diffusion coefficient (D), and subsequently the hydrodynamic diameter (D_H) of a droplet $D = \frac{kT}{3\pi\eta DH}$

The rate of intensity fluctuation is dependent upon particle size. The intensity fluctuations are passed into a digital correlator. The correlator continually adds and multiplies short time scale fluctuations in the measured scattering intensity to generate the correlation function for the sample. If the particles are large, the signal will be changing slowly and the correlation will persist for a long time. If the particles are small and moving rapidly then the correlation will disappear more rapidly. Volume size distributions are derived from the intensity distribution using Mie theory. The volume size distribution is equivalent to the mass or weight distribution, and the optical properties of the particles are required to make this transformation. Mie theory states that, when the size of the particles becomes equivalent to or greater than the wavelength of the laser, the scattering becomes a complex function with maxima and minima with respect to angle. The laser light scattering instrument we used was

Figure 16. The Malvern Mastersizer

The Malvern Mastersizer it is used to measure particle sizes in the micrometric range. It works with the principle of laser diffraction: a laser beam is directed into a diluted sample and through measuring the angular intensity of the scattered light, a particle size distribution can be obtained. Mastersizer, Malvern Instruments Ltd., Worcs., UK

3.4.7 Electrical charge/ ZetaMaster.

Zeta potential is a physical property which is exhibited by any particle in a suspension. Zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in a dispersion. It is a very good index of the magnitude of the interaction between colloidal particles. The zeta potential is the electrostatic potential at the particle slipping plane, which is the outer boundary within which all ions diffuse with the particle. The zeta potential is a function of the surface charge density, and as such, any change in the surface charge, either valence or ion condensation, will lead to changes in the measured zeta potential. Small changes in the pH or concentration of ions can lead to dramatic changes in the zeta potential. Zeta potential measurements can be used to predict dispersion stability and hence product shelf life. If all the particles have a large negative or positive zeta potential they will repel each other. A dividing line between stable and unstable dispersions is generally taken at either +30 or -30 mV. As the zeta potential moves toward 0 mV the possibility of particle aggregation increases, leading to a reduction in stability. We used ZEM5003, Zetamaster, Malvern Instruments, Worcs., UK

Figure 17. ZEM5003 Zetamaster (Malvern Instruments)

3.4.8 Oxidation measurements

The TBARS as well as peroxide values were measured in the emulsions. The primary and secondary emulsions were not stable at the same pH. At pH 3 were the primary emulsion is stable the emulsion has a positive charge, chitosan also has a positive charge at pH 3. Therefore the chitosan cannot bind to the sodium caseinate in the primary emulsion and make the second layer. At pH 5 were our secondary emulsion was the best, at that pH the primary emulsion was negatively charge and could therefore bind to the positively charged chitosan, the primary emulsion was not stable because of the isoelectric point of sodium caseinate which is around 4.6. So to be able to make some comparison emulsions two emulsions were prepared with tween20 at pH 3 and at pH 5.

3.4.8.1 Centrifuge

A centrifuge is a piece of equipment, generally driven by an electric motor that puts an object in rotation around a fixed axis, applying a force perpendicular to the axis. The centrifuge works using the sedimentation principle, where the centripetal acceleration causes more dense substances to separate out along the radial direction (the bottom of the tube). By the same token, lighter objects will tend to move to the top (of the tube; in the rotating picture, move to the center). The Thermo Scientific CL10 centrifuge that we used came from Thermo Scientific, Waltham, MA.

Figure 18. The Thermo Scientific CL10 centrifuge.

3.4.8.2 Spectrophotometer

A Spectrophotometer consists of two instruments, namely a spectrometer for producing light of any selected color (wavelength), and a photometer for measuring the intensity of light. Spectrophotometer is employed to measure the amount of light that a sample absorbs. The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector. The type of the spectrophotometer we used was Thermo Spectronic Genesys 20 also from Thermo Scientific, Waltham, MA.

Figure 19. The Thermo Spectronic Genesys 20

3.4.8.3 TBARS determination for emulsions

TBA regent is prepared from 15% TCA (trichloroacetic acid) – 0.375% TBA (2-thiobarbituric acid) in 0.25 M HCl (hydrochloric acid) and 2% BHT (butylated hydroxytoluene) in ethanol. 2 ml of TBA regents were placed in marked screw cap test tubes and 1 ml of the emulsion was added in a test tube. Then the closed test tube were placed in a boiling water bath for 15 min, the test tube was then transferred to cool water bath for 10 min. followed by

centrifugation in tin vials at 1000G for 15 min. Next the samples were allowed to sit for 10 min. and absorbance was measured at532 nm using a spectrophotometer. Blank samples were tested in the same manner for control. Concentrations of TBARS were determined from a standard curve prepared using 1,1,3,3-tetraethoxypropane.

3.4.8.4 Peroxide value determination for emulsions

Lipid hydroperoxides were measured using a method adapted from Nuchi (McClements & Decker,2001)). For the peroxide measurements the following solvents were prepared, iso-octane / isopropanol 3:1 v/v and methanol / butanol 2:1 v/v, thiocyanate solution (3.94 M) and Fe^{2+} solution (0.072M acidic solution). The thiocyanate solution was prepared by dissolving 3 g ammonium thiocyanate in water, and then fill up to volume at 10 mL. The Fe^{2+} solution was prepared from 2 other solutions. Solution 1: dissolve 0.8 g. $BaCl_2$ 2 H_2O in 25 ml 0.4 M HCl, solution 2: dissolve 0.2 g. $FeSO_4$ $7H_2O$ in 5 ml H_2O. Then 3 ml of solution 1 were mixed with solution 2 and centrifuged for 3 min and then supernatant collected for use.

First the emulsions were destabilized and lipid peroxides were collected. This was accomplished by vortexing, three times. 0.3 ml of the emulsion was added to 1.5 ml of iso-octane/isopropanol in 100 nm test tubes. Then the test tubes were centrifuged for 2 min at max speed. Following centrifugation, 0.2 mL of the isooctane:isopropanol extract (upper layer) was carefully removed and mixed in test tubes with 2.8 mL of methanol/1-butanol (2:1 vol/vol). 1 ml $BaCl_2$ (Barium chloride) was deposited into another test tube. Weight 0.2 g. of iron sulphate into a 10 ml test tube, then 5 ml of water were added and vortexed then 1 ml was transferred into the $BaCl_2$ test tube. This was centrifuged for 2 min. Next thiocyanate and the Fe^{2+} solution were mixed and 30 µl were aliquot to each sample. This was then kept in the dark for 20 min before the absorbance of the sample was measured at 510 nm using a spectrophotometer, using plastic disposable cuvettes. The concentration of hydroperoxides was determined based on a standard curve of cumene hydroperoxide (0-20 µM). The Thiocyanate solution was made by dissolving ammonium thiocyanate in water. Furthermore the Fe^{+2} solution was prepared by making two separate solutions and then mixing them together. $BaCl_2$ dissolved in water and HCl. Then $FeSO_4$ was dissolved in water and mixed together with the $BaCl_2$ solution. Iso-octane/isopropanol and methanol/butanol solvents were prepared.

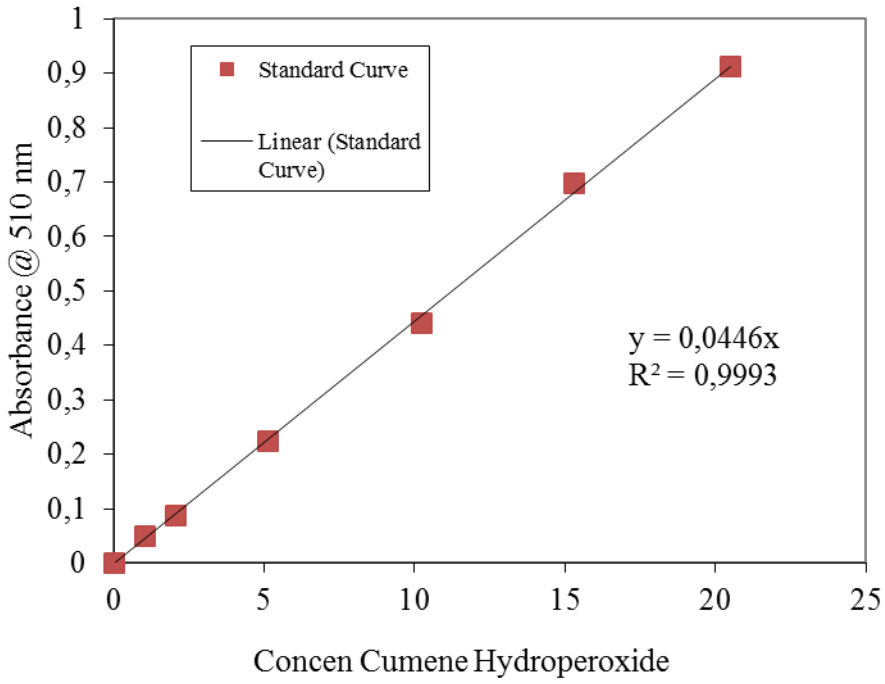


Figure 20. Standard curve prepared using 1,1,3,3-tetraethoxypropane

3.4.9 Optical Microscopy

The optical microscope, often referred to as the "light microscope", is a type of microscope which uses visible light and a system of lenses to magnify images of small samples. Basic optical microscopes can be very simple, although there are many complex designs which aim to improve resolution and sample contrast. The microstructures of selected emulsions were observed using an optical microscope (Nikon Eclipse E400, Nikon Corp., Japan). Emulsion samples were slightly vortexed in a glass test tube before analysis. A drop of emulsion was then placed on a microscope slide, covered by a cover slip. An image of the sample was acquired using digital image processing software and stored on a personal computer.

3.5 Preliminary testing

Preliminary tests were conducted to determine the number of passes required through the microfluidizer. Emulsions were made using beta-lactoglobulin, tween 20 or sodium caseinate. Corn oil was used instead of the fish oil,for the preliminary experiments because it is not as prone to oxidation, more easily available and less expensive.

First emulsions with 10% corn oil and 1% emulsifier were prepared. Three

emulsifiers; tween20, sodium caseinate and beta-lactoglobulin were compared. The oil in water emulsion was prepared by homogenizing 10% (w/w) corn oil and 90% (w/w) of the emulsifier solution. The oil and emulsifier solution were mixed together using a high speed mixer for 1 ½ min (Tissue Tearor, Model 985370-395, Biospec Products Inc.). The resulting coarse premix was finely dispersed under pressure (11 kbar) using a microfluidizer (Microfluidics, Newton, MA). Emulsions were analyzed the following day.

Measurements of the mean droplet size were used to provide information about the degree of droplet aggregation in the emulsions. Prior to analysis the emulsions were diluted using buffer solution to avoid multiple scattering effects. Zetasizer Nano (Malvern) was used to measure the particle size against volume.

Chitosan was initially used as the secondary layer, because of its cationic properties allowing it to adsorb to the surfaces of the surfactant-stabilized droplets. Experiments were conducted to establish the optimal concentration. It has to be sufficient to completely cover the oil droplets but not in excess of that, because free chitosan in the emulsion was unfavorable. It is best to use an emulsion that is stable at the widest pH range.

ζ-Potential measurements were used to provide indirect information about the interfacial composition of primary and secondary emulsions. By measuring the droplets charge after a charged biopolymer is added to an emulsion one can infer whether the biopolymer has adsorbed chitosan or not. The emulsions were diluted using a buffer solution to avoid multiple scattering effects.

Creaming stability measurements were used to provide information about droplet aggregation in the emulsions. Ten mL of emulsion at pH 2 – pH 8 were transferred into a test tube and then sealed with a cap. This was then stored in a dark place at room temperature. After 1 day of storage several emulsion separated into different layers, a cream layer at the top and a transparent serum layer at the bottom. The creaming index provided indirect information about the stability. The higher the creaming index, the lower the emulsion stability. The total height of the emulsion (H_E) and the height of the serum layer (H_S) were measured by using a ruler.

Initially preliminary experiments were conducted using Salatrim or Benefat from Dansico. Benefat is a crystallized fat but at 23°C and higher temperature it is liquid. Because Benefat is crystallized at room temperature it is more complex to make the emulsions, everything has to be heated both, the fat and all the equipment. When the Benefat is incorporated in to the emulsion it does not crystallize at a lower temperature because the layer(s) that coat the fat droplets in the emulsion prevent crystallization. SDS was used as an

emulsifier in the preliminary tests with Benefat. A good emulsion with Benefat was expected and no problems with the interaction of fat and surfactant were for seen. The initial tests were conducted with the microfluidizer for emulsification but the fat crystallized upon cooling and the traces of crystallized Benefat keept clogging the Microfluidizer rendering the method unsuitable. Therefore the following experiments with Benefat were conducted with sonicator.

3.5.1 Solution preparation

Emulsifier solution containing 1 wt% protein was prepared by dispersing powdered sodium caseinate into 10 mM acetyl acid buffer (pH 2.0), the protein was added to the buffer solution little by little and then stirred overnight to ensure complete hydration. Chitosan solution (1 wt%) was prepared by dispersing weighed amount of the power material into 10 mM acetyl acid buffer (pH 2.0) and stirring overnight to ensure complete hydration. Pectin solution (1 wt%) was prepared by dispersing weighed amounts of the powered material into the same buffer and stirring for at least 2 hours to ensure complete hydration. The pectin solution was then diluted and the pH readjusted to pH 5 using HCl or NaOH solutions.

3.5.2. Emulsion preparation

An oil in water emulsion was prepared by homogenizing 10% (w/w) fish oil and 90% (w/w) sodium caseinate solution. The oil and sodium caseinate solution were mixed together using a high speed mixer for 1 ½ min (Tissue Tearor, Model 985370-395, Biospec Products Inc.). The resulting coarse premix was finely dispersed under pressure (11 kbar and eight times) using a microfluidizer (Microfluidics, Newton, MA). This emulsion was referred to as the "stock emulsion" and contained submicron lipid droplets stabilized the by the sodium caseinate. A series of emulsions containing the same oil [1% (w/w) fish oil] and protein [0.1% (w/w) sodium caseinate] contents were prepared. 100 ml of the 1°emulsion was prepared from 10 ml of stock emulsion and 90 ml of 10 mM acetate acid buffer (pH 2), with and without 10% Sucrose. 100 ml of the 2° emulsion was prepared from 10 ml of stock emulsion 40 ml of 10 mM acetate acid buffer (pH 2), with and without 10% Sucrose and then mixed with 4 ml of Chitosan solution and 46 ml of 10 mM acetate acid buffer, with and without 10% Sucrose. 100 ml of the 3° emulsion was prepared from 10 ml of stock emulsion 15 ml of 10 mM acetate acid buffer (pH5), with and without 10% Sucrose and then mixed with 4 ml of Chitosan solution and 21 ml of 10 mM acetate acid buffer, with and without 10% Sucrose. Then 21.7 ml of the pectin solution was mixed with 28.3 ml of 10 mM acetate acid buffer, with and without 10% Sucrose, the solution was adjusted to pH 5 before mixing it to the

pectin solution. The 2° and 3° emulsions both contained [0.04% (w/w) chitosan] but chitosan with different deacetylation (50% DDA, 70% DDA and 92% DDA), the 3°emulsions then contained [0.65% (w/w) pectin]. These emulsions are prepared by mixing different ratios of stock emulsion [10% (w/w) fish oil, 1% wt% sodium caseinate], chitosan solution [0.2% (w/w) chitosan], pectin solution [1% (w/w) pectin] and buffer solution.

- *Primary emulsion*, 1% w/w fish oil, 0.1% w/w sodium caseinate, 10 mM acetate acid buffer pH 3.
- *Secondary emulsion*, 1% w/w fish oil, 0.1% w/w sodium caseinate, 0.04% w/w chitosan (50% DDA, 70%DDA and 92% DDA) 10 mM acetate acid buffer pH 5.
- *Tertiary emulsion,* 1% w/w fish oil, 0.1% w/w sodium caseinate, 0.04% w/w chitosan, 0.65% w/w pectin, 10 mM acetate acid buffer pH 5.

Sucrose was included in the acetate acid buffer solutions, used to prepare the emulsions that we used for the freeze thaw experiments, to obtain a final sucrose concentration of 10% w/w. These mixtures were then stirred using magnetic stirrer. The flocculation formed in secondary-, and tertiary emulsion was disrupted by sonication for 30 sec at 20 kHz, and 70% amplitude (Model 500, Sonic Disembrator, Fisher Scientific, Pittsburgh). The resulting emulsions were stirred for 30 min and then stored at room temperature overnight before being analyzed.

3.5.3 pH

pH is a measure of the acidity or basicity of an aqueous solution. Solution with pH less than 7 are said to be acidic and solutions with a pH greater than 7 are basic. A low pH indicates a high concentration of hydronium ions, while a high pH indicates a low concentration. pH is defined as a negative decimal logarithm of the hydrogen ion activity in a solution.

$$\mathrm{pII} - -\log_{10}(a_{\mathrm{H}^+}) - \log_{10}\left(\frac{1}{a_{\mathrm{H}^+}}\right)$$

Where $a_{\mathrm{H}}+$ is the activity of hydrogen ions in units of molar concentration.

3.5.3.1 pH measurements

The pH was adjusted with the addition of HCl or NaOH to the primary, secondary and tertiary emulsion, the pH range span from pH 2.0–8.0. The influence of pH on various solutions and emulsions with different content and concentration was compared.

Chitin/chitosan with different degrees of deacetylation (50% DDA, 70% DDA and 92% DDA) were tested. The zeta potential was measured as a function of pH.

3.6 Environmental stress

3.6.1 Freeze-thaw

A number of physicochemical phenomena that occur during frozen storage may account for the observed influence of freeze-thaw cycling on emulsions stability. Water crystallization occurs when the emulsions are placed in the freezer. As more water crystallizes the droplets are forced closer together and then there may not be sufficient free water to fully hydrate the droplet surface, thus favoring droplet to droplet interactions. (Saito and others 1999; Ausborn and others 1994; Komatsu, Okada & Handa 1997; Strauss & Hauser. 1986). Ice crystallization leads to an increase in the ionic strength of any freeze-concentrated non-frozen aqueous phase surrounding the emulsion droplets (Komat and others 1997), which may promote droplet to droplet interactions. It is also possible that ice crystals formed during freezing may penetrate into the oil droplets and disrupt their interfacial membranes, thus making them more prone to coalescence. Cooling may also have caused some of the fat in the emulsion droplets to crystallize, which may promote partial coalescence due to penetration of a fat crystal from one droplet through the membrane to another droplet. (Harada & Yokomizo. 2000; Vanapalli, Palanuwech & Coupland. 2002)

3.6.1.1 Preparation of freeze-thaw cycles and measurements

The influence of repeated freeze-thaw cycles on the mean particle diameter, microstructure, zeta-potential and creaming stability of primary, secondary and tertiary emulsions were compared. Emulsions samples (10 ml) were transferred into glass test tubes and stored for 24 hours at -18°C in the freezer. These emulsions were then stored at room temperature for 24 hours (to melt any crystalline fat and water). These freeze thaw cycle was repeated form 0 to 3 times and its influence on emulsion properties was measured after each cycle.

3.6.2 Particle size

Particle size distribution has a very high correlation between theoretical prediction and experimental measurements. Particle size measurements are reported as the volume-surface mean diameter: $d_{32} = \sum n_i^3 / \sum n_i^2$, where n_i is the number of droplets of diameter d_i. Many of the most important properties of emulsion-based food products are determined by the size of the droplets that they contain, for example, shelf life, appearance, texture, and flavor. If all the droplets in an emulsion are of the same size it is referred to as monodisperse emulsion, but if there is a range of droplet sizes present it is referred to as a polydisperse emulsion (Figure 18). The droplet size of a monodisperse emulsion can be completely characterized by a single number, such as the droplet diameter or radius. Monodisperse emulsions are sometimes prepared and used for fundamental studies because the interpretation of experimental measurements is much simpler than for polydisperse emulsions. Nevertheless, real food emulsions always contain a distribution of droplet sizes, and so the specification of their droplet size is more complicated than for monodisperse systems. In some situations, it is important to have information about the full particle size distribution of an emulsion (i.e., the fraction of droplets in different specified size ranges), whereas in other situations knowledge of the average droplet size and the width of the distribution is often sufficient. (McClements, 1999)

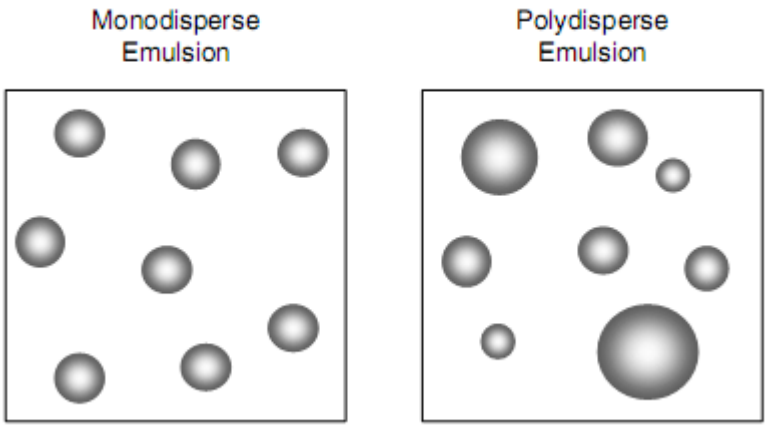


Figure 21. Schematic representation of monodisperse and polydisperse emulsions. In a monodisperse emulsion all the droplets have the same size, but in a polydisperse emulsion they have a range of different sizes

3.6.2.1 Preparation for particle size measurements

The particle size distribution of the emulsions was measured using a laser light scattering instrument (Mastersizer2000, Malvern Instruments Ltd). This instrument measures the angular dependence of the volume and light scattered from a stirred diluted emulsion. To avoid multiple scattering effects the emulsions were diluted with buffer prior to making the light scattering measurements.

3.6.3 Zeta potential

Particle size is often considered one of the most important parameters for dispersed solutions, however, as particle size reduces, the surface area increases significantly in comparison with the volume, so surface properties increasingly determine the dispersions characteristics. One of most the significant surface properties is the surface charge. This is an important factor in determining the interactions between particles, and hence dispersion characteristics such as dispersion stability, flocculation, viscosity, film forming characteristics etc. The surface charge cannot be measured directly. Instead the charge at a distance from the particle, called the zeta potential is measured. This potential is usually of more interest because particles interact according to the magnitude of this value, rather than the potential at the surface of the particle. The zeta potential is a consequence of the existence of surface charge, and can give information on electrical interaction forces between the dispersed particles. Using DLVO theory, it is possible to assess the stability of suspensions and emulsions by means of the zeta potential.

The basis of DLVO is to use the sum of the repulsive force (V_R - electrostatic BORN forces) and attractive (VA - van der WAALS forces) to calculate the particle interaction potential. $V_{TOTAL} = V_R + V_A$

In many cases, the zeta potential can be used directly as the criterion for assessing product quality. However, stability assessment using zeta potential as the measurable variable may only be useful if the system is sufficiently well understood. Electrokinetic measurements can be used to investigate the effect of each of the excipients in a formulation on the dispersed materials surface characteristics. Figure 19 shows schematic examples of zeta potential vs pH functions that are characteristic of certain surface conditions. These curves are found for example in the protolysis of acid or alkaline surface groups and in pH-dependent adsorption processes.

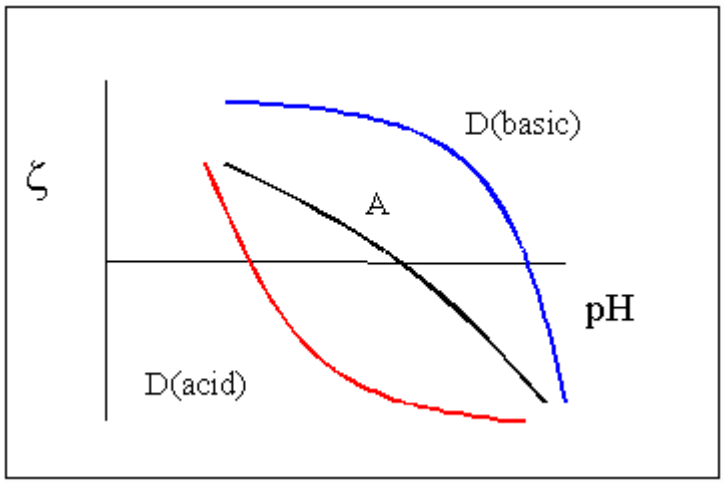


Figure 22. Model examples of pH vs zeta functions showing dissociation of acid or alkaline surface groups and adsorption

The zeta potential can be calculated from the mobility using the Henry equation.

Henry equation:

$m_E = e * x * f(k\ a)/6ph$

e - dielectric constant of the medium, h - viscosity of the medium, f(k a) - correction factor which takes into account the thickness of the double layer and particle diameter. The unit k is a reciprocal length. 1/k is frequently described as the thickness of the double layer. (Silver Colloids, 2010)

3.6.3.1 Preparation for Zeta-potential measurements

The zeta potential of emulsions was determined using a particle electrophoresis instrument (Zetasizer nano series-Zen 3600 Malvern Instruments, Worcestershire UK). The zeta-potential is determined by measuring the direction and velocity of droplet movement in a well-defined electric field, and then the Henry equation was used to calculate the ζ-potential. Emulsions were diluted using a buffer solution of the appropriate pH to avoid multiple scattering effects.

3.6.4 Creaming

Creaming is one of the most common physical mechanisms responsible for the instability of food emulsions. Creaming is a form of gravitational separation, it describes the upward movement of droplets due to the fact that they have a lower density than the surrounding liquid (McClements, 1999).

3.6.4.1 Preparation for creaming stability measurements

Ten grams of each emulsion was transferred into a test tube (15 mm internal diameter and 125 mm height) and then stored at room temperature for 1 day. After storage a number of the emulsions separated into an optically opaque "cream" layer at the top and a transparent or turbid "serum" layer at the bottom. After storage, digital images of these emulsions were made to observe creaming as an indicator of long-term stability. The creaming index was found by measuring the total height of the test tube with the emulsion (H_E) and the height of the serum layer (H_S) in the test tube measured with a ruler. The extent of creaming was then characterized by creaming index: $CI = (H_S / H_E)(\%) \times 100$.

3.7 Statistical analysis

Experiments were performed in duplicate with 3 samples each time, using freshly prepared samples. Average and standard deviation were calculated from the duplicate measurements using Excel (Microsoft Inc., USA).

4. Results and discussion

4.1 Preliminary testing

4.1.1 Surfactant

4.1.1.2 Size measurements

The emulsion that contained sodium caseinate had a better outcome than the emulsion that contained beta-lactoglobulin as the emulsifier, if the emulsion was passed 5 times through the microfluidizer (Figure 23). It was evident that tween20 was not suitable as an emulsifier because the size distribution was wider than for sodium caseinate. Tween20 was therefore not sufficiently stable. Several different concentrations of the emulsifiers and different passes through the microfluidazer were tested (Figure 24).

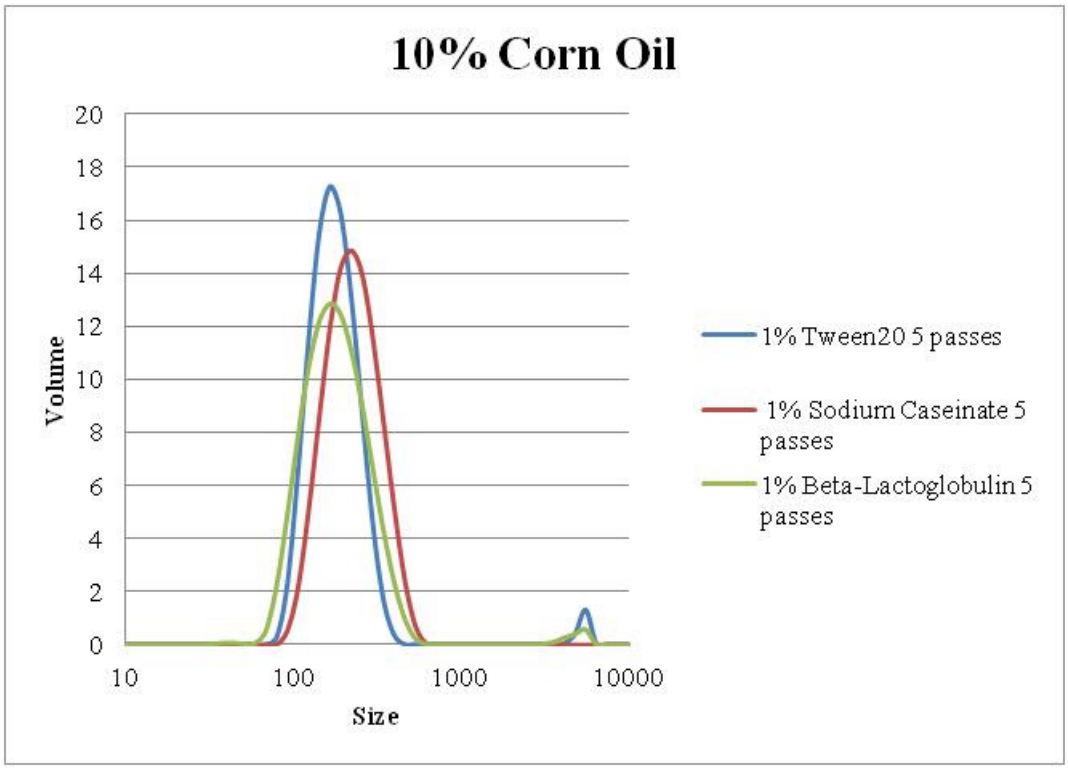


Figure 23. Dependence of volume on particle size in primary emulsion, with different surfactants

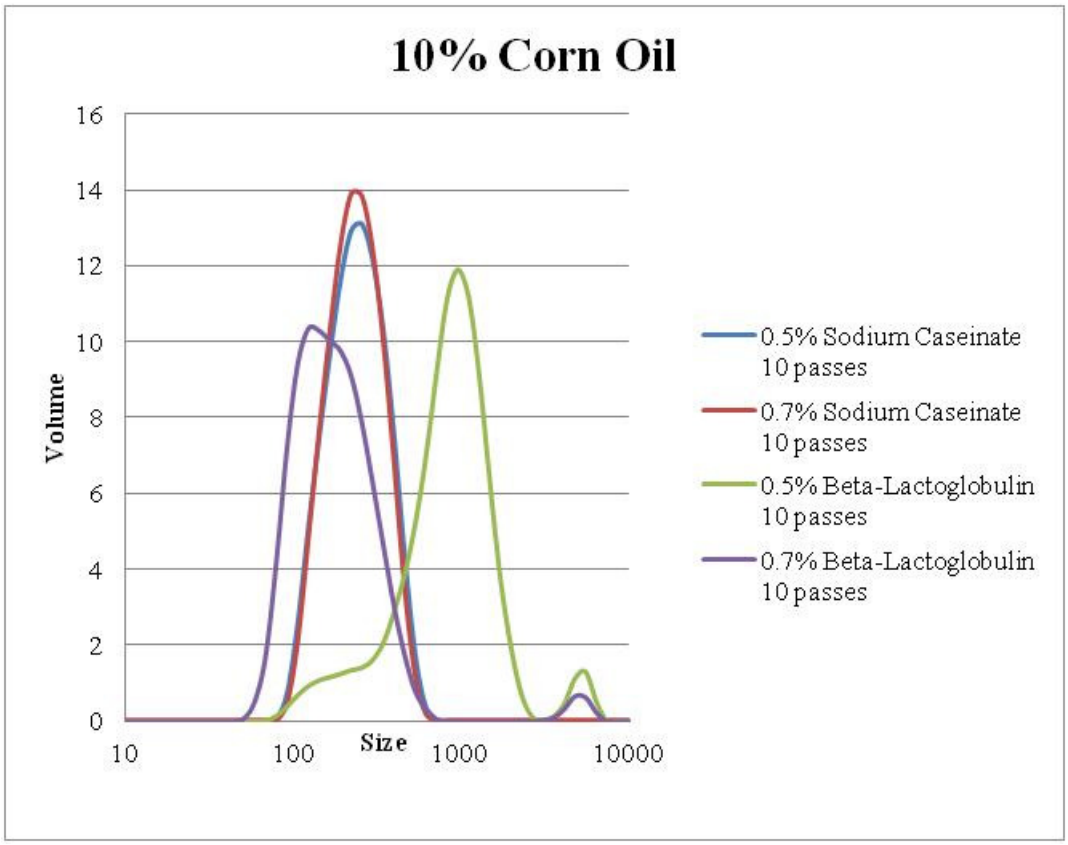


Figure 24. Dependence of volume on particle size in primary emulsion, with different surfactant, with different concentration.

4.1.2 Chitosan

Chitosan was used as the second layer. The first aim was to have just the right concentration to completely cover the oil droplets. The second aim was to find the amount that made the emulsions stable at the widest pH range. Emulsions with different concentrations of chitosan were made, pH of the emulsions were adjusted from 2 to 8 with addition of HCl and NaOH.

4.1.2.1 Charge measurements

The emulsions had to be stable at the widest pH range. The emulsions would not be stable unless the primary and the secondary surfactant had different charge, (and then secondary and tertiary layers with opposite charge). The opposite charge attract, negatively charge surfactant adsorbs to positively charge surfactants. At pH 4 and above the 1°emulsion containing sodium caseinate as the surfactant was negatively charged, the chitosan on the other hand was positively charged between pH 2 and pH 8 (Figure 25).

The tests showed that the preferred pH was 4 to 5. It was decided to to use pectin as the third layer because pectin had a negative charge from pH 2 and above (Figure 25) and should therefore bind to the positive charged chitosan.

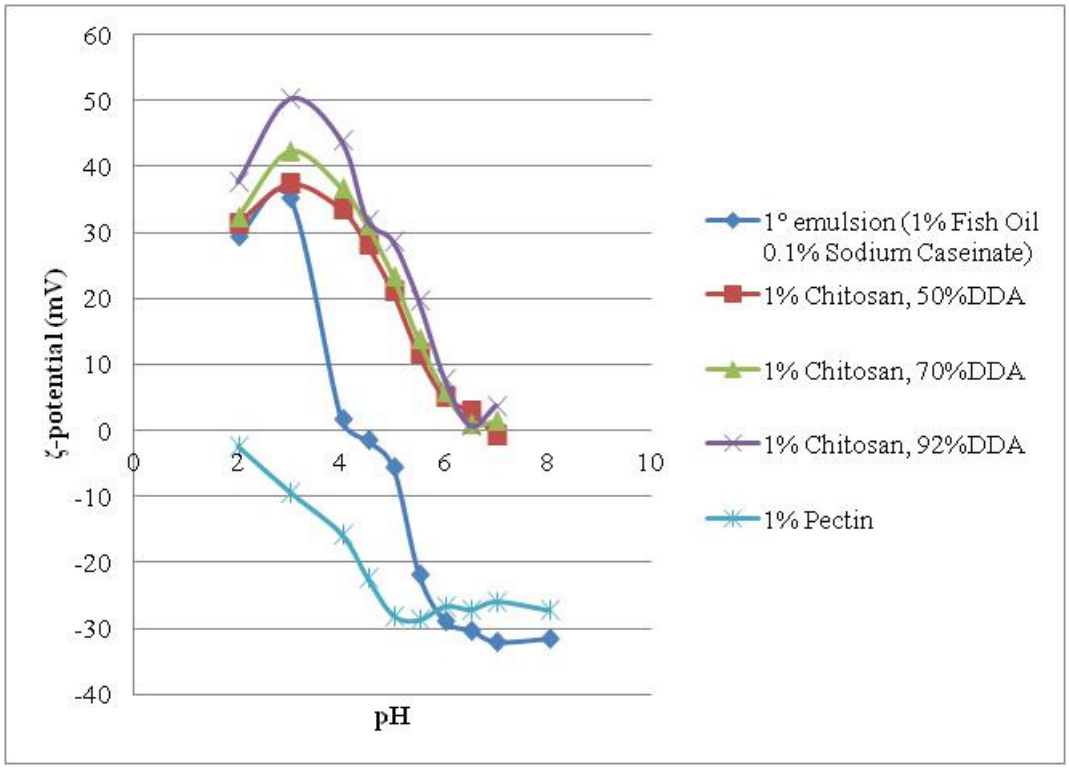


Figure 25. The influence of pH on the ζ-potential in primary emulsion, 1% Chitosan in acetate buffer with different %DDA and 1% pectin in acetate buffer.

The concentration of Chitosan with 70% DDA at three different pH levels (Figure 26) was measured. After 0.02% (w/w) the charge was more or less the same until at least 0.05% (w/w), so charge wise the concentration between 0.02% and 0.05% (w/w) seemed not to matter. In the next subsection the particle size measurements will be presented.

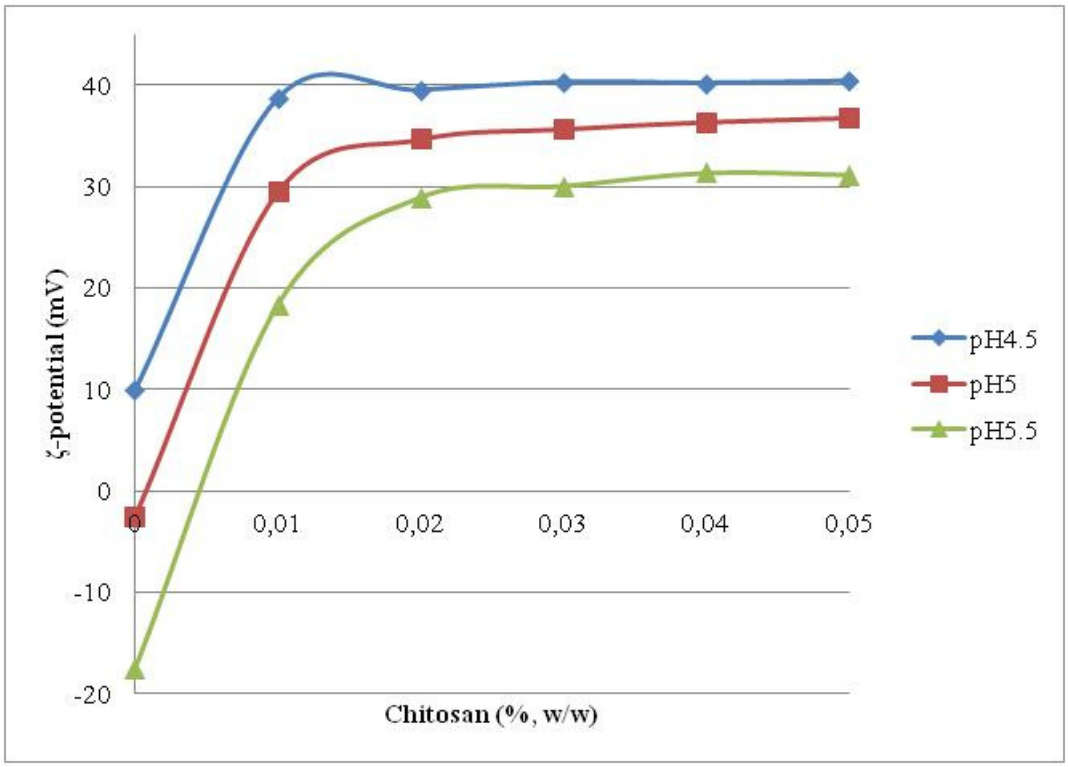


Figure 26. The influence of Chitosan concentration on the ζ-potential of diluted secondary emulsions (1% w/w fish oil, 0.1% w/w sodium caseinate, 0,04% w/w chitosan (70%DDA) 10 mM acetate acid buffer).

The ζ-Potential measurements were used to provide indirect information about the interfacial composition of primary and secondary emulsions (Figure 27). By measuring the droplet charge after a charged biopolymer was added to the emulsion it could be determined whether the biopolymer had adsorbed or not. The emulsions were diluted using a buffer solution to avoid multiple scattering effects. Diluted emulsions were then injected into the measurement chamber (Zetasizer nano) and the ζ-potential was determined by measuring the direction and velocity that the droplet moved in the applied electric field.

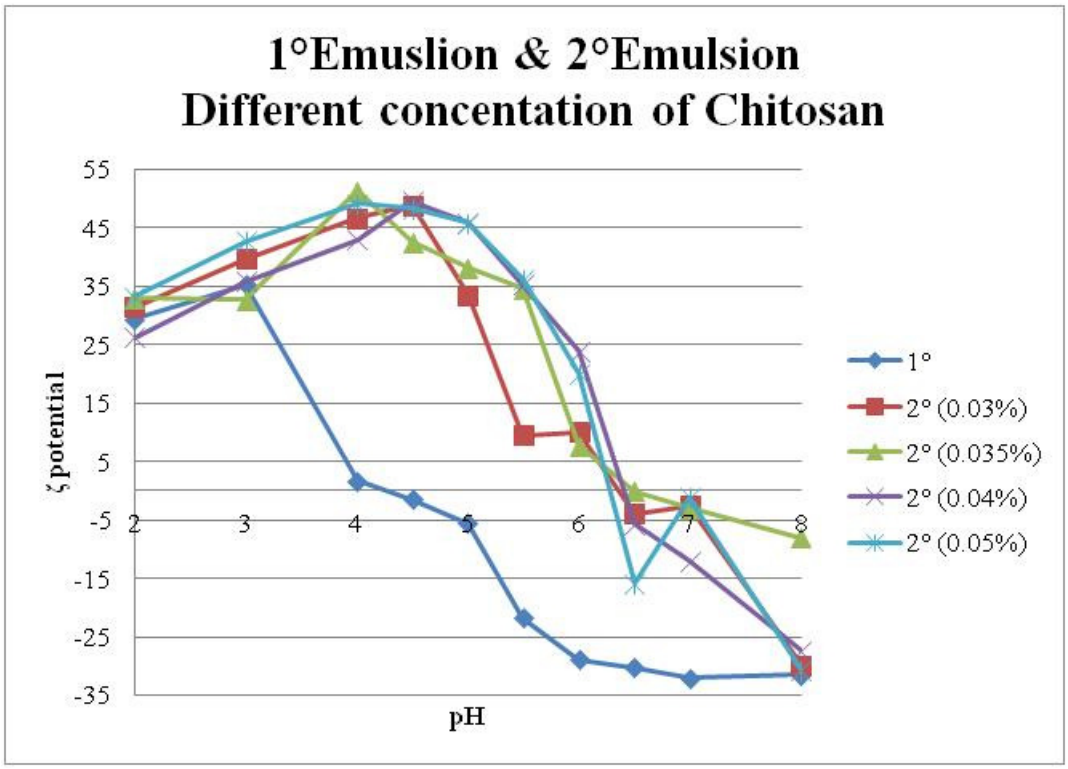


Figure 27. The influence of Chitosan concentration on the ζ-potential of primary and secondary emulsions. The secondary emulsions contained chitosan with different DDA.

4.1.2.1 Size measurements

The particle size was determined at several pH values with chitosan concentrations from 0.03% to 0.05% (w/w). (Figures 28-31).

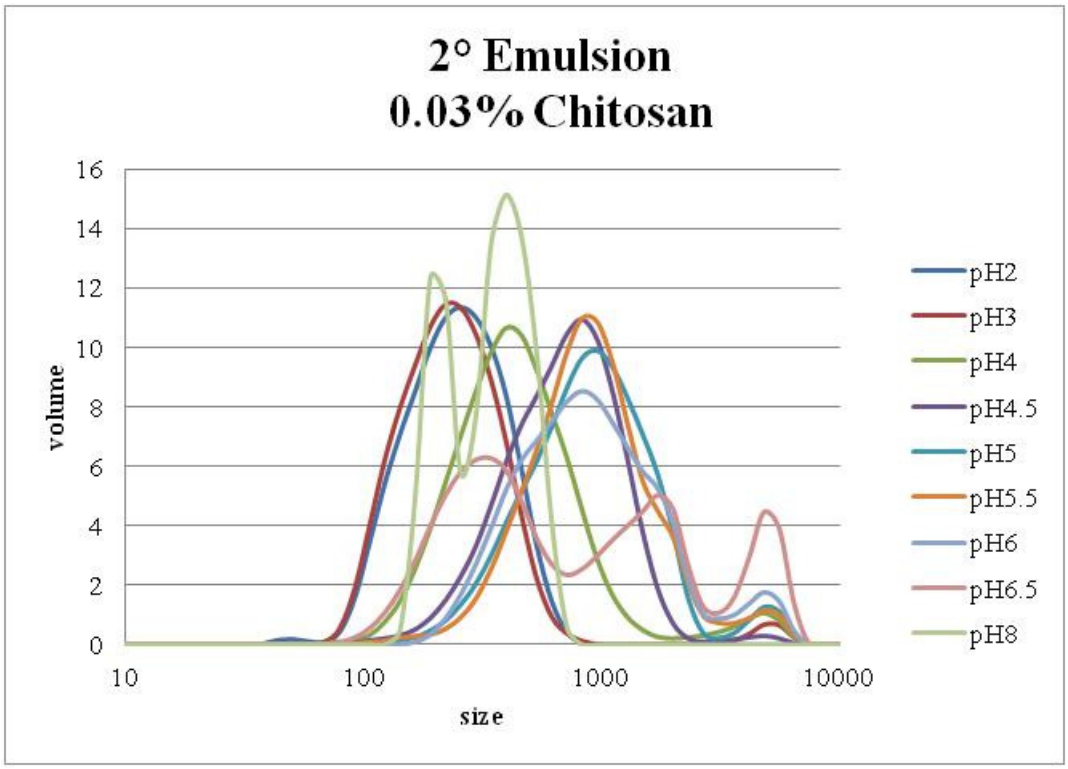


Figure 28. Dependence of volume on particle size with 0.03% Chitosan (70% DDA)

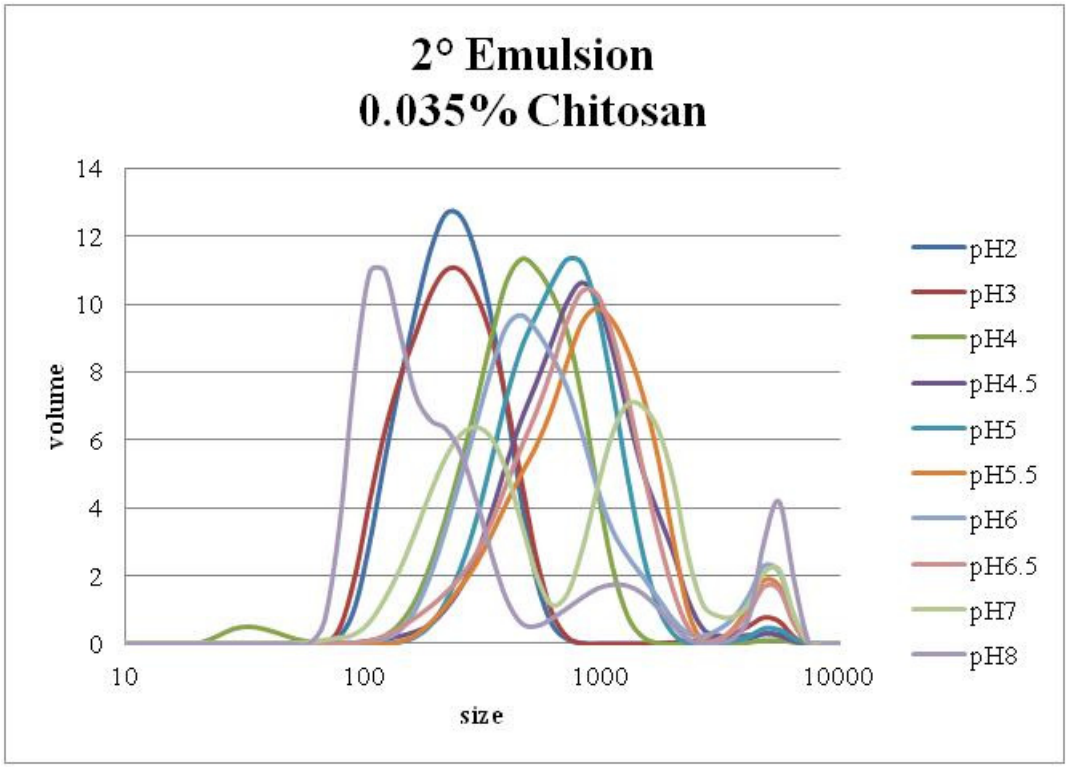


Figure 29. Dependence of volume on particle size with 0.035% Chitosan (70% DDA)

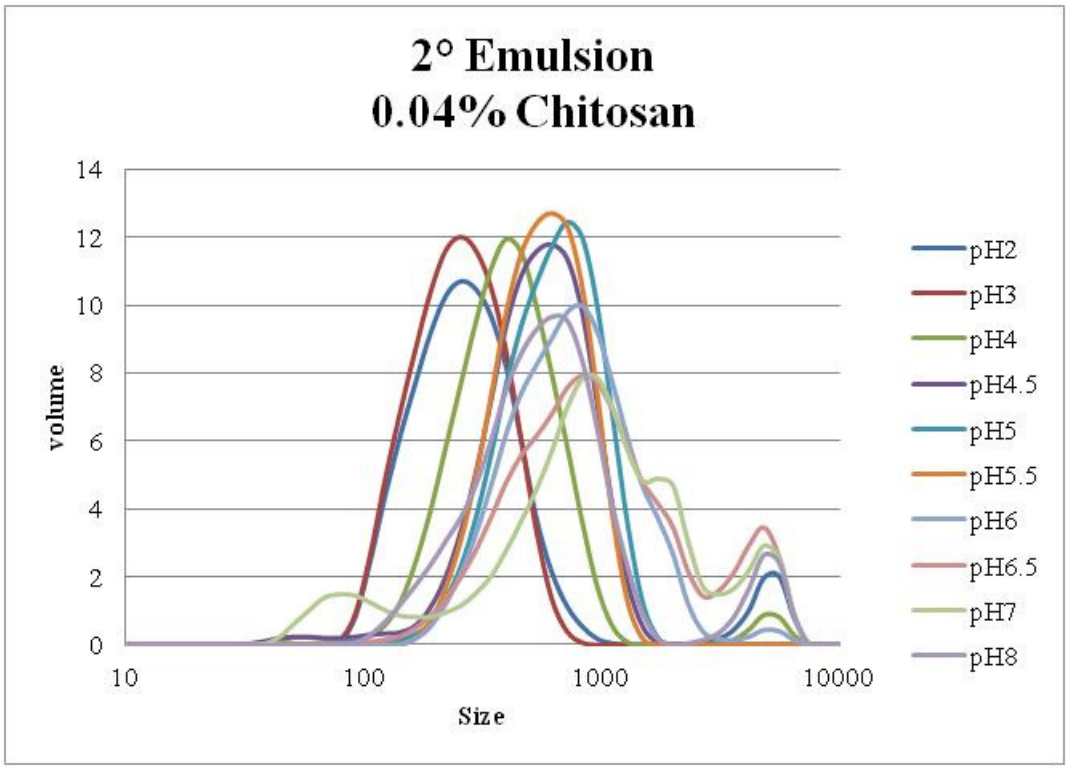


Figure 30. Dependence of volume on particle size with 0.04% Chitosan (70% DDA)

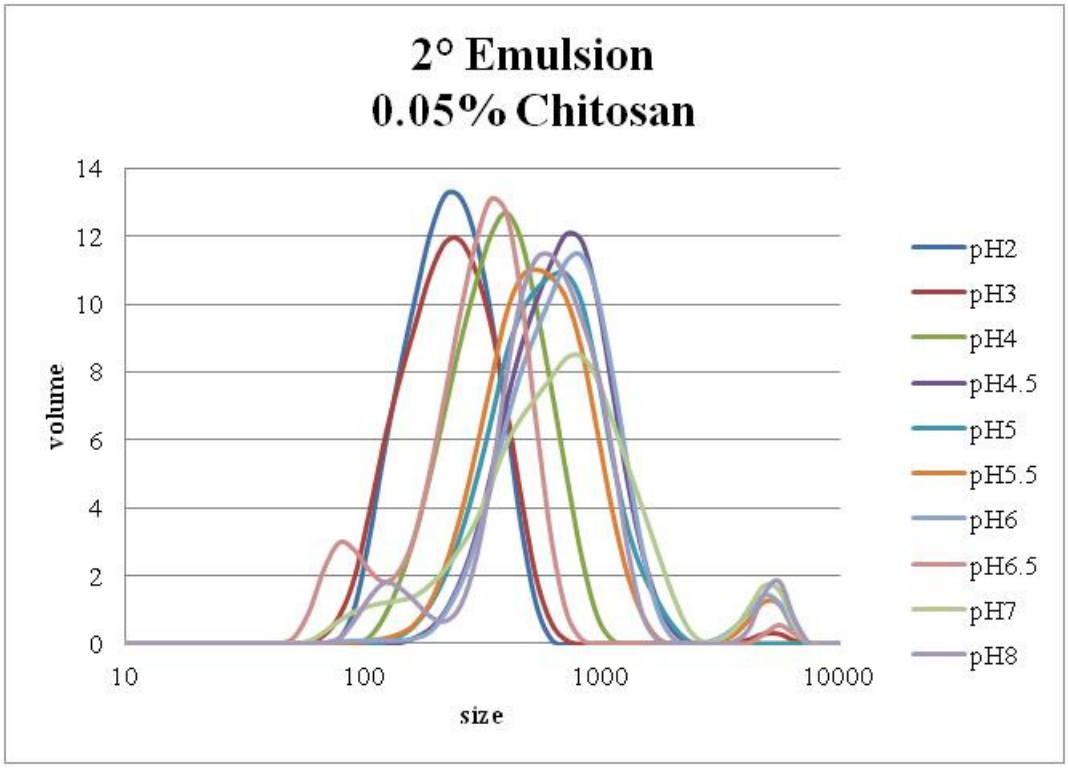


Figure 31. Dependence of volume on particle size with 0.05% Chitosan (70% DDA)

To determine which concentration of Chitosan was the optimal for this system the 2° emulsion containing 1% Fish Oil, 0.1% Sodium Caseinate and a few different concentrations of Chitosan (from 0 till 0.05% w/w) were tested. The pH levels of these emulsions were adjusted from pH 4.5 to pH 5.5 then the particle size was determined. The emulsion containing 0.04% Chitosan was the only emulsion that showed sufficient stability at pH 4.5, pH 5 and pH 5.5 (Figure 29). The results (in addition to other results) indicated that 0.04% of Chitosan in the emulsion would be most preferable for further experiments. The goal was to cover the droplets in the 1°emulsion with chitosan, but chitosan concentration could not be excessive, to avoid free chitosan in the solution. The results indicated that the emulsion containing 0.04% chitosan covered the 1° emulsion droplets. Although the emulsion containing 0.05% was also stable, it was not used to avoid the possibility of free chitosan in the emulsion.

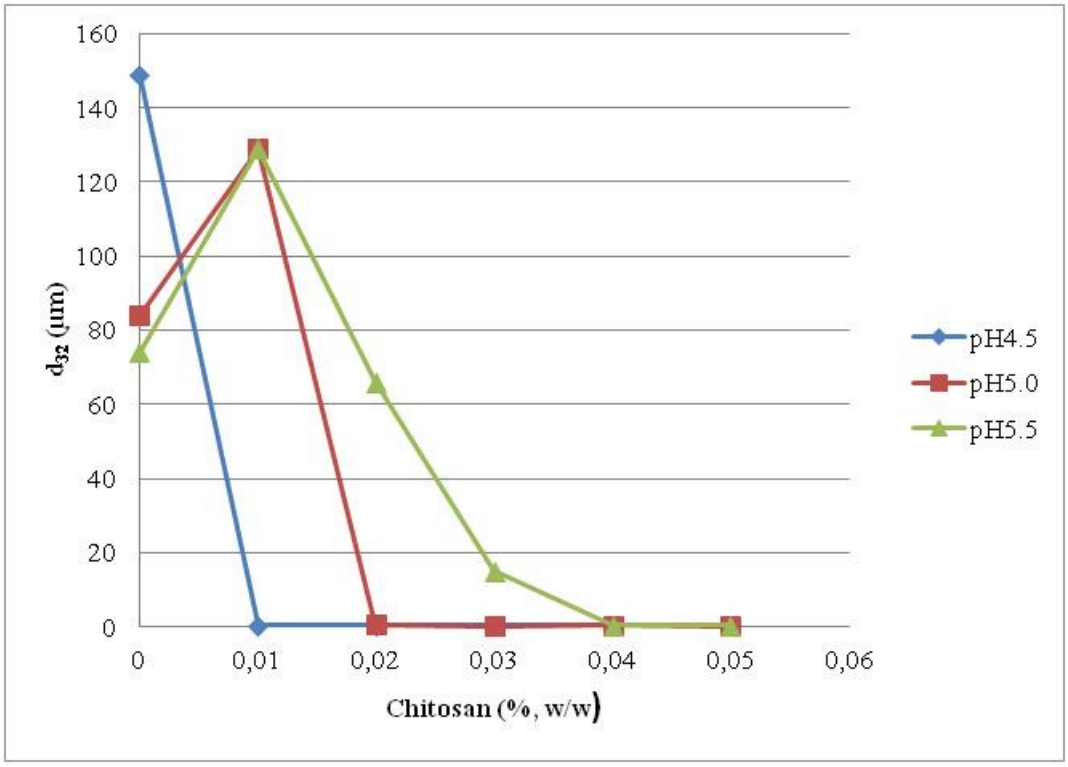


Figure 32. The influence of chitosan concentration on the mean particle diameter (d43) of diluted secondary emulsions (1% w/w fish oil, 0.1% w/w sodium caseinate, 0.04% w/w chitosan (70%DDA) 10 mM acetate acid buffer).

4.1.2.2 Creaming measurements

The extent of creaming was characterized by creaming index: $CI = (H_E / H_S)(\%) \times 100$ (graph 30). To help decide the best concentration of Chitosan the same emulsions as in subsection (4.1.2.1) was used and the creaming index was measured. The emulsion containing 0.03, 0.04 and 0.05% of Chitosan did not show any creaming (Figure 33 and Figure 34). The results confirm the results of size measurements. No creaming indicates that the emulsion is stable, in the emulsions that contained under 0.03% chitosan creaming was measured which indicates that the chitosan was not sufficient to cover the fish-oil-sodium-caseinate droplets completely.

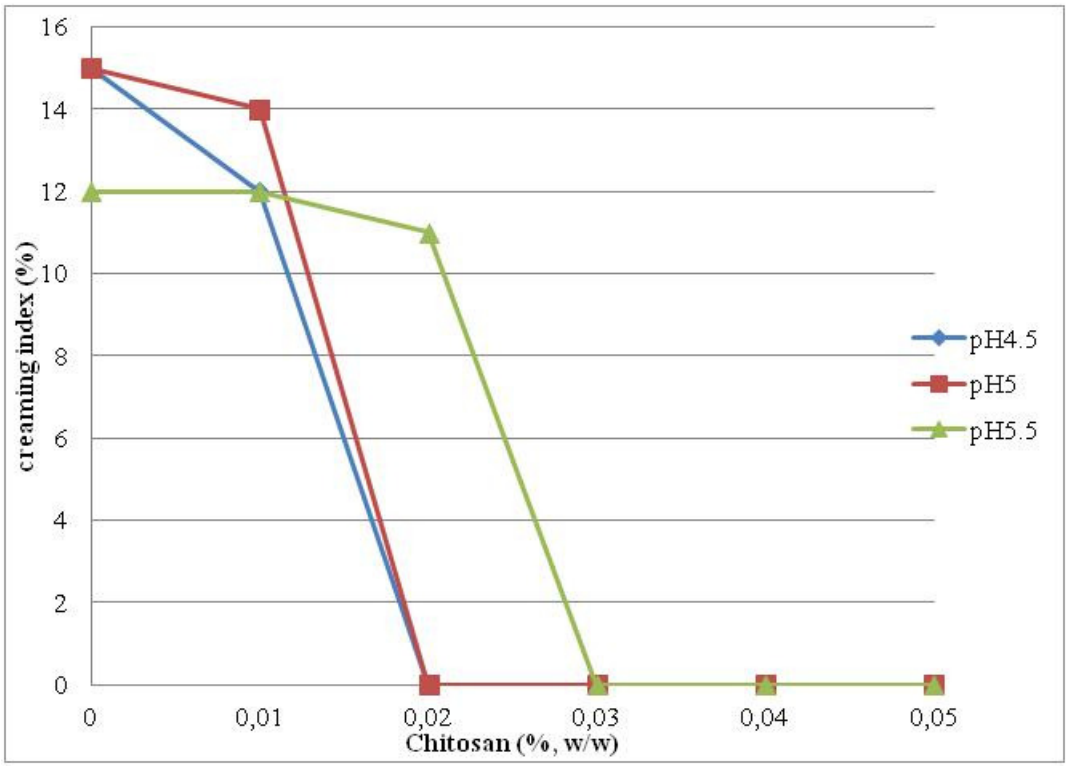
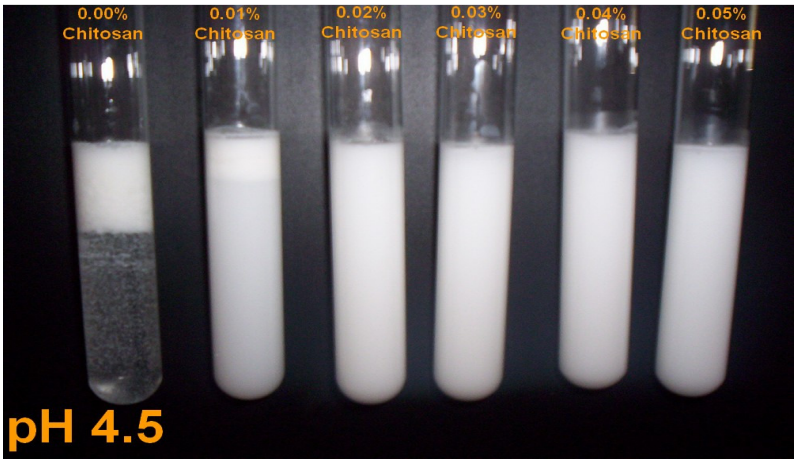
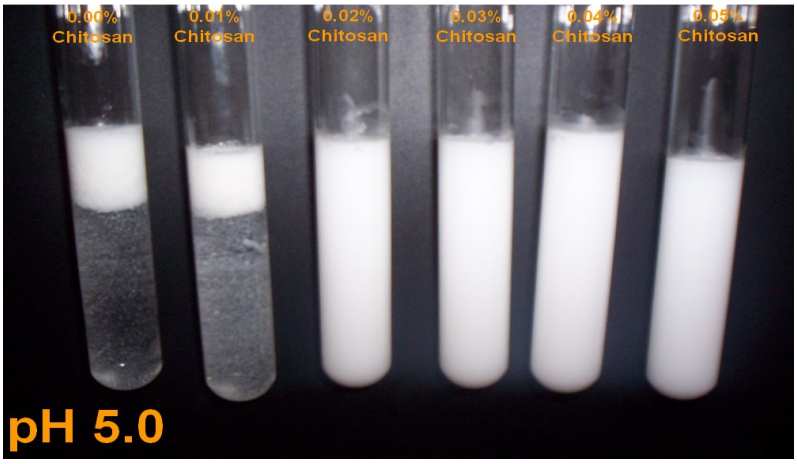


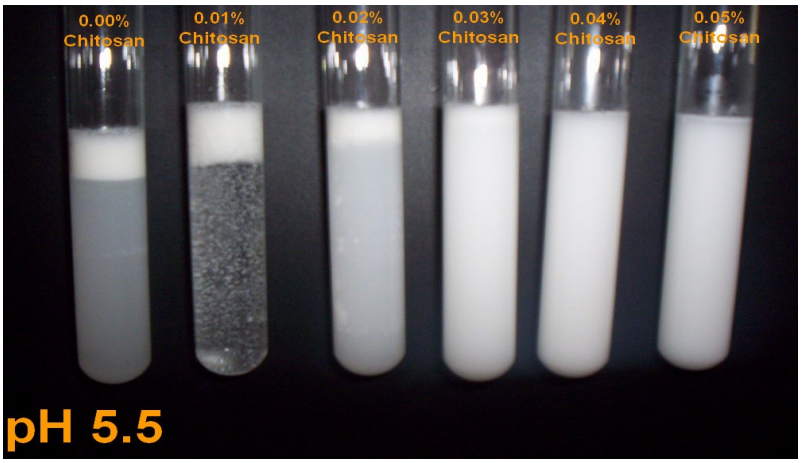
Figure 33. The influence of pectin concentration on the thickness of creamed layer formed in tertiary emulsions (1% w/w fish oil, 0.1% w/w sodium caseinate, 0.04% w/w chitosan (70%DDA) 10 mM acetate acid buffer).



(a)



(b)



(c)

Figure 34. Creaming stability images of secondary emulsions with different concentration of Chitosan. (a) at pH 4.5, (b) at pH 5.0 and (c) at pH 5.5

4.1.3 Benefat

The main problem with Benefat was that the microfluidizer clogged up. All the equipment was preheated before passing the emulsions and carefully cleaned after use, but there was always some fat that could not be cleaned out in the tubes, so when the equipment had cooled down the remaining fat crystallized. After a few passes the crystallized fat clogged the machine. This was followed by experiments applying the sonicator but the resulting emulsions were not sufficiently dispersed or homogenous.

SDS was used as an emulsifier for this test emulsion to increase the likely hood of a good emulsion with the Benefat. Because Benefat is a modified triacylgycerol, it is stable at room temperature and therefore problems came up when the microfluidizer cooled back down to room temperature. When using a sonicator, the dispersion of the fat droplets only happens around the probe and therefore it was harder to obtain homogenous emulsions.

4.1.3.1 Size measurements

The particle size of emulsions from after 4, 6 and 8 passes though the microfluidizers (Figure 35) was analyzed. The emulsion with 8 passes had the best particle size distribution.

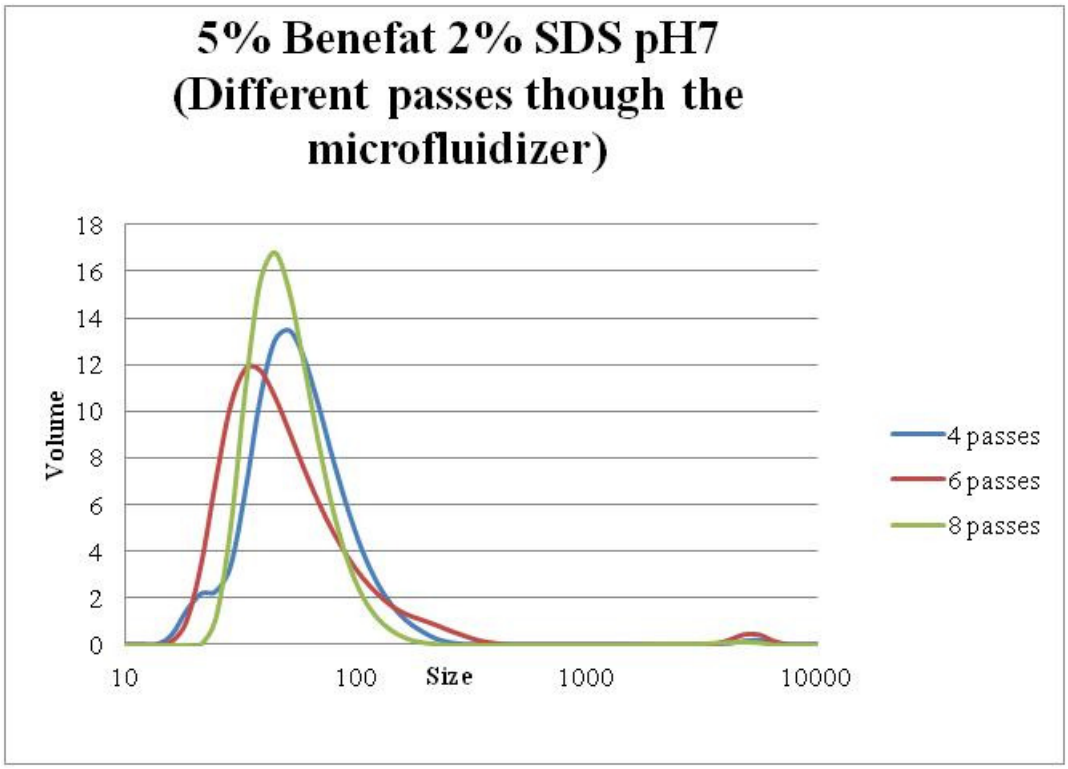


Figure 35. Volume as a function of particle size for different passes through the microfluidizer.

1° and 2°emulsion was made with preferred surfactant; Benefat, Sodium Caseinate and Chitosan. The particle size of the 1°and 2° emulsions was measured (Figure 36). After a few unsuccessful attempts to produce emulsions with an even distribution of particles, the sonicator was used instead of the microfluidizer, because the microfluidizer kept on clogging due to the heat changes in the pipes.

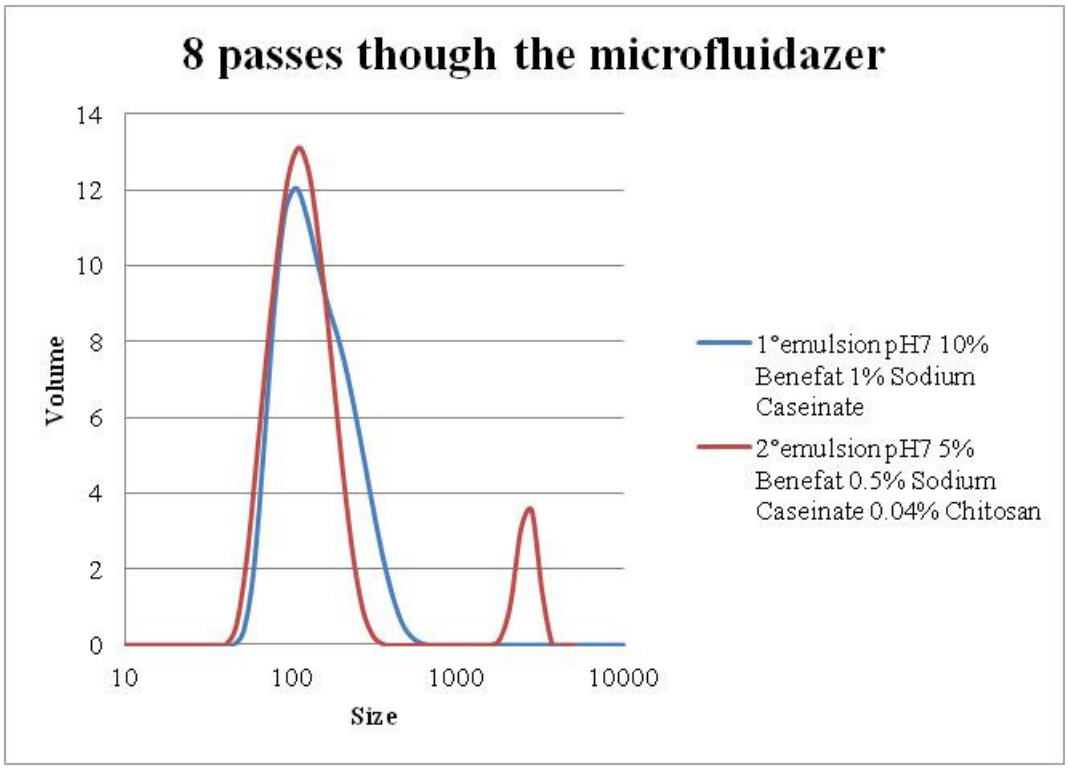


Figure 36. Particle size distributions measured by laser diffraction for diluted primary emulsion (10% w/w Benefat, 1% w/w, Sodium Caseinate, 10 mM acetate acid buffer pH 3) and secondary emulsion (5% w/w Benefat, 1% w/w, Sodium Caseinate, 0.04% w/w Chitosan (70 % DDA) 10 mM acetate acid buffer pH 3).

Optimization of time was attempted in the sonicator to be able to obtain the best distribution of particles in the emulsions. Emulsion at pH 7 and emulsion at pH 3 were tested. It was observed that the particles were much larger than in the microfluidizer, like would be expected. The emulsion at pH 7 had a more uniform distribution than the emulsion at pH 3 (Figures 37 and 38).

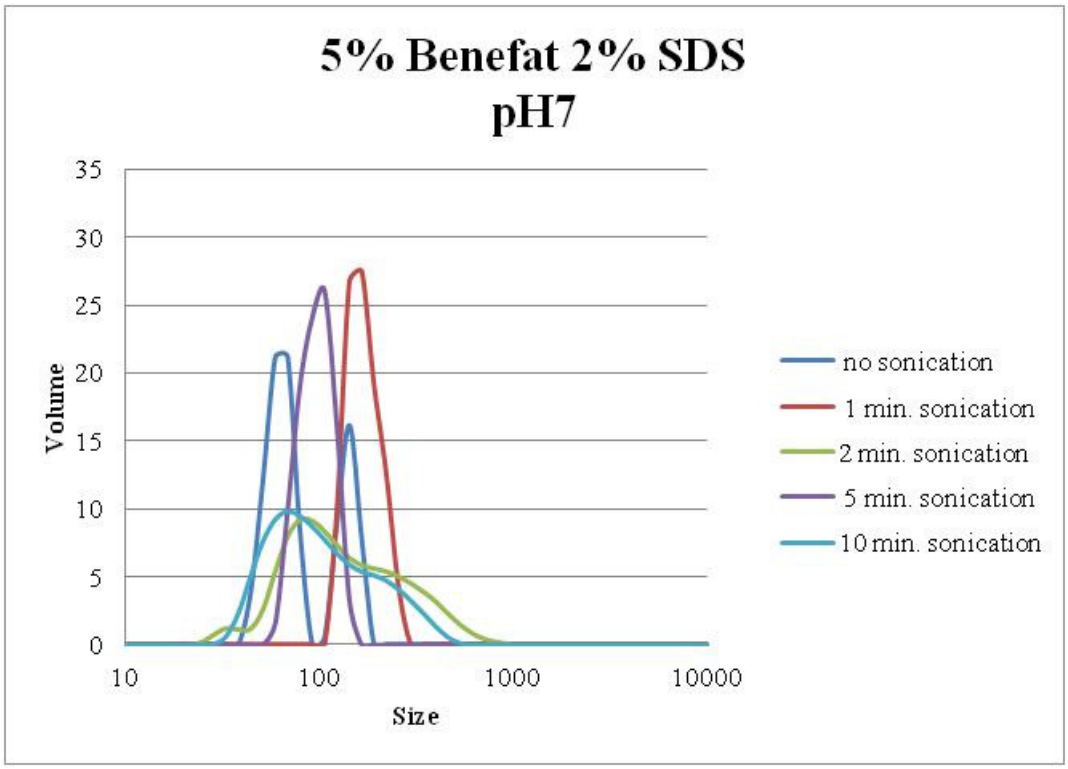


Figure 37. Particle size distributions measured by laser diffraction for diluted primary emulsions (5% w/w Benefat, 2% w/w, SDS, 10 mM acetate acid buffer pH 3).

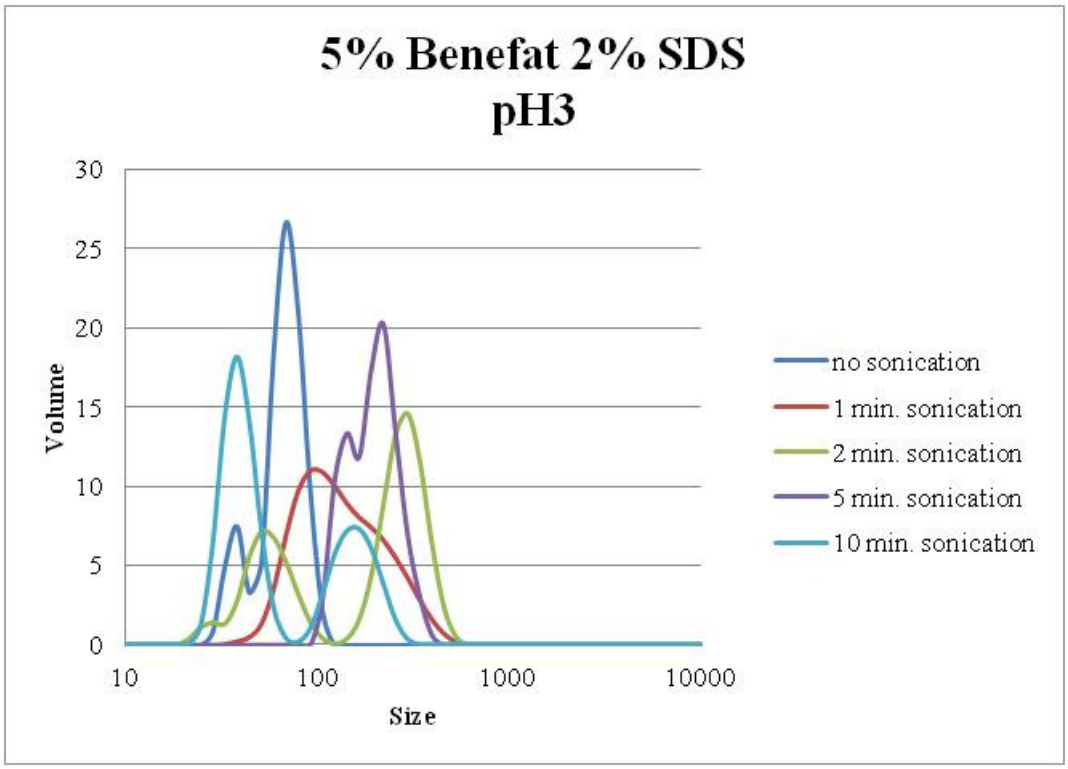


Figure 38. Particle size distributions measured by laser diffraction for diluted primary emulsions (5% w/w Benefat, 2% w/w, SDS, 10 mM acetate acid buffer pH 3).

The 2° emulsion was tested using the sonicator, but that did not work sufficiently (Figure 39). The best way to use the sonicator would be to apply it after adding the second layer to a 1°emulsion that was previously made in the microfluidizer.

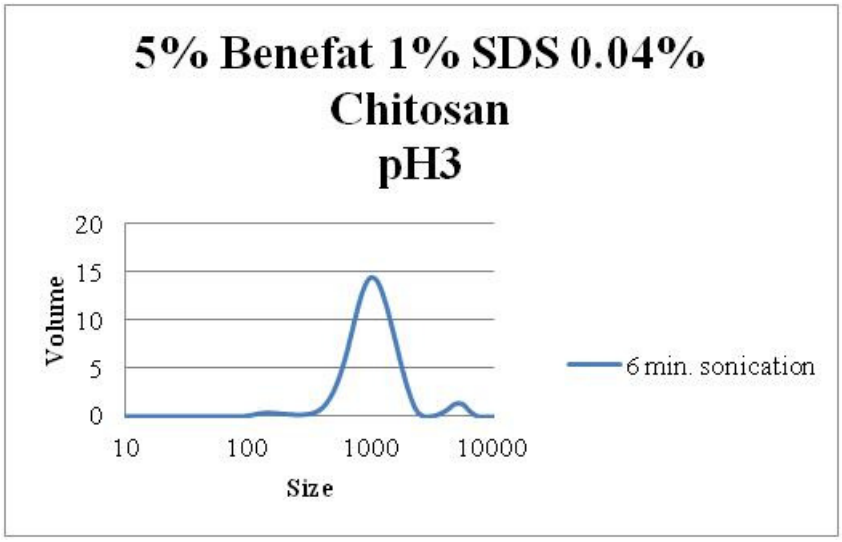


Figure 39. Particle size distributions measured by laser diffraction for diluted secondary emulsions (5% w/w Benefat, 1% w/w, SDS, 0.04% w/w chitosan (70%DDA) 10 mM acetate acid buffer pH 3).

4.1.4 Summary

In the preliminary studies were performed with oil in water (1°) emulsion stabilized with protein Two proteins, Sodium Caseinate and β-Lactoglobulin were compared at different concentrations of the proteins and oil, number of passes though the microfluidizer. The stability of emulsions at different pH was also tested. Chitosan concentration for the the second layer was optimized with the help of particle charge, size and the creaming index. The results from the preliminary study resulted in the use of 1% Fish oil, 0.1% Sodium Caseinate, 8 passes through the microfluidizer, then add 0.04% of Chitosan and adjust the emulsion to pH 5 as the parameters for further experiments. In the following studies it was decided to use Fish Oil instead of the Benefat. It was not possible to make good homogeneous emulsion containing Benefat.

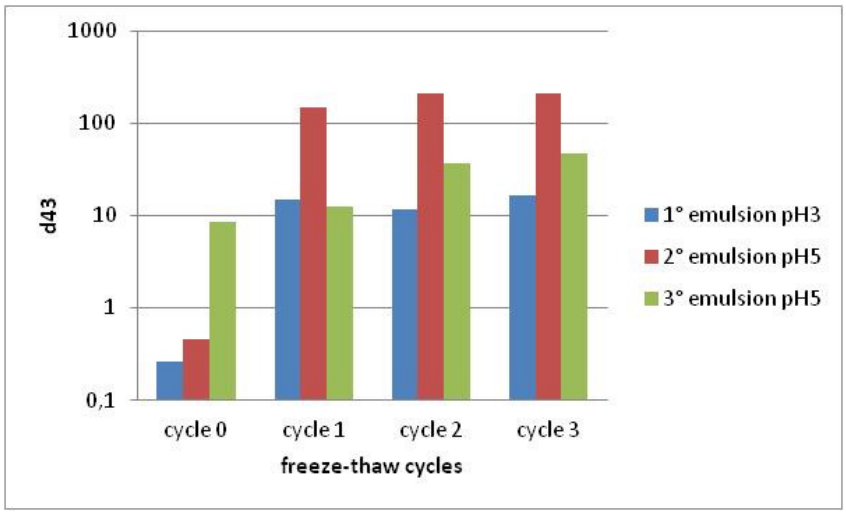
4.2 End emulsion

4.2.1 Freeze-thaw measurements

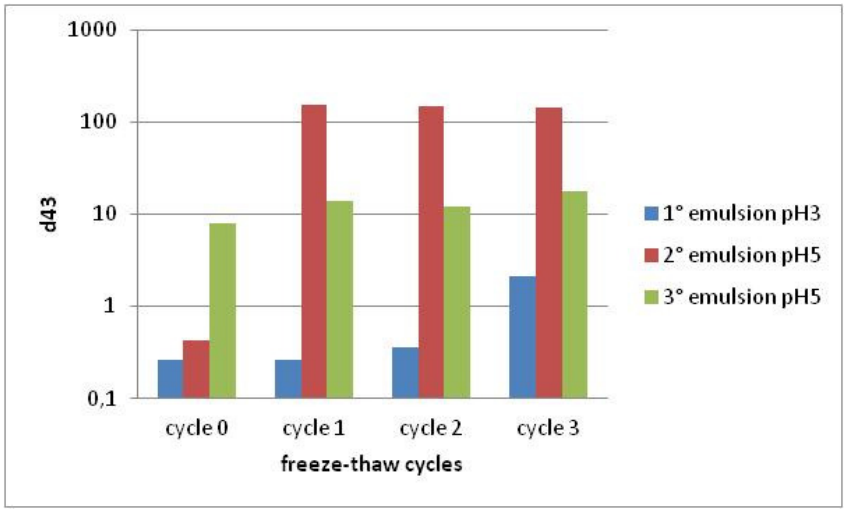
The influence of freeze-thaw cycling (-18°C / room temperature) and sucrose (0% or 10% w/w) on the mean particle diameter and microstructure of primary, secondary and tertiary emulsions was measured (Figures 40 till 44).

4.2.1.1 Particle size

In the absence of sucrose, there was an appreciable increase in the mean particle size after one freeze-thaw cycle in the primary emulsions and the secondary emulsions. On the other hand, the tertiary emulsions were much more stable to droplet aggregation. For example, after three freeze-thaw cycles the mean particle diameters (d_{43}) in the primary, secondary and tertiary emulsions were 16, 211 and 46 µm respectively (compared to 0.3, 0.5 and 8 for the untreated emulsions). The addition of 10% w/w sucrose to the primary emulsions made it more stable to droplet aggregation after 2 freeze-thaw cycles, with the mean particle diameter remaining less than 0.5 µm but after the third freeze-thaw cycle it increased to over 2 µm. The addition of sucrose did not seem to affect the droplet aggregation in the secondary and tertiary emulsions. The secondary emulsion did not show much stability to droplet aggregation showing large increase in mean particle after one freeze-thaw cycle. The tertiary emulsion showed large mean particle size even without freezing, but the mean particle size stayed more or less the same from cycle 0 to cycle 3.



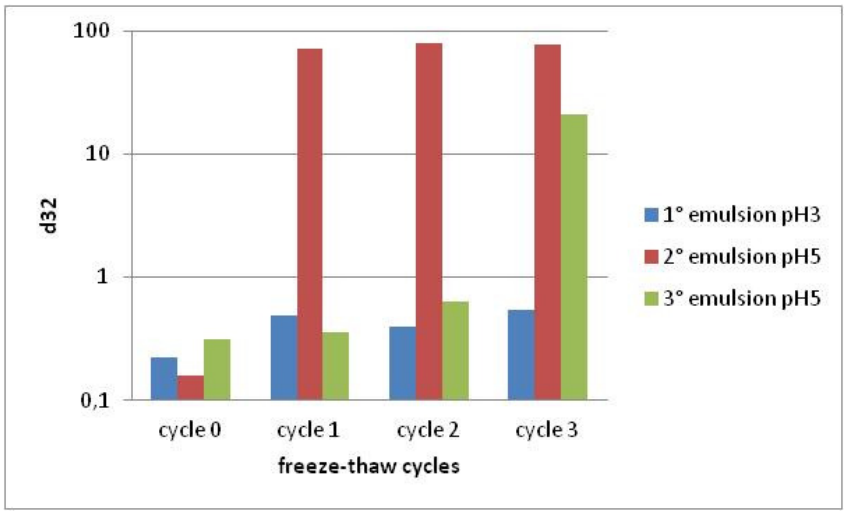
(a)



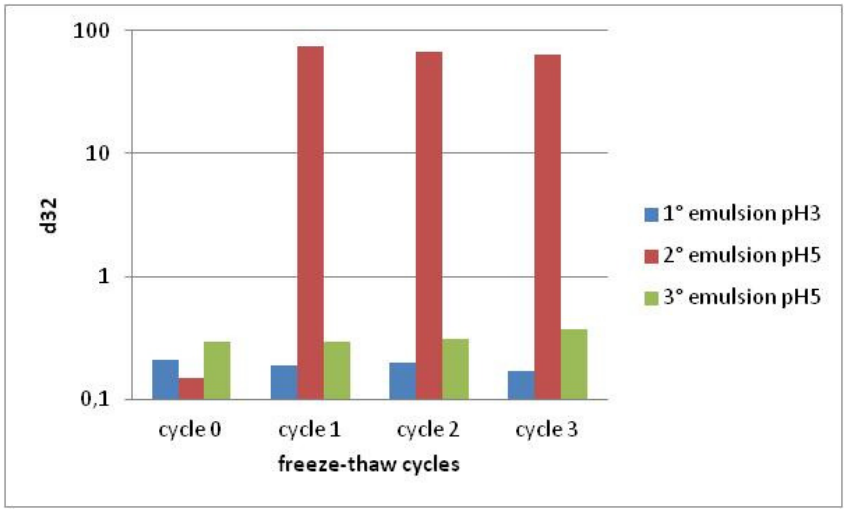
(b)

Figure 40. Dependence of the mean particle diameter (d_{32}) of primary, secondary, and tertiary emulsions (1% Fish Oil, 0.1%, 0.04% chitosan and 0.65% pectin) on number of freeze-thaw cycles and on sucrose concentration: (a) 0% sucrose; (b) 10% sucrose.

The influence of freeze-thaw cycling and sucrose (0 or 10% w/w) in the mean particle diameter and microstructure of primary, secondary and tertiary emulsions was measured. In the absence of sucrose, there was an appreciable increase in the mean particle size after one freeze-thaw cycle in the primary and tertiary emulsions. The secondary emulsion did not show much stability to droplet aggregation showing large increase in mean particle diameter after one freeze-thaw cycle, both in absence and presence of sucrose.



(a)



(b)

Figure 41. Dependence of the mean particle diameter (d_{32}) of primary, secondary, and tertiary emulsions (1% Fish Oil, 0.1%, 0.04% chitosan and 0.65% pectin) on number of freeze-thaw cycles and on sucrose concentration: (a) 0% sucrose; (b) 10% sucrose.

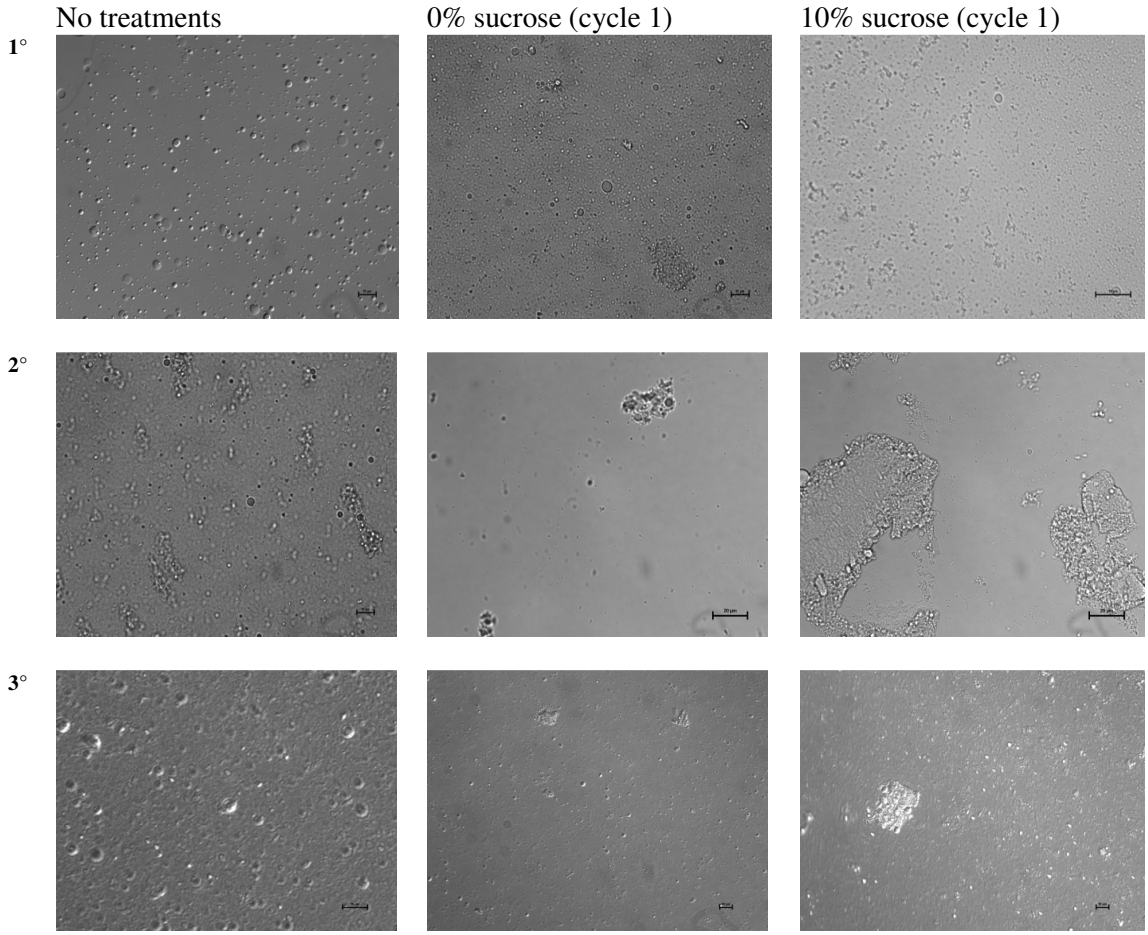
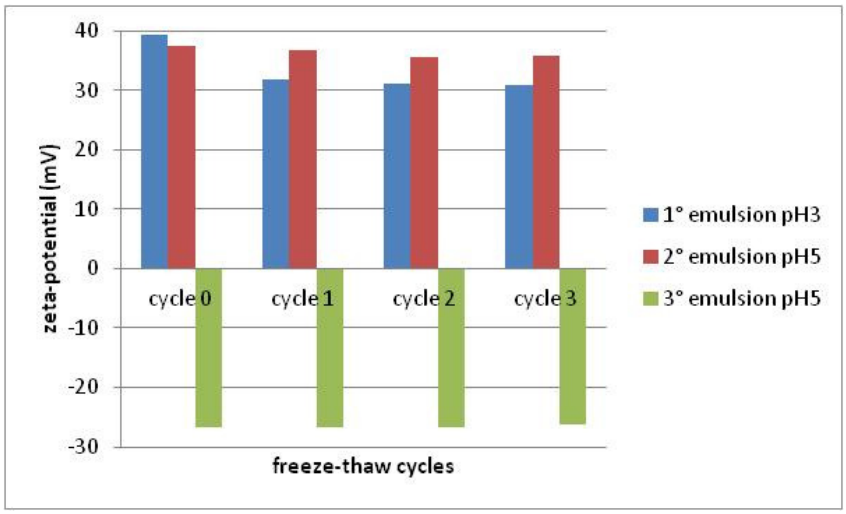


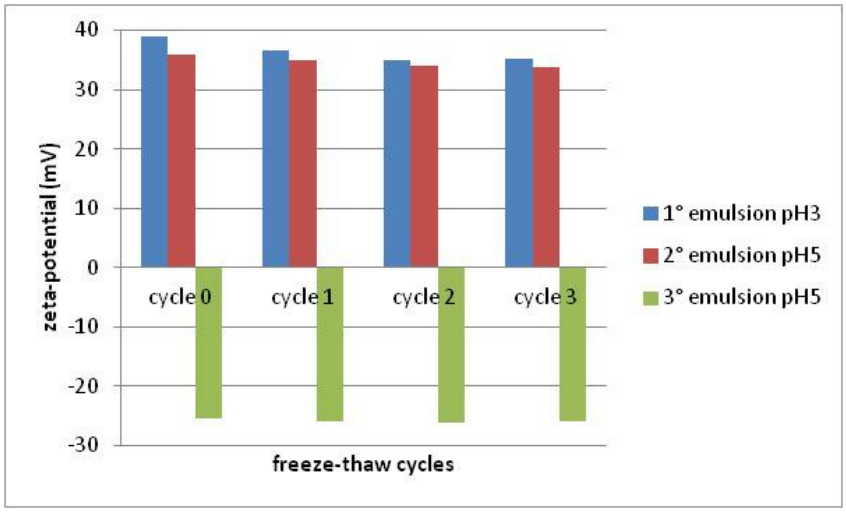
Figure 42. Emulsions microstructure of primary, secondary, and tertiary emulsions (1% Fish Oil, 0.1%, 0.04% chitosan and 0.65% pectin) before and after three freeze-thaw cycles (0% and 10% w/w sucrose). The control emulsions (no freezing/thawing) looked similar in the absence and presence of sucrose.

4.2.1.2 Charge Measurements

Zeta-potential measurements were used to provide indirect information about the interfacial composition of primary, secondary and tertiary emulsions (Figure 43).



(a)



(b)

Figure 43. Dependence of particle electrical charge (ζ-potential) of primary, secondary and tertiary emulsions (1% Fish Oil, 0.1%, 0.04% chitosan and 0.65% pectin) on number of freeze-thaw cycles and on sucrose concentration: (d) 0% sucrose; (e) 10% sucrose.

The zeta-potential of the droplets in the primary emulsions, containing sodium caseinate, was 39 ± 1 mV, that can be because the solution was at pH 3 and therefore under the isoelectric point (pI ≈ 4.6) of the absorbed sodium caseinate. The zeta-potential of the droplets in the secondary emulsions was 37 ± 2 mV. Keep in mind that the secondary emulsions were measured at pH 5, at that pH the sodium caseinate is negatively charge and the chitosan positively charged, so the chitosan molecules should attract to the negatively charge sodium caseinate molecules and make the second layer around the oil droplets. The zeta-potential of the droplets in the tertiary emulsions was −26 ± 2 mV, which indicates that the anionic pectin molecules had adsorbed to the surface of the cationic sodium-caseinate–chitosan coated droplets. Neither did the addition of 10% w/w sucrose or the freeze thaw cycles to primary secondary and tertiary cause appreciable change in the measured zeta-potential, that suggests that the interfacial composition was unaltered by these treatments.

4.2.1.3 Creaming measurements

The influence of freeze-thaw cycling and sucrose on the creaming stability of primary, secondary and tertiary emulsions was also measured (Figure 44 and Figure 45). The primary emulsions remained stable to creaming in the presence of 10% sucrose, even after three freeze-thaw cycles. In the absence of sucrose, creaming instability was observed after one freeze-thaw cycle in the primary emulsion, cream layer was measured 10%. After three freeze thaw cycles the cream layer was 8% at that point the cream layer had settled. The secondary emulsion showed creaming instability, it measured 15% after one freeze-thaw cycle in the presence and in absence of sucrose it measured 19%. On the other hand the tertiary emulsion remained stable to creaming even after three freeze-thaw cycles, both in present and absence of sucrose. The creaming stability measurements therefore supported the particle size (d_{32}) analysis. Indicating that droplets coated with one layer is more stable in the present of sucrose at pH 3 to freeze-thaw cycling and that droplets coated with 3 layers is more stable than droplet coated with 2 layers at pH 5 to freeze-thaw cycling. Rapid creaming in diluted emulsions is indicative of extensive droplet aggregation, e.g., flocculation or coalescence (McClements, 1999).

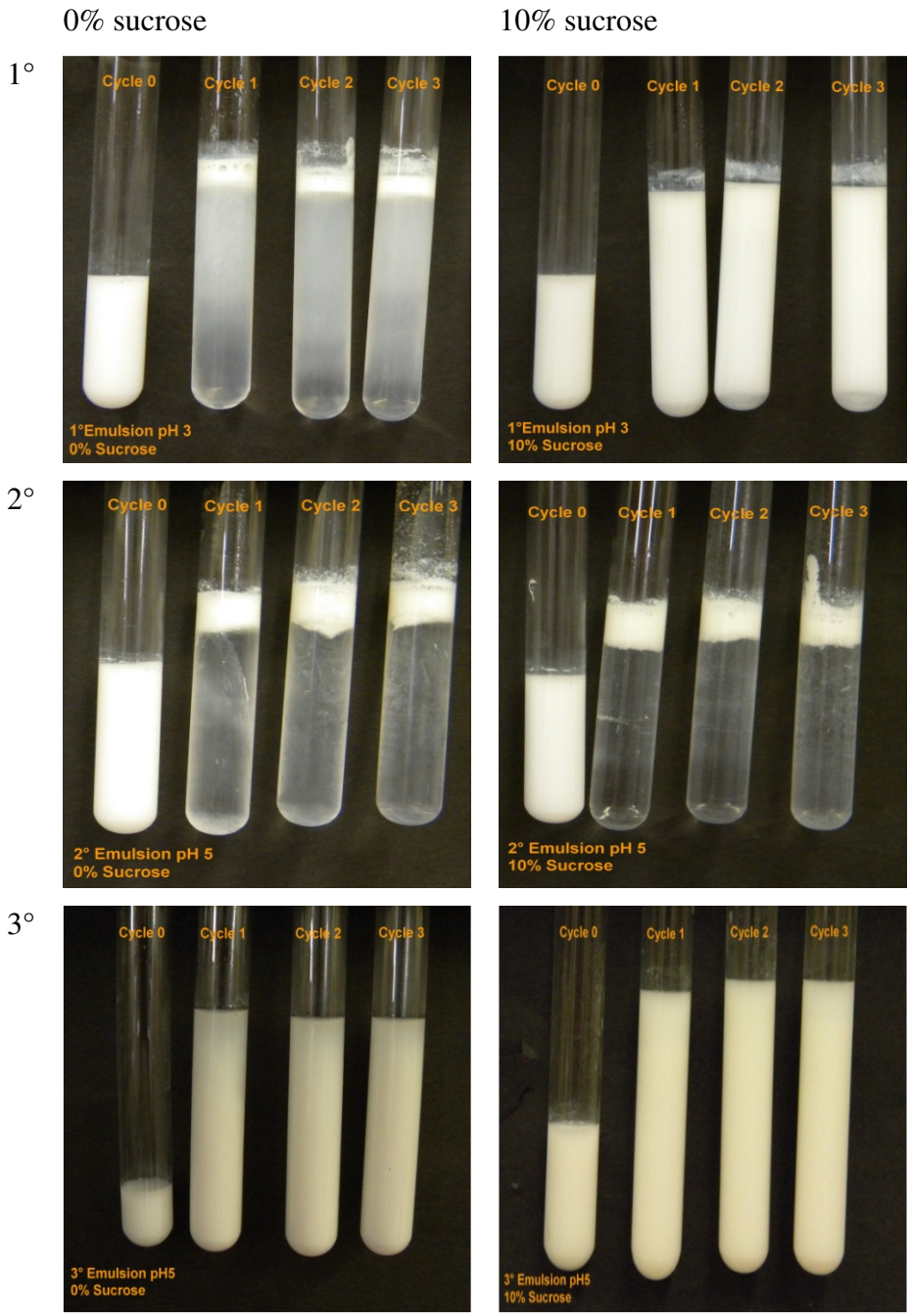
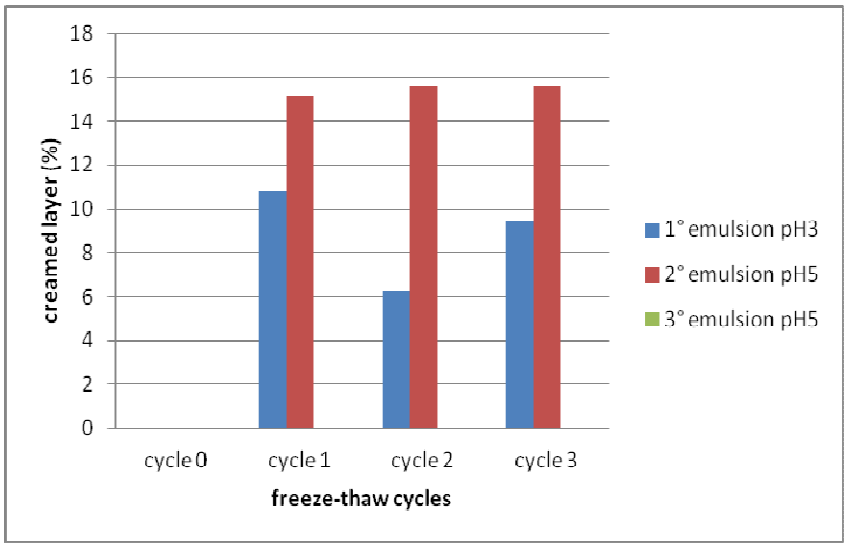
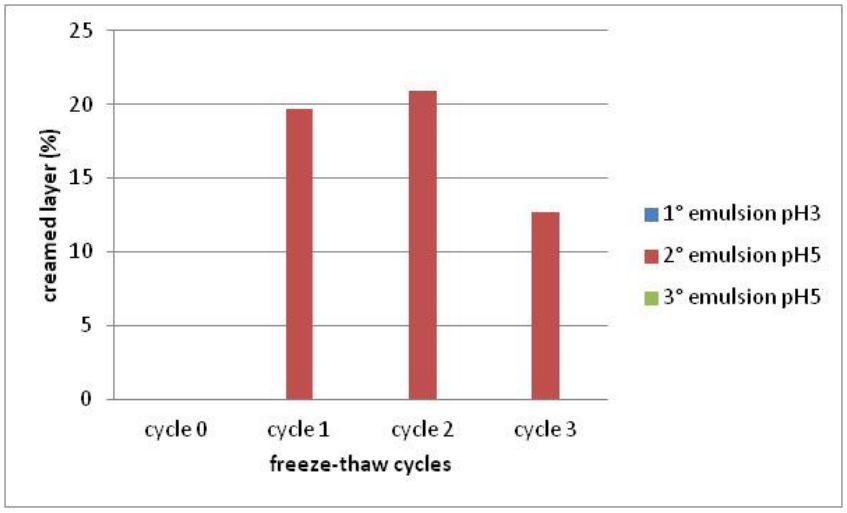


Figure 44. Digital image of original emulsions (1% Fish Oil, 0.1%, 0.04% chitosan and 0.65% pectin) number of freeze-thaw cycles and on sucrose concentration.



(a)



(b)

Figure 45. Dependence of emulsion creaming stability of primary, secondary and tertiary emulsions (1% Fish Oil, 0.1%, 0.04% chitosan and 0.65% pectin) on number of freeze-thaw cycles and on sucrose concentration: (a) 0% sucrose; (b) 10% sucrose.

4.2.5 DDA% Chitosan

The % deacetylation of Chitosan determines its number of possible positively charged sites. The effect of % DDA on emulsion stability was determined.

4.2.5.1 Zeta potential

Zeta-potential was measured as a function of pH in primary and the secondary emulsions, the secondary emulsions contained Chitosan with different % DDA (50%, 70 % and 92%). The charge of the primary emulsion went from strongly positive at pH 2 to

strongly negative at pH 8 crossing from positive to negative at pH 4 to 5, (the isoelectric point the sodium caseinate is ≈ 4.6). The primary emulsion had only one stabilizer so it would be expected that the primary emulsions were more unstable than the secondary emulsions. The secondary emulsions containing Chitosan with 50% DDA went from strongly positive (25 mV) at pH 2 to weakly positive at pH 8 (3 mV). Chitosan with 50% DDA has a lower charge density (positive groups per unit length). The secondary emulsion containing Chitosan with 70% DDA went from strongly positive (30 mV) at pH 2 to negative at pH 8 (-21 mV), the emulsion drops to negative at pH 7 (Figure 42). The secondary emulsion containing Chitosan with 92% DDA went from strongly positive (32 mV) at pH 2 to strong negative (-28 mV) at pH 8. At pH 6 the charge was 26 mV but at pH 7 the charge droped to -6 mV

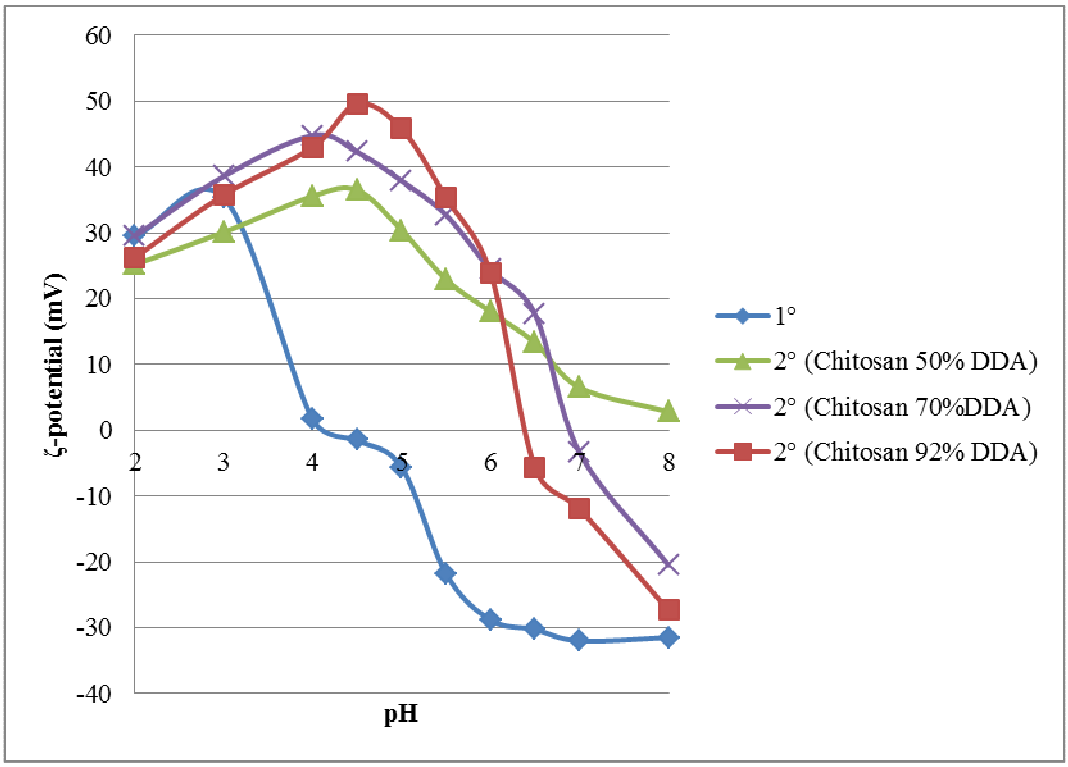


Figure 46. Particle electrical charge (ζ-potential) of primary and secondary emulsions as a function of pH.

4.2.5.2 Size measurements

The particle size was measured as a function of pH in the secondary emulsions, the secondary emulsions contained Chitosan with different DDA % (50%, 70 % and 92%). All the emulsions stayed stable from pH 2 till 4.5. The emulsion that contained Chitosan with 50% DDA was stable at the widest pH range from pH 2 till 6.5. The emulsion containing Chitosan with 70% DDA was stable from pH 2 till pH 6. Compared to the charge measurements the emulsion containing Chitosan with 70% DDA went from positive to negative at pH 6/6.5. The

emulsion containing Chitosan with 92% DDA was more unstable than the other 2, the emulsion became unstable at pH 4.5 compared to the charge measurements the emulsions when from positive to negative at pH 4/4.5 (Figure 46 and 47).

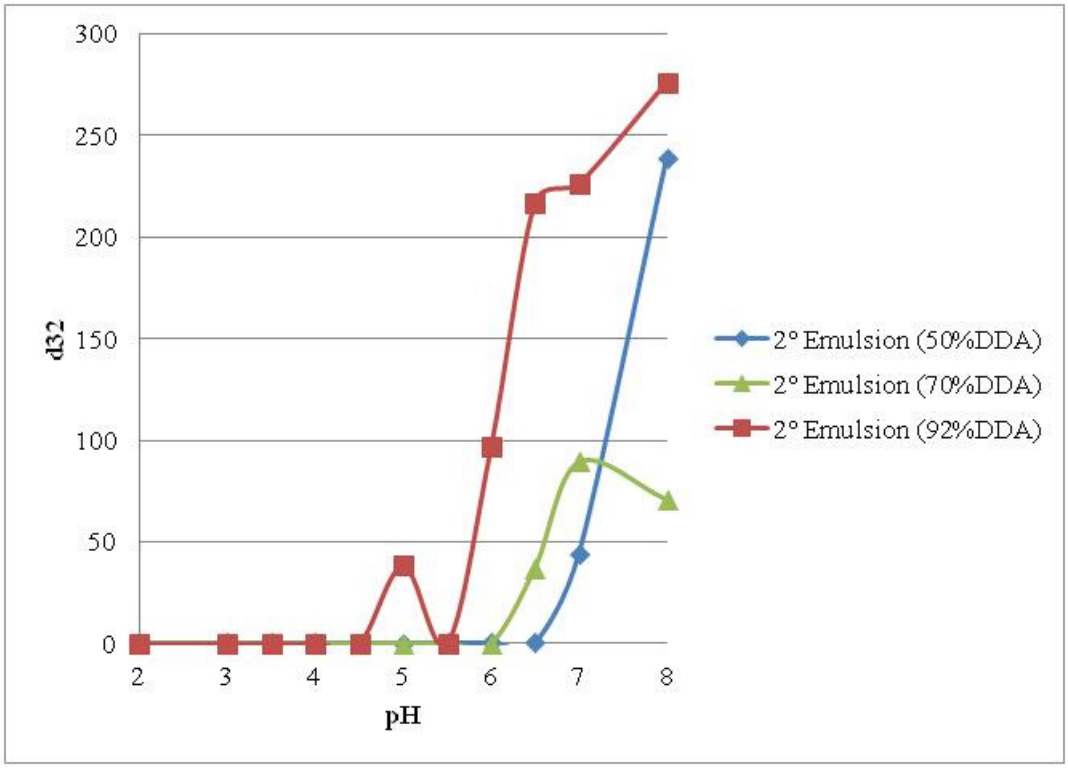


Figure 47. Particle size (d_{32}) of secondary emulsions (with different DDA% of Chitosan) as a function of pH.

4.2.5.3 Creaming stability

The creaming index was measured as a function of pH in the primary and the secondary emulsions, the secondary emulsions containing Chitosan with different DDA % (50%, 70 % and 92%). The creaming index measurements supported the particle size measurements as well as the charge measurements. No creaming was visible at pH 2 to 4.5. The emulsions containing Chitosan with 50% DDA were stable at the widest pH range, from pH 2 to 6.5, but at pH 7 the creaming index measured high, that is comparable to the size and charge measurements. The emulsion containing Chitosan with 70% DDA had no visible creaming from pH 2 to pH 6 that was comparable to both the size and creaming measurements. The emulsions containing Chitosan with 92% DDA displayed a measurable creaming index at pH 4.5 that was also comparable to the size and charge measurements (Figure 48).

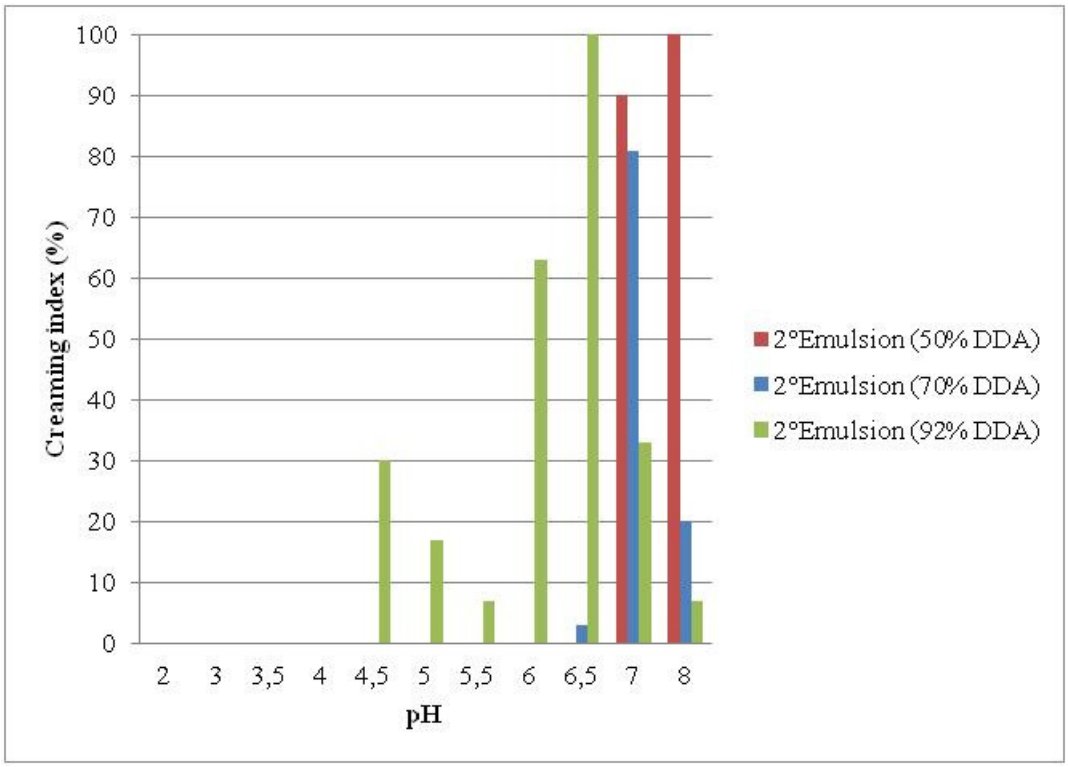


Figure 48. Creaming index (%) of secondary emulsions (with different % DDA of Chitosan) as a function of pH

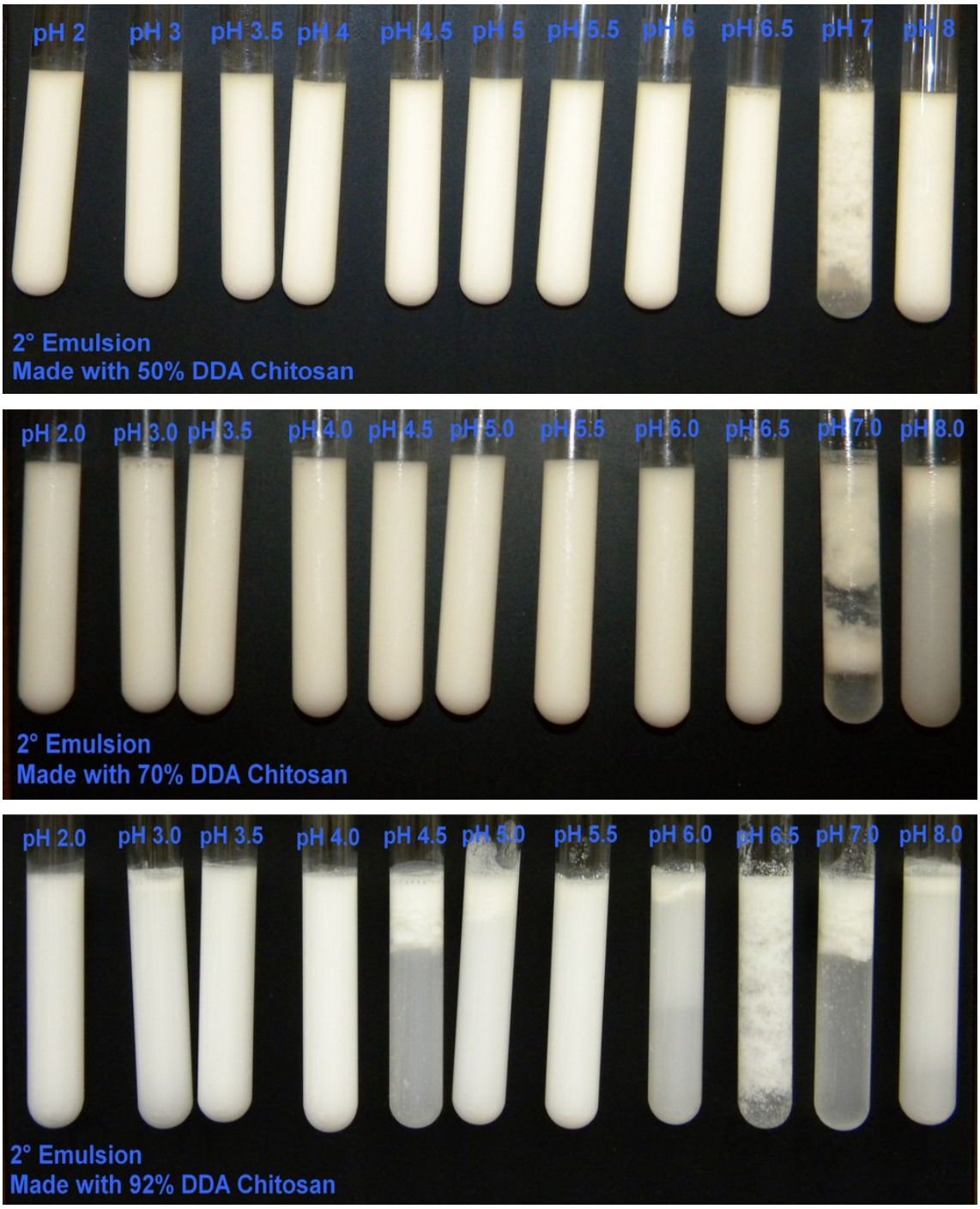


Figure 49. Creaming stability images of secondary emulsions as a function of pH. (a) 50% DDA Chitosan, (b) 70% DDA Chitosan and (c) 92% DDA Chitosan.

4.2.3 Oxidation measurements

All 4 emulsions were stored at 37°C for 14 days in order to determine difference in the oxidative stability of the samples. The lipid oxidation rates in primary (sodium caseinate at pH 3), secondary (sodium caseinate – chitosan at pH 5), primary (tween20 pH 3) and primary (tween20 pH5) emulsions containing fish oil were compared by measuring lipid hydroperoxide and TBARS. Lipid hydroperoxide and TBARS formation was much faster at in the primary emulsions containing tween20 both at pH 3 and pH than the primary emulsion containing sodium caseinate and secondary emulsion containing sodium caseinate and chitosan ($p < 0.05$). After 10 days there was almost no hydroperoxide accumulation observed ($p < 0.05$). The physical properties that may influence the rate of lipid oxidation rated in oil-in-water emulsions include particle size, which influences surface area; emulsions droplet charge, which causes either attraction or repulsion of transition metals; and thickness of the layer at interfacial regions of the emulsion droplet that can impact interactions between lipids and aqueous phase prooxidants. The emulsion droplet size can influence lipid oxidation rates because smaller particle sizes result in a larger surface area and thus a greater possibility for lipids – aqueous phase prooxidant interactions (Lethuaut, Metro & Genot. 2002). One of the major mechanisms for oxidation of emulsified lipids is the iron-promoted degradation of lipid hydroperoxides into free radicals that can oxidize unsaturated fatty acids (McClements & Decker. 2000). Thus free radicals that impact iron – hydroperoxide interaction, can have a dramatic effect on lipid oxidation rates. The greater stability of 1° and 2° emulsions containing sodium caseinate and chitosan, can be attributed to the fact that the sodium caseinate coated droplets at pH 3 and the sodium caseinate – chitosan coted droplets at pH 5 were both highly positively charged (+35 mV and +38 mV), and will therefore electrostatically repel prooxidative transition metals.

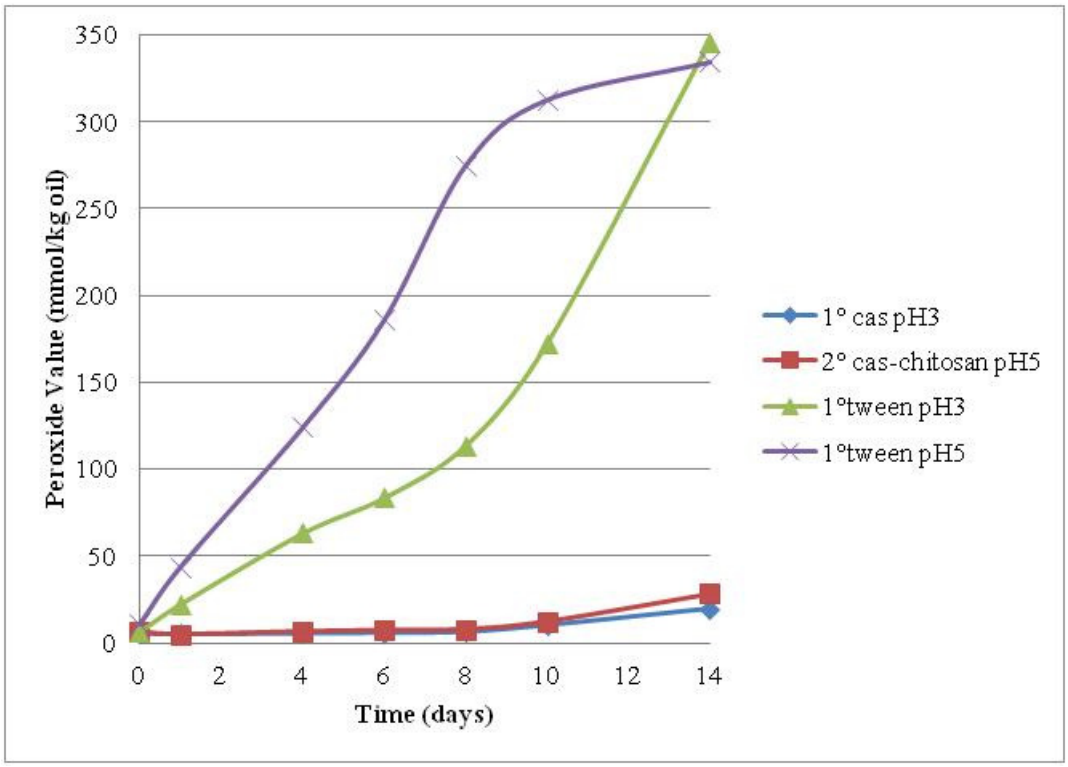


Figure 50. Formation of lipid hydroperoxides in multilayerd emulsions (1°emulsion containing sodium caseinate as the only layer at pH3, 2° emulsion containing sodium caseinate as the first layer and then chitosan (70%DDA) as the second layer at pH 5, 1° emulsion with one layer, tween20 at pH 3 and then 1°emulsion with tween 20 at pH 5) stored at 37°C.

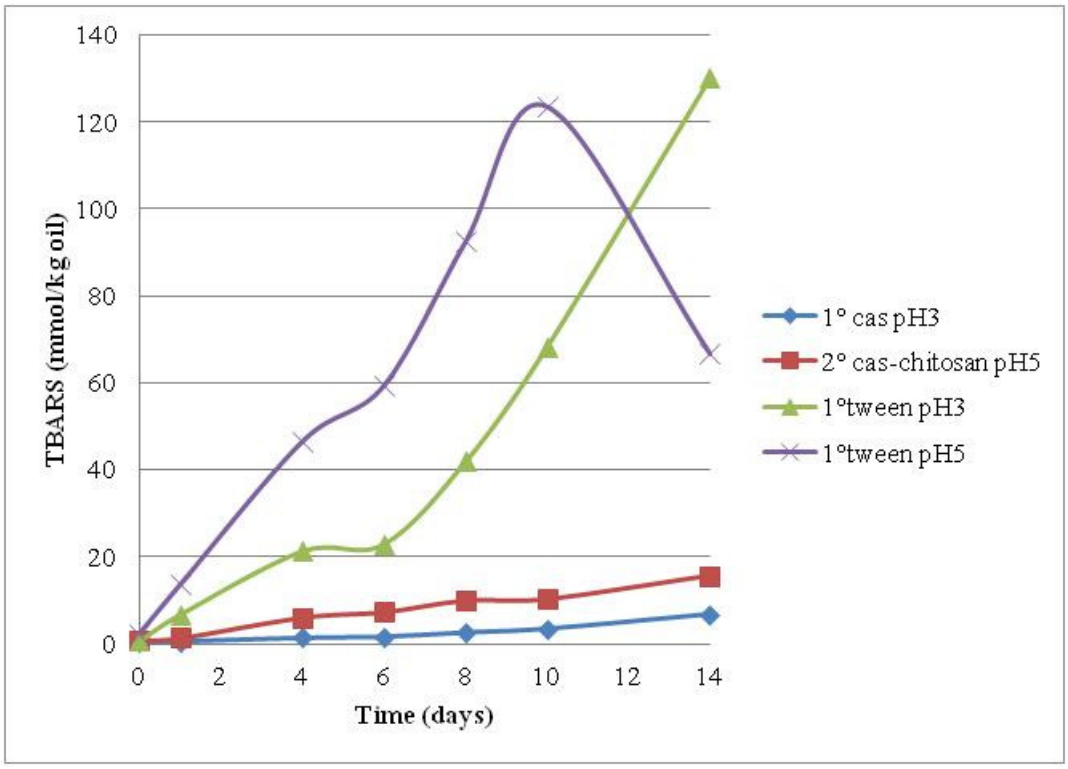


Figure 51. Formation of TBARS in multilayerd emulsions (1°emulsion containing sodium caseinate as the only layer at pH3, 2° emulsion containing sodium caseinate as the first layer and then chitosan (70%DDA) as the second layer at pH 5, 1° emulsion with one layer, tween20 at pH 3 and then 1°emulsion with tween 20 at pH 5) stored at 37°C.

5. Summary and conclusions

This study has shown that stable emulsions containing tri-layered lipid droplets can be prepared using a simple and cost-effective method. Initially, a primary emulsion containing small droplets was produced by homogenization of oil, water, and an anionic emulsifier (Sodium caseinate at pH 3.0). A secondary emulsion containing cationic lipid droplets coated with a caseinate–chitosan membrane was then produced by mixing a cationic polysaccharide (chitosan at pH 5) with the primary emulsion and agitation to disrupt any flocculation. A tertiary emulsion containing anionic droplets coated with a Caseinate–Chitosan–Pectin membrane was then produced by mixing an anionic polysaccharide (pectin at pH 5) with the secondary emulsion, without applying mechanical agitation.

By coating the fish oil droplets with layers the environmental conditions of the oil were improved. The fish oil did not oxidase as rapidly, the emulsions were stable at a wider pH range and the possibility of freeze and thaw cycles were obtained.

It has been shown that different DDA of chitosan does not have strong influence on the charge of caseinate-chitosan coated droplets, on the particle size or on the creaming index at low pH levels. As soon as the pH increases above 5 the chitosan with the highest DDA (90%) was the most unstable. Furthermore it has been shown that chitosan will adsorb to the surface of the caseinate coated oil droplets, because the droplets have appreciable negative charge. Emulsions containing droplets coated with caseinate membranes at pH 3 (with 10% sucrose) were stable to coalescence when they were frozen and thawed and these emulsions did not show any creaming. Emulsions containing droplets coated with caseinate-chitosan membranes at pH 5 were highly unstable to coalescence when either the fat droplets or the water phase crystallized. Creaming occurred after the first freeze thaw cycle. Emulsions containing droplets coated with caseinate–chitosan–pectin membranes at pH 5 (with 10% sucrose) were stable to both coalescence and creaming when they were frozen and thawed, although there may have been some weak flocculation in the system. The layer-by-layer deposition technique used in this study may be useful for developing frozen food products with improved stability to freezing and thawing.

The improved oxidative stability of the emulsion droplets was likely due to the cationic nature of the droplets that can repel prooxidative metals and possibly form a thicker interfacial region that could decrease interactions between lipids and water soluble prooxidants. The data suggest that fish oil-in-water emulsions stabilized by caseinate chitosan

membranes may be used to produce oxidative and physically stable omega-3 FAs in functional foods.

The information obtained in this study is particularly useful for the design and production of oil-in-water emulsions stabilized by multilayer membranes. These multilayer emulsions have previously been shown to have better stability to environmental stresses than conventional single-layer emulsions under certain circumstances, and may therefore be suitable for utilization in industrially for a variety of applications.

One potential limitation of this technology is the number of additional steps required to prepare the multilayered emulsions compared to conventional emulsions, which will increase production costs and therefore limit its application to certain specialized products.

6. Suggestion for further work

Future work would be to test the emulsion in a digestion system and to see if the double environmental triggers work. The final step in this experiment would be to incorporate our emulsion in some food products. The ideal food product might be a spread for breed or biscuits, cold sauce or yogurt.

7. References

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