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RATIONALIZING NANOEMULSION FORMATION FOR ENCAPSULATION, PROTECTION AND DELIVERY OF BIOACTIVE FOOD COMPONENTS

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RATIONALIZING NANOEMULSION FORMATION FOR ENCAPSULATION, PROTECTION AND DELIVERY OF BIOACTIVE FOOD COMPONENTS

A Dissertation Presented

by

YING YANG

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David Julian McClements, Chair

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DEDICATION

To my loving family who support me all the time

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ABSTRACT

RATIONALIZING NANOEMULSION FORMATION FOR ENCAPSULATION, PROTECTION AND DELIVERY OF BIOACTIVE FOOD COMPONENTS

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The objective of this thesis was to design and develop novel food-grade nanoemulsion-based delivery systems for the encapsulation, protection and delivery of lipophilic bioactive food components. These delivery systems could be widely applied in aqueous-based fortified food products, such as beverages, salad dressing and yogurt *etc.*

Both the low- and high-energy methods could be used for fabricating nanoemulsions (r < 100 nm). The microfluidization method could form nanoemulsions at low surfactant-to-oil ratios (SOR < 0.1), but it required the use of high-energy inputs and expensive equipment. On the other hand, the spontaneous emulsification method could also form ultrafine emulsions and moreover it was simple and inexpensive, but it required much higher surfactant-to-oil ratios (SOR > 0.5) for forming nanoemulsions.

Q-Naturale® is a natural food-grade surfactant, which is got from the bark of the *Quillaja saponaria* Molina tree. By using high pressure homogenization (microfluidization), Q-Naturale® could form relatively small droplets (d < 200 nm) at low

surfactant-to-oil ratios (SOR < 0.1), but the droplets were not as small as those produced using Tween 80 under similar conditions (d < 150 nm). The emulsions formed by using Q-Naturale® as the emulsifier were stable to droplet coalescence over a range of pH values (2 to 8), salt concentrations (0 to 500 mM NaCl) and temperatures (20 to 90 ºC). Thus, the design of "all-natural" delivery system for vitamin E by using Q-Naturale® as the emulsifier were then studied. Ultrafine emulsions could not be fabricated using pure α-tocopherol acetate as the oil phase due to its very high viscosity, but they could be formed when ≥ 20% MCT was incorporated into the oil phase prior to homogenization. In the absence of glycerol, Q-Naturale® was able to form emulsions containing relatively small droplets (d < 400 nm) from oil phases containing relatively high vitamin levels (60 to 80%). The addition of glycerol into the aqueous phase could help decreasing this droplet size. Q-Naturale® was more effective than Tween 80 at producing small droplets with oil phase containing high levels of vitamin E acetate.

The influence of carrier oil type on the bioaccessibility and molecular form of vitamin E encapsulated in Q-Naturale® delivery system was then examined using a simulated gastrointestinal model. The total bioaccessibility of vitamin E after digestion was higher for LCT- than MCT-emulsions, which was attributed to the greater solubilization capacity of mixed micelles formed from long chain fatty acids. The conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion was also considerably higher for LCT- than MCT-emulsions, which may impact the subsequent

absorption of the vitamin E. Morever, by using emulsion titration assay for further quantifying the kinetics and extent of vitamin E and vitamin E acetate solubilization in model mixed micelles, the solubilization capacities were depended on the composition of the mixed micelles: micelle solubilization of vitamin E was increased by the presence of phospholipid (DOPC), but did not depend strongly on the presence of free fatty acid (octanoic acid or linoleic acid). The solubilization capacity of the mixed micelles for vitamin E was higher than that for vitamin E acetate, which was attributed to differences in the ability of the vitamin molecules to be incorporated into the micelle structure.

Finally, all the key factors impacting on the bioaccessibility of emulsified vitamin E, such as calcium ions, phospholipids, carrier oil type *etc*., were then overall studied using a simulated small intestine model. The addition of calcium ($CaCl_2$) to the SSIF increased the extent of lipid digestion in LCT-emulsions, but had little impact in MCT-emulsions. The bioaccessibility of vitamin E increased in the presence of calcium and phospholipids (DOPC) in LCT-emulsions, but decreased in MCT-emulsions. The highest bioaccessibility (≈ 66%) was achieved for LCT-emulsions when the SSIF contained both calcium and DOPC. The conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion was considerably higher for LCT-emulsions when calcium ions were present in the SSIF, but was not strongly affected by SSIF composition for MCT-emulsions.

Overall, the results obtained in these studies will provide guidelines for rationally

designing effective nanoemulsion-based delivery systems for encapsulating, protecting and delivering lipophilic bioactive components. These delivery systems could be used in various industrial products, such as fortified foods, pharmaceuticals, cosmetics, and personal care products in the future.

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CHAPTER 1

INTRODUCTION

Nowadays consumers pay more attention to the safety and healthiness of food products, which leads to an increasing interest within the food industries in the development of delivery systems for the encapsulation, protection and delivery of lipophilic bioactive components, such as ω-3 fatty acids, carotenoids, flavonoids and vitamins [1-4]. These days, food industries are interested in fortifying food and beverages with various kinds of functional food ingredients, such as flavors, colors, vitamins and nutraceuticals and the growth of fortified beverages had been estimated to be around 19 % from 2009 to 2014 [5].

Since most of these bioactive food components (*e.g.* vitamins, carotenoids, and polyunsaturated triglycerols *etc.*) are lipophilic compounds, O/W emulsions are one of the most effective delivery systems for helping these ingredients incorporated into the aqueous-based food products. Typical conventional emulsions have droplets with mean diameters between 100 nm and 100 μm. The droplet sizes in conventional emulsions are close to the light wavelength so that they can scatter light strongly which will lead to the turbid or opaque appearance of the emulsions [6]. Moreover, conventional emulsions have a relatively low thermodynamic stability. Thus, a considerable amount of research has been done to optimize the design and fabrication of emulsion-based delivery systems.

Recently, food industries are very interested in avoiding using synthetic ingredients by using natural "label friendly" alternatives instead [7, 8]. Hence, there has been a focus on identifying and characterizing natural emulsifiers that can be successfully used in emulsion-based food and beverage products. Biopolymer-based emulsifiers, such as proteins (whey, soy and egg) and polysaccharides (gum arabic and modified starch), have been shown to be effective at forming and stabilizing emulsions [8-11]. However, biopolymer emulsifiers often have limitations in their functional performance. Protein stabilized emulsions have lower stability at pH values around their isoelectric point, at high ionic strengths, and at elevated temperatures [12]. On the other hand, it is often difficult to produce oil droplets with very small sizes (d < 200 nm) using polysaccharide-based emulsifiers [9, 10, 13]. Q-Naturale® is an extract isolated from the bark of the *Quillaja saponaria* Molina tree and which contains surface active components that are capable of forming surfactant micelles and stabilizing O/W emulsions [14-16]. The major components within this extract have been reported to be saponins [17, 18], which are high molecular weight glycosides consisting of a sugar moiety attached to a triterpene or a steroid aglycone [19].

Vitamin E is an essential micronutrient for both humans and animals due to its antioxidant and non-antioxidant biological activities. Vitamin E could work as an antioxidant by inhibiting the formation of reactive oxygen species (ROS) during the lipid oxidation. According to the activities of vitamin E in quecing free radicals, vitmain E

is believed to prevent some chronic diseases that are related with the generation of free radicals. Due to its antioxidant or non-antioxidant activities, vitamin E provides a lot of health benefits for human beings, such as enhancing immune systems, anti-inflammation by inhibiting the release of reactive oxygen species (ROS) and preventing cardiovascular diseases by inhibiting the oxidation of low-density (LDL) [20].

The term "vitamin E" actually refers to a group of fat-soluble vitamins that are widely used as functional ingredients in food, pharmaceutical, and cosmetic preparations [21]. Vitamin E is a kind of highly hydrophobic vitamin that cannot be directly dispersed into aqueous solutions [22]. Therefore, appropriate emulsion-based delivery systems need to be developed for helping dispersing vitamin E into the aqueous environment [23].

A major objective of this study was to carry out research to develop a novel emulsion-based delivery systems for lipophilic bioactive food components by using a newly available natural emulsifier – Q-Naturale®. Following fabrication, these delivery systems will be evaluated for its physical properties, its chemical and environmental stability. The impact of different delivery systems on the stability and release properties of bioactive compounds will also be investigated. The results gained from this research should facilitate the utilization of nanotechnology to design functional delivery systems, thereby enhancing bioactive utilization and bioavailability in the diet.

CHAPTER 2

LITERATURE REVIEW

2.1 Emulsion-based Delivery System

2.1.1 Emulsions

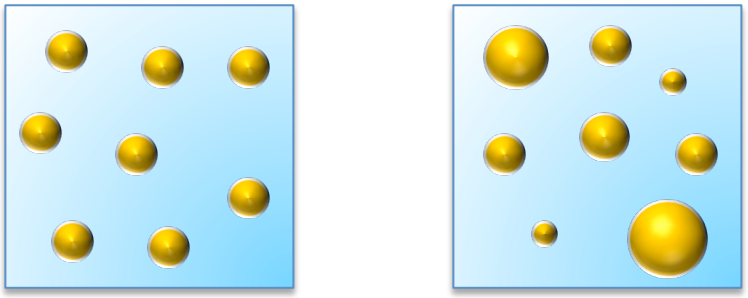
Emulsions are systems containing two non-mixable liquids (usually oil and water) and in the system one of the liquids is dispersed as small droplets in the other [24-26]. Emulsions could be found in all kinds of natural and processed foods, such as milk, cream, beverages, salad dressings, mayonnaise, sauces, ice cream, coffee whitener, spreads, butter, and margarine *etc*. Emulsion technology is particularly suitable for the encapsulation, protection and delivery of lipophilic functional food ingredients, *e.g.* ω-3 fatty acids, oil-soluble vitamins and nutraceuticals. Variety of emulsion-based delivery systems, such as conventional oil-in-water (O/W), multilayer (O_M/W), water-in-oil-in-water (W/O/W), and oil-in-water-in-water (O/W/W) emulsions, could be used for incorporating these food components [26, 27].

2.1.2 Emulsion Particle Characteristics

2.1.2.1 Particle Size

The particle size of the emulsion droplets would greatly influence some emulsion properties, such as the appearance, stability and rheology *etc*. Either volume-weighted ($d_{4,3}=\sum d_i n_i^4/\sum d_i n_i^3$) or surface-weighted mean diameter ($d_{3,2}=\sum d_i n_i^3/\sum d_i n_i^2$) could be used for representing the droplet sized, where n_i is the number of droplets of diameter d_i. The

mean particle size of emulsion droplets depends on homogenization conditions (e.g., intensity or duration of energy input) and system composition (e.g., emulsifier type and concentration) [28]. The particle size distribution could be measured by light scattering or microscopy methods. A *monodisperse* emulsion refers to the emulsion with droplets of the same size while a *polydisperse* emulsion means the emulsions with a range of different droplet sizes (**Figure 2.1**). Real food emulsions are usually *polydisperse* emulsions so that the droplet size description of real food is more complicated.



Monodisperse Emulsion *Polydisperse* Emulsion

Figure 2.1 Schematic representation of *monodisperse* and *polydisperse* emulsions [28].

2.1.2.2 Particle Charge

Due to the adsorption of ionized emulsifiers, mineral ions, or biopolymers to the surface, the droplets in the emulsions often have an electrical charge, which impacts many emulsion properties. The characteristics of the emulsifiers are the key factor impacting the droplet electrical charge. Emulsions formed by using non-ionic emulsifier (*e.g.*, Tweens and Spans) usually have no or small charge while emulsions formed by using anionic emulsifier (*e.g.*, lecithin and fatty acids) are negative charged

and emulsions stabilized by cationic emulsifier (*e.g.*, lauric arginate) have positive charge.

2.1.2.3 Particle Concentration

Particle concentration refers to the number, mass, or volume of droplets per unit volume or mass of emulsion [28]. Because of the core-shell structure of emulsion droplets, the overall effective volume fraction ($C_{effective}$) is including both the volume fraction of the core (C_{core}) and the shell (C_{shell}). The effective volume fraction of emulsion droplets could be calculated by the following equation [29, 30]:

$$C_{effective} = C\left(1 + \frac{t}{r}\right)^3$$

According to the equation, the real effective volume fraction of the oil droplets ($C_{effective}$) in emulsions may be much larger than the real volume fraction of the oil phase (C_{core}). This effect could be applied for developing novel emulsion-based food products, such as low fat foods with same viscosity at much lower oil concentration.

2.2 Emulsion Formation

Generally, there are two main approaches for emulsions fabrication: high-energy methods and low-energy methods.

2.2.1 High Energy Emulsification Methods

In the food industry, the most common ways for producing food-grade emulsions are high-energy methods, such as microfluidization, high-pressure valve homogenization, and sonication. These high-energy methods can be used with a wide variety of different

oil and emulsifier types [31-35]. The principles of these high-energy methods are to mechanically breakup the oil phase into small droplets which could be dispersed evenly within the aqueous phase by applying intense disruptive forces [36].

2.2.1.1 High Pressure Valve Homogenizer

High-pressure valve homogenizers (HPVHs) are the most popular emulsification method in the food industry. Usually, a coarse emulsion needs to be prepared first before using HPVHs. As shown in **Figure 2.2**, after fed into HPVHs, the coarse emulsion experiences a combination of intense shear, cavitational and turbulent flow conditions, which cause the larger droplets to break into small droplets as homogenized products [37]. The final product are strongly depends on the number of passes and the pressure applied. Meanwhile, the characteristics of the material used for homogenization (e.g., the disperse-to-continuous phase viscosity ratio, concentration of emulsifier) also greatly impact the fine emulsion formation [38, 39].

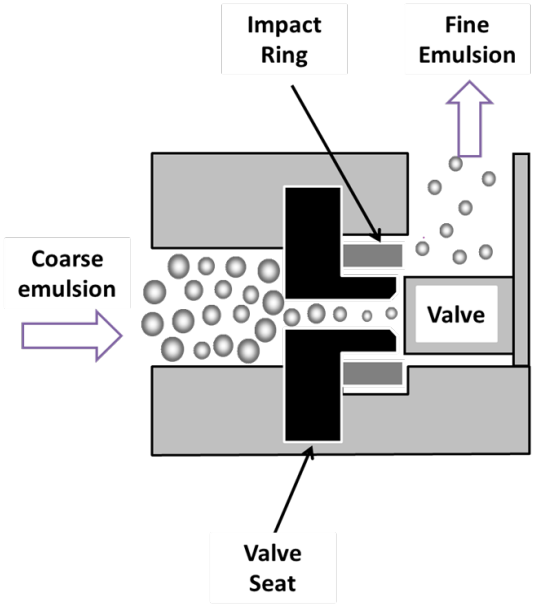


Figure 2.2 Schematic representation of High-pressure valve homogenizers

2.2.1.2 Microfluidizer

Microfluidizer can be used for fabricating very small emulsion droplets directly from individual oil phase and water phase. Microfluidizer has similar principle as high-pressure valve homogenizers but microfluidizer could generate higher intense shear forces. After fed into microfluidizer, the coarse emulsion flowing was divided into two streams and each stream passed through a separate fine channel. The two streams of coarse emulsions would then impinge on each other in an interaction chamber. As a result of that, intense disruptive forces are generated, which lead to highly efficient droplet disruption. Previous researches have shown that the microfluidization is more effective at creating more uniform emulsion droplets with smaller droplet size. The major factors for the products of microfluidizer include pressure, number of passes,

oil-water interfacial tension, oil-water viscosity ratio, and emulsifier type and concentration [8, 33, 40, 41].

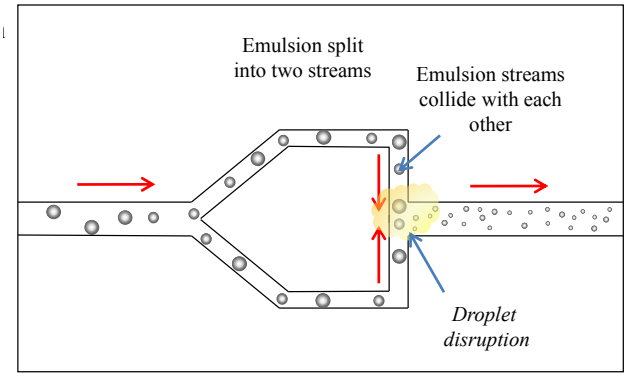


Figure 2.3 Schematic representation of microfluidizer

2.2.1.3 Sonication

Ultrasonic emulsification is also a kind of high-energy emulsification methods commonly used for ultrafine emulsion formation. Wood and Loomis first reported ultrasonic emulsification in 1927 and the first patent of sonication emulsification was granted in 1944 in Switzerland [42, 43]. Sonication emulsification was recognized as fast and efficient technique for formulating stable, uniform and small emulsion droplets [44].

Sound waves (frequency > 20 kHz) will be used to cause ultrasound-driven mechanical vibrations, followed by the formation of acoustic cavitation. Thus, the coarse emulsions will be broken into tiny emulsion droplets by the powerful shocks from cavitation bubble collapse [45]. There are several factors which influence the output droplet size of the sonication emulsification system, such as ultrasonic power input,

processing time, oil concentration, emulsifier type and concentration, mixing ratio of oil and surfactant, viscosity of continuous phase, processing time [46].

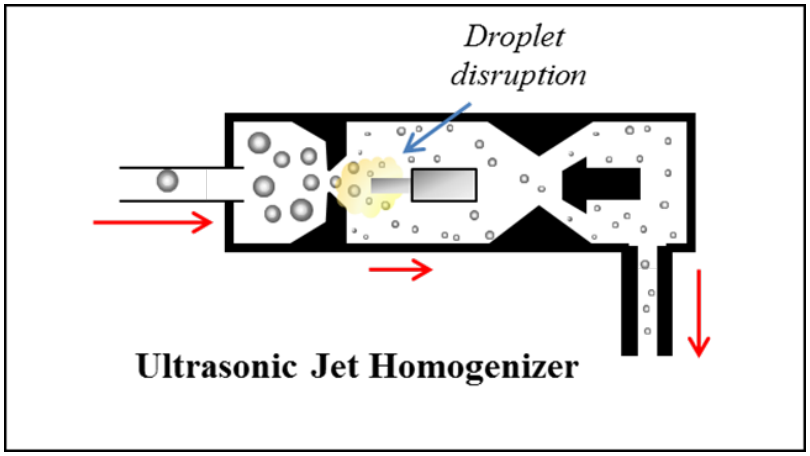


Figure 2.4 Schematic representation of Sonication

2.2.2 Low Energy Emulsification Methods

Ultrafine emulsions can also be produced using a variety of low-energy methods, such as phase inversion and spontaneous emulsification methods.

Compared with high-energy methods, low-energy approaches may have advantages for certain applications within the food and beverage industries: they are often more effective at producing very fine droplets; they have lower equipment and energy costs; they are simpler to implement. However, at the same time, some potential disadvantages may also exist in low-energy systems, such as higher concentration of emulsifier, limitations of oil types and surfactant types that could be applied in low-energy systems *etc.*

2.2.2.1 Spontaneous Emulsification

Spontaneous emulsification is a process that emulsions are spontaneously formed without the aid of external energy supply when two immiscible fluids with very low interfacial tension get in contact with each other [47]. The spontaneous emulsification method is originally widely used in the pharmaceutical industry [48, 49]. Systems prepared using this method is often referred to as self-emulsifying drug delivery systems (SEDDS) or self-nano-emulsifying drug delivery systems (SNEDDS) based on some literature. In the spontaneous emulsification method, emulsions will be formed immediately as two immiscible fluids placed in contact with each other (**Figure 1**) [50-52]. Practically, this method can be carried out in a number of different ways to form ultrafine emulsions: the compositions of the two phases can be varied; the environmental conditions can be varied (*e.g.,* temperature, pH and ionic strength); and/or, the mixing conditions can be varied (*e.g.,* stirring speed, rate of addition, and order of addition) [53].

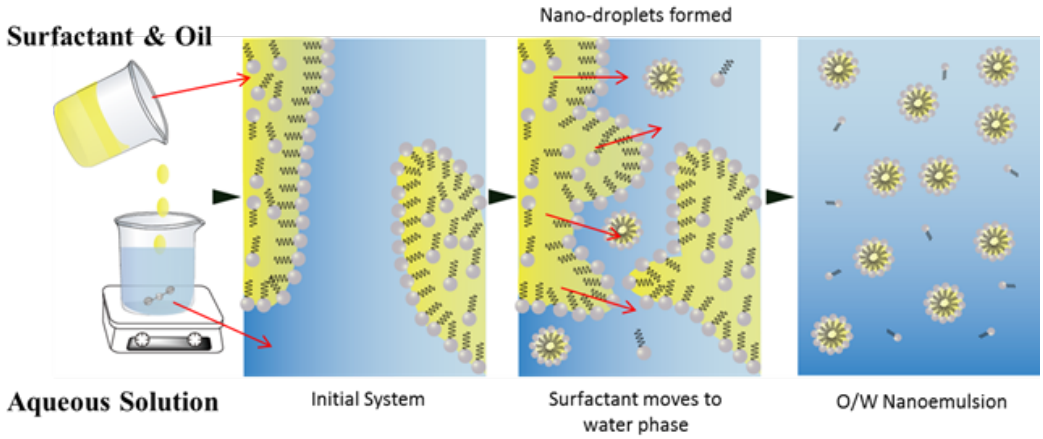


Figure 2.5 Schematic representation of spontaneous emulsification

2.2.2.2 Phase Inversion Temperature (PIT) Method

The phase inversion temperature (PIT) method is also a low-energy method which has attracted quite a lot of interests nowadays since it is an organic, solvent-free method [54]. Shinoda and Saito first introduced the PIT concept. The PIT method realizes the formation of ultrafine emulsions by utilizing the specific ability of surfactants, usually nonionic surfactants such as polyethoxylated surfactants. Their affinities for water and oil were modified in function of the temperature, and therefore to undergo a phase inversion. Indeed, the so-called transitional emulsion phase inversion occurs when, at fixed composition, the relative affinity of the surfactant for the different phases is changed, resulting in a gradual modification of the temperature [55, 56]. Oil, water and nonionic surfactants are all mixed together at room temperature and slightly stirred. Then, the mixture is gradually heated up. As a result, the surfactant solubility progressively changes from the aqueous to the oily phase. Above the phase inversion temperature the surfactant is fully solubilized in oil and thus the mixture undergoes a phase inversion, from an oil-in-water (o/w) to a water-in-oil (w/o) emulsion.

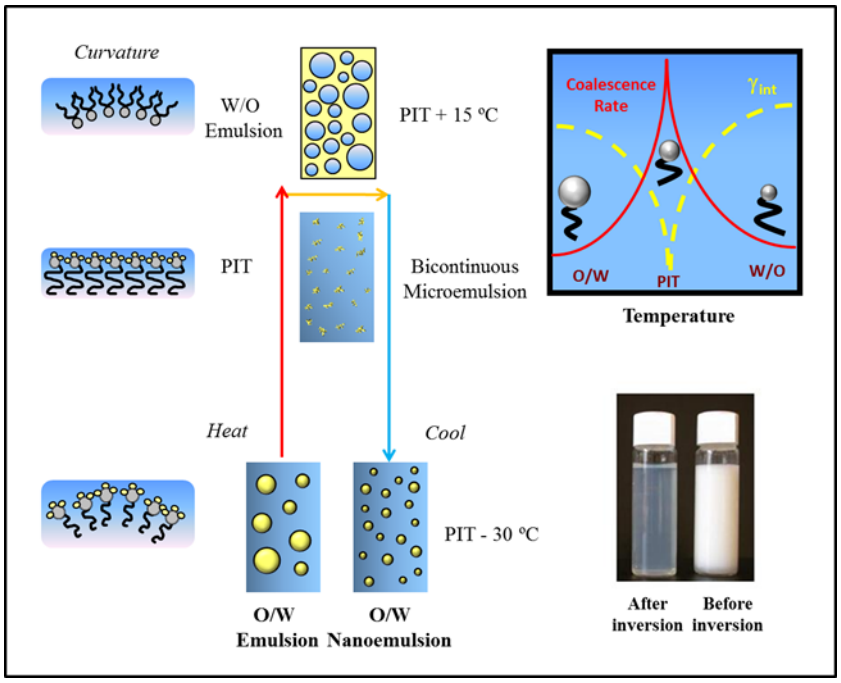


Figure 2.6 Schematic diagram of the formation of emulsions by the Phase Inversion Temperature (PIT) method [57].

2.3 Emulsifier

Beeswax used in skin lotion by a Greek physician named Galen of the 2nd century (in AD 131–203) was the earliest emulsifier on record whereas egg yolk was the first emulsifier on record used for food applications and egg yolk was used to help mixing liquid oil phase into an acidified aqueous phase of mayonnaise in the early 19th century [58].

Emulsifiers facilitate the formation of emulsions by adsorbing to the interfaces between oil and water during homogenization [59, 60]. Emulsifier molecules are amphoteric molecules containing both nonpolar and polar regions. The nonpolar group could be aromatic, aliphatic or alicyclic groups while the polar groups include

heteroatoms like oxygen, nitrogen, and sulfur and the polar groups in the emulsifier molecules are the main reason for making the emulsifier anionic, cationic or nonionic [61].

The emulsifier plays a critical role in both droplet break-up and coalescence. The most important functions during the homogenization process for emulsifiers include: (i) aiding in the droplet break-up by decreasing the interfacial tension between oil an aqueous phase by reducing free energy needed for breaking large droplets; (ii) forming a protective coating around the droplets to prevent the droplets from aggregation and re-coalescence [62]. Thus small molecule emulsifiers are the most suitable emulsifiers for the nanoemulsion formation according to the requirements of both droplet break-up and coalescence as they may quickly absorbed to the interfaces and they have lower interfacial tensions.

Every year approximately 500,000 metric tons of emulsifiers, valued at USD 600-700 million, are produced and sold in the global market [63].

Each type of emulsifier has its unique chemical structure so that each type of emulsifier has its special functional properties. There is no single emulsifier that could be applied in all kinds of situations. The thermodynamic stabilities of emulsions are quite low due to the unfavorable contact between the two non-mixable liquids [64]. Therefore stabilizers will be necessary to prevent the emulsions from breaking down.

The type and concentration of emulsifier will greatly influence the droplet size

distribution and stability of the emulsion [65]. Various emulsifiers, such as small molecular weight surfactants, phospholipids and biopolymers, could all be used for food application. Compared with biopolymers, small molecular surfactants are usually more effective in fabricating fine emulsions since they can be adsorbed to the newly formed droplet surface fast and prevent droplets from re-coalescence. Small molecular surfactants can be used in both low- and high-energy emulsification procedures while biopolymers can only be used in high-energy emulsification systems [46].

Hydrophilic–lipophilic balance (HLB) is a concept for categorizing emulsifiers. Usually, surfactants with a high HLB (8–18) could be used for forming O/W emulsions while low HLB surfactants (3-8) could be used for W/O emulsion formation (**Figure 2.7**) [66].

W/O emulsions
Low HLB (3~8)

O/W emulsions
High HLB (8~18)

Figure 2.7 Emulsions formed by emulsifiers with different HLB value

Emulsifier could also be categorized by their electric properties: cationic surfactants (lauric arginate), anionic surfactants (rhamnolipid) and non-ionic surfactants (sorbitan fatty acid esters (spans), polyoxyethylene sorbitan fatty acid esters (tweens), sugar esters and monoglycerides) [67-69].

Table 2.1 Emulsifiers in food products [58, 70]

Food Products	Emulsifiers	Functionality
Peanut butter	Mono/diglycerides	Dispersion stabilization
Chocolate	Lecithin	Viscosity modification
Citrus beverages	Polyglycerol esters, SAIB	Clouding (weighting)
Sponge cakes, desserts	Mono/diglycerides	Aeration
Ice cream, whipped toppings	Polysorbate 80, polyglycerol esters	Controlled fat agglomeration

The surface load (Γ) of a surfactant can be calculated from the interfacial tension (γ) versus surfactant concentration (c) data using the following formula [16]:

$$\Gamma = -\left(\frac{1}{RT}\right)\frac{d\gamma}{d\ln c} \quad (1)$$

Here R is the gas constant, T is the absolute temperature, and c is expressed in moles per liter [71].

The surface activity (K) of an emulsifier can be conveniently characterized by the following relationship [26]:

$$K = \frac{1}{c_{1/2}} = \exp\left(-\frac{\Delta G_{ads}}{RT}\right) \quad (2)$$

Here $c_{½}$ is the surfactant concentration where half of the available adsorption sites on the oil-water interface are covered by surfactant, and ΔG_{ads} is the free energy change associated with surfactant adsorption.

2.3.1 Synthetic Emulsifiers

Most of synthetic emulsifiers are petroleum/hydrocarbon derivatives and many of

them have been used in the food industry for a long time without any evidence of harmful effects. Synthetic emulsifiers were designed to contain naturally or non-naturally occurring molecules which prevented them from being metabolized their passage through the human bodies GI tract [70].

- *Mono- and Diacylglycerols:* Mono- and diacylglycerols are the most common used synthetic emulsifiers in the food industry (about 70 % of total emulsifier used in the food industry) and they were first synthesized in 1853 by Berthelot [70]. There are small quantities of mono- and diacylglycerols in natural fats and oils because of the hydrolysis reaction. The use of mono- and diacylglycerols in the 1930s in the margarine industry on a large scale was a great breakthrough in the application history of mono- and diacylglycerols [2]. High ratio shortenings containing mono- and diglycerides made oil droplets dispersed into the dough better, resulting in the increase of the strength in cake batters. Moreover, the application of mono- and diglycerides in ice cream was then patented in 1936.
- *Sorbitan esters and polysorbates:* Sorbitan esters and polysorbates (**Figure 2.8**) are both non-ionic emulsifiers. Sorbitan esters are generated from the simultaneous esterification and cyclization reactions that are caused by heating a mixture of fatty acids, sorbitol and a catalyst under an inert atmosphere (**Figure 2.9)**. Sorbitol, which is initial material for sorbitan esters, was generated from the hydrogenation of sucrose. These days, there is an alternative way for producing sorbitol from D-glucose.

D-glucose, which is got from corn syrups (maize) or tapioca, reacts with hydrogen gas under pressure in the presence of a catalyst (*e.g.*, nickel phosphate) [72].

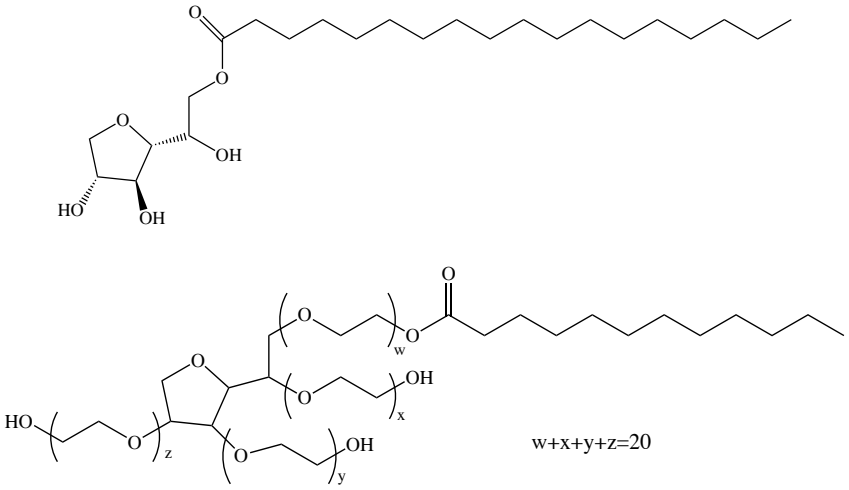


Figure 2.8 Structures of sorbitan monostearate (Span 60) and Polyoxyethylene (20) sorbitan monolaurate (Tween 20)

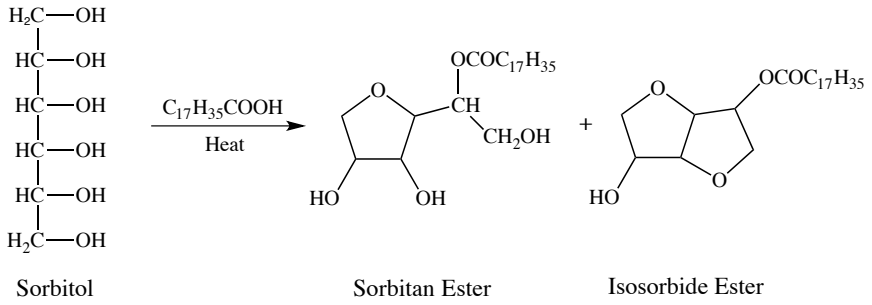


Figure 2.9 Cyclization and esterification of sorbitol

2.3.2 Natural Emulsifiers

For more than 20 years, the food industry has a growing interest in replacing synthetic ingredients with natural "label friendly" alternatives such as lecithin, protein and polysaccharide in an effort to produce natural food products [7, 13]. Consequently, there have been many studies that focus on identifying and characterizing natural

emulsifiers that can be successfully used in emulsion-based food and beverage products. Natural emulsifiers are obtained from various nuts, berries and leaves.

- *Protein:* Protein is one of the most frequently used emulsifier in the food industry. Proteins have been considered to be a kind of good emulsifiers because of their ability to absorb fast enough to the interface between the oil droplets and the aqueous phase to lowering the interfacial tension [26]. The protein film around the lipid droplets is highly viscoelastic preventing the rupture or displacement by other surface active component [73]. Depending on the protein molecular structure and the ionic content of the aqueous medium, the stabilisation of dispersed particles and droplets by adsorbed protein can be explained in terms of a combination of polymeric (steric) and electrostatic interactions [24, 74]. Emulsions stabilized by proteins, however, are often unstable to aggregation at pH values around their isoelectric point, at high ionic strengths, and at elevated temperatures [12]. Adsorbed proteins lose their net charge around the isoelectric point thereby promoting extensive droplet aggregation. Conformation changes of proteins occur above their thermal denaturation temperature (T_m) and after interfacial adsorption, which exposes non-polar and sulfhydryl groups [75].
- *Polysaccharides*: Some polysaccharides such as gum arabic and modified starch adsorb at oil–water interfaces. Hence they have the capacity to function as emulsifiers and emulsion stabilizers by means of interfacial action. Polysaccharides

may increase emulsion stability to aggregation and environmental stresses by the formation of a thick interfacial layer, increasing the steric repulsion and decreasing the van der Waals attraction [28]. However, it is often difficult to produce oil droplets with very small sizes (d < 200 nm) using polysaccharide-based emulsifiers [9, 10, 13]. In addition, due to the low efficiency of polysaccharides for lowering surface tension, emulsions stabilized by polysaccharides, require a considerable higher amount of polysaccharide, which will increase the manufacturing cost.

- *Lecithin:* Certain types of lecithin are also suitable for utilization as natural emulsifiers in food and beverage products, e.g., phospholipids derived from egg, soy, or milk [67, 76]. However, natural lecithin (which has two non-polar tails) usually has to be chemically or enzymatically modified to lysolecithin (which has one non-polar tail) before it can be successfully used to stabilize oil-in-water emulsions.
- *Q-Naturale*®: Q-Naturale® is a food ingredients just marketed by the National Starch company (Bridgewater, NJ) and it is an extract isolated from the bark of the *Quillaja saponaria* Molina tree (an evergreen native to Chile and Peru) and it has surface activities of forming surfactant micelles and stabilizing oil-in-water emulsions [14-16]. The major components within this extract have been reported to be saponins [17, 18], which are high molecular weight glycosides consisting of a sugar moiety attached to a triterpene or a steroid aglycone [19]. The saponins are surface active substances because they contain both hydrophilic regions (such as rhamnose, xylose, arabinose,

galactose, fucose, and glucuronic acid) and hydrophobic regions (such as quillaic acid and gypsogenic acid) on the same molecule [16, 77].

2.4 Vitamin E as Lipophilic Bioactive Food Components

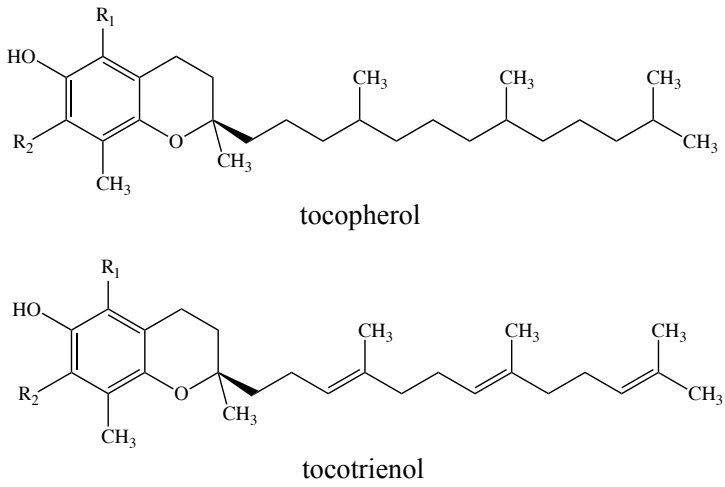
The term "Vitamin E", which was first characterised in wheat-germ oil and lettuce, introduced in 1922 by Evans and Bishop described a dietary factor in animal nutrition considered at the time to be especially important for normal reproduction [78]. "Vitamin E" refers to a group of fat-soluble vitamins that have been widely used as functional ingredients in food, pharmaceutical, and cosmetic industries [21]. Vitamin E is a highly lipophilic molecule that cannot be directly dispersed into aqueous solutions [22]. Thus, it must be incorporated into an appropriate colloidal delivery system prior to its dispersion into the aqueous-based food products [23]. A number of previous studies have shown that vitamin E can be successfully incorporated into emulsion-based delivery systems, such as microemulsions [21, 79], nanoemulsions [80-82], emulsions [23, 83] and liposomes . One major problem of vitamin E is that it is only partially absorbed at the intestinal site, which reduces its bioavailability. Former studies have shown that the absorption of fat-soluble vitamins, including vitamin E, will be increased with the presence of surfactants or emulsions, compared to when they are incorporated into bulk oils [84-86].

2.4.1 Structure and Chemical Properties of vitamin E

Natural vitamin E encompasses eight different forms, the α-, β-, γ- and

δ-tocopherols and the α-, β-, γ- and δ-tocotrienols (**Figure 2.10**). Eight different vitamin E molecule forms have a common structural feature: a chromanol ring and a phytol side chain. α-tocopherol is the only one active form of vitamin E that could be found in human bodies so that it is also vitamin E with the largest quantities found in blood and tissues [25].

α-tocopherol molecules can help protecting membrane lipids against the lipid oxidation since α-tocopherol can trap peroxyl radicals which are responsible of the initiation of lipid oxidation [87, 88]. The principle oxidation metabolites of α-tocopherol are α-tocopherol quinone, α-tocopherol hydroquinone, 2,3-epoxy-α-tocopherol quinone, and 5,6-epoxy-α-tocopherol quinone (**Figure 2.11**). α-tocopherol molecules could also help stabilizing membranes mechanically due to Van der Waals interactions [69]. Vitamin E quinone has the activities of preventing blood clotting since it can help inhibiting the activities of vitamin K dependent carboxylase, which regulates blood clotting [71]. The digestion procedure of vitamin E is similar to vitamin A and carotenoids digestion and α-tocopherol can utilize SR-B1 to enter into enterocytes [72].



R_1	R_2	
CH_3	CH_3	α
CH_3	H	β
H	CH_3	γ
H	H	δ

Figure 2.10 Chemical structure of vitamin E

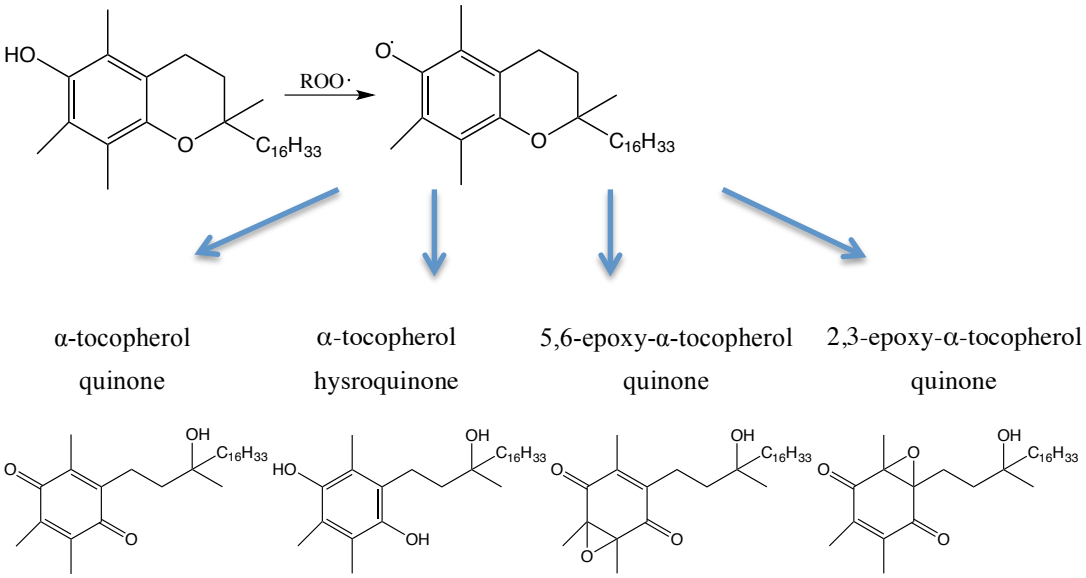


Figure 2.11 α-Tocopherol and its principle oxidation products [89]

2.4.2 Food Source and Intake

Vegetable oils such as olive oil, sunflower oil, safflower oil and nuts are the main natural source of tocopherol (Toc) while tocotrienol, a minor plant constituent, is abundant especially in rice bran, cereals, grains, palm oil, green vegetables and annatto seeds [90]. α-, β-, γ-, and δ-tocopherols and tocotrienols can all be found in natural

foods but the amounts of each kinds of vitamin E are quite different.

Table 2.2 Distribution of tocotrienol and tocopherol in food sources (mg/kg) (http://www.ars.usda.gov/ba/bhnrc/ndl)

Food	α-tocopherol (mg)	ϒ-tocopherol (mg)
Olive oil (1 tablespoon)	1.9	0.1
Soybean oil (1 tablespoon)	1.1	8.7
Corn oil (1 tablespoon)	1.9	8.2
Canola oil (1 tablespoon)	2.4	3.8
Safflower oil (1 tablespoon)	4.6	0.1
Sunflower oil (1 tablespoon)	5.6	0.7
Almonds (1 ounce)	7.4	0.2
Hazelnuts (1 ounce)	4.3	0
Peanuts (1 ounce)	2.4	2.4
Spinach (1/2 cup, raw)	0.3	0
Carrots (1/2 cup, raw chopped)	0.4	0
Avocado (1 fruit)	2.7	0.4

Vitamin E uptake into plasma is similar to fat uptake through the small intestine. Bile acid secreted into the small intestinal facilitate the formation of micelles containing vitamin E and oil hydrolysis products, which passed through the membrane of intestinal cells and were then transported as lipoproteins (*e.g.*, chylomicrons) in the lymph and blood system [91, 92]. The RDA of vitamin E is 15 mg/day and deficiency might occur in case of fat malabsorption or for premature infants.

Table 2.3 The Recommended Dietary Allowance (RDA) for α-Tocopherol [20]

Age	Males (mg/day)	Females (mg/day)
0-6 months	4 mg	4 mg
7-12 months	5 mg	5 mg
1-3 years	6 mg	6 mg

4-8 years	7 mg	7 mg
9-13 years	11 mg	11 mg
14-18 years	15 mg	15 mg
19 years and older	15 mg	15 mg
Pregnancy (all ages)	-	15 mg
Breast-feeding (all ages)	-	19 mg

2.4.3 Health Benefits of vitamin E

Previous researches show that tocopherols have quite a lot health benefits for human beings, such as reducing heart disease, delaying Alzheimer's disease, and preventing cancer [93]. Compared with polyunsaturated fatty acids, vitamin E works 1000 times faster in quenching peroxyl radicals ($ROO\cdot$) [94]. As vitamin E may functions in scavenging peroxyl radicals, it may works as an antioxidant which slows down the propagation of free radicals in membranes and in plasma lipoproteins and breaks the chain of lipid oxidation [95]. Severe vitamin E deficiency may lead to neuromuscular abnormalities characterized by spin cerebellar ataxia and myopathies in human beings [96-102].

2.4.3.1 Inflammatory

Inflammation was related with the release of some reactive oxygen species, such as $O_2^{-\bullet}$, $OH^{\cdot}$, H_2O_2 and singlet oxygen ($^1O_2$). Previous researches indicated that the symptoms in patients who were suffering from several types of acute or chronic inflammatory conditions could be relieved with the consumption of vitamin E [103]. In many occasions vitamin E works effectively as standard drug treatment but the

side-effects were much lower [104].

O^{2-} generation will be inhibited as a result of the addition of α-tocopherol to polymorphonuclear cells [105-107]. Vitamin E supplements have significant effects on inhibiting the release of reactive oxygen species and the section of IL-1 beta from monocytes and preventing the monocyte-endothelial cell adhesion. The inhibition of protein kinase C activities, which are caused by α-tocopherol, will result in the inhibition of the release of reactive oxygen species and of lipid oxidation [105].

2.4.3.2 Cardiovascular Diseases (CVD)

The increase of vitamin E consumption in daily diet has been demonstrated to help reducing the risk of death from heart disease in both males and females. Vitamin E shows several potentially health benefits for cardiovascular diseases, such as preventing the oxidation of low-density lipoproteins (LDL) and inhibiting the proliferation of smooth muscle cell [108]. During the i*n-vitro* studies, vitamin E were found to inhibit the oxidation of low-density lipoprotein (LDL) cholesterol, which is the main reason for causing atherosclerosis since its atherogenicity will be enhanced due to the oxidative damage to LDLs [20].

Previous researches have been done to study vitamin E uptake in presumably healthy people and these people were then studied for several years to find out how many of them were diagnosed with or died as a result of heart disease. People who had more than 7 mg/day of α-tocopherol from daily diet were less risky (~35 %) to die from heart

disease compared with those people who got less than 3-5 mg/day of α-tocopherol [109, 110]. Moreover, based on another two large studies, a significant reduce in the risk of heart disease will happen in women and men who consumed at least 100 IU of supplemental *RRR*-α-tocopherol (67 mg of *RRR*-α-tocopherol) daily [111, 112]. According to a study bases on approximately 90,000 nurses, the highest intakes of vitamin E primarily from supplements would prevent heart disease by 30% to 40% [112].

2.4.3.3 Immune Function

Adequate vitamin E has been proved to be essential for the maintenance of the immune system. Vitamin E consumption with higher than recommended dietary levels will result in stronger immunity response of human bodies while the deficiency of vitamin E will lead to weaker immunity response [113]. For elderly adults who are given 200 mg of synthetic α-tocopherol (equivalent to 100 mg of *RRR*-α-tocopherol or 150 IU of *RRR*-tocopherol) per day for several months, an increased formation of antibodies in response to hepatitis B vaccine and tetanus vaccine injection could be observed [114].

2.5 Bioavailability of Vitamin E Encapsulated in the designed delivery system

96% of American women and 93% of men do not meet the current vitamin E recommended dietary allowance (RDA), which is 15 mg (22 IU RRR-) α-tocopherol per day [20, 115]; the average intake of α-tocopherol in the US are only s 6.9 mg/day [116]. Because of vitamin E's deficiencies of poor solubility either in water or in most of

inorganic solvents and poor stability to O_2, light or temperature, vitamin E is treated as capsules with the disadvantages of low bioavailability and being hardly dispersed in aqueous environemnt. Conversion of the capsules to water-soluble liquid is the ideal ways to overcome the above problems [79]. Vitamin E absorption is greatly influenced by the digestion processes and the absorption of diet fat. The procedure of the absorption of vitamin E through the small intestine is quite similar to fat uptake. Bile salts secreted by the intestinal lumen could induce the formation of micelles to incorporate vitamin E and the micelles could pass through the small intestinal cell membranes and then be transported as lipoproteins in the lymph and blood [91, 95].

The isomer *RRR*-α-tocopherol is the form of α-tocopherol exsiting in all natural food. However, although vitamin E supplements usually contain 100 IU to 1,000 IU of α-tocopherol, not all the α-tocopherol are isomer *RRR*-α-tocopherol (*d*-alpha-tocopherol), Which is the most favorable α-tocopherol for human beings. Actually the most common one in fortified foods and supplements is synthetic α-tocopherol (*all-rac*-α-tocopherol or *dl*-α-tocopherol) containing all eight isomers of α-tocopherol. Compared with *RRR*-α-tocopherol, synthetic α-tocopherol is less bioavailable since part of α-tocopherol isomers present in *all-rac*-α-tocopherol cannot by utilized by human bodies.

Previous researches have proved that O/W emulsion which encapsualtes vitamin E inside could help increasing the bioavailability of vitamin E in human bodies. The bioavailability of the guest molecules incorporated in the emulsions will be greatly

improved expecially as the surfactants stablizing the droplets could enhance transmembrane passage across the digestive tract and transdermal drug permeability [117].

During the digestion, fat-soluble nutrients including α-tocopherol need to be packaged into micelles to get to be absorbed. The *in vitro* model mimics the digestion precedure in human bodies and can be used to quantify the fraction of a nutrient that has been micellarised and thereby made bioaccessible [118]. According to previous *in vitro* digestion study, the bioaccessibility of α-tocopherol will greatly depend on the food source. The bioaccessibility of α-tocopherol from bananas and bread was 100 % whereas the bioaccessible α-tocopherol were only 29% and 22% from cheese and milk respectively [119].

Vitamin E is a kind of fat soluble vitamin transported by lipoproteins so that tissue vitamin E transportation will be greatly impacted by lipid and lipoprotein metabolism and its tissue depletion takes decades rather than weeks [92]. The concentration of α-tocopherol in plasma are regulated by the liver (the α-tocopherol transfer protein (α-TTP)) and also by vitamin E metabolism and excretion [92].

The absorption and disposition of labeled vitamin E were studied by using short-term studies (from 3 to 72 h). Longer protocols were used for evaluing the delivery and transportation of labeled vitamin E to peripheral tissues by administering large oral doses (*e.g.*, 400 IU) [120]. Although various tocopherols are absorbed and

transported in chylomicrons, α-tocopherol is the only form of vitmain E that will be maintained in plasma and tissues [121, 122]. The plasma half-life of RRR-α-tocopherol's plasma half-life (48 to 60 h) is much longer than systhetic α-tocopherol (15 h) [123, 124]. Due to the rapid recirculation between the liver and the plasma, the entire circulating α-tocopherol pool nearly gets to be replaced everyday[124]. The fraction of vitamin E absorbed were found to be around 55 to 79% according to the study taken from 1960-1970 by utilizing radioactive α-tocopherol during the study [125, 126]. These days, there are some more accurate studies in which the results were got from the observed plasma-labeled α-tocopherol concentrations and absorption ratio of α-tocopherol in humans was only 33 % [127]. These studies are very important for suggesting people's daily uptake of vitamin E since only part of the vitmain E consumed would be absorbed.

The fraction of the ingested food ingredients that reaches the systemic circulation could be defined as *bioavailability* (*F*), which can be got by: $F = F_B \times F_T \times F_M$. In this equation, F_B - the bioaccessibility coefficient or the lipid fraction released from the food matrix into the the GI tract juices; F_T - the transport coefficient or the released lipid component fraction which is transported across the intestinal epithelium; F_M – the lipid component fraction reaching the systemic circulation without experiencing metabolization [128].

As the food components reach the systemic circulation, they will be distributed

into different tissues, where they may be stored, utilized or excreted [129, 130]. Nowadays the *in-vitro* studies are still good ways for examining the relative bioavailability of α-tocopherol from various food sources and also for fast screening the effectiveness of different α-tocopherol delivery systems as it is relatively difficult in assessing the *in-vivo* bioavailability of α-tocopherol.

2.5.1 *In-vitro* digestion model

Emulsions will experience diffenent conditions after ingestion, such as dilution by mixing with mucin in the mouth, expose to acidic environment in the stomach *etc.* *In-vitro* digestion models have been widely applied for studying the bioaccessibility of encapsulated lipophilic nutraceuticals. The ideal *in-vitro* digestion method should provide accurate results in a short time and it should act as a useful alternative to animal and human models. In this way, it should work as a rapid screening tool for foods or delivery systems with different compositions and structures (**Figure 2.12**) [131].

- ***Mouth:*** Mouth is the first environment that an emulsion encounters after ingestion. After consumption, emulsions will experience a series of processing, such as mixing with saliva, temperature change to body temperature, changes in ionic strength and pH, contacting with biological surfaces (tongue, teeth, and cheeks) and breaking down during mastication [132, 133]. Emulsions will react with digestive enzymes in mouth (e.g. lingual lipase, amylase and protease). As food first passes through the mouth, saliva and mucin would induce emulsion flocculation and then a rapid

depletion flocculation was observed in all kinds of emulsions.

- ***Stomach****:* Food product passes from the mouth through to the esophagus and then enters into the stomach. The time that food product stays in stomach mainly depends the nature of the food, such as composition, size, pH and ionic properites *etc.* [134, 135]. Gastric fluids containing enzymes (pepsin and gastric lipase), minerals (Na^+, Ca^{2+} *etc.*) and various surface-active components are going to mix with food products and food products will undergo a very acidic condition (pH ≈ 1–3) [136-138]. Meanwhile, food products will also experience mechanical agitation because of the stomach motility [139, 140]. The pH value in human stomach was between 1 and 3 under fasting conditions and an apparent increase in stomach content pH value will be observed after food ingestion and then the pH value will gradually decrease over the next hour or so to reach around pH 2 [137, 138, 141]. The natural properties (initial pH, composition, structures *etc.*) of food ingested are the key factor that influence the pH-time profile [141]. The high acidic environment in the stomach enables enzymes activation, food components hydrolysis, microorganisms inactivation. The ionic strength in stomach will also significantly increase after consuming food because of the ions from the food. Furthermore, gastric lipase could also hydrolyze some emulsified triacylglycerols (TAGs) to diacylglycerols (DAGs), monoacylglycerols (MAGs), and free fatty acids (FFAs) [142-144]. Usually when 10–30% of the fatty acids are released from the TAGs, lipid hydrolysis

will stop.

- ***Small Intestine**:* Food emulsions will get into the small intestine after passing through the stomach. The secretion of bile salts, phospholipid, cholesterol ester and pancreatic fluids will be motivated by the entering of lipids into the small intestine. Pancreatic lipase complexing with co-lipase acts at the surface of the emulsified TG droplets to hydrolyze TAG to MAG (sn-2 position) plus FFA [128, 145]. Pancreatic lipase/co-lipase, called carboxylic-ester hydrolase or bile salt stimulated lipase along with pancreatic lipase, is able to split TAG molecule [146]. During the digestion procedure in small intestine, natural surface-active components such as bile salts impact the digestion and absorption of lipids significantly and bile salts will facilitate the formation of micelles containing MAG, FFA and phospholipids and their transport by passive diffusion across the intestinal mucosa [147] (**Figure 2.13**). These days, the pH-stat method has been widely used in pharmaceutical and food area for mimicking the small intestinal condition and *in vitro* digestion study [30,31,37,38]. The principle of pH-stat is to record the amount of alkali solution (0.25 M NaOH) that had to be added to the reaction chamber to maintain the pH at 7.0 and then determine the percentage of free fatty acids (FFA) released from lipids, usually triacylglycerols [148].

Figure 2.12 Overview of physicochemical processes that occur when a bioactive component trapped within an emulsified carrier lipid is exposed to the small intestine [149]

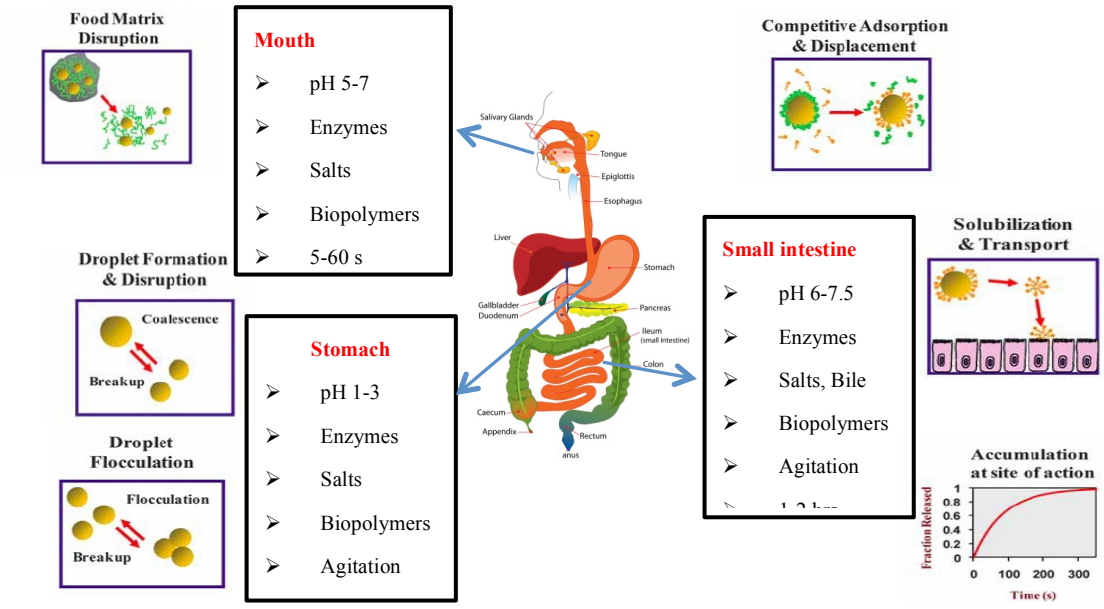


Figure 2.13 Schematic representations of the possible changes of emulsions as they pass through the GI tract and the physicochemical conditions in digestive tract [128]

2.5.2 Cell Culture Study of Bioaccessibility of Vitamin E

Bioavailability, which is defined as the fraction of an ingested nutrient available for physiologic use or storage in the body, may be estimated by using an *in-vitro* model

to apply the micelles got from an *in-vitro* digestion to a Caco-2 intestinal cell culture (**Figure 2.14**) [150]. These days cell culture models have also been commonly utilized as part of *in-vitro* digestion models. In particular, the Caco-2 cell culture model has been widely used as a predictive tool for the absorption of bioactive components from foods and nutrient supplements.

Nowadays the major steps and molecular mediators of vitamin E transportation from the luminal micelle phase into the enterocyte have not been well defined yet. Vitamin E uptake is rapid, saturable and temperature dependent in Caco-2 cells [151]. Intestinal cellular uptake of vitamin E from mixed micelles has been traditionally recognized as a simple process of passive diffusion but the recent researches indicated the importance of the scavenger receptor class B type I (SR-BI) in the cellular uptake of vitamin E [152]. α-tocopherol is absorbed by the intestinal absorptive cells *via* diffusion or protein-mediated mechanisms [153]. Vitamin E will be temperately stored in the microsome fraction before being secreted by chylomicrons or HDL after an ATP binding cassette A1-mediated efflux [151]. The same route as α-tocopherol may probably also be used for the transportation of α-tocopheryl acetate in human bodies if it is not converted into the latter compound by the intestinal or pancreatic esterase(s) [154-157].

The *in-vitro* digestion/Caco-2 cell culture model provides a rapid, low-cost method for fast screening foods and food matrix for studying the bioavailability of bioactive components before more definitive human trials [158, 159]. In the present

study, we have employed an *in-vitro* digestion procedure coupled with a Caco-2 cell model system to compare the micellarisation, cell uptake and secretion of α-tocopherol from supplemented minced meat (pork, beef and turkey) with other supplemented food products (apple sauce, bread and mayonnaise). The Caco-2 transwell model is a well-established method for the assessment of nutrient bioavailability from a range of foodstuffs [160-162].

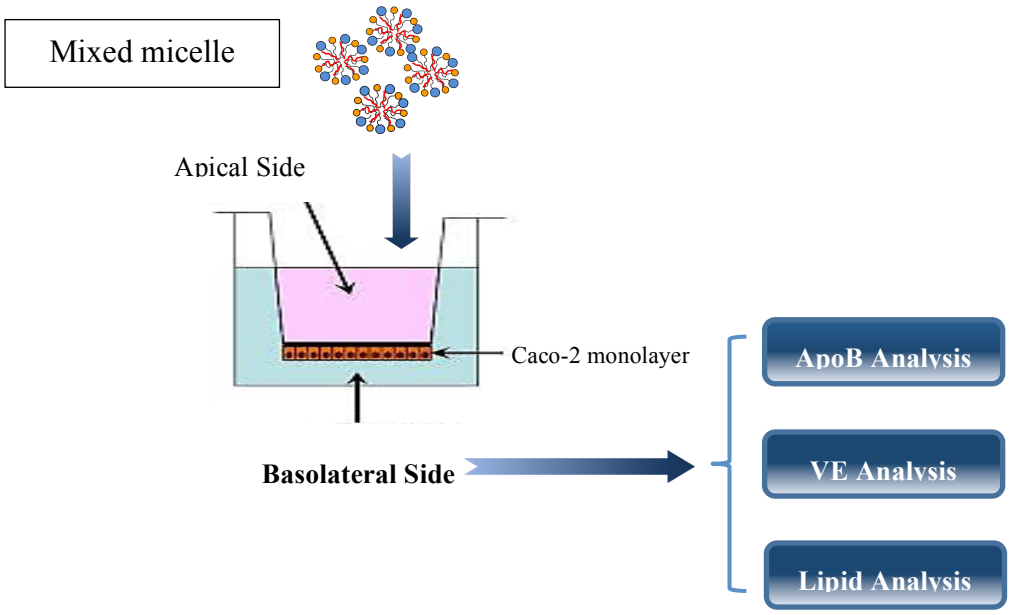


Figure 2.14 Schematics for the Caco-2 cell monolayer model for absorption and transport of vitamin E across the intestinal epithelial.

CHAPTER 3

FABRICATION OF ULTRA-FINE EDIBLE EMULSIONS: COMPARISON OF HIGH-ENERGY AND LOW-ENERGY HOMOGENIZATION METHODS

3.1 Introduction

There is considerable interest within the food and beverage industries in the utilization of ultrafine emulsions (also referred to as nanoemulsions) to encapsulate and deliver lipophilic functional agents, such as flavors, colors, antimicrobials, micronutrients, and nutraceuticals [3, 22, 27, 163, 164]. Ultrafine emulsions can be fabricated from food-grade ingredients using relatively simple processing operations, such as mixing, shearing, and homogenization [29, 165]. Ultrafine emulsions are thermodynamically unstable systems that typically consist of surfactant, oil, and water [34, 39, 166]. Oil-in-water emulsions contain surfactant-coated lipid droplets dispersed within an aqueous continuous phase. The radius of the droplets in ultrafine emulsions (r < 100 nm) is relatively small compared to the wavelength of light (λ = 390 to 750 nm) and so they tend to be either transparent or only slightly turbid [163, 167]. One of the most promising potential applications of ultrafine emulsions is therefore to incorporate lipophilic active ingredients (*e.g.,* vitamins, nutraceuticals, and antimicrobials) into aqueous-based foods or beverages that need to remain optically transparent, such as some fortified waters, soft drinks, sauces, and dips [163]. Another potential advantage of these systems is that they often have better stability to particle aggregation and

gravitational separation than conventional emulsions due to their small particle size [39]. In particular, Brownian motion effects become important for small particles ($r < 100$ nm), which counterbalances the tendency for gravitational separation to occur [29]. The fact that the interfacial layer of ultrafine droplets makes up an appreciable part of the total particle volume means that ultrafine emulsions can be produced that are much more viscous than conventional emulsions with the same oil content [34, 39, 166], which may have interesting applications in food products where a paste or gel is required. The small size of the droplets in ultrafine emulsions has also been shown to increase the bioavailability of certain types of lipophilic substances [168], which may be useful for increasing the bioactivity of some nutraceuticals.

In the food industry, ultrafine emulsions are usually produced using high-energy methods, such as microfluidization, high pressure valve homogenization, and sonication [31-35]. These methods generate intense disruptive forces that mechanically breakup the oil phase into tiny droplets that are dispersed within the aqueous phase [36]. There are a number of limitations in using high-energy methods to produce ultrafine emulsions, such as high initial equipment and operating costs, high power requirements, potential for equipment breakdown, and difficulties in producing very fine droplets from certain kinds of food ingredients (*e.g.,* highly viscous oils or slowly-adsorbing emulsifiers) [29].

Ultrafine emulsions can also be produced using a variety of low-energy methods, such as phase inversion and spontaneous emulsification methods [29, 169, 170]. These

low-energy methods are based on the spontaneous formation of fine oil droplets in surfactant-oil-water (SOW) systems under specific environmental conditions (composition, temperature, stirring), which are then trapped in a metastable state [57, 171]. Low-energy approaches may have advantages over high-energy approaches for certain applications within the food and beverage industries: they are often more effective at producing very fine droplets; they have lower equipment and energy costs; they are simpler to implement. On the other hand, there are also some potential disadvantages of low-energy systems, including limitations on the types of oils and surfactants that can be used to form stable ultrafine emulsions, and the fact that relatively high surfactant-to-oil ratios (SOR) are typically needed to produce them.

The purpose of this study was to compare the effectiveness of a high-energy method (microfluidization) that is widely used in the food industry to produce ultrafine emulsions with a low-energy method (spontaneous emulsification) that has been shown to work for many non-food applications. The microfluidizer works on the principle of dividing an emulsion flowing through a channel into two streams, passing each stream through a separate fine channel, and then directing the two streams at each other in an interaction chamber. Intense disruptive forces are generated within the interaction chamber when the two fast moving streams of emulsion impinge upon each other, leading to highly efficient droplet disruption. A number of previous studies have shown that microfluidization is particularly efficient at creating ultrafine emulsions from food grade

ingredients, and have identified some of the major factors that influence homogenization efficiency, including oil-water interfacial tension, oil-water viscosity ratio, and emulsifier type and concentration [8, 33, 40, 41]. In the spontaneous emulsification method an emulsion is immediately formed when two liquids are mixed together (**Figure 3.1**) [50-52]. Practically, this method can be carried out in a number of different ways to form ultrafine emulsions: the compositions of the two phases can be varied; the environmental conditions can be varied (*e.g.,* temperature, pH and ionic strength); and/or, the mixing conditions can be varied (*e.g.,* stirring speed, rate of addition, and order of addition) [53]. The spontaneous emulsification method has been widely used to produce ultrafine emulsions in the pharmaceutical industry, where they are usually referred to as self-nano-emulsifying drug delivery systems (SNEDDS) [48, 49].

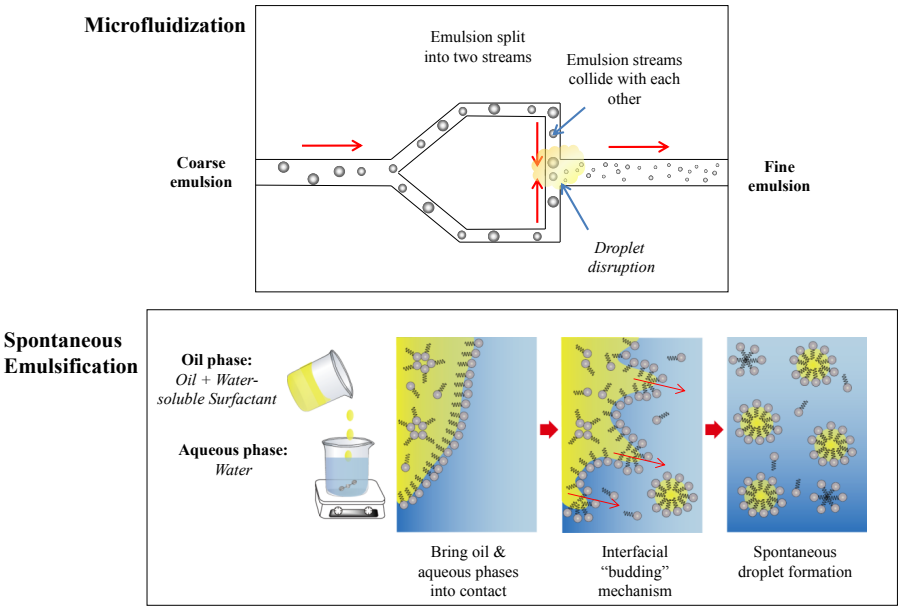


Figure 3.1 Underlying principles for droplet formation in high-energy (microfluidization) and low-energy (spontaneous emulsification) methods of producing emulsions.

In this study, we compared the efficacy of the microfluidization and spontaneous emulsification methods at producing ultrafine emulsions from food grade oils (MCT) and surfactants (Tweens), and we then tested the stability of the resulting emulsions to environmental stresses that they might encounter in the food industry. This research may be useful for identifying the most appropriate homogenization method for different food and beverage applications.

3.2 Materials and methods

3.2.1 Materials

Non-ionic surfactants, POE(20) sorbitan trioleate (Tween 85) and POE(20) sorbitan monoolieate (Tween 80), were purchased from Sigma-Aldrich Co. (St. Louis, MO). Medium chain triglyceride (MCT) oil (Miglyol 812) was purchased from Coletica (Northport, NY).

3.2.2 Emulsion preparation

3.2.2.1 Emulsions produced by microfluidization

Oil, water and surfactant were mixed together in a beaker, and then blended together using a high-shear mixer (Bamix, Biospec Products, Bartlesville, OK) for two minutes to form a coarse emulsion. The total weight of each sample was 100 g, and the overall composition of the samples was 20 wt% oil (MCT), 0.1 to 20 wt% surfactant

(Tween 80 and/or Tween 85), and 79.9 to 60 wt% water (pH 3.5, buffer solution). The emulsion premix samples were then passed through a microfluidizer (Microfluidics, Newton, MA) three times at 10,000 psi (68.95 MPa) at ambient temperature.

3.2.2.2 Emulsions produced by spontaneous emulsification

Initially, oil and surfactant were placed in a container and then blended using a high shear mixer for two minutes to prepare a homogeneous solution. The oil-surfactant mixture was then poured into a water phase (pH 3.5 buffer solution) at a rate of about 10 g per minute with continuous stirring (500 rpm) using a magnetic stirrer for 15 minutes to keep the system homogeneous. At this pouring rate, the formation of an optically opaque emulsion was observed to occur as soon as the oil-surfactant mixture came into contact with the water phase. However, it was noticed that if the oil-surfactant mixture was poured into the water phase too quickly (> 20 g per minute) then large clots were formed that were difficult to disperse, and therefore we always used slower rates.

3.2.3 Emulsion characterization

The particle size distributions of the samples were measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Malvern, U.K.). All measurements were performed on at least two freshly prepared samples (*i.e.,* new samples were prepared for each series of experiments) and were reported as means and standard deviations.

3.2.4 Surfactant – Oil – Water (SOW) Phase behavior

The phase behavior of surfactant, oil, and water mixtures was determined using a dilution experiment: increasing amounts of water were added to a surfactant-oil mixture. Initially, MCT was mixed with surfactant (Tween 80, Tween 85, or 1:1 Tween 80/Tween 85) at a mass ratio of 1 part oil to 1 part surfactant. A series of samples with different water contents were then prepared by adding increasing amounts of double distilled water drop-wise into the surfactant-oil mixtures (25 ± 1 °C). The resulting SOW systems were then mixed at a low speed using a magnetic stirrer to ensure the different components were thoroughly mixed. After equilibration, the appearance of the systems was observed visually and using optical microscopy (Nikon Eclipse 80i, Nikon Instrument Inc., Melville, NY).

3.3 Results and discussion

3.3.1 Comparison of emulsions formed by high- and low-energy methods

Initially, we examined the influence of homogenization method and system composition on the formation of oil-in-water emulsions. Our aim was to establish the influence of these parameters on the size of the droplets produced using the microfluidization and spontaneous emulsification methods, as well as to establish the minimum amount of surfactant needed to produce emulsions containing small droplets (r < 100 nm).

Microfluidizaiton: For the microfluidization method, there was a steep decrease in

mean particle radius of the 20 wt% MCT oil-in-water emulsions when the surfactant concentration was increased from 0 to 2 wt% (SOW from 0 to 0.2), after which the particle size reached a relatively constant level (**Figure 3.2**). The minimum particle radius attained at high surfactant levels was 55 ± 2 nm for all three systems (averaged over the data from 5 to 20 wt% surfactant). The dependence of particle size on surfactant concentration can be conveniently divided into two distinct regimes for high pressure homogenization [172, 173]: the "surfactant-limited zone" and the "surfactant-rich zone". At low surfactant concentrations, there was insufficient surfactant present to cover the new surfaces created within the homogenizer when larger droplets were broken down to smaller ones (**Figure 3.1**). Consequently, two or more small oil droplets that collided with each other would tend to coalesce because there was insufficient surfactant present at their surfaces to prevent them from coming into close contact. Coalescence tends to continue until all of the droplets are covered by sufficient surfactant to prevent them from merging together. Hence, the droplet size in the surfactant-limited zone is primarily determined by the type and amount of surfactant present. The minimum mean droplet radius (r_{32}) that is obtained in this region is given by the following equation [174]:

$$r_{\min} = \frac{3 \cdot \Gamma \cdot \phi}{c_S} = \frac{3 \cdot \Gamma \cdot \phi}{c'_S (1 - \phi)} \qquad (1)$$

Here, Γ is the surface load of the surfactant (in kg m^{-2}), ϕ is the disperse phase

volume fraction, c_S is the total surfactant concentration in the emulsion (in kg m^{-3}) and c'_S is the total surfactant concentration in the continuous phase (in kg m^{-3}). This equation assumes that all of the surfactant adsorbs to the droplet surfaces, and that the droplets do not grow after formation *e.g.,* due to Ostwald ripening or coalescence. In our study, the emulsions contained 20 wt% oil droplets ($\phi \sim 0.20$) and 0.1 to 20 wt% surfactant in the aqueous phase ($c'_S \sim 1$ to 200 kg m^{-3}). If we assume that all of the surfactant adsorbed to the oil-water interface, then we can use the measured values of r_{min} (55 nm) to predict the surface load of the surfactants. The value of c'_S was taken to be the minimum surfactant concentration required to reach the smallest droplet radius (*i.e.,* the value at the end of the surfactant-limited zone), which was approximately 2 wt% (20 kg m^{-3}). Consequently, the value of the surface load calculated for the surfactants using Equation 1 was: $\Gamma = 1.5$ mg m^{-2} (1.5×10^{-6} kg m^{-3}), which is close to published values for this parameter [174, 175]. In practice, the surfactant molecules will be distributed through many different environments (*e.g.,* oil-water interface; micelles and monomers in the aqueous phase: and, reverse micelles and monomers in the oil phase), and so not all of the surfactant will go to the oil-water interface. Nevertheless, this amount would be expected to be relatively small since the critical micelle concentrations of the Tweens are typically very low < 0.002 wt% [176]. In addition, the droplet surfaces may not need to be completely covered by surfactant in order for protection against coalescence to occur. The above value should therefore only be seen as a rough estimate of the actual surface

load.

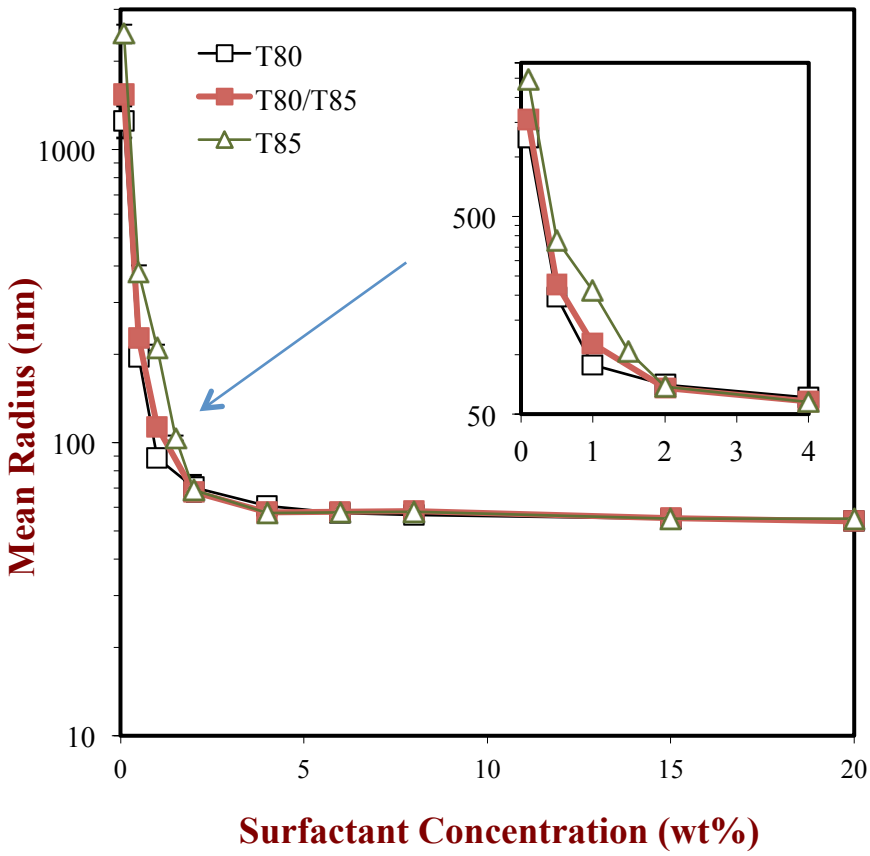


Figure 3.2 Influence of surfactant concentration on the mean particle radius (r_{32}) of 20 wt% MCT oil-in-water emulsions formed by a high-energy method (microfluidization). Emulsions were passed through the homogenizer three times at 10,000 psi.

At relatively high surfactant concentrations there is sufficient surfactant present to rapidly cover all of the small oil droplets created within the homogenizer, and so the droplet size is limited by the maximum disruptive forces generated by the homogenizer rather than by surfactant properties. In this regime, adding more surfactant therefore has little impact on the size of the droplets produced during homogenization. Previous

studies have shown that the particle size can be decreased in this regime by increasing the homogenization pressure (P) to facilitate further droplet disruption [8, 41, 60]. Typically, there is a linear decrease in $\log(d)$ with increasing $\log(P)$ for oil-in-water emulsions prepared using small molecule surfactants in a microfluidizer, with the slope depending on homogenizer design.

In general, the two surfactants and their mixture showed similar trends in their particle size *versus* surfactant concentration dependences, but there were some differences (**Figure 3.2**). In the surfactant-limited zone, the decrease in mean particle size with increasing surfactant concentration was steeper for the Tween 80 than for the Tween 85, and the mixture was somewhere in between. Thus, for the same surfactant concentration smaller droplets were produced for Tween 80 than for Tween 85 in the surfactant-limited zone. There are a number of possible explanations to account for this difference, *e.g.,* there were differences in the adsorption rate of the surfactants to the oil-water interface, or in the ability of the surfactants to stabilize the droplets against coalescence within the homogenizer. Tween 85 is more lipophilic than Tween 80 and therefore there will have been a greater fraction of this surfactant initially present within the oil phase. The oil phase is more viscous than the aqueous phase and therefore Tween 85 in the oil phase may have moved to the droplet interface more slowly than Tween 80 in the water phase.

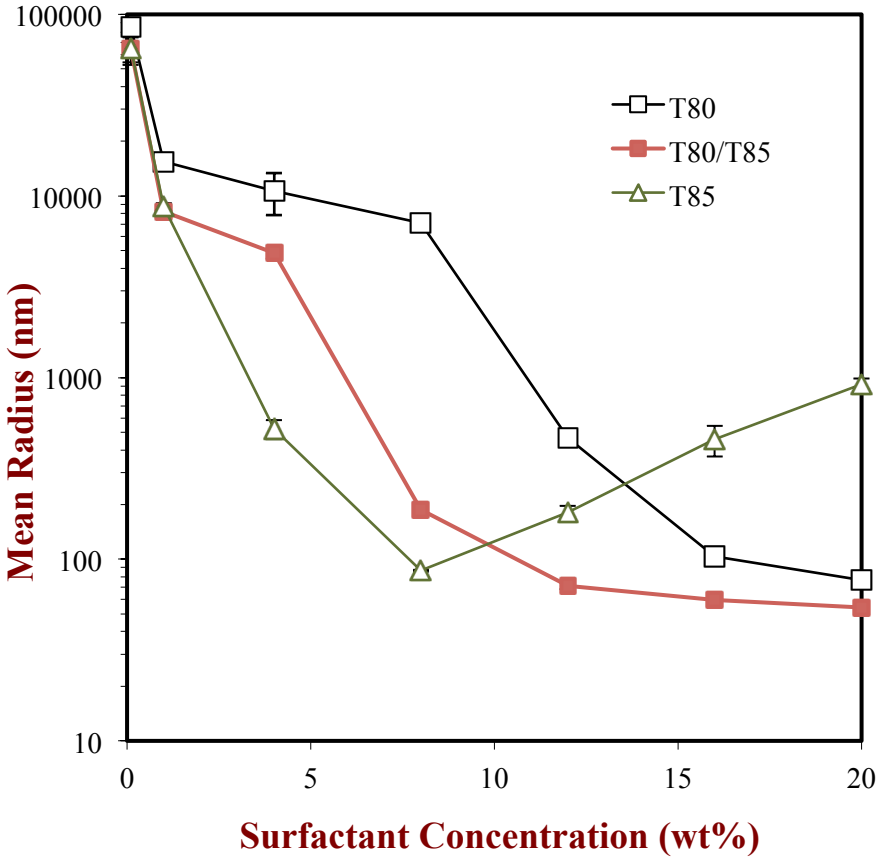


Figure 3.3 Influence of surfactant concentration on the mean particle radius (r_{32}) of 20 wt% MCT oil-in-water emulsions formed by a low-energy method (spontaneous emulsification). The preparation procedure is described in the manuscript.

Spontaneous emulsification: The spontaneous emulsification (SE) method was also used to prepare 20 wt% MCT oil-in-water emulsions using the same type and concentrations of surfactant (**Figure 3.3**), so as to directly compare the results with those obtained using the microfluidization method (**Figure 3.2**). The influence of surfactant concentration on the full particle size distribution of the samples prepared using the SE method is also reported (**Figure 3.4**). The SE method was capable of forming

emulsions with small mean droplet radius (r < 100 nm), but the amount of surfactant required to form small droplets with a monomodal distribution was considerably higher than the microfluidization method (**Figures 3.3 and 3.4**). For example, a surfactant-to-oil ratio (SOR) of < 0.1 was typically required to form small droplets (r < 100 nm) using the microfluidization method, but a SOR > 1 was required to form small droplets using the SE method. Thus, approximately ten-fold more surfactant was needed to produce similarly-sized droplets using the low-energy approach than using the high-energy approach. This clearly highlights one of the major disadvantages of the spontaneous emulsification method for forming food-grade ultrafine emulsions. For the Tween 80 and the Tween 80/Tween 85 system, the particle size progressively decreased with increasing surfactant concentration with the smallest mean droplet radius being obtained at 20% surfactant: r = 77 nm for Tween 80 and r = 55 nm for the Tween 80/Tween 85 mixture. For the Tween 85 system there was a decrease in mean particle radius from 0 to 8% surfactant, followed by an increase at higher surfactant concentrations (**Figure 3.3**). A minimum droplet radius of around 87 nm was observed at 8% surfactant for this system. These results show that at low and intermediate surfactant concentrations (< 8%) the smallest lipid droplets can be formed with Tween 85, but at higher surfactant concentrations the smallest droplets can be formed with the Tween 80/Tween 85 mixture. The full particle size distributions showed that the droplets formed were monomodal at sufficiently high surfactant concentrations for the

Tween 80 (**Figure 3.4a**) and mixed systems (**Figure 3.4b**), and at intermediate concentrations for the Tween 85 system (**Figure 3.4c**).

Figure 3.4a

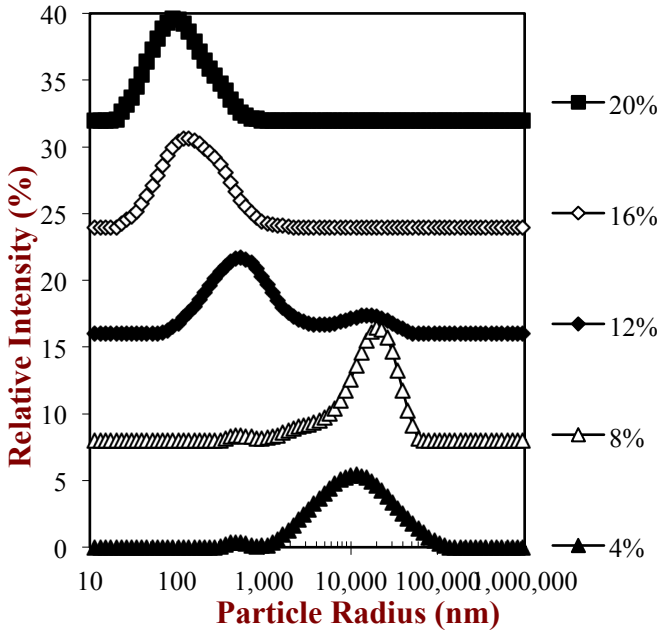


Figure 3.4c

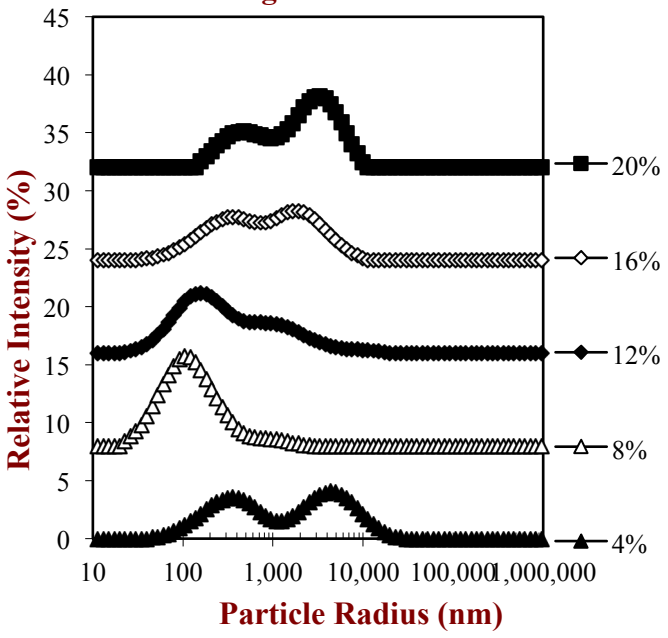


Figure 3.4 The particle size distributions of MCT oil-in-water emulsions formed by using different concentrations of surfactants prepared by the spontaneous emulsification method: (a) Tween 80; (b) Tween 80/Tween 85; (c) Tween 85.

The fundamental relationship between system composition and the size of the droplets produced by the spontaneous emulsification method is less well understood than for high energy methods such as microfluidization. A number of studies have empirically examined the relationship between particle size, system composition and preparation conditions [177-180]. Fernandez and co-workers proposed that ultrafine oil droplets were formed when water was added to a surfactant/oil mixture due to phase inversion: the system went from a W/O microemulsion to a bicontinuous system to an O/W emulsion [1]. Recently, Mercuri and co-workers proposed the following mechanism for the spontaneous formation of emulsion droplets based on a study of the phase behavior and microstructure of ternary mixtures of oil (soybean oil), surfactant (Tween 80 and Span 80) and water [181]. When an aliquot of surfactant/oil mixture is brought into contact with a water phase a boundary is initially formed between the S/O and W phases. Water then penetrates into the S/O phase causing it to swell, leading to the formation of W/O microemulsions and then to liquid crystalline phases. Eventually, a fragment (≈ 50 μm) of the liquid crystalline S/O phase breaks off from the S/O-W boundary and moves into the water phase. These relatively large fragments may then further dissociate into the nano-sized lipid droplets eventually formed. An alternative

hypothesis proposed by Anton and co-workers to account for spontaneous emulsification is the movement of surfactant molecules from the S/O phase into the water phase after the two phases come into contact, which leads to spontaneous formation of nano-sized droplets at the boundary due to a budding mechanism (**Figure 3.1**) [50]. In reality, it is likely that water will penetrate into the S/O phase and that surfactant will move into the water phase, although the relative rate and influence of these two processes is currently unknown. Nevertheless, neither of these mechanisms can currently be used to quantitatively account for the influence of system composition on the size of the droplets formed by the SE method.

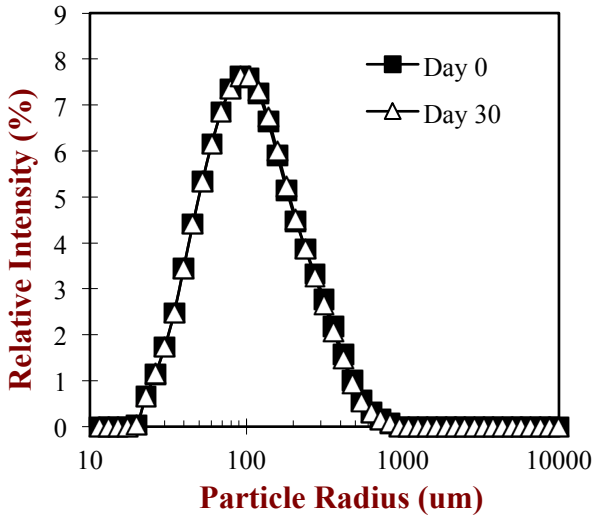
The fact that a minimum in droplet size was observed for the Tween 85 system at around 8 wt% is currently not well understood. Previous studies have also reported a minimum in the droplet size for surfactant-oil-water systems containing Tween 85 and MCT [182, 183]. These authors reported that the mean particle size obtained using the SE method had a minimum value at a surfactant-to-oil ratio of Tween 85-to-MCT of about 3-to-7 (0.43-to-1), which is very similar to the value of 8-to-20 (0.40-to-1) found in our work. Pouton and co-workers hypothesized that high concentrations of this surfactant led to the formation of viscous gels at the S/O-W boundary that retarded self-emulsification by limiting molecular diffusion processes [182, 183]. The influence of system composition on the physicochemical characteristics of the SOW systems is reported in Section 3.3. It is clear that further work is required to rationalize the

formation of oil-in-water emulsions with specific droplet sizes using the self-emulsification approach.

3.3.2 Stability of the emulsion formed by spontaneous emulsification

The long-term stability of an emulsion is one of the most important factors determining its suitability for application within the food and beverage industries [184, 185]. For this reason, the stability of the emulsions produced in this study was assessed by measuring changes in their droplet sizes and appearance during storage at ambient temperature (~20 ºC) for one month. Emulsions formed using microfluidization remained stable to droplet aggregation (there was no change in their particle size distribution) and gravitational separation (there was no visible evidence of creaming) throughout a 30 day period, which can be attributed to their relatively small particle sizes (data not shown).

(a)



(b)

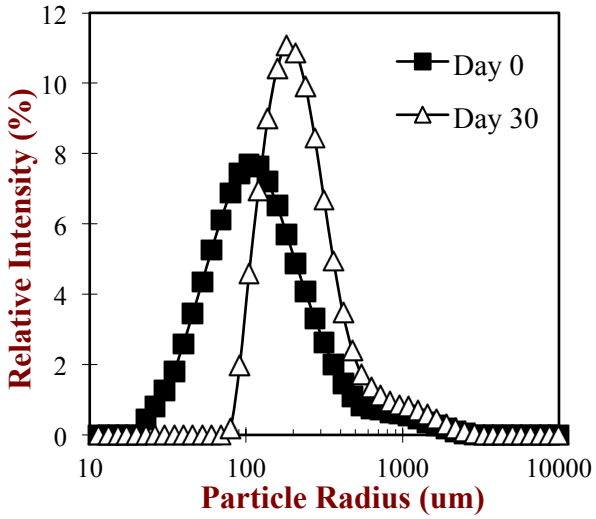
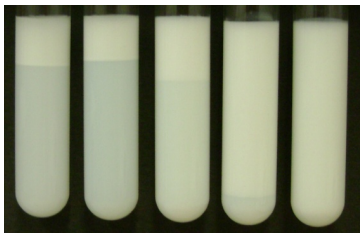


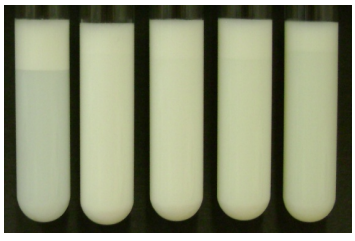
Figure 3.5 Influence of storage at ambient temperature on the particle size distribution of 20 wt% MCT oil-in-water emulsions formed by using different surfactants: (a) Tween 80; (b) Tween 85. Like the Tween 80 system, the mixed surfactant system (Tween 80/Tween 85) was also stable to droplet growth during storage (data not shown).

For the emulsions formed using the spontaneous emulsification method, light scattering measurements indicated that the emulsions stabilized by Tween 80 and by Tween 80 / Tween 85 were stable to particle growth during one month storage, with no significant change in mean particle radius (**Table 3.1**) or particle size distribution (**Figure 3.5a**). On the other hand, there was an appreciable increase in the mean particle radius of the emulsions stabilized by Tween 85 during storage (**Table 3.1**), which can be attributed to an increase in the fraction of larger droplets present in the particle size distribution (**Figure 3.5b**). The observed differences in emulsion stability can be

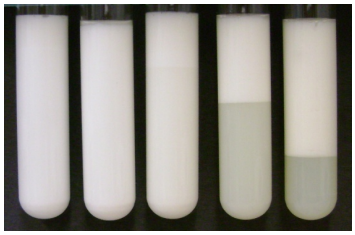
attributed to differences in the influence of the molecular characteristics of the surfactants on droplet coalescence. It is known that the rate of droplet coalescence tends to increase as the hydrophile-lipophile balance (HLB) of non-ionic surfactants decreases from high to intermediate values [186]. Surfactants with high HLB numbers (> 15) tend to be water soluble, form micelles, and stabilize oil-in-water emulsions, whereas surfactants with intermediate HLB numbers (5 to 9) tend to be soluble in both the oil and water phases, form lamellar structures, and are not particularly good at stabilizing either O/W or W/O emulsions [187]. The physicochemical origin of this effect is that the interfacial tension at the oil-water interface tends to become very low as the HLB number tends towards 7 (the surfactant curvature tends towards unity), which means the interface becomes highly mobile and susceptible to coalescence through "hole" formation [188].



Tween 80



Tween 80 /Tween 85



Tween 85

4% 8% 12% 16% 20%

Figure 3.6 Photographs of the influence of surfactant type and concentration on the stability of 20 wt% MCT oil-in-water emulsions produced by the spontaneous emulsification method to gravitational separation after 30 days storage.

Visual observation of the 20 wt% MCT oil-in-water emulsions after 30 days storage indicated that homogenization method, surfactant type, and surfactant concentration also had a major impact on their stability to gravitational separation. As mentioned earlier, all the emulsions prepared using microfluidization remained stable to gravitational separation during storage, which may be attributed to their relatively small droplet sizes (data not shown). A number of the samples prepared by the spontaneous emulsification method exhibited evidence of creaming instability after storage, with an optically opaque cream layer being observed at the top of the samples and a slightly turbid serum layer at the bottom (**Figure 3.6**). For the Tween 80 samples, the height of the cream layer increased as the surfactant concentration increased, suggesting the samples were more stable to creaming at higher surfactant concentrations. For the Tween 85 samples, the emulsions were fairly stable to creaming at low and intermediate surfactant concentrations (4 to 12 %), but showed extensive creaming at higher surfactant

concentrations (16 and 20%). For the mixed surfactant samples, the emulsions were unstable at the lowest surfactant concentration (4%), were stable at intermediate concentrations (8 and 12%), and showed some evidence of creaming again at the higher values (16 and 20%).

A number of factors may contribute to the creaming stability of the emulsions. First, the creaming velocity of a lipid droplet increases as its radius increases, so that emulsions containing larger droplets will cream more rapidly than those containing smaller ones [174]. Consequently, the emulsions prepared with low surfactant concentrations are likely to be unstable to creaming because they contain relatively large droplets. The emulsions prepared with relatively low levels of Tween 85 had smaller droplet sizes than those prepared with Tween 80 or the mixed system (**Figure 3.3**), which may account for their better creaming stability at low surfactant concentrations (**Figure 3.6**). On the other hand, the relatively poor creaming stability of the Tween 85 samples at high surfactant concentrations (**Figure 3.6**) may be due to their relatively large particle size (**Figure 3.3**). Second, creaming may be induced at intermediate surfactant levels due to the presence of non-adsorbed surfactant micelles that generate a depletion attraction between the droplets [189]. The strength of the depletion attraction increases with increasing surfactant concentration, and above a critical surfactant level (which increases with decreasing droplet size) flocculation may be induced, leading to rapid creaming. This phenomenon may account for the fact that some creaming was observed

in the Tween 85 and mixed surfactant systems at high surfactant concentrations, even though they had relatively small droplet sizes.

Table 3.1 Surfactant properties and influence of surfactant type on mean particle radius of emulsions formed by spontaneous emulsification after 0 and 30 days storage. The surfactant concentration is the value where the minimum droplet size was produced by the SE method. Data for the surfactants were taken from the literature [176].

Surfactant Type	**MW (g/mol)**	**HLB**	**CMC (mg/100g)**	**Surfactant Concentration**	**Radius** *Day 0*	**Radius** *Day 30*
Tween 85	1310	15	1.31	8 %	87 nm	183 nm
Tween 80	1839	11	0.033	20 %	78 nm	77 nm
Tween 80:Tween 85	1575	13	-	20 %	55 nm	55 nm

3.3.4 Phase behavior of SOW systems

The phase behavior of the system at the oil/surfactant-water boundary plays a key role in the formation of lipid droplets by the spontaneous emulsification process [190]. We therefore used dilution experiments and optical microscopy measurements to provide some information about the phase behavior of the SOW systems used in this study.

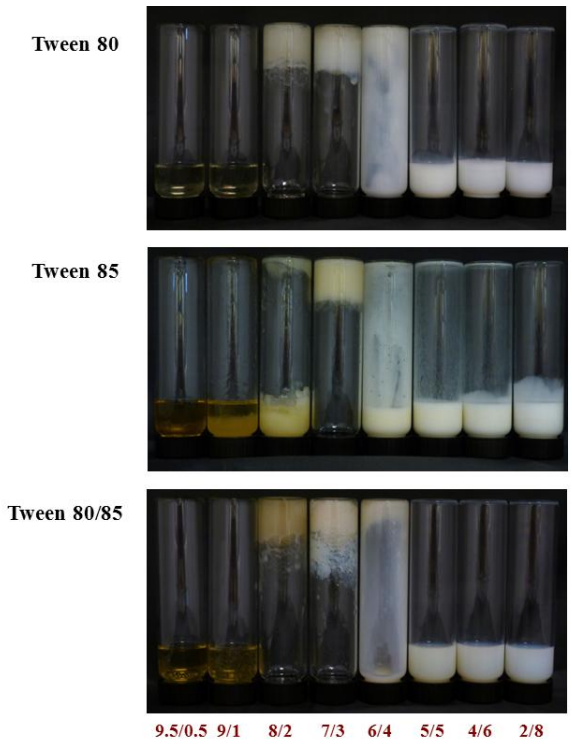


Figure 3.7 Photographs of the influence of surfactant type on the appearance and properties of surfactant-oil-water (SOW) mixtures containing different SO-W ratios, but a fixed SO ratio (1:1). *Annotation*: the numbers x/y represent the mass ratio of SO to W, *e.g.,* 9/1 means there was 9 g of surfactant-oil mixture and 1 g of water.

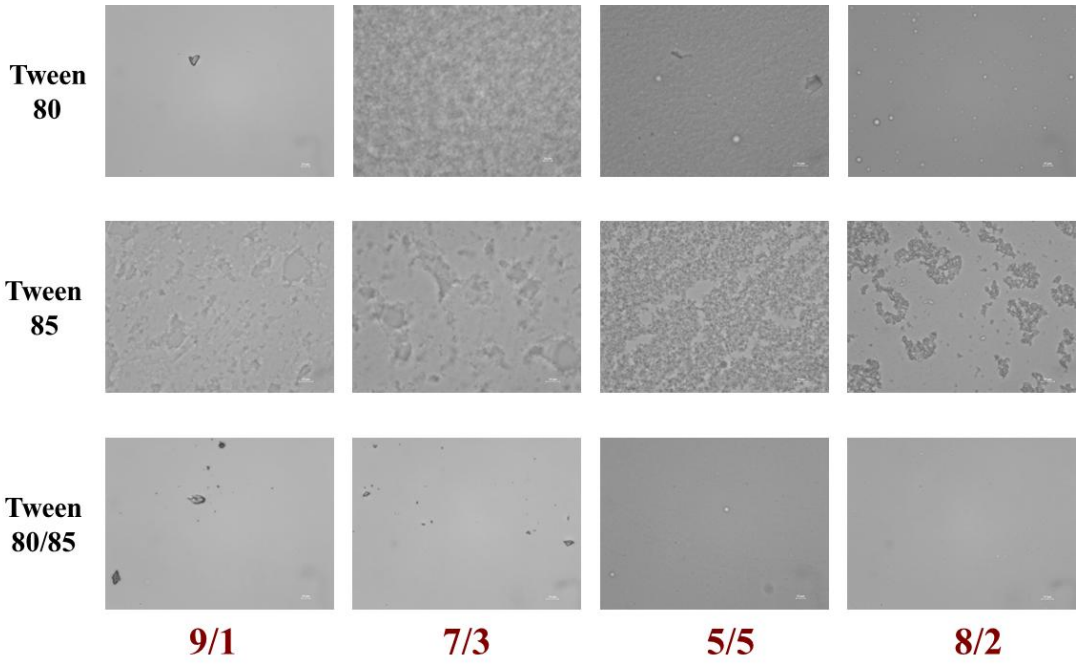


Figure 3.8 Micrographs of the influence of surfactant type on the appearance of surfactant-oil-water (SOW) mixtures containing different SO-W ratios, but a fixed SO ratio (1:1). *Annotation*: the numbers x/y represent the mass ratio of SO to W, *e.g.,* 9/1 means there was 9 g of surfactant-oil mixture and 1 g of water. The small white bar in the lower right hand corner of each image represents 10 μm.

A series of mixtures with different mass fractions of surfactant (S), oil (O), and water (W), but with a fixed surfactant-to-oil ratio (S:O = 1:1) was prepared, and then their overall appearance (**Figure 3.7**) and microstructure (**Figure 3.8**) was measured. Visual observations suggested that all of the surfactants exhibited relatively similar behavior when the amount of water present in the system was increased: at high SO-to-W ratios (low water contents) a viscous transparent oily solution was formed; at intermediate SO-to-W ratios a gel-like translucent material was formed; at high SO-to-W ratios (high water contents) a low viscosity milky liquid was formed (**Figure 3.7**).

Examination of the SOW systems using optical microscopy indicated that surfactant type did have an appreciable influence on their microstructures (**Figure 3.8**). The Tween 80 sample was relatively homogeneous at low and high SO-to-W ratios, suggesting that large particles were not present in the oil or water continuous systems. At high SO-to-W ratios, this corresponds to the transparent oily liquid observed at low water contents. At low SO-to-W ratios, this corresponds to the milky liquid formed at high water contents. A highly heterogeneous microstructure was observed under the microscope at intermediate SO-to-W ratios *i.e*. 7:3 (**Figure 3.8**), which corresponded to the gel-like structure observed visually (**Figure 3.7**). The Tween 85 showed quite different behavior, with large heterogeneous structures being formed at all surfactant/oil to water ratios studied (**Figure 3.8**). The precise nature of these structures is currently unknown, although it is likely that they are some kind of liquid crystalline phase. Large heterogeneous structures were only observed in the Tween 80/85 system at a SO-to-W ratio of 8:2 (data not shown), otherwise there was no large structures formed (**Figure 3.8**). Observation of the same systems using cross-polarized optical microscopy indicated that they had some order, *i.e.,* the structures rotated polarized light and gave a white appearance against a black background (data not shown). This birefringent behavior suggests that the surfactants formed liquid crystalline structures at intermediate SO-to-W ratios.

3.4 Conclusions

This study has highlighted some of the advantages and disadvantages of low and high energy approaches for creating oil-in-water emulsions containing ultrafine oil droplets. The high energy method (microfluidization) was able to produce emulsions with small droplets (r < 100 nm) using low surfactant-to-oil ratios (< 1:10), but it required relatively expensive specialized equipment and has relatively high operating costs. On the other hand, the low energy method (spontaneous emulsification) is inexpensive and can be implemented using simple equipment (stirring), but it requires high surfactant-to-oil ratios (> 5:10) to produce small droplets. The usage of relatively high surfactant levels may cause problems in some food and beverage products due to high ingredient costs, off flavors, and safety concerns. In addition, high surfactant levels may lead to emulsion instability due to depletion flocculation or due to accelerated droplet growth through Ostwald ripening effects. Further work is required to establish whether low energy methods can be developed that produce small droplet sizes without the need for high surfactant levels.

CHAPTER 4

FORMATION AND STABILITY OF EMULSIONS USING A NATURAL SMALL MOLECULE SURFACTANT: QUILLAJA SAPONIN (Q-NATURALE®)

4.1 Introduction

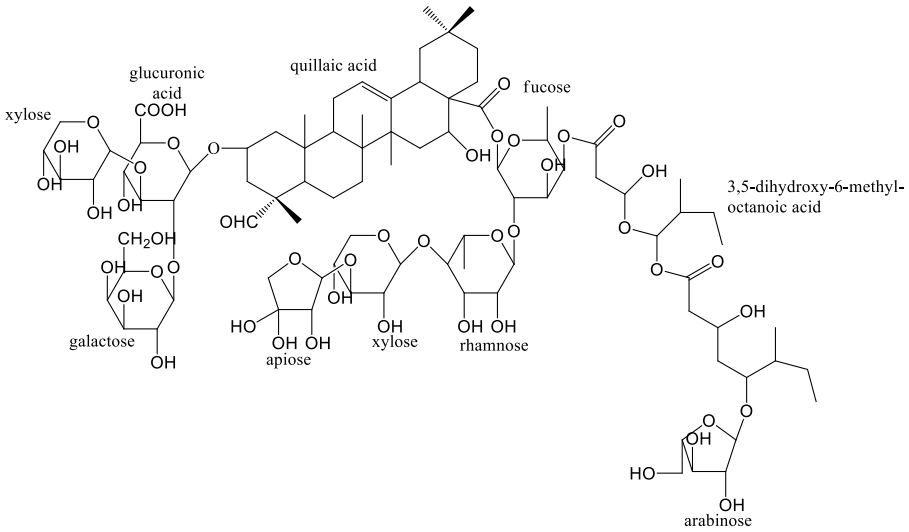
Small molecule surfactants are widely used in the food industry to form and stabilize emulsion-based food and beverage products [67, 191]. Surfactants facilitate the formation of oil-in-water emulsions by adsorbing to oil-water interfaces during homogenization, thereby reducing the interfacial tension and enhancing further droplet disruption [59, 60]. In addition, adsorbed surfactants provide a protective coating around oil droplets, which inhibits their aggregation and improves the long-term stability of emulsions [24, 192]. A large number of surfactants are available for utilization within the food industry, which vary according to their molecular, physicochemical, and functional properties, as well as their ease of utilization, legal status, and costs [67]. Some of the most important practical characteristics of surfactants for commercial use are: (i) the minimum amount required to produce small droplets; (ii) the smallest droplet size that can be achieved under specified homogenization conditions; (iii) the stability of the droplets formed to environmental stresses, such as pH, ionic strength, and temperature [193]. There are wide variations in the performance of surfactants depending on their molecular characteristics, *e.g.,* head group and tail group structure [194]. Consequently, food and beverage manufacturers must select the most appropriate surfactant for a

particular application [193].

Recently, there has been increasing interest within the food industry in replacing synthetic ingredients with natural "label friendly" alternatives [7, 13]. Consequently, there has been a focus on identifying and characterizing natural emulsifiers that can be successfully used in emulsion-based food and beverage products. Biopolymer-based emulsifiers, such as proteins (whey, soy and egg) and polysaccharides (gum arabic and modified starch), have been shown to be effective at forming and stabilizing emulsions [9-11, 13]. Nevertheless, biopolymer emulsifiers often have limitations in their functional performance. Protein-coated oil droplets are often unstable to aggregation at pH values around their isoelectric point, at high ionic strengths, and at elevated temperatures [12]. On the other hand, it is often difficult to produce oil droplets with very small sizes (d < 200 nm) using polysaccharide-based emulsifiers [9, 10, 13]. Certain types of lecithin are also suitable for utilization as natural emulsifiers in food and beverage products, *e.g.,* phospholipids derived from egg, soy, or milk [67, 76]. However, natural lecithin (which has two non-polar tails) usually has to be chemically or enzymatically modified to lysolecithin (which has one non-polar tail) before it can be successfully used to stabilize oil-in-water emulsions. In this study, we investigated the suitability of a natural saponin-based surfactant that has recently become commercially available as a food ingredient to form and stabilize oil-in-water emulsions.

An extract isolated from the bark of the *Quillaja saponaria* Molina tree (an

evergreen native to Chile and Peru) has been found to contain surface active components that are capable of forming surfactant micelles and stabilizing oil-in-water emulsions [14-16]. The major components within this extract have been reported to be saponins [17, 18] , which are high molecular weight glycosides consisting of a sugar moiety attached to a triterpene or a steroid aglycone [19]. The saponins are surface active substances (**Figure 4.1**) because they contain both hydrophilic regions (such as rhamnose, xylose, arabinose, galactose, fucose, and glucuronic acid) and hydrophobic regions (such as quillaic acid and gypsogenic acid) on the same molecule [16, 77]. A food ingredient based on the quillaja saponin extract has recently been marketed by the National Starch company (Bridgewater, NJ) under the trade name Q-Naturale®.



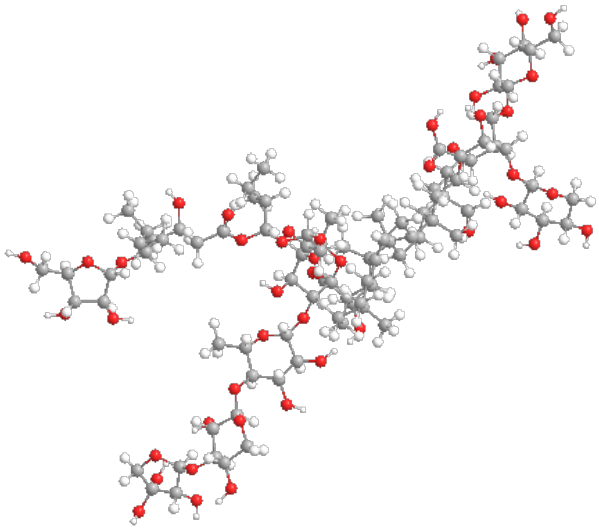


Figure 4.1 Structural representations of quillaja saponin – a proposed major surface active component of the food ingredient Q-Naturale.

In this study, we characterized the interfacial properties of influence of homogenization conditions on the formation of oil-in-water emulsions using Q-Naturale®, and then studied the influence of environmental stresses (pH, ionic strength, and temperature) and long-term storage on the stability of the resulting emulsions to droplet growth and gravitational separation. The performance of Q-Naturale® was compared to that of a synthetic surfactant (Tween 80) that is currently widely used in the food and beverage industry to formulate emulsion-based products.

4.2 Materials and methods

4.2.1 Materials

The non-ionic surfactant polyoxyethylene (20) sorbitan monooleate (Tween 80) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Quillaja saponin (Q-Naturale®

200) was provided by National Starch LLC (Bridgewater, N.J.). The molecular weights of Tween 80 (Sigma-Aldrich, St. Louis, MO) and quillaja saponin [16] have been reported to be 1310 and 1650 g mol^{-1}, respectively. Medium chain triglyceride (MCT) oil (Miglyol 812) was purchased from Coletica (Northport, NY).

4.2.2 Emulsion preparation

Oil-in-water emulsions were prepared by homogenizing 10 wt% lipid phase (MCT) with 90 wt% aqueous phase. The aqueous phase consisted of surfactant (Tween 80 or Q-Naturale®) and buffer solution (10 mM sodium phosphate buffer, pH 7.0). A coarse emulsion premix was prepared by blending the lipid and aqueous phases together using a high-speed mixer (Bamix, Biospec Products, Bartlesville, OK) for 2 min at room temperature. Fine emulsions were formed by passing the coarse emulsions through an air-driven microfluidizer (Microfluidics, Newton, MA, USA). The coarse emulsions were fed into the microfluidizer through a 100 ml glass reservoir, and were passed through the homogenization unit for different numbers of passes at various homogenization pressures. The type and amount of surfactant added to the aqueous phase prior to homogenization was varied in some experiments. The commercial Q-Naturale® ingredient contained 14% of surfactant (active ingredient) dispersed within an aqueous solution, and therefore we reported its concentration based on the amount of active surfactant present (rather than the overall ingredient).

4.2.3 Emulsion stability testing

The stability of the prepared emulsions to a series of environmental stresses that might be encountered in commercial practice was tested:

Thermal processing: Emulsion samples were placed in glass test tubes and then incubated in water baths set at different temperatures (30 to 90 ºC) for 30 min. The samples were then cooled to room temperature and stored for 24 hours at ambient temperature prior to analysis.

pH and salt: The pH and ionic strength of emulsions was adjusted to a specified level using NaOH, HCl, and/or NaOH solutions. Emulsion samples were then placed in glass test tubes and incubated for 24 hours at ambient temperature prior to analysis.

Long-term storage: Emulsion samples were placed in glass test tubes and then incubated in temperature controlled rooms (5, 37, and 55 °C) for one month prior to analysis.

4.2.4 Particle characterization

The particle size distributions of the samples were measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Malvern, U.K.). The droplet charge (ζ-potential) of emulsions was determined using a particle electrophoresis instrument (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, UK). Samples were diluted with buffer solutions of the appropriate pH prior to analysis to avoid multiple scattering effects.

4.2.5 Interfacial tension measurements

The interfacial tension *versus* surfactant concentration was measured at the MCT oil – water interface using a drop shape analysis instrument (DSA 100, Kruss GmbH, Hamburg Germany). The surface pressure (π) was calculated from the interfacial tension (γ) data using the following formula: $\pi = \gamma - \gamma_0$, where γ and γ_0 are the interfacial tensions measured in the presence and absence of surfactant.

4.2.6 Optical microscopy measurements

A Nikon optical microscope (Nikon Eclipse 80i, Nikon Instrument Inc., Melville, NY) with a 60× objective lens (NA 0.75) was used to capture images of emulsion samples.

4.2.7 Statistical analysis

All measurements were performed on at least two freshly prepared samples (*i.e.,* new samples were prepared for each series of experiments) and were reported as means and standard deviations.

4.3 Results and discussion

4.3.1 Interfacial characteristics

The interfacial activity of surfactants plays an important role in determining their ability to form and stabilize emulsions [195, 196], and therefore we measured the interfacial tension *versus* surfactant concentration profiles for the two surfactants used (**Figure 4.2**). For both surfactants, the interfacial tension decreased appreciably with

increasing surfactant concentration indicating that the surfactants adsorbed to the oil-water interface. A fairly constant interfacial tension was reached at high surfactant levels ($\gamma_{\infty} \approx 5$ mN m^{-1}), which indicated that the interfaces had become saturated with surfactant [71]. Q-Naturale and Tween 80 both had fairly similar interfacial tension *versus* surfactant concentration profiles, which indicated that they had similar surface activities. The critical micelle concentration (CMC) of the two surfactants was determined from the interfacial tension *versus* logarithm of surfactant concentration profiles using the method described previously, *i.e.,* determining the intersect between linear plots extrapolated from high and intermediate surfactant concentrations [16]. We estimated the CMC values from the data shown in **Figure 4.2** to be around 0.003 and 0.01 wt% for the Tween 80 and Q-Naturale, respectively. These values is in reasonable agreement with reported values of 0.0022 wt% for Tween 80 [197] and 0.01 to 0.07 wt% for quillaja saponin from various sources [16].

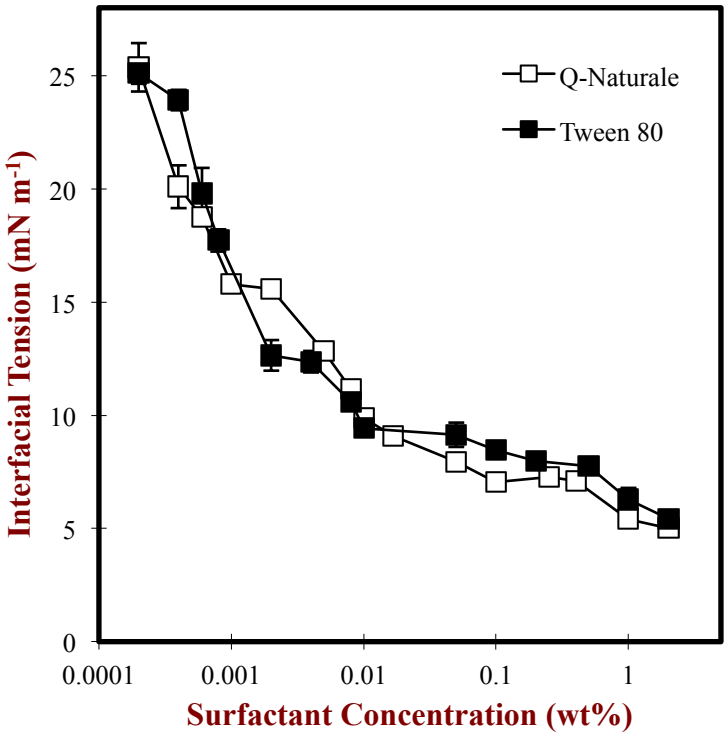


Figure 4.2 Influence of Tween 80 and Q-Naturale concentration on the interfacial tension measured at the MCT oil-water interface.

The surface load (Γ) of a surfactant can be calculated from the interfacial tension (γ) versus surfactant concentration (c) data using the following formula [16]:

$$\Gamma = -\left(\frac{1}{RT}\right)\frac{d\gamma}{d\ln c} \qquad (1)$$

Here R is the gas constant, T is the absolute temperature, and c is expressed in moles per liter [71]. We used this expression to determine the surface load of the two surfactants from the slope of the γ *versus* ln c data (**Figure 4.2**): Γ = 3.1 and 2.9 mg m^{-2} for Tween 80 and Q-Naturale, respectively. The area (A) occupied per molecule at the oil-water interface can be calculated from the surface load: $A = 1/(\Gamma N_A)$, where N_A is

Avogadro's number [16]. We obtained values of A = 72 and 96 Å^2 per molecule for Tween 80 and Q-Naturale, respectively. The value for Q-Naturale is in good agreement with the value of 83 Å^{-2} reported for quillaja saponin in previous studies [16].

The surface activity (K) of an emulsifier can be conveniently characterized by the following relationship [26]:

$$K = \frac{1}{c_{1/2}} = \exp\left(-\frac{\Delta G_{ads}}{RT}\right) \quad (2)$$

Here $c_{½}$ is the surfactant concentration where half of the available adsorption sites on the oil-water interface are covered by surfactant, and ΔG_{ads} is the free energy change associated with surfactant adsorption. To a rough approximation, $c_{½}$ can be taken to be equal to the surfactant concentration where the surface pressure is half its maximum value (π_∞). In this study, we calculated the following parameters from the interfacial pressure *versus* surfactant concentration profiles: for Tween 80, $c_{½}$ = 0.0014 wt% (10.7 μM), K = 94,000, and ΔG_{ads} = -11.4 RT; for Q-Naturale, $c_{½}$ = 0.0010 wt% (6.1 μM), K = 165,000, and ΔG_{ads} = -12.0 RT.

Overall, our measurements show that both surfactants were highly effective at reducing the interfacial tension at an oil-water interface, and that both had fairly similar interfacial characteristics (**Figure 4.2**).

4.3.2 Influence of surfactant type and concentration on emulsion formation

In this section, the influence of surfactant type and concentration on the particle

size of 10 wt% MCT oil-in-water emulsions produced using a high pressure homogenizer (microfluidizer) under standardized homogenization conditions (10,000 psi, 4 passes) was examined (**Figure 4.3**). As expected, there was a decrease in mean particle diameter (d_{32}) with increasing surfactant concentration for both types of surfactant used. This effect can be attributed to two main factors: (i) a higher surfactant concentration means that a larger surface area can be stabilized during homogenization; (ii) a higher surfactant concentration leads to faster coverage of the droplet surfaces by surfactant molecules, and therefore better protection against recoalescence [192]. The dependence of oil droplet size on surfactant concentration can be conveniently divided into two distinct regimes for emulsions prepared by high pressure homogenization [172, 173]: the "surfactant-limited zone" and the "surfactant-rich zone". At low surfactant concentrations, there is insufficient surfactant present to cover the new surfaces created within the homogenizer when larger droplets are broken down to smaller ones. Consequently, two or more small oil droplets that collide tend to coalesce because there is insufficient surfactant present at their surfaces to prevent them from coming into close contact. Coalescence tends to continue until all of the droplets are covered by sufficient surfactant to prevent them from merging together. Hence, the droplet size in the surfactant-limited zone is primarily determined by the type and amount of surfactant present. At sufficiently high surfactant concentrations, the droplet size is primarily governed by the maximum disruptive energy that can be generated by the homogenizer, rather than the

amount of surfactant present. Nevertheless, the droplet size may still depend somewhat on surfactant type since this will determine interfacial characteristics that influence droplet disruption such as interfacial tension and rheology.

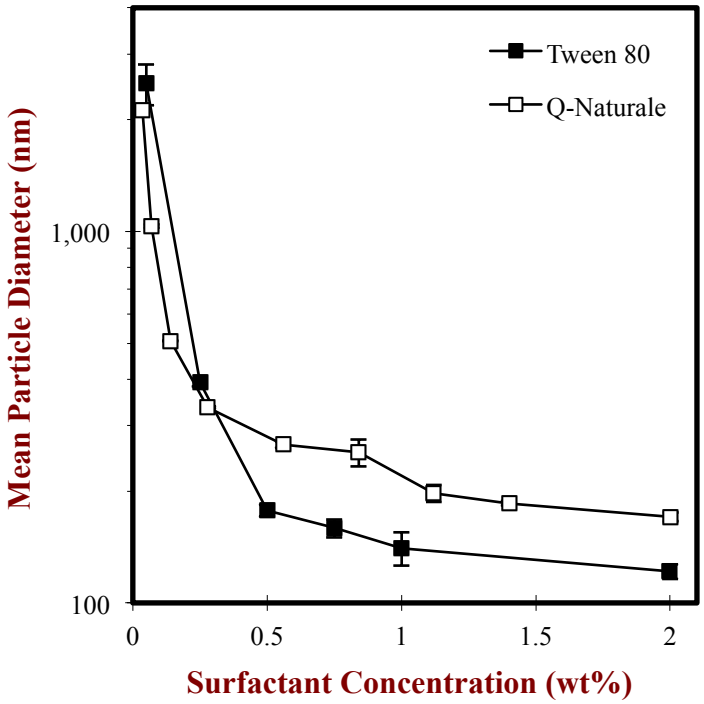


Figure 4.3 Influence of surfactant concentration on the mean particle diameter (d_{32}) of 10 wt% MCT oil-in-water emulsions containing either Tween 80 or Q-Naturale produced using standardized homogenization conditions (10,000 psi, 4 passes).

There were appreciable differences in the particle size *versus* surfactant concentration profiles obtained for the two types of surfactants (**Figure 4.3**). Smaller droplets could be produced at high surfactant concentrations using Tween 80 ($d \approx 120$ nm) than using Q-Naturale ($d \approx 170$ nm). In addition, a lower surfactant concentration was required to produce small droplets ($d < 200$ nm) for Tween 80 (≈ 0.3 to 0.5%) than for

Q-Naturale (≈ 0.8 to 1.1 %). These experiments indicate that both types of surfactants were effective at producing small droplets, but that Tween 80 was more efficient than Q-Naturale. The differences in the effectiveness of the two surfactants may be due to a number of factors that impact droplet disruption and coalescence within homogenizers, including the speed at which they adsorb to the oil-water interface, their ability to reduce the oil-water interface tension, their influence on the dynamic interfacial rheology, and their effectiveness at generating repulsive interactions between droplets [59, 60, 192]. The surface activities of the two surfactants (measured under static conditions) were fairly similar (**Section 3.1**), which suggest that some of the other factors mentioned may be more important.

The minimum droplet size that can be theoretically produced during homogenization given a fixed amount of surfactant can be calculated from the following expression [174]:

$$d_{\min} = \frac{6 \cdot \Gamma \cdot \phi}{c_S} = \frac{6 \cdot \Gamma \cdot \phi}{c'_S (1 - \phi)} \tag{3}$$

Here, Γ is the surface load (in kg m^{-2}), ϕ is the disperse phase volume fraction, c_S is the concentration of emulsifier in the emulsion (in kg m^{-3}) and c'_S is the concentration of emulsifier in the continuous phase (in kg m^{-3}). In our study, the emulsions contained 10 wt% of oil droplets ($\phi \sim 0.1$) and a maximum surfactant concentration of 2 wt% in the aqueous phase ($c'_S \sim 20$ kg m^{-3}). The surface load of the two surfactants was calculated

from the interfacial measurements in the previous section to be around 3 mg m^{-2} ($\Gamma = 3 \times 10^{-6}$ kg m^{-2}). Hence, the minimum mean droplet diameter (d_{32}) that could be achieved assuming that all of the surfactant adsorbed to the droplet surfaces during homogenization is about d_{min} = 100 nm. The mean droplet diameters produced in our study were somewhat higher than this value, which suggested that there was more than enough emulsifier initially present to cover all of the droplets formed by the microfluidizer. The fact that the observed minimum droplet diameter (~120 - 170 nm) achieved was higher than the predicted value may have been because the surfactant did not adsorb quickly enough during homogenization, or because the homogenizer was incapable of generating sufficiently intense disruptive forces at the pressures used.

4.3.3 Influence of homogenization conditions on emulsion formation

Previous studies have shown that the size of the droplets in emulsions produced by high pressure homogenization depends on processing conditions such as homogenization pressure and number of passes [8, 33, 198, 199]. We therefore examined the influence of these experimental parameters on the size of the droplets produced by the two surfactants used in this study. The mean droplet diameter decreased with increasing homogenization pressure (**Figure 4.4**) and number of passes (**Figure 4.5**) for both types of surfactant, which is in agreement with previous studies [13, 41, 173, 200].

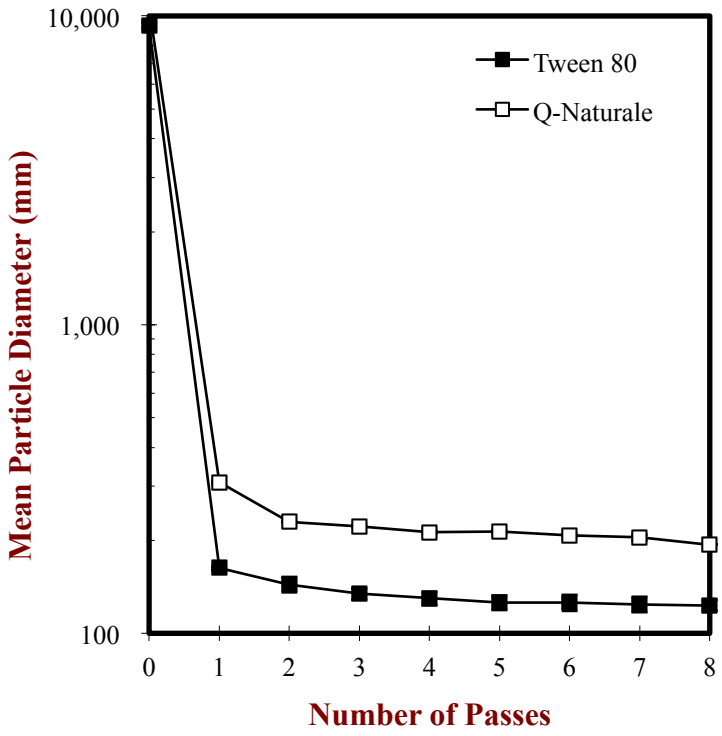


Figure 4.4 Influence of number of passes through a high pressure homogenizer on the mean particle diameter (d_{32}) of 10 wt% MCT oil-in-water emulsions containing either 1 wt% Tween 80 or Q-Naturale produced at a constant homogenization pressure (10,000 psi).

At a fixed homogenization pressure, the mean droplet diameter decreased with increasing number of passes (**Figure 4.4**), which can be attributed to the fact that a higher proportion of the droplets will have had a chance to pass through the most disruptive zone within the homogenizer. The largest decrease in mean droplet diameter occurred when the coarse emulsion premix (zero passes) was initially passed through the homogenizer (one pass). After one pass through the homogenizer, there was only a slight decrease in droplet size upon further passes indicating that this type of

homogenizer is highly effective at droplet disruption. This is commercially important since food and beverage manufacturers aim to minimize the number of passes required to form an emulsion since this reduces processing time and cost. The limiting droplet size obtained after a high number of passes is determined by the maximum disruptive energy that can be generated by the homogenizer at the pressure used (provided there is sufficient surfactant present to cover all the droplet surfaces produced).

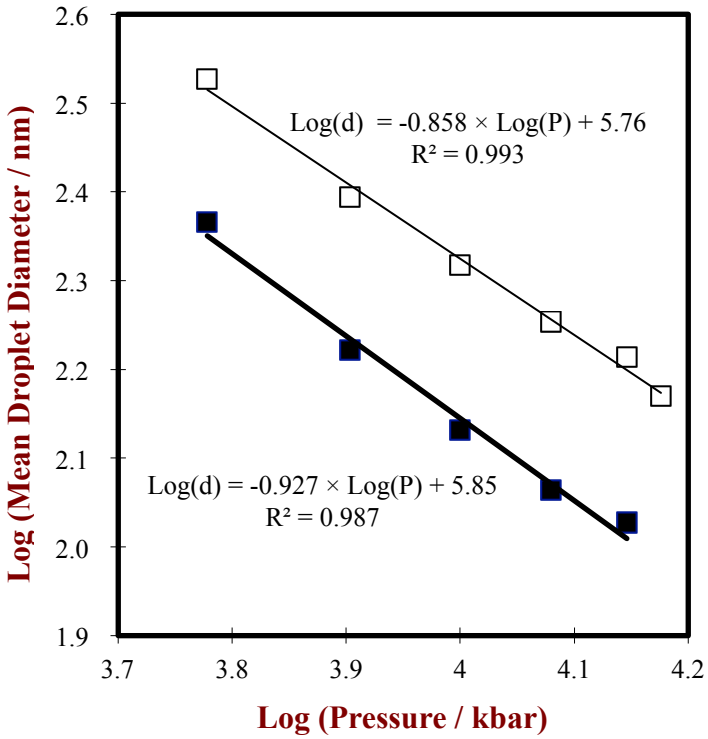


Figure 4.5 Influence of homogenization pressure on the mean particle diameter (d_{32}) of 10 wt% MCT oil-in-water emulsions containing either 1 wt% Tween 80 or Q-Naturale passed four times through the homogenizer.

There was a linear decrease in the logarithm of the mean droplet diameter with the logarithm of the homogenization pressure for emulsions stabilized by either Tween 80 or

Q-Naturale (**Figure 4.5),** which is in agreement with previous studies that indicate there should be a linear relationship providing there is sufficient emulsifier present [201]. The slope determined from the $\log_{10}(d)$ versus $\log_{10}(P)$ plots was around -0.93 when Tween 80 was used as the surfactant and around -0.86 when Q-Naturale was used (**Figure 4.5**). These values are somewhat higher than previously reported values of around -0.6 to -0.8 for high pressure homogenizers [202, 203]. This difference may be due to differences in the homogenizer design, homogenization valve, or particle sizing methods used in different studies.

4.3.4 Influence of environmental stresses on emulsion stability

Practically, an emulsion-based food or beverage should remain stable throughout the anticipated shelf life of the final product. Emulsions may become unstable through a number of different instability mechanisms (*e.g.,* flocculation, coalescence, Ostwald ripening, and gravitational separation), which depend on storage conditions such as pH, ionic strength, and temperature [28]. We therefore examined the influence of environmental stresses on the stability of emulsions stabilized by the two different kinds of surfactant.

4.3.4.1 Influence of Thermal Processing

Emulsions prepared using either Tween 80 or Q-Naturale were both stable to thermal processing across the entire temperature range studied (30 to 90 ºC), with little change in mean particle diameter (**Figure 4.6**) or visible evidence of gravitational

separation, *i.e.,* all the emulsions appeared white and homogeneous (data not shown). Lipid droplets stabilized by non-ionic surfactants may become unstable to droplet coalescence when they are heated close to a critical temperature (the "phase inversion temperature" or PIT) because of dehydration of the polar surfactant head groups [50, 204]. Dehydration causes a change in the optimum curvature of the surfactant molecules at the oil-water interface, as well as their relative solubilities (and partitioning) in the oil and water phases, which favors droplet coalescence [205]. The PIT depends on the chemical structure of the surfactant, as well as the nature of the oil phase [177, 178]. Our results suggest that both Tween 80 and Q-Naturale are stable to droplet coalescence during relatively short term heating when MCT is used as the oil phase, presumably because the PIT is sufficiently far above the highest temperature used in this study. Nevertheless, we did seem some instability in the Tween 80 stabilized emulsions when they were heated at elevated temperatures for prolonged times (see **Section 4.3.4.4**), which suggests that they may have been unstable to coalescence at elevated temperatures.

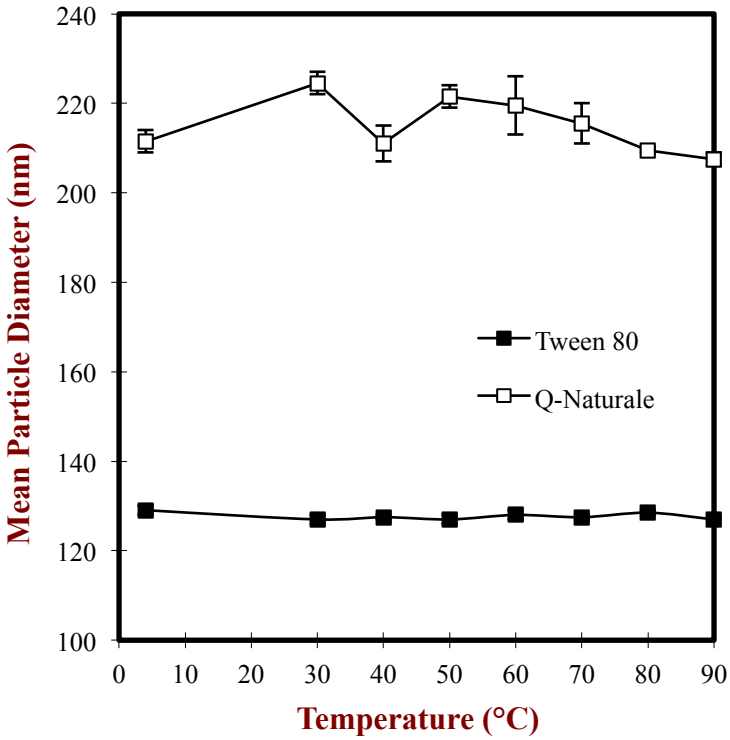


Figure 4.6 Influence of thermal processing on the mean particle diameter (d_{32}) of 10 wt% MCT oil-in-water emulsions containing either 1 wt% Tween 80 or Q-Naturale. Emulsions were held for 30 minutes at the specified temperature, and then cooled to ambient temperature prior to analysis.

4.3.4.2 Influence of pH

The influence of pH on the mean particle diameter, ζ-potential, creaming stability, and microstructure of the emulsions stabilized by the two different surfactants was examined. For the Tween 80 stabilized emulsions, the electrical charge on the droplets went from slightly negative (- 9 mV) at pH 9 to slightly positive (+ 2 mV) at pH 2 with a point of zero charge around pH 3.5 (**Figure 4.7a**). Tween 80 is a non-ionic surfactant and would therefore not be expected to have any charge, although previous studies have

shown that droplets stabilized by non-ionic surfactants may be charged due to the presence of ionizable surface active impurities (such as free fatty acids) or due to preferential adsorption of hydroxyl ions (OH^-) at high pH or hydrogen ions (H^+) at low pH to the droplet surfaces [26]. Emulsions containing Tween 80 appeared to be stable to droplet aggregation and gravitational separation at all pH values studied, with no evidence of droplet growth in the light scattering data (**Figure 4.7b**), no change in microstructure in optical microscopy images (data not shown), and no visible evidence of creaming (data not shown). The fact that the droplets had little net charge across the entire pH range studied suggests that droplet aggregation was mainly prevented by steric repulsion due to the relatively large hydrophilic (polyoxyethylene) head groups of the adsorbed Tween molecules [76].

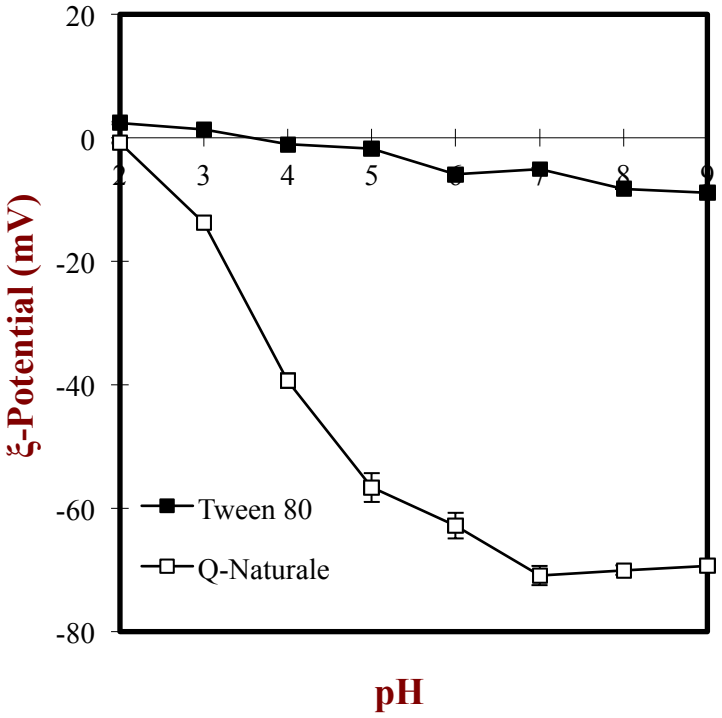


Figure 4.7a Influence of storage pH on the droplet charge (ζ-potential) of 10 wt% MCT oil-in-water emulsions containing either 1 wt% Tween 80 or Q-Naturale.

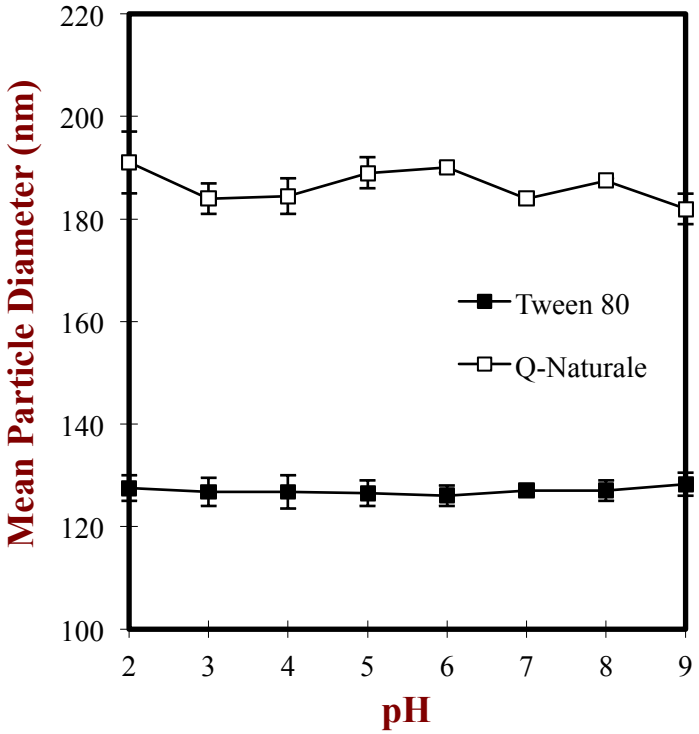
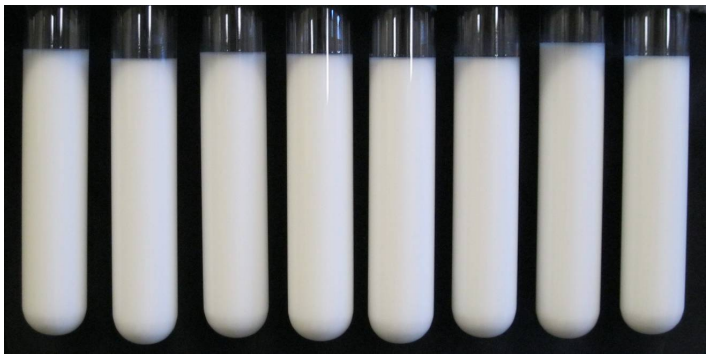
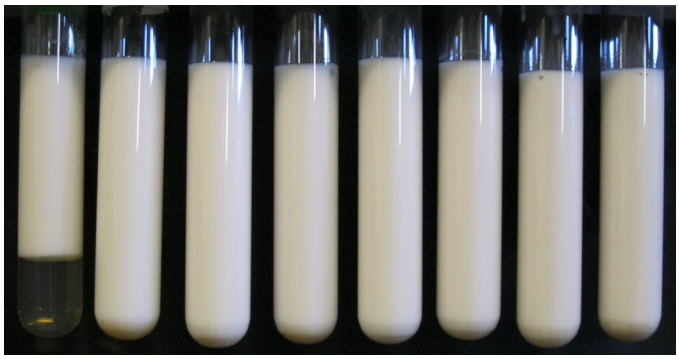


Figure 4.7b Influence of storage pH on the mean particle diameter (d_{32}) of 10 wt% MCT oil-in-water emulsions containing either 1 wt% Tween 80 or Q-Naturale.



Tween 80



Q-Naturale

Figure 4.7c Influence of storage pH on the appearance of 10 wt% MCT oil-in-water emulsions containing either 1 wt% Tween 80 or Q-Naturale. Note that extensive creaming occurred at pH 2 in the Q-Naturale stabilized system.

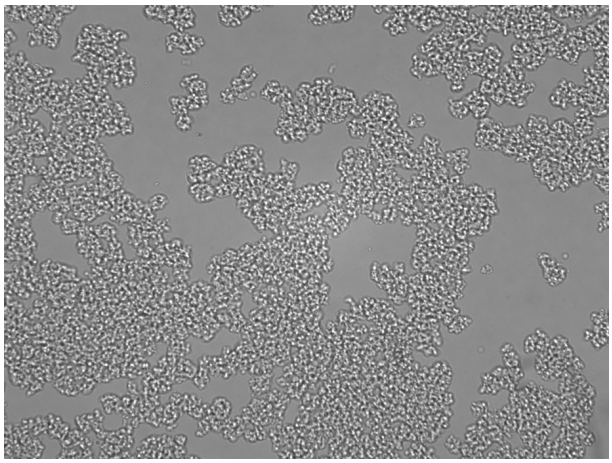


Figure 4.7d Microstructure of 10 wt% MCT oil-in-water emulsions containing 1 wt% Q-Naturale at pH 2. Extensive droplet flocculation can clearly be observed. All other emulsions appeared uniform in appearance under the microscope.

The Q-Naturale stabilized emulsions had a high negative charge (-69 mV) at pH 9, which decreased in magnitude as the pH was increased until it reached a value close to zero at pH 2 (- 1 mV) (**Figure 4.7a**). The origin of the negative charge can be attributed to the presence of a carboxylic acid group within the structure of quillaja saponin (**Figure**

4.1). Typically, carboxylic acid groups have pK_a values around pH 3.5, which would account for the large decrease in ζ-potential observed around this value (**Figure 4.7a**). At high pH values (pH >> pK_a) this group would be fully charged ($-COO^-$) whereas at low pH values (pH << pK_a) it would be uncharged (-COOH). The presence of a strong negative charge on the droplets might play an important role in determining the functional properties of this surfactant for certain applications: (i) a high negative charge may make emulsions susceptible to flocculation due to electrostatic bridging by cationic multivalent ions (such as calcium) [206, 207]; (ii), a high negative charge may attract cationic pro-oxidants (such as transition metals) to the droplet surfaces and thereby promote lipid oxidation [208, 209]; (iii) a high negative charge may cause droplets to adhere to cationic surfaces [210]. Our light scattering measurements indicated that the droplets were stable to droplet aggregation across the entire pH range studied (**Figure 4.7b**). On the other hand, visual observation of the emulsions indicated that extensive creaming occurred at pH 2 (**Figure 4.7c**), and optical microscopy images showed that the emulsions containing Q-Naturale were highly aggregated at pH 2 (**Figure 4.7d**). Taken together these results suggest that the emulsions were highly flocculated at pH 2, but that they were stable to coalescence (otherwise we would have seen an increase in the mean droplet diameter at pH 2 in the light scattering data). The light scattering measurements were performed on highly diluted samples, and so any flocculated droplets may have become dissociated prior to analysis. These results indicate that emulsions stabilized by

quillaja saponin are stable across most of the pH range present within food products (pH 3 to 8), but that they might become unstable to flocculation in highly acidic products or within the human stomach after ingestion.

4.3.4.3 Influence of ionic strength

The influence of ionic strength was examined by adding different amounts of salt (0 to 500 mM NaCl) to the samples and then measuring their stability after storage overnight (**Figure 4.8**). Emulsions containing Tween 80 were stable at all salt concentrations studied, exhibiting no change in mean particle diameter (**Figure 4.8a**), no evidence of creaming (data not shown), and no change in microstructure (data not shown). These results highlight the fact that the oil droplets in the Tween 80 emulsions are mainly stabilized against aggregation by steric (rather than electrostatic) repulsion [26]. Emulsions containing Q-Naturale appeared to be stable to droplet aggregation at all salt concentrations studied when analyzed by light scattering, with no evidence of an increase in droplet diameter at any NaCl concentration (**Figure 4.8b**). However, creaming stability and optical microscopy measurements indicated that Q-Naturale emulsions were stable at low salt concentrations (< 300 mM NaCl), but unstable to creaming (**Figure 4.8b**) and aggregation (**Figure 4.8c**) at higher salt concentrations. The fact that the mean particle size data determined by light scattering indicated that the droplets remained small at all salt concentrations studied suggests that droplet aggregation was due to flocculation rather than coalescence in the presence of high salt levels. These results

suggest that both electrostatic and steric repulsion play an important role in stabilizing Q-Naturale® coated droplets against aggregation. At low salt concentrations the long-range electrostatic repulsion between the droplets is strong enough to prevent aggregation, but at high salt concentrations the long-range attractive interactions (*e.g.,* van der Waals) become stronger than the long-range repulsion interactions (*e.g.,* electrostatic), leading to droplet association. Nevertheless, at high salt concentrations the short-range steric repulsion between droplets still seems to be sufficiently strong to prevent the droplets from coming close enough together to merge together and coalesce. The fact that the Tween 80 emulsions were stable to flocculation at high salt concentrations whereas the Q-Naturale emulsions were unstable suggests that the polymeric head groups of Tween 80 are thicker than those of Q-Naturale.

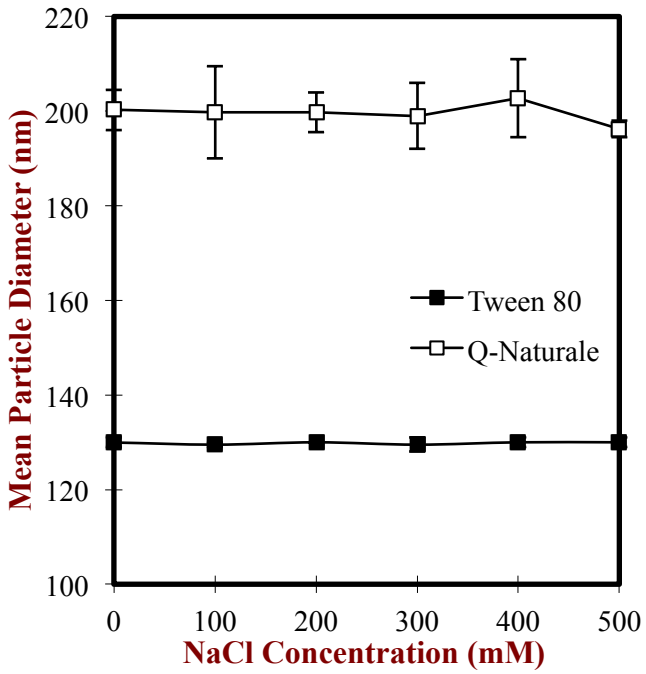


Figure 4.8a Influence of salt concentration on the mean particle diameter (d_{32}) of 10 wt% MCT oil-in-water emulsions containing either 1 wt% Tween 80 or Q-Naturale.

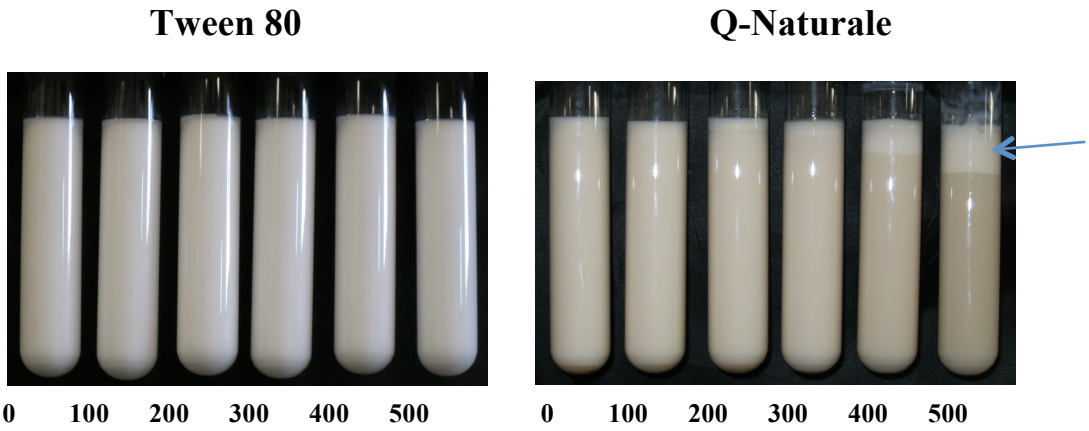


Figure 4.8b Influence of salt concentration on the appearance of 10 wt% MCT oil-in-water emulsions containing either 1 wt% Tween 80 or Q-Naturale. Creaming was observed at the higher salt concentrations in the Q-Naturale emulsions (arrow).

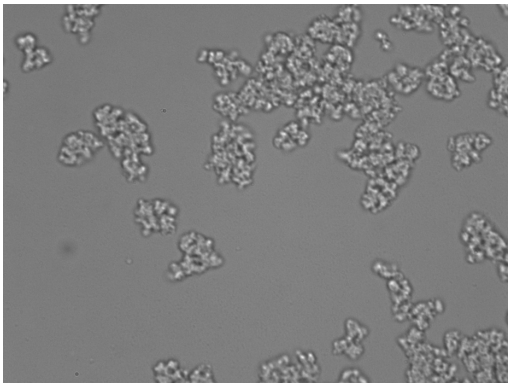


Figure 4.8c Influence of high salt concentration (500 mM) on the microstructure of 10 wt% MCT oil-in-water emulsions containing 1 wt% Q-Naturale.

4.3.4.4 Influence of storage time and temperature

The long-term stability of emulsions is one of the most important factors determining their shelf life in commercial food and beverage applications [28]. We

therefore compared the stabilities of emulsions formed using Q-Naturale with those formed using Tween 80 during storage at different temperatures (5, 37, and 55 °C) for one month. These experiments were carried out using emulsions at neutral pH in the absence of added salt. We observed no change in the visible appearance of emulsions containing Q-Naturale at any of the storage temperatures (data not shown) and little change in particle size distribution (**Figure 4.9**). We also found only a small increase in mean droplet diameter of the Q-Naturale emulsions after one month storage (**Table 4.1**), indicating that they were fairly stable to droplet growth and gravitational separation. We also did not observe any change in the appearance (data not shown) or mean particle diameter (**Table 4.1**) of the Tween 80 stabilized emulsions stored at 5 or 37 ºC for one month indicating that they were also stable to droplet growth and creaming. On the other hand, we did observe a thin layer of oil on top of the Tween 80 stabilized emulsions stored at 55 °C for one month, and an appreciable increase in mean droplet diameter (**Table 4.1**). Interestingly, we did not observe an appreciable change in mean droplet diameter or oiling off after heating the Tween 80 stabilized emulsions at high temperatures for short times (*e.g.,* 60-90 ºC for 30 minutes) as described earlier (**Section** 4.**3.4.1**). These results suggest that emulsions stabilized by this non-ionic surfactant may be unstable to long-term storage at elevated temperatures, but may be stable during short-term thermal processing operations. A possible explanation for this phenomenon is that Tween surfactants may chemically degrade (hydrolyze) when stored at elevated

temperatures for prolonged periods and therefore lose their surface activity [211].

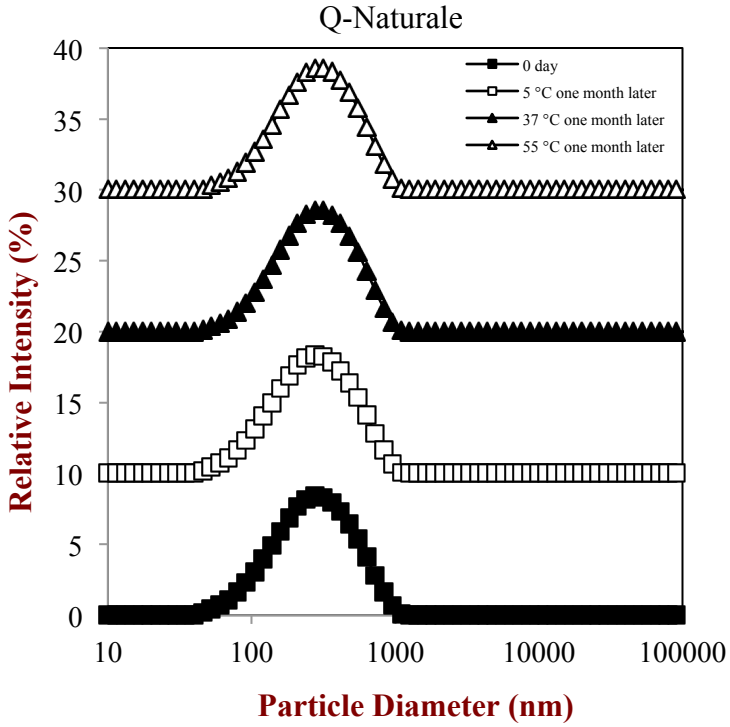


Figure 4.9 Influence of long-term storage (one month) on the particle size distribution of 10 wt% MCT oil-in-water emulsions containing Q-Naturale.

Table 4.1 Particle size changes of emulsions formed by Tween 80 and Q-Naturale respectively after one month storage

		0 day	One month later
Tween 80	5 °C	133 nm	129 nm
	37 °C		132 nm
	55 °C		155 nm
Q-Naturale	5 °C	188 nm	200 nm
	37 °C		204 nm
	55 °C		209 nm

4.4 Conclusions

We have carried out a detailed study of the interfacial, emulsion forming, and emulsion stabilizing properties of Q-Naturale, a natural food-grade surfactant based on quillaja saponin that has recently become commercially available. Q-Naturale was found to be highly surface active, and had similar interfacial properties as Tween 80 a synthetic non-ionic surfactant widely used in the food industry. Q-Naturale-coated droplets had a large negative charge at neutral pH, which decreased in magnitude at acidic pH values, which was attributed to the presence of a carboxylic acid group. Q-Naturale was able to form oil-in-water emulsions with relatively small droplet sizes (d < 200 nm) at low surfactant-to-oil ratios (SOR ≈ 1:10), which were stable to a range of thermal treatments (30 to 90 ºC), salt concentrations (≤ 300 mM NaCl), and pH conditions (pH 3 to 8). In addition, they had good long-term stability (one month) when stored at various holding temperatures (5, 37, and 55 °C). The emulsions were unstable to flocculation and creaming (but stable to coalescence) when stored at highly acidic (pH 2) or high ionic strength (400 or 500 mM NaCl) conditions, which was attributed to the reduction in the electrostatic repulsion operating between the droplets. Overall, our results show that Q-Naturale is an effective natural surfactant that may be suitable for utilization in a variety of food and beverage products.

CHAPTER 5

ENCAPSULATION OF VITAMIN E IN EDIBLE EMULSIONS FABRICATED USING A NATURAL SURFACTANT

5.1 Introduction

There is increasing interest within the food and biomedical industries in the creation of delivery systems to encapsulate, protect, and release lipophilic bioactive compounds, such as ω-3 fatty acids, phytosterols, flavonoids, carotenoids, vitamins, and drugs [3, 212-214]. These delivery systems should be designed so that they can incorporate appreciable amounts of the bioactive component, protect it from physical or chemical degradation during storage, and deliver it to the appropriate site of action after ingestion. They should also be designed so that they are compatible with the delivery vehicle in which they are to be incorporated, *e.g.,* for food and beverage products they should not adversely affect their appearance, stability, texture or flavor. In addition, for food and beverage applications delivery systems must be fabricated entirely from food-grade ingredients using economic and commercially viable processing operations. Emulsion-based delivery systems are particularly suitable for this type of application, since they can be fabricated entirely from generally recognized as safe (GRAS) food ingredients and processing operations [128, 215].

The term "vitamin E" refers to a group of fat-soluble vitamins that are widely used as functional ingredients in food, pharmaceutical, and cosmetic preparations [216].

Vitamin E comes in eight different molecular forms that have a common structural feature: a chromanol ring and a phytol side chain [23]. The different molecular forms of vitamin E are classified as either tocopherols (α, β, γ, and δ) or tocotrienols (α, β, γ, and δ), with α-tocopherol being the most biologically active form [217]. The major biological function of Vitamin E appears to be as an oil-soluble antioxidant [218]. Various other health benefits of vitamin E have also been claimed, such as reducing cardiovascular disease, diabetes and cancer [219-221]. For these reasons, there has been interest in fortifying many processed foods and beverages with Vitamin E [22]. Some studies suggest that the bioavailability of Vitamin E may be increased when it is delivered in colloidal form rather than bulk form [222]. Vitamin E is unstable to oxidation in food many food products and may therefore be lost during processing, storage, and utilization [223, 224]. For this reason, vitamin E acetate (rather than vitamin E) is used in many food and beverage applications since it has a higher oxidative stability. After consumption vitamin E acetate is broken down to vitamin E in the gastrointestinal tract by the action of pancreatic esterases [225]. The recommended daily intake (RDI) of Vitamin E is 15 mg/day, although deficiency only usually occurs in the case of individuals with diseases that inhibit fat absorption or in premature infants [23, 218].

Vitamin E is a highly lipophilic molecule that cannot be directly dispersed into aqueous solutions [22]. Instead, it must be incorporated into an appropriate colloidal delivery system prior to dispersion [23]. A number of previous studies have shown that

vitamin E can be successfully incorporated into emulsion-based delivery systems, such as microemulsions [79, 216], nanoemulsions [80-82] and emulsions [23, 83]. Encapsulation of vitamin E has been reported to improve its physicochemical stability during storage and its biological activity after consumption [226, 227]. In the current study, we focus on the development of oil-in-water emulsions suitable for incorporating vitamin E in functional food and beverage products designed to improve human health and wellness.

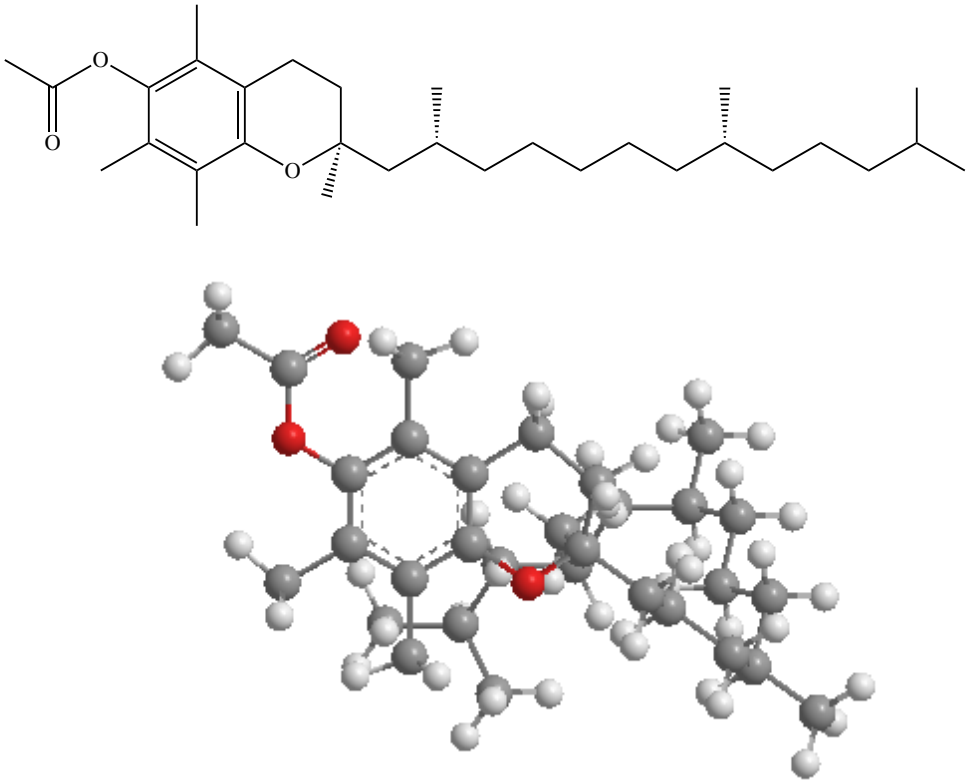


Figure 5.1 Structure of Vitamin E acetate – a highly lipophilic bioactive molecule.

Recently, there has been increasing interest within the food industry in replacing synthetic ingredients with natural "label friendly" alternatives [7, 8]. Consequently, there

has been a focus on identifying and characterizing natural emulsifiers that can be successfully used in emulsion-based food products. In this study, we investigate the emulsifying properties of a recently developed compound extracted from the bark of the *Quillaja saponaria* Molina tree. The major components within quillaja extract have been reported to be saponins [17, 18] , which are high molecular weight glycosides consisting of a sugar moiety attached to a triterpene or a steroid aglycone [19]. The surface activity of saponins arises from the fact that they contain both hydrophilic regions (sugar groups) and hydrophobic regions (such as quillaic and gypsogenic acids) on the same molecule [16, 77]. Saponins have previously been shown to form surfactant micelles in aqueous solutions and to stabilize oil-in-water emulsions [228, 229]. A food ingredient based on a quillaja saponin extract has recently been marketed by the National Starch Company (Bridgewater, NJ) under the trade name Q-Naturale®.

We investigate a number of factors expected to influence the size of the oil droplets formed in oil-in-water emulsions produced by high pressure homogenization: oil phase composition (Vitamin E to medium chain triglyceride (MCT) ratio); aqueous phase composition (glycerol to water ratio); and, surfactant type (Q-Naturale® *versus* Tween 80 ®). Our aim was to identify optimum conditions for fabricating all-natural emulsion-based delivery systems for encapsulating vitamin E. These delivery systems may be suitable for application in a variety of industrial applications, including food, beverage, pharmaceutical, cosmetic, and healthcare products.

5.2 Materials and methods

5.2.1 Materials

The non-ionic surfactant polyoxyethylene (20) sorbitan monooleate (Tween 80) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Quillaja saponin (Q-Naturale® 200) was provided by National Starch LLC (Bridgewater, N.J.). The molecular weights of Tween 80 (Sigma-Aldrich, St. Louis, MO) and quillaja saponin [228] have been reported to be 1310 and 1650 g mol-1, respectively. Medium chain triglyceride (MCT) oil (Miglyol 812) was purchased from Coletica (Northport, NY). Vitamin E acetate (**Figure 1**) was obtained from BASF (Florham Park, NJ).

5.2.2 Emulsion preparation

Oil-in-water emulsions were prepared by homogenizing 10 wt% lipid phase (MCT) with 90 wt% aqueous phase. The aqueous phase consisted of surfactant (1 wt% Tween 80 or Q-Naturale®) and buffer solution (10 mM sodium phosphate buffer, pH 7.0). A coarse emulsion premix was prepared by blending the lipid and aqueous phases together using a high-speed mixer (Bamix, Biospec Products, Bartlesville, OK) for 2 min at room temperature. Fine emulsions were formed by passing the coarse emulsions through an air-driven microfluidizer (Microfluidics, Newton, MA, USA). The coarse emulsions were passed through the homogenization for 4 passes at 9,000 psi. The commercial Q-Naturale® ingredient contained 14 % of surfactant (active ingredient) dispersed within an aqueous solution, and therefore we reported its concentration based

on the amount of active surfactant present (rather than the amount of overall ingredient).

5.2.3 Particle characterization

The particle size distributions of the samples were measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Malvern, UK) after samples had been diluted with buffer solution.

5.2.4 Rheological properties measurements

The rheological behavior of samples was measured using a dynamic shear rheometer (Kinexus Rotational Rheometer, Malvern Instruments, Malvern, U.K). A cup and bob geometry consisting of a rotating inner cylinder (diameter 25 mm) and a static outer cylinder (diameter 27.5 mm) was used in viscosity and oscillation measurements. The samples were loaded into the rheometer measurement cell and allowed to equilibrate at 25 °C for 5 min before beginning all experiments. Shear viscosity (η) measurements were carried out at different shear rates (0.01 to 10 s^{-1}).

5.2.5 Interfacial tension measurements

The interfacial tension was measured at oil – water interfaces using a drop shape analysis instrument (DSA 100, Kruss GmbH, Hamburg Germany).

5.2.6 Turbidity measurement

The turbidity of selected samples was measured using a UV-visible spectrophotometer (Agilent 7010 Particle Size Analyzer, Agilent Technologies. Inc, CA, USA). Samples were diluted in buffer solution to a range of different oil droplet

concentrations, and the turbidity was determined at 600 nm using buffer solution as a blank.

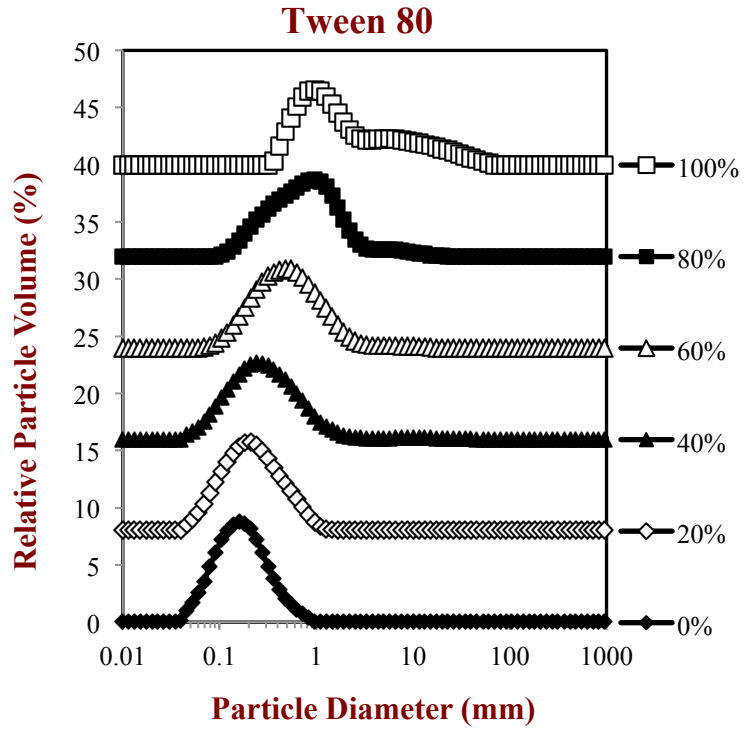
5.2.7 Statistical analysis

All measurements were performed on at least two freshly prepared samples (*i.e.,* new samples were prepared for each series of experiments) and were reported as means and standard deviations.

5.3 Results and discussion

5.3.1 Impact of droplet composition: Vitamin E to MCT ratio

Initially, we examined the influence of oil phase composition on the formation of oil-in-water emulsions by high-pressure homogenization. Ideally, we aimed to produce emulsions containing a high percentage of vitamin E acetate within the oil phase so as to increase their loading capacity. However, vitamin E acetate is an oily material that is difficult to homogenize alone due to its relatively high viscosity. We therefore examined the effect of mixing it with medium chain triglycerides (MCT), which is edible oil with a relatively low viscosity. The influence of oil phase composition on emulsion formation was compared for systems prepared using either Q-Naturale or Tween 80.


Tween 80
Relative Particle Volume (%)
50
45
40
35
30
25
20
15
10
5
0
0.01
0.1
1
10
100
1000
Particle Diameter (mm)
100%
80%
60%
40%
20%
0%

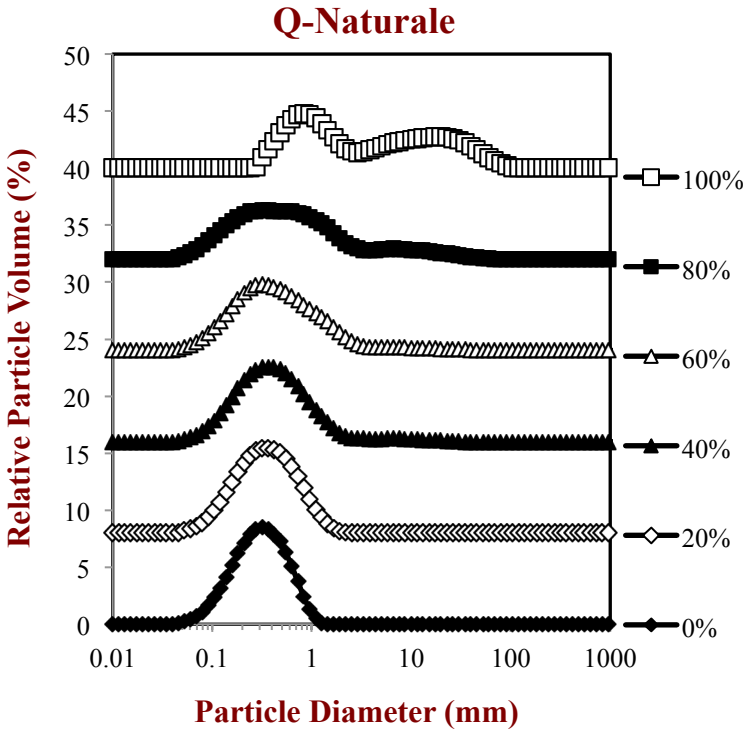

Q-Naturale
Relative Particle Volume (%)
50
45
40
35
30
25
20
15
10
5
0
0.01
0.1
1
10
100
1000
Particle Diameter (mm)
100%
80%
60%
40%
20%
0%

Figure 5.2 Influence of Vitamin E acetate concentration in the oil phase (Vitamin E acetate + MCT) on the particle size distributions of 10 wt% oil-in-water emulsions produced using high pressure homogenization. Emulsions were created using either Tween 80 or Q-Naturale or as surfactant

A series of 10 wt% oil-in-water emulsions with different oil phase compositions were produced using the microfluidizer (9,000 psi, 4 passes) and then the particle size distributions (**Figure 5.2**) and mean particle diameters (**Figure 5.3**) were measured. Monomodal particle size distributions were observed for both surfactant types when the oil phase contained from 0 to 80% vitamin E acetate, *i.e.,* 100 to 20% MCT (**Figure 5.2**). On the other hand, broad bimodal distributions were observed for the two surfactants when the oil phase contained 100% vitamin E acetate. For both surfactants, there was a slight increase in mean droplet diameter (d_{32}) with increasing vitamin E acetate concentration from 0 to 80%, followed by a much steeper increase from 80 to 100% (**Figure 5.3**). Tween 80 appeared to be more effective at forming small droplets at relatively low vitamin E acetate concentrations (≤ 40 wt%), but Q-Naturale was more effective at relatively high vitamin E acetate concentrations (60 - 80 wt%).

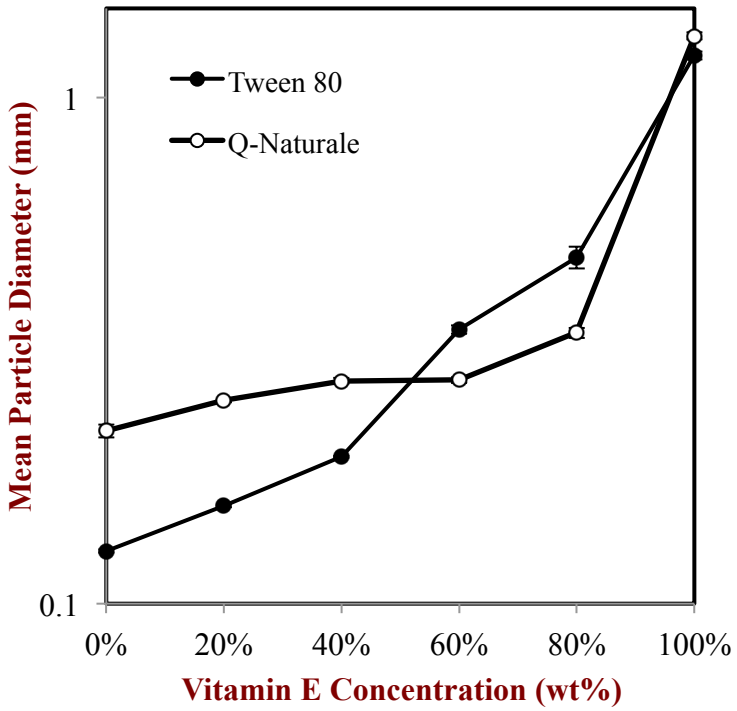


Figure 5.3 Influence of Vitamin E acetate concentration in oil phase (Vitamin E acetate + MCT) on mean particle diameter (d_{32}) of 10 wt% oil-in-water emulsions produced using high pressure homogenization. Emulsions were created using either Q-Naturale or Tween 80 as surfactant

Previous research has shown that droplet disruption becomes more difficult as the viscosity of the disperse phase increases for various types of mechanical homogenizers [201, 230, 231]. This effect can be attributed to the increase in time required for the droplets to become deformed within the disruption zone of a homogenizer as the disperse phase viscosity increases [201, 203]. Hence, more viscous oil droplets are difficult to breakup within a homogenizer because they may leave the disruption zone before they have had time to become deformed and disrupted.

An empirical expression has been proposed to relate measurements of mean droplet diameter (d) to disperse phase viscosity (η_D) for emulsions produced by high pressure homogenizers [232, 233]:

$$d = A\eta_D^B \quad (1)$$

A and B are empirically determined constants, with B being reported to lie between 0.2 and 0.9 for high pressure homogenizers with the actual value depending on the major disruption mechanism involved [232]. To examine the influence of disperse phase viscosity on droplet size we measured the viscosities of a series of oil phases containing different amounts of Vitamin E acetate and MCT (**Figure 5.4**). The shear viscosity of all the oil phases was Newtonian over the shear rates studied (data not shown). The shear viscosity of pure vitamin E acetate ($\eta \approx 2.95$ Pa.s) was over two orders of magnitude higher than that of pure MCT ($\eta \approx 0.028$ Pa.s). The viscosity increased with increasing vitamin E acetate concentration, particularly steeply between 80 and 100%. The relationship between droplet diameter and the disperse phase viscosity for emulsions prepared using the two different surfactants is shown in **Figure 5.5**. The empirical parameters in Equation 1 were calculated from linear regression analysis of the $\log[d]$ *versus* $\log[\eta_D]$ data: $A = 0.681$, $B = 0.476$ for Tween 80; $A = 0.622$, $B = 0.335$ for Q-Naturale. The B values lie within the range reported previously for high pressure homogenizers [232]. The observation that the droplet diameter was somewhat less dependent on disperse phase viscosity for Q-Naturale® than for Tween 80

may be due to a number of physicochemical phenomena. First, since the molecule of Q-Naturale® is a little bigger than Tween 80, Q-Naturale® may have been adsorbed more slowly to the surfaces of droplets than Tween 80 and therefore more droplet re-coalescence may occurre within the homogenizer. Second, Q-Naturale® may have formed a coating around the oil droplets that was more resistant to disruption within the homogenizer, therefore retarding droplet breakup within the disruption zone.

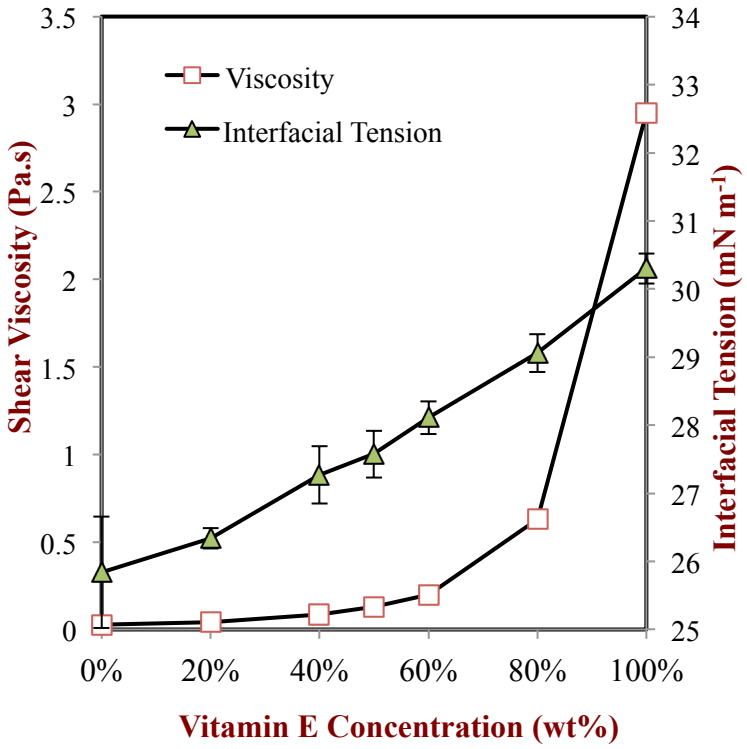


Figure 5.4 Shear viscosities and interfacial tensions of oil phases containing different amounts of vitamin E acetate and MCT. The interfacial tension was measured against buffer solution.

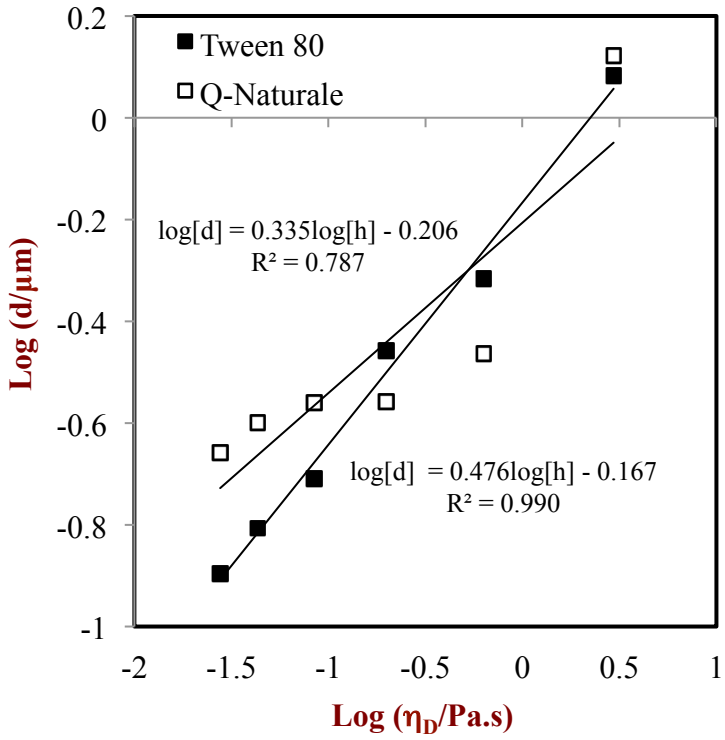


Figure 5.5 Influence of disperse phase viscosity (η_D) on the mean droplet diameter of 10 wt% oil-in-water emulsions produced using high pressure homogenization. Emulsions were created using either Q-Naturale or Tween 80 as surfactant.

Interfacial tension also plays an important role in the formation of small droplets during homogenization, since it is proportional to the force required to deform and disrupt droplets within the homogenization zone [234, 235]. We therefore measured the interfacial tension at the oil-water interface of oil phases with different compositions (**Figure 5.4**). The interfacial tension increased appreciably as the Vitamin E acetate concentration in the oil phase increased, changing from ≈ 25.8 mN m^{-1} for pure MCT to ≈ 30.3 mN m^{-1} for pure vitamin E acetate. This increase suggests that vitamin E acetate is a more hydrophobic molecule than MCT, *i.e.,* there is a greater free energy penalty to

increase the oil-water interface. The observed increase in mean droplet diameter with increasing vitamin E acetate concentration in the oil phase may therefore be partly due to this increase in interfacial tension, since this would make it harder to disrupt the droplets in the homogenizer. However, one also has to consider how quickly the surfactants adsorb to the oil-water interface during homogenization, since this will determine the actual interfacial tension at the droplet surfaces.

5.3.2 Impact of aqueous phase properties on particle size

The size of the droplets in an oil-in-water emulsion produced using high pressure homogenization also depends on aqueous phase properties, such as viscosity and interfacial tension. Previous studies have found that smaller droplets can be formed when the disperse-to-continuous phase viscosity ratio (η_D/η_C) is close to unity and the disperse-to-continuous phase interfacial tension is lowered [60, 199, 236]. We therefore examined the possibility of producing smaller vitamin E droplets by changing the composition of the aqueous phase to alter the viscosity ratio and interfacial tension. And this aim was achieved by adding different amounts of glycerol to the aqueous phase.

The shear viscosity of the aqueous phase increased as the glycerol concentration increased, with their being over a 5-fold increase when the glycerol level was raised from 0 to 50% (**Figure 5.6**). This effect can be attributed to the fact that the viscosity of pure glycerol is much higher than that of pure water. On the other hand, there was a decrease in the interfacial tension of the aqueous phase as the glycerol concentration increased

(**Figure 5.7**), which occurs because glycerol is less polar than water.

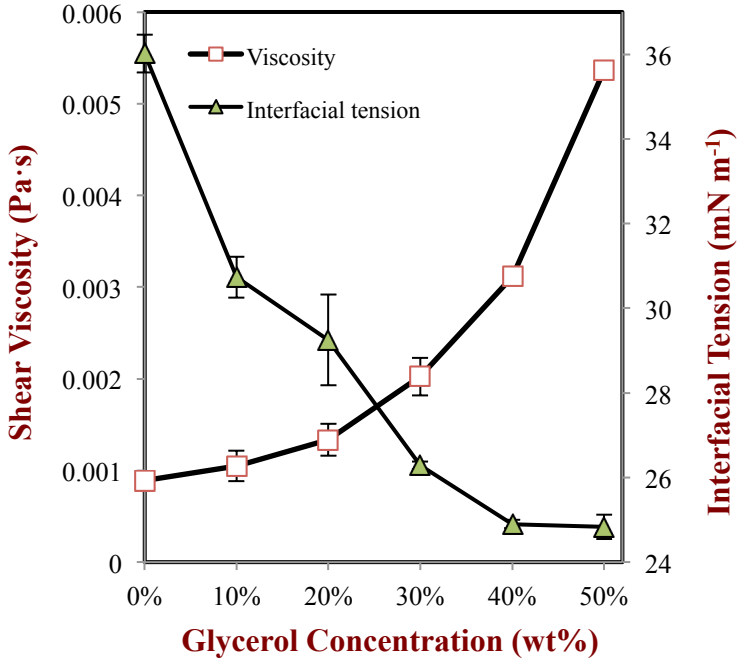


Figure 5.6 Shear viscosities and interfacial tensions of aqueous phases containing different glycerol concentrations. The interfacial tension was measured between an oil phase (60 % Vitamin E: 40% MCT) and aqueous phase

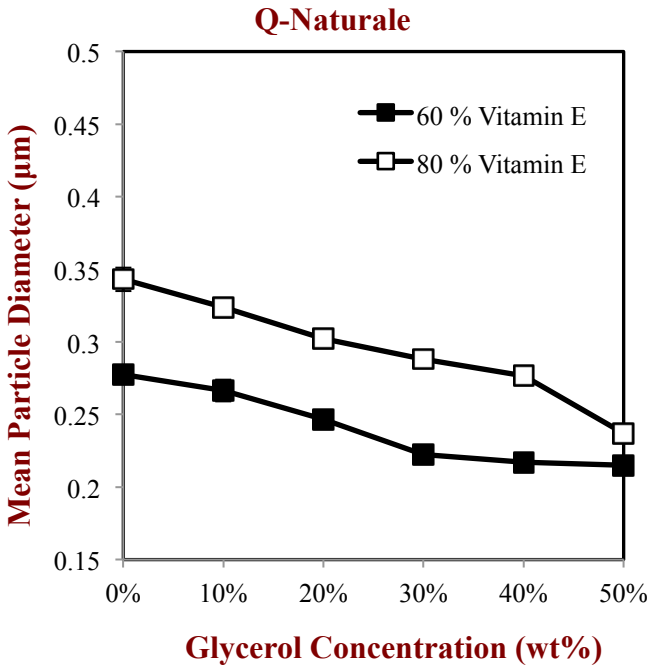


Figure 5.7a Influence of aqueous phase concentration (glycerol:water) on the mean particle diameter of 10 wt% oil-in-water emulsions (pH 7) stabilized by 1 wt% Q-Naturale.

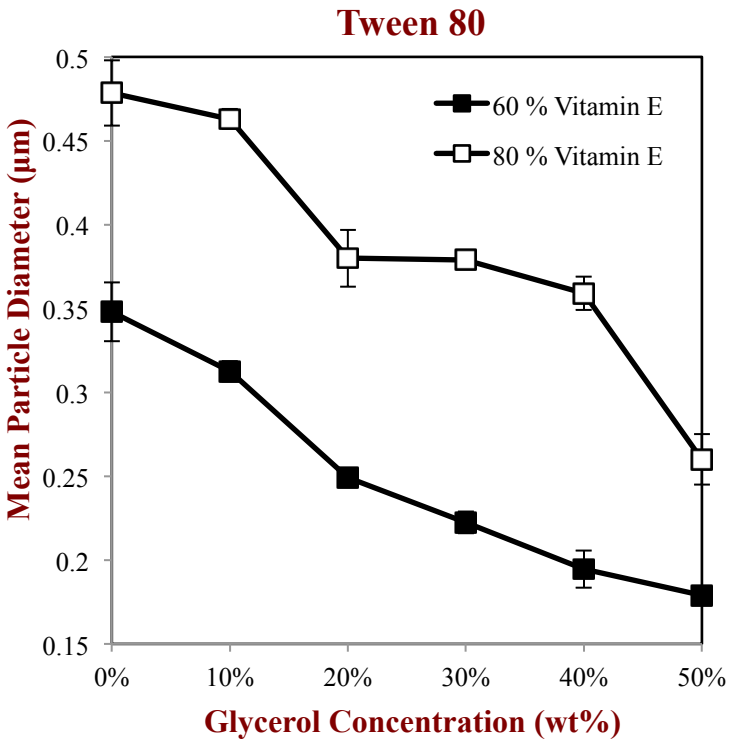


Figure 5.7b Influence of aqueous phase concentration (glycerol:water) on the mean particle diameter of 10 wt% oil-in-water emulsions (pH 7) stabilized by 1 wt% Tween 80.

The influence of aqueous phase properties on droplet size was studied by preparing a series of 10 wt% oil-in-water emulsions with the same oil phase compositions (60 or 80% vitamin E acetate) and same concentrations of emulsifier (1 wt% Tween 80 or Q-Naturale), but different aqueous phase compositions (0 to 50% glycerol) using fixed homogenization conditions (9,000 psi, 4 passes). For all systems studied, there was a decrease in the mean particle diameter with increasing glycerol concentration (**Figure**

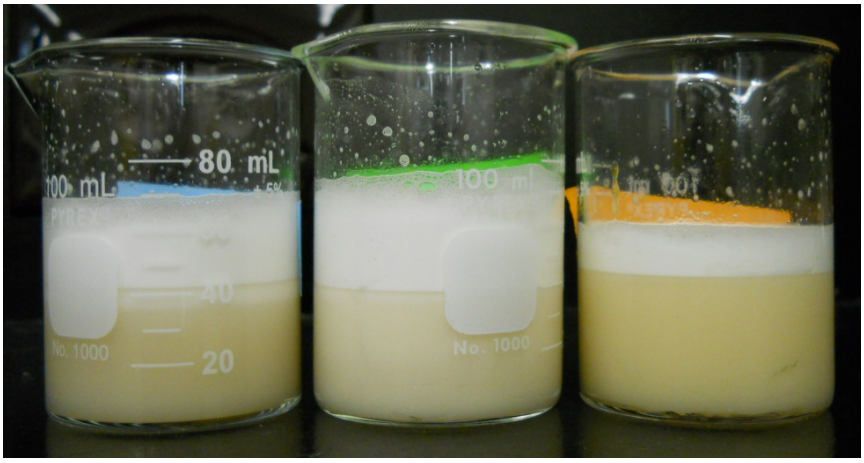
5.8). This decrease can be attributed to two different effects of glycerol on the physicochemical properties of the aqueous phase: as the glycerol concentration increases, the viscosity ratio decreases and the interfacial tension decreases, both of which favor the formation of smaller droplets. At the same glycerol concentration, smaller droplets were produced using Q-Naturale than using Tween 80, which supports the experiments described in Section 3.1 at high vitamin E acetate concentrations. Smaller droplets were produced when the dispersed phase contained 60% rather than 80% vitamin E acetate, which can be attributed to the lower viscosity of the former system facilitating droplet disruption within the homogenizer (**Section 5.3.1**).

An expression similar to Equation 1 can be used to model the influence of disperse phase viscosity on droplet size produced by high pressure homogenization [233]:

$$d = A'\eta_C^{B'} \quad (2)$$

Here A' and B' are empirical constants and η_C is the continuous phase viscosity. In homogenizers where droplet disruption is primarily due to turbulence, droplet size is not believed to be strongly dependent on continuous phase viscosity, *i.e.,* $B' < -0.03$ [237]. On the other hand, in homogenizers where shear forces play an important role in droplet disruption, the droplet diameter should decrease with increasing continuous phase viscosity [203]. In this study, we found $B' \approx -0.32$ for Tween 80 and ≈ -0.13 for Q-Naturale (at 60% vitamin E acetate) and $B' \approx -0.30$ for Tween 80 and ≈ -0.18 for Q-Naturale (at 80% vitamin E acetate). The fact that the B' values are appreciably

smaller than -0.03 suggests that shear forces played an appreciable role in droplet disruption [203]. The emulsions containing Q-Naturale as a surfactant appeared to be less sensitive to changes in continuous phase viscosity than those containing Tween 80. The values measured in this study are in reasonable agreement with those reported previously of $B' \approx -0.24$ for an anionic surfactant (SDS) using a similar homogenizer [236].



Q-Naturale **Q-Naturale + Glycerol** **Q-Naturale + Glycerol + Lysolecithin**

Figure 5.8 Foaming problem observed during emulsion preparation when Q-Naturale® was used solely as a surfactant. From left to right: Images of 10 wt% oil-in-water emulsions prepared using Q-Naturale dissolved in buffer solution, buffer + 50% glycerol, or buffer + 50% glycerol + 0.1% lecithin, respectively.

Overall, the ability to produce smaller droplets at higher glycerol concentrations can be attributed to a number of different physicochemical mechanisms [28, 201]. First, ease of droplet disruption within the homogenizer decreases as the viscosity ratio tends

towards unity [238]. Second, the amount of re-coalescence occurring within the homogenizer decreases as the continuous phase viscosity increases since this retards droplet encounters [45, 231]. Third, the ease of droplet disruption decreases as the interfacial tension decreases since the Laplace pressure resisting droplet deformation is reduced [60].

Despite the advantages of using glycerol to produce smaller droplets in the Q-Naturale stabilized emulsions, we should note a practical difficulty that we encountered that could limit its commercial application. We found that adding glycerol to the continuous phase caused foaming of the emulsions containing Q-Naturale®, which sometimes caused the homogenizer to be become blocked. This effect was attributed to the ability of the high viscosity aqueous phase to trap air bubbles formed during the mixing and homogenization stages. We observed that this effect could be greatly reduced by adding a small amount of lysolecithin (0.1%) as an antifoaming agent to the aqueous phase prior to homogenization (**Figure 5.8**).

5.3.3 Influence of viscosity ratio on particle size

Our previous experiments indicated that both the dispersed phase and continuous phase viscosities had a major impact on the size of the droplets formed. In this section, we directly examined the influence of the viscosity ratio (η_D/η_C) on the droplet size produced during homogenization. The mean droplet diameters were plotted against the viscosity ratio for the various emulsions studied (**Figure 5.9**). We included data for

emulsions that had different oil phase compositions (60 % & 80 % Vitamin E) as well as different aqueous phase compositions (0 to 50 wt% glycerol). All the systems studied were 10 wt% oil-in-water emulsions stabilized by 1 wt% emulsifier prepared using fixed homogenization conditions (9,000 psi, 4 passes).

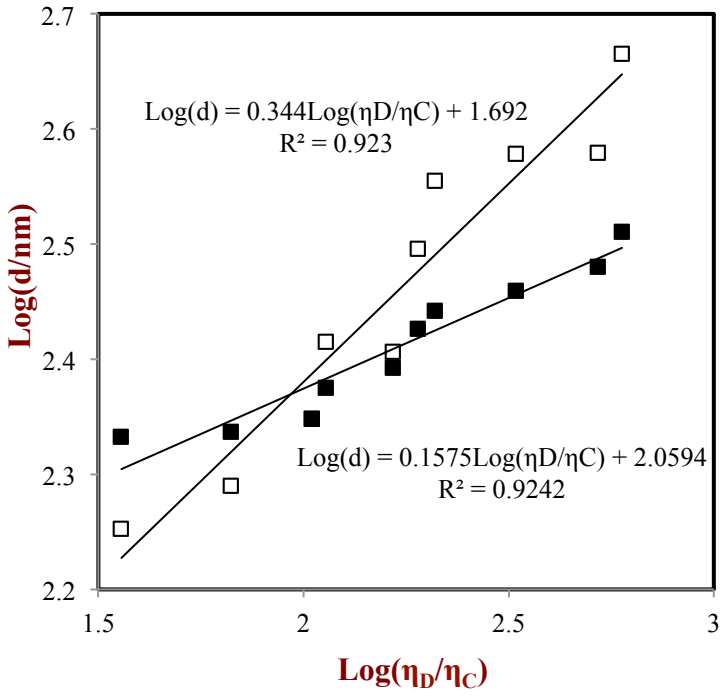


Figure 5.9 Influence of ratio of oil-to-aqueous phase viscosity ratio on the mean particle diameter for 10 wt% oil-in-water emulsions (pH 7) prepared by high pressure homogenization (9,000 psi, 4 passes) for systems stabilized by 1 wt% Tween 80 or Q-Naturale.

Previous studies suggest that there is an optimum range of disperse-to-continuous phase viscosity ratios ($0.1 < \eta D/\eta C < 5$) for producing small droplets in various kinds of homogenizers [201, 239, 240]. In this study, we were able to cover a viscosity ratio

ranging from about ηD/ηC = 35 to 600 by in the vitamin E acetate emulsions. It was not possible to reach lower viscosity ratios due to the very high viscosity of the disperse phase. The slope of a log(d) versus log(ηD/ηC) was around 0.34 for emulsions containing Tween 80 and around 0.16 for emulsions containing Q-Naturale® (**Figure 5.9**). These results again highlight that emulsions containing Tween 80 are more sensitive to viscosity changes of the dispersed and continuous phases than those containing Q-Naturale®. The smallest droplets that we could prepare were for emulsions containing 60 % Vitamin E in the oil phase and 50% glycerol in the aqueous phase: *d* = 179 nm for Tween 80 and *d* = 215 nm for Q-Naturale®. The smallest droplets that we could prepare for emulsions containing 80 % Vitamin E in the oil phase and 50% glycerol in the aqueous phase were: *d* = 260 nm for Tween 80 and *d* = 237 nm for Q-Naturale®. These droplet sizes are relatively small and should lead to the formation of stable colloidal dispersions of vitamin E. Indeed, storage tests showed no increase in the mean particle diameter of any of the emulsions after being kept at ambient temperature for one month (data not shown).

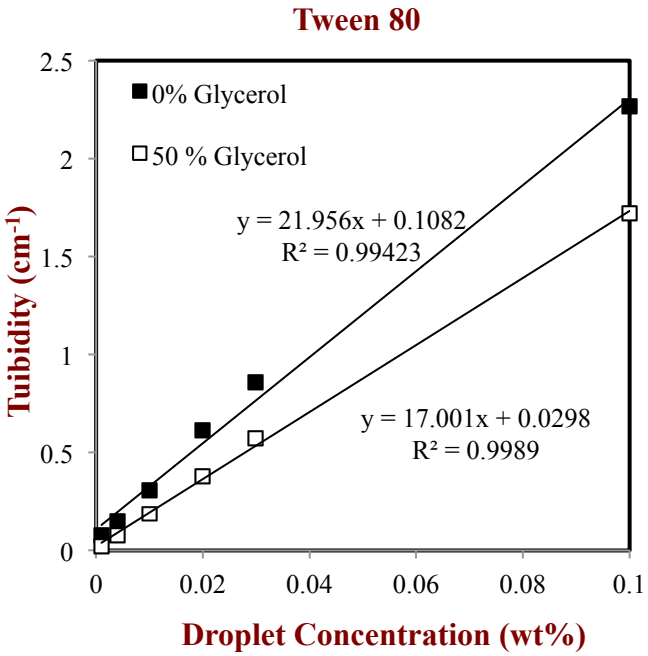
5.3.4 Optical properties of emulsions

For many practical applications, the optical properties of emulsion-based delivery systems are important. For example, for the application in clear beverage, it is necessary to use an optically transparent delivery system, whereasto opaque foods this is unimportant and to impart cloudiness to some soft drinks it may even be desirable to

have a turbid delivery system. The overall appearance of emulsion-based products is determined by the way in which they interact with light waves through absorption and scattering phenomenon [241, 242].

In this section, the optical properties of the vitamin E emulsions were characterized in terms of their turbidity increments, *i.e.,* the increase in turbidity per unit increase in droplet concentration. Emulsions were prepared using 60 wt% Vitamin E in the oil phase and either 0 % or 50 % glycerol in the aqueous phase, using both Tween 80 and Q-Naturale. The turbidity of these emulsions was then measured at 600 nm as a function of droplet concentration (**Figure 5.10**). These emulsions were diluted with buffer solution (at least 1:100) so that the final glycerol concentration (< 0.5%) would not affect the refractive index of the aqueous phase and therefore the overall light scattering characteristics of the final emulsions. For both surfactants, the turbidity increment was higher for emulsions containing 0% glycerol than those containing 50% glycerol during homogenization, which can be attributed to differences in droplet size. The emulsions prepared at higher glycerol concentrations had smaller droplet sizes, which therefore scattered light less strongly in this particle size range [167]. The differences in the turbidity increments between emulsions containing 0% and 50% glycerol were greater for Tween 80 than for Q-Naturale, which is because the size of the droplets in Tween 80 emulsions was more sensitive to changes in continuous phase composition (Section 3.2). At 0% glycerol, the turbidity increment was higher for Tween 80 (d = 0.35 μm) than for

Q-Naturale (d = 0.28 μm) emulsions because Tween 80 produced the largest droplets. On the other hand, at 50% glycerol the turbidity increment was higher for Q-Naturale (d = 0.22 μm) than for Tween 80 (d = 0.18 μm) emulsions because Tween 80 produced the smallest droplets. Thus, there was a close correlation between the particle size data and the turbidity data for these systems.



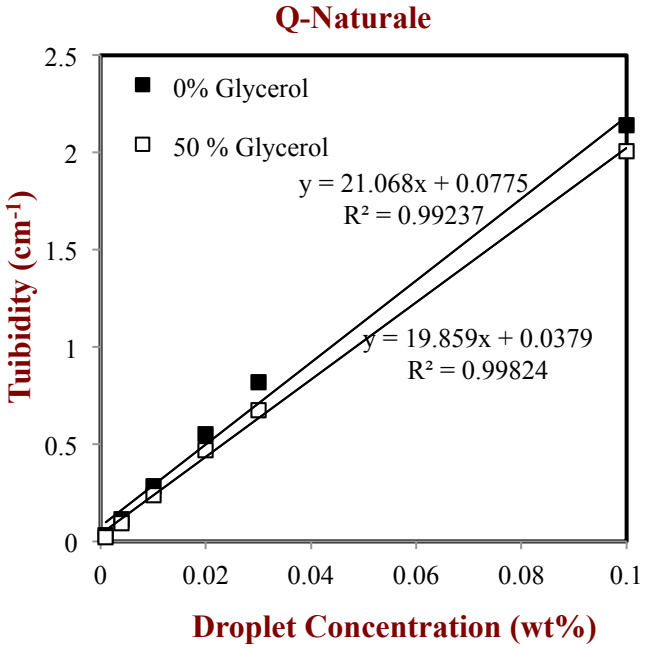


Figure 5.10 Impact of glycerol content and surfactant type on turbidity of 10 wt% oil-in-water emulsions diluted to different droplet concentrations (1 wt% Tween 80 or Q-Naturale, 9,000 psi, 4 passes).

To prepare beverage emulsions that appear visually transparent the turbidity should be < 0.05 cm^{-1} [243]. The turbidity increments reported in **Figure 5.10** can be used to estimate the oil droplet concentrations required to reach this level: 0.0018 and 0.0022 wt% for Q-Naturale and 0.0017 and 0.0026 wt% for Tween 80, for emulsions prepared from aqueous phases initially containing either 0 or 50% glycerol, respectively. These emulsions contained 60% vitamin E acetate in the oil phase, and so the maximum amount of the vitamin that can be incorporated into a transparent beverage is around 0.0010 to 0.0016%. If we assume that the serving size for a typical beverage product is 8 ounces (240 mL), then this amount would correspond to about 2.4 to 3.8 mg of vitamin

E acetate per serving. The daily recommended intake of vitamin E for adults is 15 mg [218], and therefore it should be possible to prepare clear beverages containing biologically active amounts of vitamin E using the emulsions described in this work.

5.4 Conclusions

The purpose of this study was to determine whether a natural surfactant (Q-Naturale®) based on quillaja saponin isolated from tree bark could be used to form and stabilize oil-in-water emulsions containing vitamin E acetate. We compared the performance of Q-Naturale® with a synthetic non-ionic surfactant that is currently used in the food, pharmaceutical and cosmetic industries to form emulsions (Tween 80). Tween 80 was more effective at producing small droplets at relatively low vitamin loadings (≤ 40%), whereas Q-Naturale® was more effective at relatively high vitamin loadings (60% - 80%). The size of the droplets in the emulsions could be reduced by decreasing the vitamin concentration in the oil phase, or increasing the amount of glycerol in the aqueous phase. These effects were attributed to changes in the viscosities and interfacial tensions of the dispersed and continuous phases, since these parameters are known to influence droplet disruption and coalescence during homogenization. This study has important implications for the development of "label friendly" delivery systems for incorporating lipophilic nutraceuticals (such as oil-soluble vitamins) into foods and beverages, as well as other commercial products.

CHAPTER 6

VITAMIN E BIOACCESSIBILITY: INFLUENCE OF CARRIER OIL TYPE ON DIGESTION AND RELEASE OF EMULSIFIED ALPHA-TOCOPHEROL ACETATE

6.1 Introduction

Vitamin E is known to be an essential micronutrient for maintaining the health and wellbeing of humans and other animals due to its antioxidant and non-antioxidant biological activities [244-246]. Nevertheless, the precise biological mechanisms by which it exerts its beneficial effects are still unclear, and are currently the focus of extensive research efforts [246, 247]. The term "vitamin E" actually refers to a group of related fat-soluble molecules that are found in many natural sources and are widely used as functional ingredients in food, pharmaceutical, and cosmetic preparations [216, 248]. The most important biologically active form of Vitamin E is α-tocopherol, and therefore this form tends to be used in commercial products. There has been considerable interest in fortifying foods, beverages, and supplements with vitamin E due to its beneficial nutritional qualities [215, 249]. However, there are a number of challenges associated with incorporating vitamin E into commercial products due to its chemical instability, poor water-solubility, and variable bioavailability.

The α-tocopherol form of Vitamin E is highly unstable to oxidation and may therefore be lost during the processing, storage, and utilization of commercial products

due to its chemical degradation [223, 224]. For this reason, a more chemically stable esterified form (α-tocopherol acetate) is typically used in commercial products rather than the free form [250]. Another challenge associated with the utilization of vitamin E is that it is a highly lipophilic molecule with a low very water-solubility, which means that it cannot be directly dispersed into aqueous media [163, 251]. Instead it must either be dispersed into a lipid phase or incorporated into a suitable delivery system [23, 215]. A well-designed delivery system can encapsulate lipophilic nutraceuticals in a form that that does not adversely affect product quality (such as appearance, taste, texture, or stability), protect them from chemical degradation during storage, and increase their bioavailability after ingestion [215, 252]. A wide variety of delivery systems have been developed for encapsulating lipophilic nutraceutical ingredients, including microemulsions, nanoemulsions, emulsions, solid lipid nanoparticles, and polymer particles [128, 163, 212, 253, 254]. Each of these delivery systems has specific advantages and disadvantages related to the nature of the nutraceutical to be encapsulated, the food matrix that it is to be dispersed within, and the processing, storage, and transport conditions it is exposed to.

After ingestion, it is important that a nutraceutical component has a high bioavailability so that it can effectively deliver its beneficial biological effects. Previous studies have shown that the bioavailability of vitamin E may be highly variable (ranging from < 1% to 100%) depending on its chemical form and the nature of the food matrix it

is incorporated within [119, 255]. Typically, vitamin E must be first released from the food matrix, then incorporated into mixed micelles, and then transported across the mucous layer before it is absorbed by epithelium cells in the small intestine [248, 256]. The mixed micelles are comprised of bile salts and phospholipids from the bile and pancreatic juices, as well as lipid digestion products (free fatty acids and monoacylglycerols) from the action of lipases on ingested triacylglycerol oils [257]. The bioaccessibility of lipophilic nutraceuticals and drugs has previously been shown to increase as the total amount of mixed micelles increases, as well as to depend on nature of the mixed micelles formed after lipid digestion [257-259], which may account for some of the observed variations in vitamin E bioavailability. It has also been reported that the esterified form of vitamin E (α-tocopheryl acetate) is less bioaccessible than the free form (α-tocopherol) [260-263], and so it has to be converted into α-tocopherol by digestive enzymes (*e.g.,* pancreatic esterases) within the gastrointestinal tract prior to absorption [244, 264]. However, studies using an *in vitro* digestion model have shown that α-tocopheryl acetate may be absorbed by Caco-2 cells to a similar extent as α-tocopherol [225]. Some of the observed variations in vitamin E bioaccessibility may therefore also be due to differences in the chemical nature of the ingested form, *e.g.,* free *versus* esterified. The wide variability reported in the bioavailability of vitamin E makes it difficult to interpret the results of clinical trials on the efficacy of vitamin E supplementation, and to develop appropriate guidelines on the consumption levels of

vitamin E [248]. The development of suitable delivery systems for vitamin E may be able to overcome some of the challenges associated with its varying bioavailability.

In this study, we utilized an emulsion-based delivery system fabricated from a natural surfactant (quillaja saponin) that we have recently shown to be suitable for encapsulating vitamin E acetate [265]. There is a major drive in the food industry towards developing consumer friendly labels *e.g.,* "all natural". There are few natural surfactants currently available for utilization within the food industry. Hence, the availability of a natural surfactant to form and stabilize emulsions would be an advantage for many functional food and beverage applications. Our primary focus was to examine the influence of carrier oil composition (medium chain *versus* long chain fatty acids) on the bioaccessibility of vitamin E acetate using an *in vitro* gastrointestinal tract (GIT) model, since previous studies have shown that the nature of the ingested fatty acids influences the bioaccessibility of lipophilic nutraceuticals and drugs [266-269]. We measured the change in the microstructure and properties of vitamin E-enriched fat droplets throughout the simulated GIT (mouth, stomach, and small intestine)C. We also monitored the bioaccessibility and conversion of α-tocopherol acetate to α-tocopherol after the small intestine stage. The results of this study provide valuable information about the potential biological fate of ingested emulsion-based delivery systems for vitamin E, as well as their potential for controlling the bioavailability of this important nutrient in functional foods and beverages.

6.2 Materials and methods

6.2.1 Materials

Quillaja saponin (Q-Naturale® 200) was provided by National Starch LLC (Bridgewater, NJ). Corn oil (Mazola, ACH Food Companies, Inc., Memphis, TN) was used as an example of a long chain triglyceride (LCT) and was purchased from a local supermarket. Corn oil we used in our experiment contains 2 g saturated fat, 8 g polyunsaturated fat and 4 g monounsaturated fat in every serving size (14 g). Medium chain triglyceride (MCT) oil (Miglyol 812) was purchased from Coletica (Northport, NY). Vitamin E acetate was kindly supplied by BASF (Florham Park, NJ). Lipase (from porcine pancreas pancreatin, activity>2.0 USP units/mg, Type II, L3126, Batch # 020M1589) and bile extract (porcine) were also obtained from the Sigma Chemical Company (St. Louis, MO). Type II lipase contains amylase and protease, as well as lipases. Lipase activity is one unit will hydrolyze 1.0 microequivalent of fatty acid from triacetin in 1 hr at pH 7.4 at 37°C. All other chemicals used were of analytical grade. Double distilled water was used for the preparation of all solutions and emulsions. Double distilled water was got by using Milli-Q®.

6.2.2 Emulsion preparation

Oil-in-water emulsions were prepared by homogenizing 10 wt% lipid phase and 90 wt% aqueous phase. The lipid phase consisted of vitamin E acetate dispersed in either corn oil (LCT) or medium chain triglyceride oil (MCT). The aqueous phase

consisted of surfactant (1 wt% Q-Naturale®) and buffer solution (10 mM sodium phosphate buffer, pH 7.0). A coarse emulsion premix was prepared by blending the lipid and aqueous phases together using a high-speed mixer (Bamix, Biospec Products, Bartlesville, OK) for 2 min at room temperature. Emulsions containing fine droplets were formed by passing the coarse emulsions through an air-driven microfluidizer (Microfluidics, Newton, MA, USA) four times at 9,000 psi. The Q-Naturale® ingredient contained 14 % of surfactant (active ingredient) dispersed within an aqueous solution, and therefore we reported its concentration based on the amount of active surfactant present (rather than the amount of overall ingredient).

6.2.4 Particle characterization

The particle size distributions of the samples were measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Malvern, U.K.). The droplet charge (ζ-potential) of emulsions was determined using a particle electrophoresis instrument (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, UK). Samples were diluted with buffer solutions of the appropriate pH prior to analysis to avoid multiple scattering effects.

6.2.5 *In vitro* digestion

Each emulsion sample was passed through a three-step simulated GIT model, which includes a mouth, gastric, and small intestine stage.

Mouth stage: Simulated saliva fluid (SSF) was prepared according to a

previous study [270]. 10 mL of emulsion (1 w/w% oil) was placed in a 250 mL flask containing 10 mL of SSF. This mixture was adjusted to pH 6.8 and then shaken continuously at the rate of 100 rpm in a temperature controlled incubator (37 ºC) for 10 min (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA).

Gastric stage: Simulated gastric fluid (SGF) was prepared by placing 2 g NaCl, 7 mL HCl, and 3.2 g pepsin into a flask, adding water to make up to 1 L, and then adjusting the pH to 1.2 using 1.0 M HCl (United States Pharmacopeial Convention, 2000). The sample from the mouth stage was then mixed with SGF at a 1:1 mass ratio so that the final mixture contained 0.5 % (w/w) oil. The mixture was then adjusted to pH 2.5 by using 1M NaOH and incubated with continuous shaking at 100 rpm at 37 ºC for up to 2 h to mimic stomach conditions.

Small intestinal stage: Digesta samples (30 mL) from the simulated gastric stage were added to a clean beaker, then incubated in a water bath (37 ºC) for 10 min, and then adjusted to pH 7 using NaOH solution (range from 0.05 to 1 M). The mixture was then incubated for 2 h at 37 ºC with simulated small intestinal fluid (SIF) containing 2.5 mL pancreatic lipase (4.8 mg mL^{-1}), 4 mL bile extract solution (5 mg mL^{-1}) and 1 mL calcium chloride solution (750 mM). A pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at pH 7.0) of the digestion solution after the SIF was added [271]. The amount of alkali solution (0.25 M NaOH) that had to be added to the reaction

chamber to maintain the pH at 7.0 was recorded, and used to determine the percentage of free fatty acids (FFA) released from the system [148]. Samples were also taken for physicochemical and structural characterization after 2 h *in vitro* digestion in the small intestinal stage.

6.2.6 Bioaccessibility Determination

The bioaccessibility of lipophilic bioactive components is normally defined as the fraction that is solubilized within the mixed micelle phase after lipid digestion [119, 272]. After the full *in vitro* digestion was completed, 10 ml of sample was collected and centrifuged (4000 rpm, Thermo Scientific, CL10 centrifuge) at 25 ºC for 40 min. The emulsions separated into an opaque sediment phase at the bottom, a clear micelle phase in the middle, and sometimes a thin oily or creamed phase at the top. An aliquot (0.5 ml) of the micelle phase or the raw digesta was vortexed with an organic solvent mixture (1:3 isopropanol and isooctane) at 1:5 to extract the vitamin E and then centrifuged at 1750 rpm for another 10 min. 1 mL of the top layer was removed and dried using a vacuum centrifuge drier and stored in the -80 ºC refrigerator prior to further analysis. Before detection by HPLC, samples were dissolved in 200 μL methanol.

HPLC Detection: Vitamin E concentrations in the samples were determined using HPLC (Shimadzu, Kyoto, Japan). A C18 reverse-phase column (150 - 4.6 mm, 5 μm, Beckman Coulter) was used for the chromatographic separation of α-tocopherol acetate and α-tocopherol. The flow rate of the mobile phase was 1.0 mL/min and the

sample injection volume was 20 μL. An isocratic elution was carried out using HPLC-grade solvent (95 % methanol and 5 % double distilled water containing 0.5 % phosphoric acid). The α-tocopherol acetate and α-tocopherol contents were determined using a PDA detector at 295 nm. Tocopherol quantification was determined using external standards. The overall bioaccessibility of vitamin E was estimated using the following expression:

$$Bioaccessibility = \frac{C_{micelle}}{C_{Total}} \times 100\% \quad (1)$$

Here C_{micelle} and C_{Total} represent the total concentration of vitamin E (α-tocopherol acetate + α-tocopherol) in the micelle phase and in the overall system after digestion, respectively. The percentage of specific forms of vitamin E solubilized within the micelle phase was also calculated using the same expression, but for α-tocopherol acetate and for α-tocopherol separately. The conversion of α-tocopherol acetate to α-tocopherol in the overall system after digestion was calculated from the following expression:

$$Conversion = \frac{C_{VE}}{C_{Total}} \times 100\% \quad (2)$$

Here C_{VE} and C_{Total} represent the concentration of α-tocopherol and the total concentration of vitamin E (α-tocopherol acetate + α-tocopherol) in the overall system after digestion, respectively.

6.2.7 Statistical analysis

All measurements were performed on at least two freshly prepared samples (*i.e.,*

new samples were prepared for each series of experiments) and are reported as means and standard deviations.

6.3 Results and discussion

6.3.1 Impact of carrier oil type on biological fate of emulsions

6.3.1.1 Influence on particle size

Initially, we studied the impact of vitamin E encapsulation and carrier oil type on the potential biological fate of emulsion-based delivery systems using a simulated gastrointestinal tract (GIT) model. A series of emulsions containing 2.5 wt% lipid phase were produced using either long chain triglycerides (LCT) or medium chain triglycerides (MCT). Emulsions containing lipid phases with four different compositions were prepared: LCT (100% LCT); vitamin E in LCT (25% α-tocopherol acetate + 75% LCT); MCT (100% MCT); and, vitamin E in MCT (25% α-tocopherol acetate + 75% MCT).

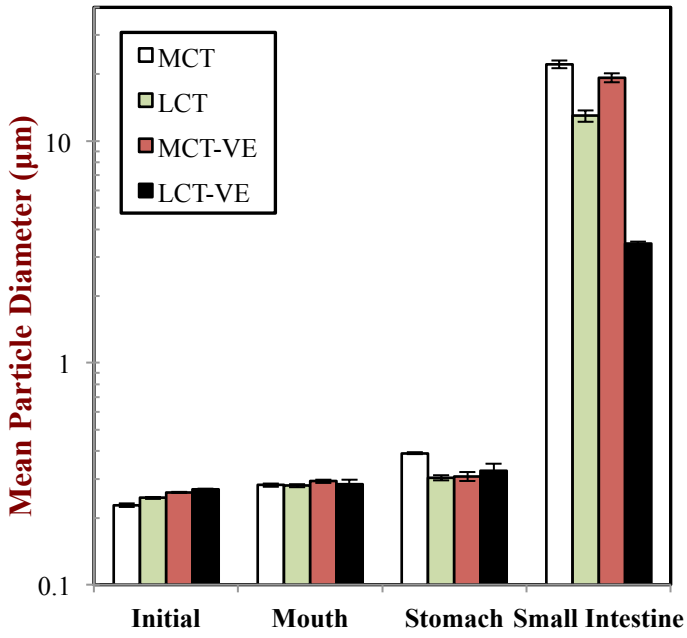


Figure 6.1a Influence of vitamin E encapsulation and carrier oil type on the mean particle diameter (d_{32}) of oil-in-water emulsions passing through different stages of a simulated gastrointestinal tract. *Key:* LCT = 100% LCT; LCT-VE = 25% α-tocopherol acetate + 75% LCT; MCT = 100% MCT; MCT-VE = 25% α-tocopherol acetate + 75% MCT.

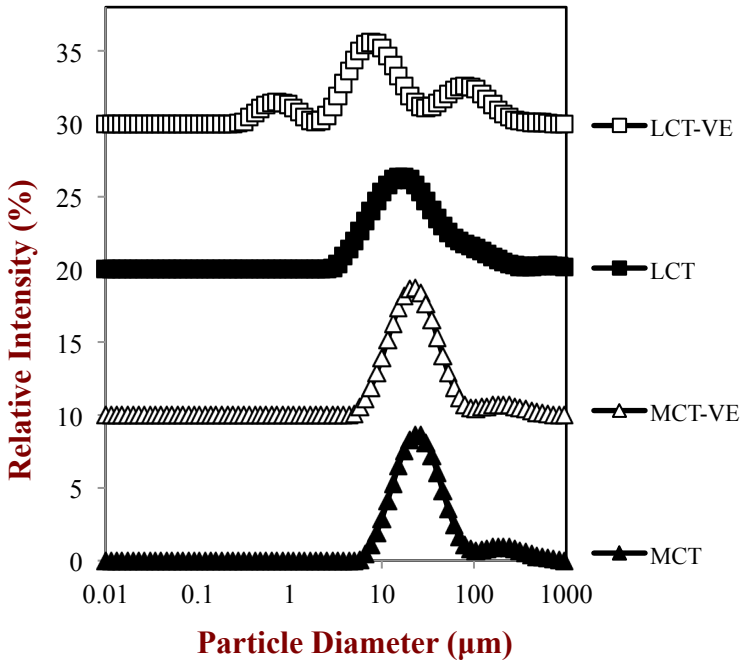


Figure 6.1b Influence of vitamin E encapsulation and carrier oil type on the particle size distributions of emulsions after passage through the small intestinal stage of a simulated gastrointestinal model. See caption to **Figure 6.1a** for *Key*.

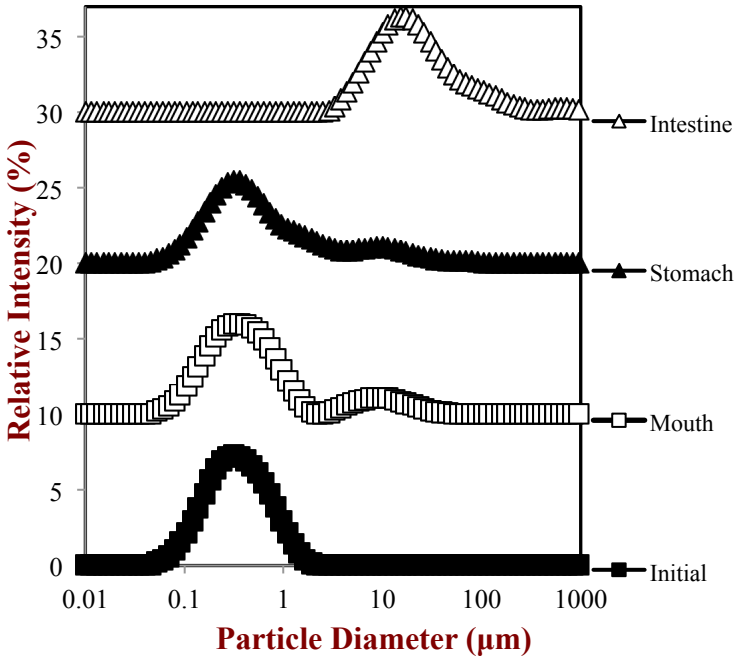


Figure 6.1c Particle size distributions of emulsions containing LCT at different stages of a simulated gastrointestinal tract.

The influence of vitamin E encapsulation and carrier oil type on the mean particle diameter after each stage of the simulated GIT model was measured (**Figure 6.1a**). The full particle size distributions of selected samples were also compared: (i) emulsions containing different carrier oil types after the small intestine stage (**Figure 6.1b**); emulsions containing LCT at different stages of the GIT model (**Figure 6.1c**). All the initial emulsions had monomodal particle size distributions and relatively small mean particle diameters (d_{32} = 228 to 270 nm). The droplets in the LCT emulsions were slightly larger than those in the MCT emulsions, and the droplets in the emulsions containing vitamin E were slightly larger than those containing no vitamin E, which may have been due to differences in oil phase viscosities. The size of the droplets produced by high pressure homogenization is known to increase as the viscosity of the oil phase increases [60, 273]. After passage through the simulated mouth and gastric stages, the mean particle diameter remained relatively small (d_{32} < 400 nm), suggesting that the oil droplets were fairly stable to aggregation under these conditions (**Figures 6.1a** and **6.1c**). Recent studies have shown that oil droplets coated by the same quillaja saponin extract used in this work (Q-Naturale®) were relatively stable to changes in pH and salt concentration [274]. Any saponin molecules adsorbed to fat droplet surfaces would not be expected to be hydrolyzed by proteases (pepsin) in the gastric juices, which can cause

instability of protein-coated fat droplets. In addition, we would not have expected any saponin molecules adsorbed to the fat droplets surfaces to be displaced due to competitive adsorption processes since the oral and gastric fluids used in this study did not contain any highly surface-active substances.

After passage through the simulated small intestinal stage there was a large increase in mean particle diameter (**Figure 6.1a**) and evidence of large particles in the particle size distributions (**Figure 6.1b**) for all emulsions studied. These results indicate that there was a marked reduction in the physical stability of the emulsions after exposure to small intestinal conditions. Several physicochemical phenomena may explain the observed reduction in emulsion stability in the small intestinal stage. First, the intestinal juices contained bile salts and phospholipids that are highly surface active molecules that may have displaced some or all of the quillaja saponins from the oil droplet surfaces. Second, the intestinal juices contained pancreatic lipase that is capable of adsorbing to oil droplet surfaces and converting triacylglycerols (such as those in LCT or MCT oils) into free fatty acids (FFAs) and monoacylglycerols (MAGs). The digestion products of lipolysis may accumulate at the oil-water interface or move into the surrounding aqueous phase depending on their molecular weight and the presence of bile salts, phospholipids, and calcium [257, 259]. The oil droplet size may therefore change appreciably as a result of alterations in their internal and interfacial compositions. One would expect that the removal of FFA and MAG digestion products from the fat droplet surfaces would

lead to a reduction in particle size [275, 276]. On the other hand, one would expect that droplet coalescence promoted by lipid digestion would lead to an increase in droplet size [275, 276]. Consequently, the overall influence of lipid digestion on fat droplet size may be complicated. It should also be noted that particle size measurements made on complex colloidal systems containing mixtures of different particles (such as those produced by lipid digestion) should be treated with some caution. The software used by commercial particle sizing instruments to analyze light scattering measurements usually assumes that the particles are spherical, dilute, and have well-defined refractive indices. In practice, the colloidal suspension resulting from lipid digestion may contain undigested fat droplets, partially digested fat droplets, mixed micelles and various other colloidal structures.

6.3.1.2 Influence on particle charge characteristics

Changes in the electrical characteristics of the particles in the emulsions were also measured after each stage in the simulated gastrointestinal tract to provide some information about changes in interfacial properties (**Figure 6.2**). Initially, all of the oil droplets coated by quillaja saponin were highly negatively charged (-40 to -46 mV), which has previously been attributed to the ionization of carboxyl groups ($-COO^-$) on the saponins at neutral pH [274]. All of the droplets remained highly negative after passage through the simulated oral stage, although there was some reduction in the magnitude of their negative charge (**Figure 6.2**). The fact that the charge remained highly negative

can be attributed to the neutral conditions within the mouth, whereas the slight changes in charge may be due to adsorption of some ionized components from the simulated saliva to the droplet surfaces, *e.g.,* minerals or mucin [270].

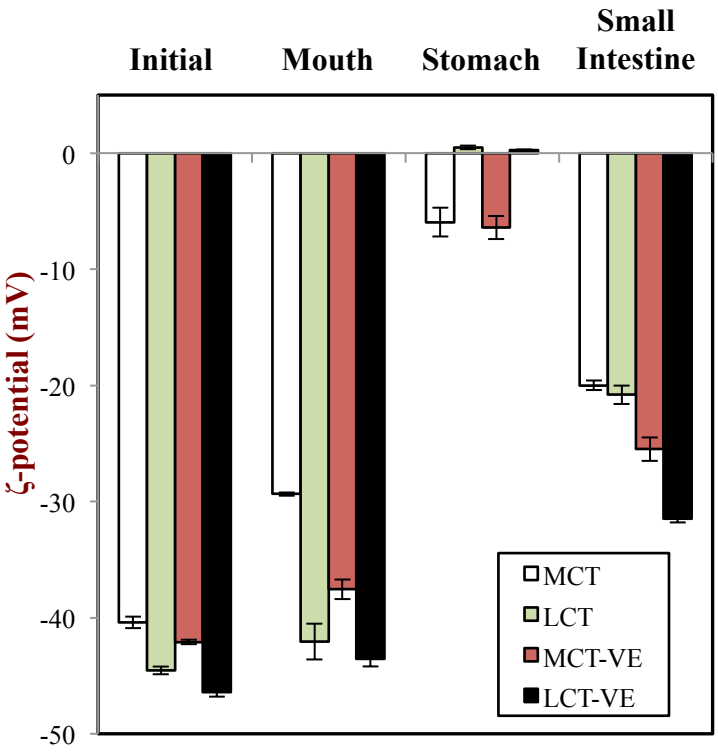


Figure 6.2 Influence of vitamin E encapsulation and carrier oil type on the electrical characteristics ζ-potential of the particles in oil-in-water emulsions passed through different stages of a simulated gastrointestinal tract. See caption to **Figure 6.1a** for *Key*.

The magnitude of the electrical charge on the droplets decreased appreciably after they were incubated in simulated gastric fluid, and the charge depended somewhat on carrier oil type (**Figure 6.2**). The emulsions containing MCT had a slightly negative charge (≈ -6 mV), whereas those containing LCT had a slightly positive charge (≈ +0.5 mV). The origin of this difference in the charge on droplets containing different types

of oils is currently unknown. One possible explanation is that the different oils phases contained different types and concentrations of ionic impurities (such as free fatty acids or phospholipids). Previous studies have shown that the electrical charge on oil droplets coated by quillaja saponin goes from highly negative at pH 7 to close to zero at pH 2, which was attributed to protonation of the carboxyl groups (-COOH) at pH values below their pK_a values [274]. The low net charge on the oil droplets under gastric conditions can therefore be largely attributed to this effect. The fact that there was little change in the mean particle diameter of the droplets under simulated gastric conditions (**Figure 6.1a**), suggests that the droplets were still coated by a layer of surface active material that prevented aggregation.

The electrical charge on the particles in all of the emulsions became strongly negative after they were incubated within simulated small intestinal fluid, and the charge depended somewhat on encapsulation of vitamin E and carrier oil type (**Figure 6.2**). The negative charge on the droplets in the small intestine can be attributed to a number of different factors. First, the quillaja saponin has a relatively high negative charge at neutral pH [274], so if any of these molecules remained at the particle surfaces they would impart a negative charge. Second, the small intestinal fluids contained phospholipids and bile salts which are anionic surface active substances that may adsorb to particle surfaces and generate a negative charge. Third, lipase converts neutral triacylglycerol molecules into anionic free fatty acids, so if any of these molecules remain

at the droplet surfaces they will also produce a negative charge. As mentioned earlier, it is not clear exactly what types of particles are detected by an electrophoresis instrument in a complex medium that contains many different types of charged particles that can scatter light.

6.3.2 Impact of encapsulation and carrier oil type on lipid digestion

The influence of vitamin E encapsulation and carrier oil type on the rate and extent of lipid digestion was measured using a pH-stat method. This approach is widely used by pharmaceutical and food researchers for the *in vitro* characterization of lipid digestion under simulated small intestinal conditions [148, 277, 278]. The pH-stat method measures the amount of alkali (0.25 M NaOH) that had to be titrated into the digestion mixture to maintain neutral pH after addition of simulated small intestinal fluids containing lipase, bile salts, and $CaCl_2$.

In general, there was a rapid initial production of free fatty acids during the first few minutes of the digestion reaction, followed by a more gradual increase at longer times (**Figure 6.3**), indicating that lipase was able to rapidly interact with the emulsified TAGs and convert them into FFAs and MAGs. Nevertheless, there were some distinct differences between emulsions prepared using different oil phases: (i). The extent of lipid digestion was higher for MCT- than LCT-emulsions; (ii) the extent of lipid digestion was higher for emulsions containing vitamin E than those containing no vitamin E. A number of earlier studies have reported that lipids containing medium-chain fatty acids

are digested more rapidly and extensively than those containing long-chain fatty acids [257, 259, 279]. A number of physiochemical explanations have been proposed to account for this phenomenon. First, medium-chain FFAs have a higher water dispersibility than long-chain FFAs [257, 259]. Consequently, the medium-chain FFAs produced during MCT digestion rapidly migrate into the aqueous phase surrounding the droplets, thereby allowing lipase to continue reacting with undigested MCT molecules within the oil droplet core. Conversely, the long-chain FFAs produced during LCT digestion tend to accumulate at the droplet surfaces, thereby inhibiting the ability of lipase to interact with the undigested LCT molecules within the droplet core [266]. Efficient digestion of LCT requires that there are enough bile salts and free calcium ions present within the aqueous phase to ensure that the long-chain fatty acids are effectively removed from the droplet surfaces through solubilization or precipitation [279]. The fact that the measured FFAs released was higher for the emulsions containing vitamin E than the equivalent emulsions containing no vitamin E may be attributed to the ability of lipase to cleave the ester bond of the α-tocopherol acetate molecule, thereby releasing α-tocopherol (**Figure 6.4**).

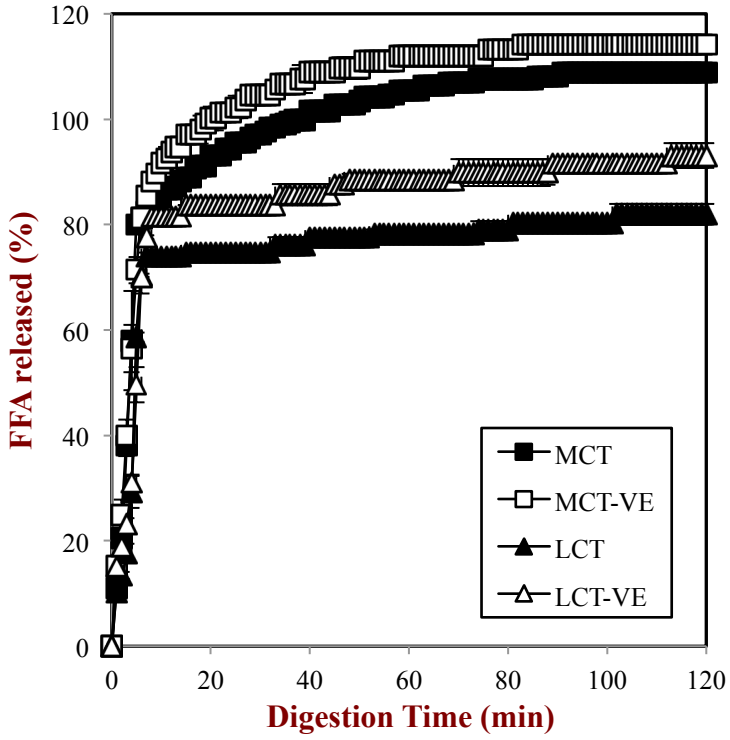


Figure 6.3 Effect of vitamin E encapsulation and carrier lipid type on rate and extent of lipid digestion measured using an i*n vitro* digestion model.

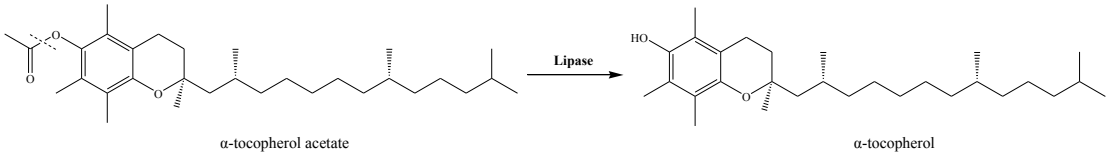


Figure 6.4 Conversion of α-tocopherol-acetate to α-tocopherol during *in vitro* digestion.

Our results suggest that >100% of FFAs were released from the MCT samples at longer digestion times (**Figure 6.3**). This apparent discrepancy can be attributed to the way that the percentage of FFAs released from the lipid droplets during *in vitro* digestion was calculated. There are two main assumptions underlying this calculation: (i) only two FFA molecules are released per TAG molecule; (ii) there are no other components within the system that release protons during the *in vitro* digestion process. In practice,

these assumptions may not be completely valid [269]. Some of the MAGs produced during digestion may have been further hydrolyzed to FFAs and glycerol, so that more than two FFA molecules were formed from each initial TAG. There may have been other components within the system that produced protons during the digestion reaction (*e.g.,* proteins, phospholipids, or vitamin E), which would require the addition of extra alkali to neutralize them.

6.3.3 Impact of carrier oil type on vitamin E bioaccessibility

In this section, we examined the impact of carrier oil type on the bioaccessibility of vitamin E after passage through the simulated GIT. After the small intestine stage, digesta were collected and centrifuged, which led to the formation of two distinct layers: a white "sediment" phase at the bottom and a watery "micelle" phase above it. Visually, the micelle phases were either optically transparent (MCT & MCT-VE) or slightly turbid (LCT & LCT-VE). The micelle phase was collected and its particle size distribution was measured before and after filtration. The bioaccessibility of the vitamin E was determined by measuring the concentrations of α-tocopherol and α-tocopherol acetate in the micelle phase and the total digesta using solvent extraction and HPLC.

The particle size distribution of the micelle phase isolated from the digesta of the LCT-emulsion before and after filtration using a 0.45 μm pore size filter is shown in **Figure 6.5**. In the unfiltered samples, a broad range of differently sized particles was observed with diameters ranging from around 140 to 5,500 nm, which could have been

due to the presence of mixed micelles, vesicles, and non-digested oil droplets. As expected, there was an appreciable decrease in the number of large particles present in the micelle phase after filtration. In practice, the mixed micelles and vesicles containing lipid digestion products must pass through the mucous layer that coats the epithelium cells prior to absorption. The mucous layer can be considered to act like a biological filter, with a critical cut-off point for particle penetration being reported to be around 400 nm [280]. Filtering the micelle layer prior to analysis may therefore provide a more accurate representation of the potential bioavailability of a lipophilic compound that must be carried by colloidal structures through the mucous layer to the epithelium cells. For this reason, we filtered the micelle phases collected from the digesta prior to further analysis.

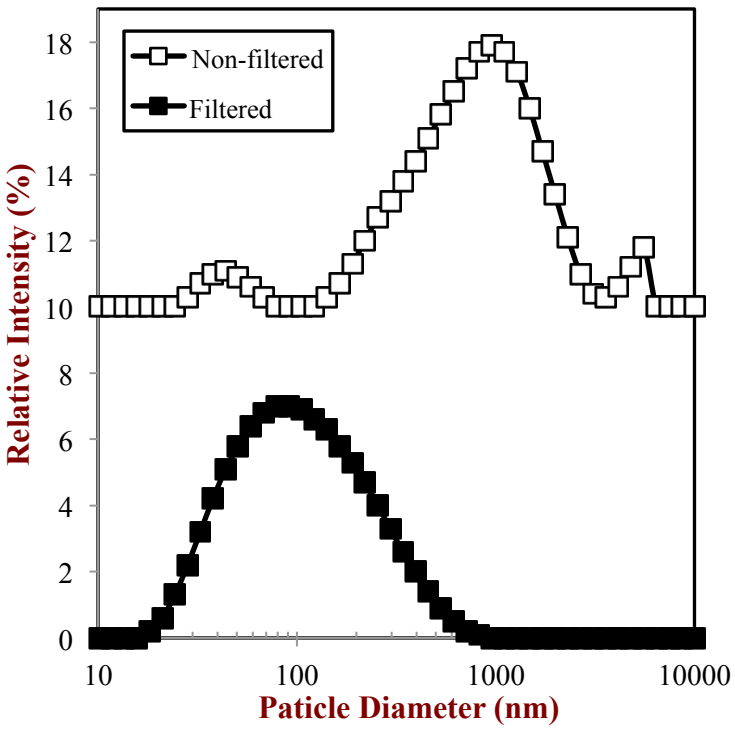


Figure 6.5 Particle size distribution of the "micelle" phase collected after digestion and centrifugation of LCT-emulsions. Measurements were made before and after filtration (0.45 μm pore size) of the micelle phase.

The mean particle diameters and turbidities of the micelle phases collected from the LCT-emulsions were larger than those collected from the MCT-emulsions (**Figure 6.6**), which indicates that they contained larger particles. These larger particles may have been comprised of non-digested triacylglycerol oil, since the pH-stat measurements indicated that not all of the LCT was digested after the small intestine stage (**Figure 6.3**). Alternatively, they may have been due to the formation of larger colloidal structures by the LCT digestion products. The mean droplet diameters were slightly smaller for emulsions containing vitamin E than for emulsions containing no vitamin E, which suggested that the presence of this lipophilic molecule altered the structure of the non-digested oil droplets or the colloidal particles formed from digestion products (**Figure 6.6**).

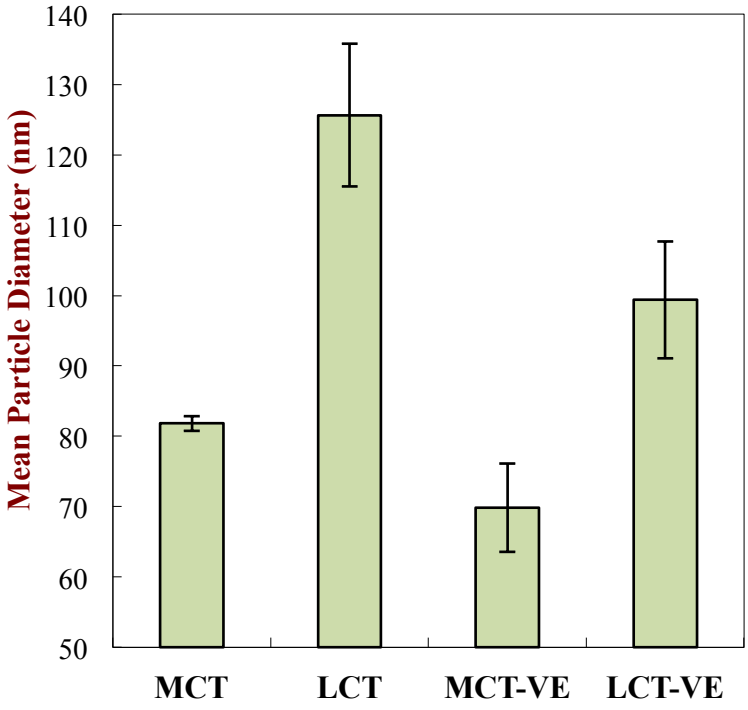


Figure 6.6 Influence of carrier oil type on the mean particle diameter (measured after filtration) and appearance of the micelle phase after *in vitro* digestion.

The concentrations of α-tocopherol acetate and α-tocopherol in the micelle phase and within the total digesta were measured after *in vitro* digestion (**Figure 6.7**). These measurements indicated that some of the α-tocopherol acetate was converted to α-tocopherol during the *in vitro* digestion process, and that the amount of vitamin E

solubilized within the micelle phase depended on carrier oil type and the molecular form of the vitamin E. The influence of carrier oil type on the overall bioaccessibility of vitamin E and the total amount of α-tocopherol acetate converted to α-tocopherol is shown in **Figure 6.8**. The overall bioaccessibility of vitamin E was around 17 % when MCT was used as the carrier oil but around 39 % when LCT was used. The solubilization of lipophilic components in the micelle phase usually increases as the total amount of mixed micelles available increases. Our lipid digestion experiments indicated that MCT was actually digested to a greater extent than LCT (**Figure 6.3**) and therefore the total concentration of FFAs and MAGs that could form mixed micelles should have been larger for MCT. On the other hand, the solubilization capacity of mixed micelles also depends on the nature of the FFAs and MAGs present. Long chain fatty acids can form mixed micelles that have a larger solubilization capacity for some lipophilic substances than medium chain fatty acids, presumably because they can accommodate larger non-polar molecules. Corn oil contains relatively long chain fatty acids (*e.g.,* C_{16} and C_{18}) while MCT contains medium chain ones (*e.g.,* C_8 and C_{10}), which would therefore account for the observed difference in solubilization capacity for vitamin E. The α-tocopherol molecule has a non-polar chain that is 14 carbon atoms in length (**Figure 6.4**). This non-polar chain may be too long to easily be accommodated within the monolayers formed by the medium chain fatty acids resulting from MCT digestion, but it may be able to fit within the monolayers formed by the long chain fatty

acids resulting from LCT digestion. The fact that the colloidal structures were larger in the LCT-emulsions than in the MCT-emulsions after lipid digestion (**Figure 6.6**) provides some support for the differences in the structure of the mixed micelles formed. Our results are in agreement with research in the pharmaceutical industry that has shown that many highly lipophilic drugs have a higher bioaccessibility when administered with LCT than with MCT [281, 282].

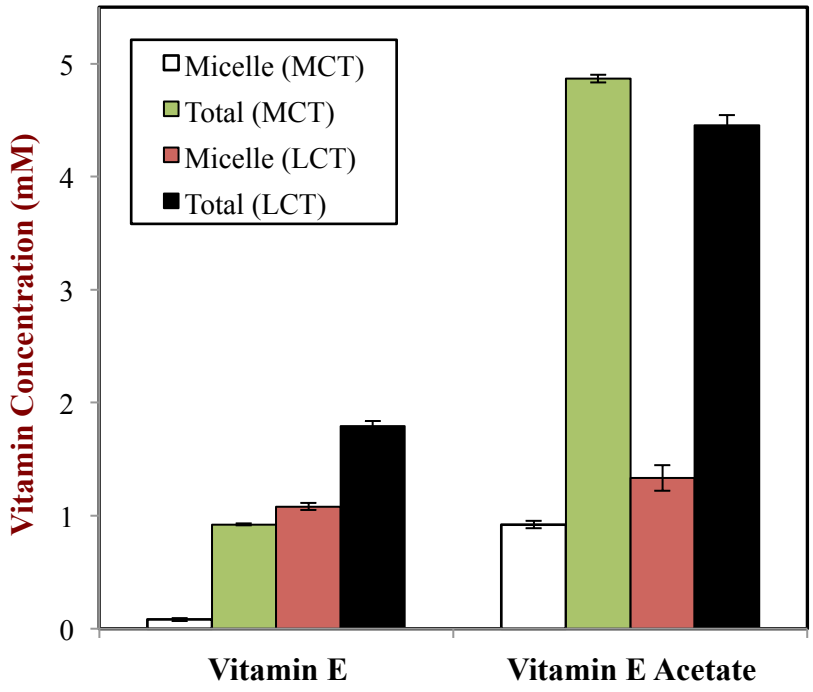


Figure 6.7 Influence of carrier oil type on the concentrations of α-tocopherol and α-tocopherol acetate within the micelle phase and the total digesta after *in vitro* digestion of emulsions.

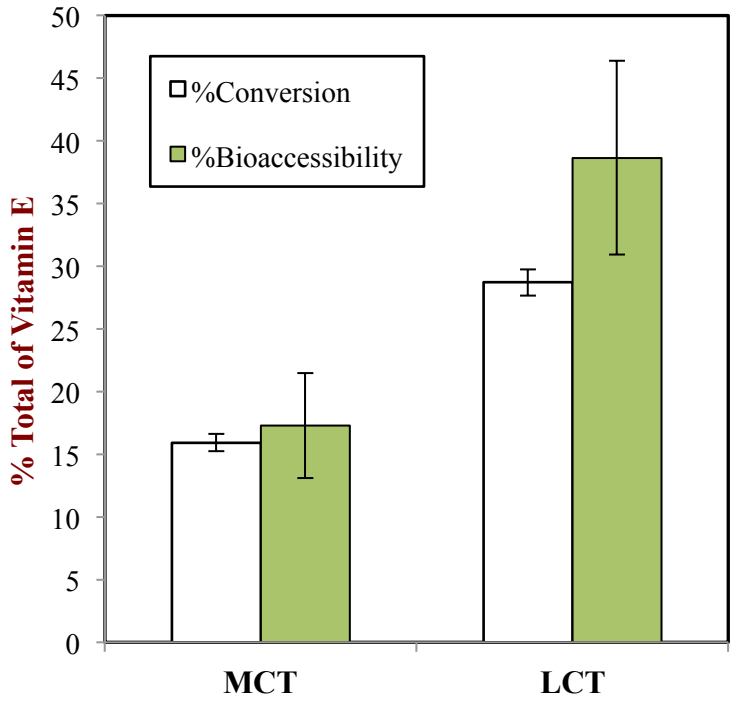


Figure 6.8 Influence of carrier oil type on the total bioavailability of Vitamin E (α-tocopherol + α-tocopherol acetate) and the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion of emulsions.

There were appreciable amounts of sediment formed in many of the samples after centrifugation (**Figure 6.6**). This sediment contains dense insoluble matter formed during the passage of the samples through the simulated gastrointestinal tract, such as aggregated digestive enzymes, bile salts, minerals, and calcium salts of free fatty acids. It is possible that some of the vitamin E acetate or vitamin E was trapped within the sediment phase.

6.3.4 Impact of carrier oil type on α-tocopheryl acetate to α-tocopherol conversion

As mentioned earlier, vitamin E is often used in an esterified form within foods

and other commercial products because the free form is highly unstable to oxidation [223, 224]. Previous studies suggest that the bioaccessibility of vitamin E may depend on its molecular form, *i.e.,* α-tocopherol *versus* α-tocopherol acetate [260-263]. We therefore calculated the amount of α-tocopherol acetate that had been converted to α-tocopherol after *in vitro* digestion (**Figure 6.8**). Carrier oil type had an appreciable influence on this process, with the extent of conversion being about 29% for the LCT-emulsion and 17% for the MCT-emulsion. This result suggests that the conversion of α-tocopherol acetate to α-tocopherol occurred more readily when LCT was used as the carrier oil than when MCT was used. Interestingly, there was a close correlation between the extent of conversion and overall vitamin E bioaccessibility (**Figure 6.8**). A possible explanation for this phenomenon is that vitamin E acetate incorporated into mixed micelles is more susceptible to de-esterification by lipase than vitamin E acetate encapsulated within oil droplets. The fact that a greater fraction of vitamin E was present within the micelle phase for the LCT-emulsion would then account for the observed increase in the hydrolysis of the acetate form. Alternatively, there may have been a competition between the triacylglycerol and the vitamin molecules for hydrolysis by lipase. The MCT molecules were digested more readily than the LCT molecules (**Figure 6.3**), and therefore the rate of vitamin E acetate hydrolysis may have been less when MCT molecules were present in the oil phase due to this competitive effect.

Table 6.1. Percentage of vitamin E present in the micelle phase after lipid digestion and centrifugation of the emulsions.

	MCT	**LCT**
Vitamin E	8.6 ± 0.1	60.3 ± 3.4
Vitamin E Acetate	18.9 ± 0.9	29.9 ± 4.3

The data presented in **Figure 6.7** was used to calculate the fraction of vitamin E present in the micelle phase for α-tocopherol and α-tocopherol acetate for both the LCT- and MCT-emulsions (**Table 6.1**). This data showed that a higher fraction of the free form (α-tocopherol) was present in the micelle phase than the esterified form (α-tocopherol acetate) for LCT, but the opposite was true for MCT. At present the molecular or physicochemical basis of this phenomenon is unknown, but it may be related to the compatibility of vitamin E molecules for mixed micelles and other different kinds of colloidal structures. For example, the removal of the acetate group may change the interaction of the vitamin E molecule with the polar head groups of the surface-active molecules that form the palisade layer of the mixed micelles.

6.4 Conclusions

The purpose of this study was to determine the influence of carrier lipid type on the bioaccessibility and hydrolysis of emulsified α-tocopherol acetate using a simulated gastrointestinal tract. We have shown that α-tocopherol acetate can be effectively encapsulated within oil-in-water emulsions using either medium chain triacylglycerols

(MCT) or long chain triacylglycerols (LCT) as a carrier oil. The rate and extent of lipid digestion was higher for MCT-emulsions than for LCT-emulsions, which was attributed to differences in the water dispersibility of the medium and long chain fatty acids formed during lipolysis. On the other hand, the total bioaccessibility of vitamin E after digestion was higher for LCT-emulsions than for MCT-emulsions, which was attributed to the greater solubilization capacity of mixed micelles formed from long chain fatty acids due to their ability to better accommodate lipophilic vitamin E molecules. Moreover, the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion was also considerably higher for LCT-emulsions than for MCT-emulsions.

In summary, our results suggest that LCT was a more effective carrier lipid than MCT for increasing the overall bioaccessibility of emulsified vitamin E. These results have important implications for designing effective emulsion-based delivery systems for oil-soluble vitamins.

CHAPTER 7

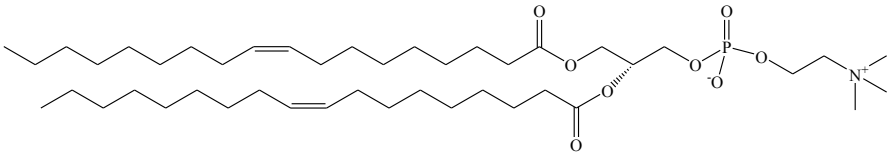
VITAMIN E AND VITAMIN E ACETATE SOLUBILIZATION IN MIXED MICELLES: PHYSICOCHEMICAL BASIS OF BIOACCESSIBILITY

7.1 Introduction

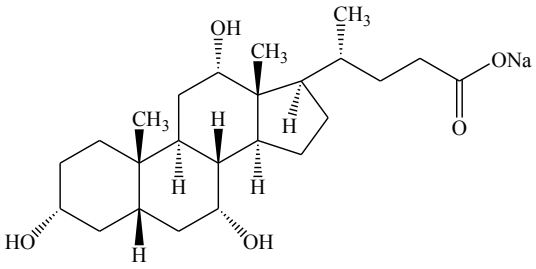
Vitamin E has various antioxidant and non-antioxidant biological activities that have been reported to provide health benefits to humans, such as reducing cardiovascular disease, diabetes, and cancer [244-246]. However, the physiological basis of many of these claimed health effects have still not been clearly elucidated [246, 247]. Vitamin E is widely used as an ingredient by the pharmaceutical, food, and supplement industries due to its perceived health benefits [216, 248].

The expression "vitamin E" actually refers to a class of molecules that have related chemical structures and biological activities, with the most widely used being α-tocopherol (**Figure 7.1**). The incorporation of α-tocopherol into commercial products is often challenging due to its chemical instability, poor water-solubility, and highly variable bioavailability [215, 249]. α-Tocopherol is a polyunsaturated lipid that is highly susceptible to oxidation and it may therefore be lost during the processing, transport, storage, or utilization of commercial products [223, 224]. Consequently, the esterified form of α-tocopherol (α-tocopherol acetate) is often used as a source of vitamin E in pharmaceutical, food, and supplement products because it has better chemical stability than the free form [250]. Both the free and esterified forms of α-tocopherol are highly

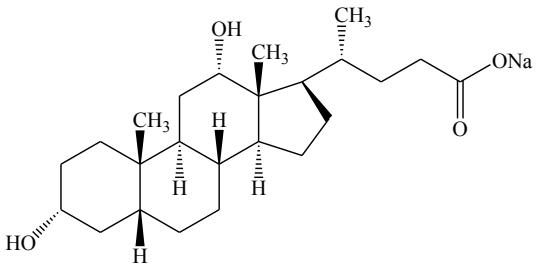
non-polar molecules that have low water-solubilities, which means they cannot simply be dispersed within aqueous-based products [163, 251]. Instead, they must be encapsulated within a suitable delivery system, such as a microemulsion, nanoemulsion, or emulsion [23, 212, 215]. After a product has been ingested, it is important that the vitamin E has a high oral bioavailability so that it can deliver its potentially beneficial health effects. However, the oral bioavailability of vitamin E may vary widely (< 1% to 100%) depending on the chemical form ingested and the nature of the delivery system used [119, 255]. This wide variability causes problems in determining an appropriate level of vitamin E to incorporate into a commercial product, and so it is critical to understand the major factors influencing bioavailability.



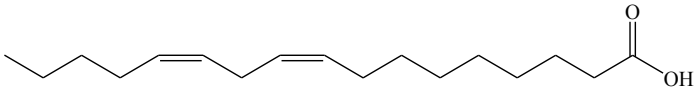
DOPC



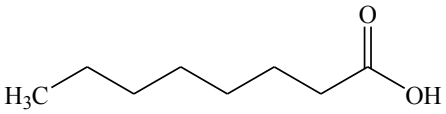
NaC



NaDC



Linoleic acid



Octanoic acid

Figure 7.1 Structures of the various molecules used to form mixed micelles in this study.

The oral bioavailability of vitamin E depends on a number of physicochemical and physiological processes that occur within the human gastrointestinal tract. Initially, the vitamin E should be released from the delivery system, then incorporated into "mixed micelles" in the small intestine, and then transported across the mucus layer where it is absorbed by epithelium cells [248, 256]. The solubilization of vitamin E into mixed micelles plays a critical role in determining its overall oral bioavailability. The term "mixed micelles" actually refers to a group of different colloidal species that are capable of solubilizing lipophilic components, such as micelles, vesicles, bilayers, and liquid crystals [283-285]. Mixed micelles are formed from bile salts and phospholipids present in the bile and pancreatic juices secreted by the small intestine, as well as from

any free fatty acids (FFA) and monoacylglycerols (MAG) produced due to hydrolysis of ingested triacylglycerol molecules by lipase [257]. Studies have shown that the bioaccessibility of highly lipophilic components tends to increase as the total amount of mixed micelles available in the small intestine increases, but also depends on the structural organization of the mixed micelles formed after lipid digestion [257-259]. The chemical form of the vitamin E has also been shown to play an important role in its bioaccessibility. Some studies have reported that α-tocopheryl acetate is less bioaccessible than α-tocopherol [260-263], whereas others have reported little difference [225].

In a recent study using a simulated gastrointestinal tract model, we found that the bioaccessibility of vitamin E was higher in emulsions prepared using a long chain triglyceride (LCT) than those prepared using a medium chain triglyceride (MCT) (Yang and McClements, 2013). We also found that the bioaccessibility of α-tocopheryl acetate was lower than that of α-tocopherol. These differences were attributed to differences in the ability of the mixed micelles formed by lipid digestion to solubilize the vitamin E molecules: long chain fatty acids form larger mixed micelles than medium chain fatty acids, and can therefore solubilize larger lipophilic molecules; α-tocopheryl acetate is longer than α-tocopherol and is therefore more difficult to be incorporated into smaller mixed micelles. The purpose of the current study was to better understand the role of the chemical state of vitamin E (α-tocopherol *versus* α-tocopheryl acetate) and the

composition of the mixed micelles (bile salts, phospholipids, and fatty acids) on vitamin bioaccessibility. A well-defined *in vitro* model (emulsion titration assay) was used to quantify the kinetics and extent of vitamin solubilization in mixed micelles so as to provide a more detailed mechanistic understanding of the physicochemical processes involved [286, 287]. A number of previous researchers have also used mixed micelle solutions as model systems for solubilization of lipophilic components, such as free fatty acids [288], carotenoids [289-291] and vitamin E [154]. The current study should provide information about the major factors influencing the bioaccessibility of vitamin E that will facilitate the rational design of more effective delivery systems.

7.2 Materials and methods

7.2.1 Materials

Sodium cholate (NaC), sodium deoxycholate (NaDC), vitamin E, oleic acid, and linoleic acid (**Figure 7.1**) were purchased from the Sigma Chemical Company (St. Louis, MO). 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All other chemicals used were of analytical grade. Double distilled water was used for the preparation of all solutions and emulsions.

7.2.2 Mixed micelle solution preparation

7.2.2.1. Optimization of Preparation Method

Initially, a series of experiments was carried out to determine the optimum method for preparing mixed micelle solutions to use in the emulsion titration assay. All

the final mixed micelle solutions contained a bile salt concentration of 20 mM (40% NaDC and 60% NaC), but they varied in the preparation method used and in the presence of other constituents: bile salts (BS); phospholipids (PL); long chain fatty acids (LCFA); medium chain fatty acids (MCFA).

BS: Bile salts (20 mM) were dispersed within an aqueous buffer solution (PBS buffer, pH 7.0).

BS+PL (*After*): Phospholipid (1.6 g/L lecithin, lysolecithin, or DOPC) was dissolved in an organic solvent (methanol : chloroform, 1:1, v/v), placed in a glass flask, and then the organic solvent was removed by nitrogen evaporation. An aqueous bile salt solution (20 mM, pH 7.0) was then added to the flask and the system was sonicated for 30 min.

BS+PL (*Before*): Phospholipid (1.6 g/L lecithin, lysolecithin, or DOPC) and bile salt (10 mM) were dissolved in an organic solvent (methanol : chloroform, 1:1, v/v), placed in a glass flask, and then the organic solvent was removed by evaporation. An aqueous bile salt solution (10 mM, pH 7.0) was then added to the flask and the system was sonicated for 30 min.

BS+PL + LCFA or MCFA (*Before*). In this case, phospholipid (2 mM, 1.6 g/L DOPC), bile salt (10 mM) and fatty acid (2 mM, 1.6 g/L linoleic acid or octanoic acid) were dissolved in an organic solvent (methanol : chloroform, 1:1, v/v), placed in a glass flask, and then the organic solvent was removed by evaporation. An aqueous bile salt

solution (10 mM, pH 7.0) was then added to the flask and the system was sonicated for 30 min.

7.2.2.2. Optimized System

Once an optimum system had been identified, this was used in the remainder of the studies. The type and levels of constituents used to prepare the mixed micelle solutions were based on those previously reported in the literature [292-294], with some slight modifications as reported below. First, a solution of 10 mM bile salt (NaC, NaDC), 2 mM DOPC and 4 mM FAs in chloroform:methanol (1:1, v/v) solvent was prepared. The organic solvent was then removed by blowing nitrogen on the sample at ambient temperature so as to form a thin lipid film on the glass vessel. Phosphate buffer (10 mM, pH 7.4) was then added to the glass vessel containing the lipid film, and the resulting mixture was sonicated for 30 min at room temperature, which resulted in the formation of a transparent aqueous solution containing the mixed micelles.

7.2.3 Vitamin emulsion preparation

Vitamin emulsions were prepared by homogenizing 10 wt% lipid phase (Vitamin E or Vitamin E acetate) with 90 wt% aqueous phase. The aqueous phase consisted of surfactant (2 wt% bile salt) and buffer solution (10 mM sodium phosphate buffer, pH 7.0). A coarse emulsion premix was prepared by blending the lipid and aqueous phases together using a high-speed mixer (Bamix, Biospec Products, Bartlesville, OK) for 2 min at room temperature. Fine vitamin emulsions were formed by passing the coarse

emulsions through an air-driven microfluidizer (Microfluidics, Newton, MA, USA). The coarse emulsions were passed through the homogenization for 4 passes at 9,000 psi.

7.2.4 Particle characterization

The mean particle diameter, particle size distribution, and electrical charge of mixed micelles were determined. The mean particle diameter and particle size distribution were measured using dynamic light scattering (Nano-ZS, Malvern Instruments, Malvern, UK). The electrical charge (ζ-potential) was determined using electrophoretic mobility measurements (Nano-ZS, Malvern Instruments, Worcestershire, UK). Samples were placed in a capillary test tube that was loaded into the instrument. Samples were equilibrated for 1 min inside the instrument before data were collected over at least 10 sequential readings and analyzed using the Smoluchowski model.

7.2.5 Emulsion titration assay

An *emulsion titration assay* [286] was used to quantify the rate and extent of vitamin solubilization in mixed micelle solutions (**Figure 7.2**). Aliquots of vitamin emulsion were injected into mixed micelle solutions and then the turbidity was measured over time, or after 3 h storage (mixed micelles containing fatty acids) or overnight storage (mixed micelles without fatty acids) at room temperature.

7.2.6 Turbidity measurements

Turbidity measurements were used to monitor the solubilization of the injected vitamin emulsions after they were added to the mixed micelle solutions. The turbidity

was determined using an UV-visible spectrophotometer at 600 nm (Ultra-spec 3000 pro, Biochrom Ltd, Cambridge, UK). The turbidity was measured over time after an aliquot of vitamin E emulsion was added to the micelle solution (solubilization kinetics) or after a specified storage period.

7.2.7 Optical microscopy measurements

A Nikon optical microscope (Nikon Eclipse 80i, Nikon Instrument Inc., Melville, NY) with a 40× objective lens was used to capture images of selected emulsion samples. An aliquot of sample was placed on a glass microscope slide and then a digital image was captured and stored.

7.2.8 Statistical analysis

All measurements were performed on at least two freshly prepared samples (*i.e.,* new samples were prepared for each series of experiments) and were reported as means and standard deviations.

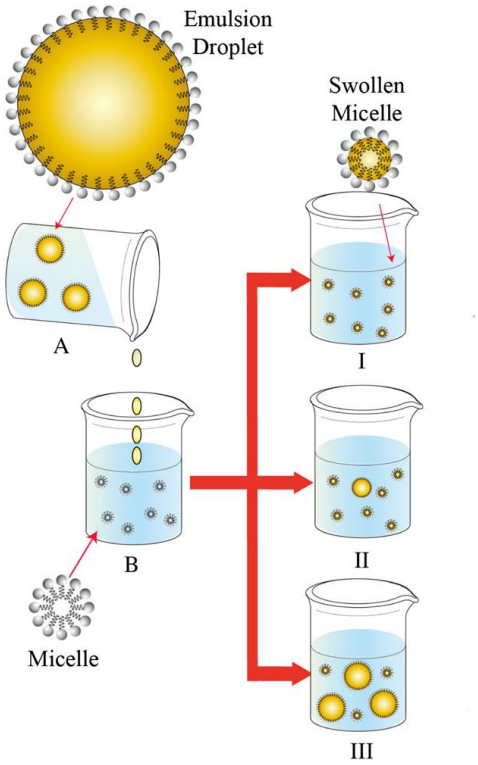


Figure 7.2 Schematic representation of *emulsion titration assay* used to monitor the solubilization of vitamin droplets in mixed micelle solutions. **A**: Stock vitamin emulsion (1 wt% bile salt, 10 wt% vitamin, 89 wt% aqueous phase). **B**: mixed micelle solution. *Regime I*: vitamin concentration < C_{Sat} - all vitamin solubilized in swollen micelles; *Regime II*: vitamin concentration $\approx$ C_{Sat} - mixture of swollen micelles and non-dissolved vitamin droplets; *Regime III*: vitamin concentration > C_{Sat} – predominance of non-dissolved vitamin droplets [295]

7.3 Results and discussion

7.3.1 Influence of preparation method on mixed micelle properties

The purpose of this series of experiments was to determine the most appropriate method of preparing mixed micelle solutions for studying vitamin solubilization using the emulsion titration assay. For this method it is important that the initial mixed micelle

solution is optically transparent, and so we examined the influence of composition and preparation method on the formation of clear mixed micelle solutions.

The mixed micelles formed in the human body are compositionally and structurally complex, and their properties depend strongly on the nature of an ingested food, particularly the type and amount of lipids present [257, 296]. Mixed micelles contain bile salts, phospholipids, and cholesterol from the small intestinal fluids, as well as monoacylglycerols (MAG) and free fatty acids (FFA) from any lipid digestion products. These surface-active lipids self-assemble into a "mixed micelle" colloidal dispersion, which may contain micelles, vesicles, and liquid crystalline phases that vary in composition, dimensions, and structure [297, 298].

For fundamental studies of the physicochemical phenomenon associated with the solubilization process it is advantageous to use simulated mixed micelle solutions with well-defined properties. A number of different methods of preparing simulated mixed micelle solutions have been reported in the literature [292-294, 299, 300]. These methods differ in the types and amounts of constituents used, as well as in the methods used to prepare the mixed micelles. The composition of model mixed micelle systems is usually chosen to be much simpler than that of real mixed micelles for a number of reasons: (i) to improve the ease and reproducibility of sample preparation; (ii) to facilitate data interpretation; (iii) to enable comparison of data between different research groups. Hence, they typically contain one or two bile salt components, a single phospholipid

component, and a limited number of lipid digestion products (such as FFA and MAG), rather than a highly complex mixture of many different components. Previous studies indicate that the properties of the mixed micelle phase formed also depend on the preparation method. In this study, we therefore examined the influence of composition and preparation method on the formation of mixed micelle solutions. In particular, we investigated the influence of order of bile salt addition (before or after solvent evaporation) and phospholipid type (lecithin, lysolecithin, or DOPC) on the appearance, particle size, and electrical charge of mixed micelles.

7.3.1.1 Influence of order of addition

Initially, we examined the influence of adding the bile salts to the phospholipids (lecithin) before or after evaporation of the organic solvent. In the absence of phospholipids, the bile salt solution was optically transparent (**Figure 7.3a**). When the bile salt solution was added to a vessel containing dried phospholipids (*i.e.,* after solvent evaporation), the resulting solution was highly turbid (**Figure 7.3a**) and contained a population of relatively large particles, *i.e., d* > 100 nm (**Figure 7.3b**). On the other hand, when the bile salts were mixed with the phospholipids first and then the mixture was dried (*i.e.,* before evaporation), the resulting solution was fairly clear (**Figure 7.3a**) and the majority of particles were relatively small, *i.e., d* < 10 nm (**Figure 7.3b**). These results indicate that mixing a portion of the bile salts with the phospholipids prior to solvent evaporation leads to the formation of smaller colloidal particles in the system.

Presumably, the bile salts and phospholipids intermingled more easily when they were dispersed in organic solvent and then dried together, thus enabling them to form mixed micelles upon reconstitution with an aqueous solution. When the bile salts were added after the phospholipids had been dried, it may have been difficult for them to be incorporated into the colloidal structures formed when the phospholipids were rehydrated (*e.g.,* vesicles).

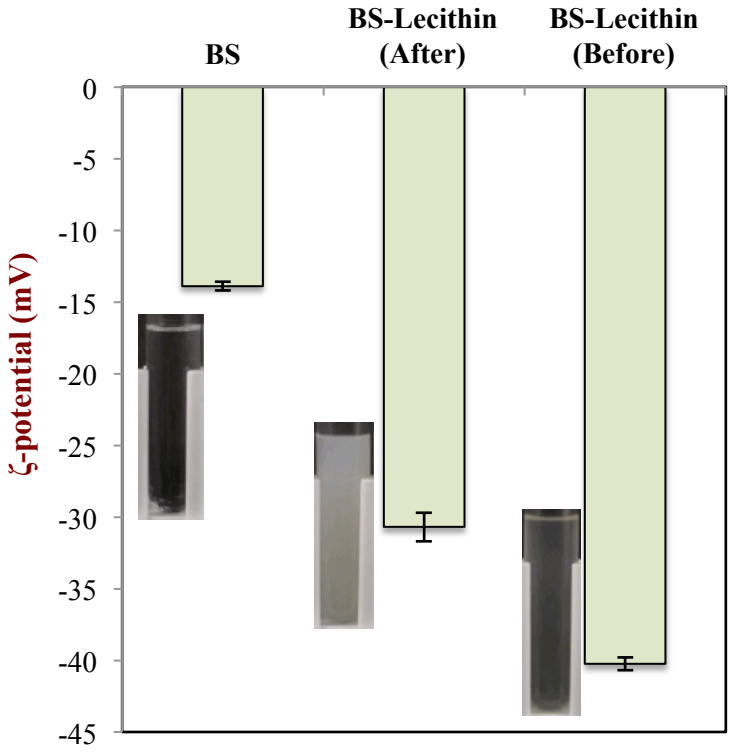


Figure 7.3a Electrical characteristics and appearance of mixed micelle solutions formed by mixing bile salts with phospholipids (lecithin) either after or before evaporation of the organic solvent.

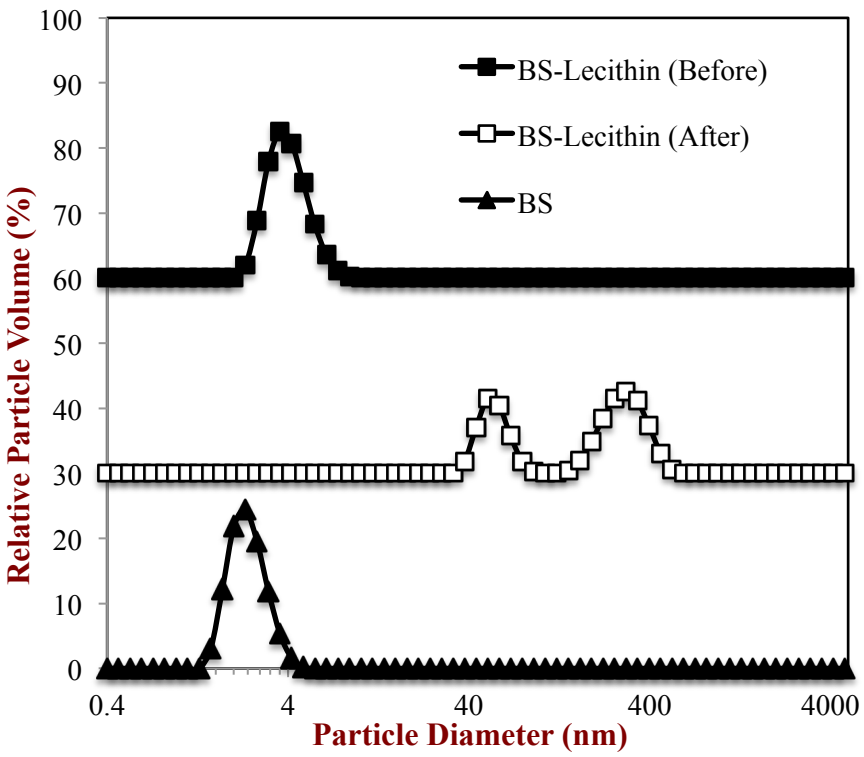


Figure 7.3b Particle size distributions (measured by dynamic light scattering) of mixed micelle solutions formed by mixing bile salts with phospholipids (lecithin) either after or before evaporation of the organic solvent.

The electrical characteristics of the colloidal structures formed also depended on the preparation method (**Figure 7.3a**). In the absence of phospholipids, the bile salts had an appreciable negative charge, which can be attributed to the presence of anionic carboxyl groups ($-COO^-$) [301]. The negative charge on the mixed micelles containing bile salts and phospholipids (≈ -30 to -40 mV) was appreciably higher than that on the bile salts alone (≈ -14 mV), which is due to the fact that lecithin contains anionic components such as phosphatidic acid. The mixed micelles formed by adding the bile salts before solvent evaporation were considerably more negatively charged than those

formed by adding bile salts after solvent evaporation, suggesting that there were some changes in the structural organization of the ionized groups in the different types of colloidal particles formed.

Similar results were found when lysolecithin or DOPC were used as the phospholipid rather than lecithin, *i.e.,* the mixed micelle solutions were turbid and contained large particles when the bile salts were added before solvent evaporation, but were clear and contained small particles when the bile salts were added after solvent evaporation (data not shown). For this reason, we determined that adding the bile salts to the phospholipids prior to solvent evaporation was the most suitable approach for preparing clear mixed micelle solutions.

7.3.1.2 Influence of phospholipid type

We then compared the influence of phospholipid type on the properties of mixed micelle solutions. In this case, a fraction of the bile salts were mixed with the phospholipids before solvent evaporation to facilitate the formation of clear mixed micelle solutions containing small particles. Mixed micelle solutions prepared using a pure phospholipid (DOPC) were less turbid (**Figure 7.4a**) and contained smaller particles (**Figure 7.4b**) than those prepared using either lecithin or lysolecithin. This phenomenon may have been due to some water-insoluble impurities in the lecithin and lysolecithin samples (*e.g.,* triacylglycerols and sterols) that could not easily be incorporated into the mixed micelle structures [302]. In addition, lecithin and

lysolecithin contain a number of different types of phospholipids and some of these may promote the formation of colloidal structures other than simple micelles, *e.g.,* phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid [303]. For this reason, we concluded that DOPC was the most suitable phospholipid for preparing transparent mixed micelle solutions.

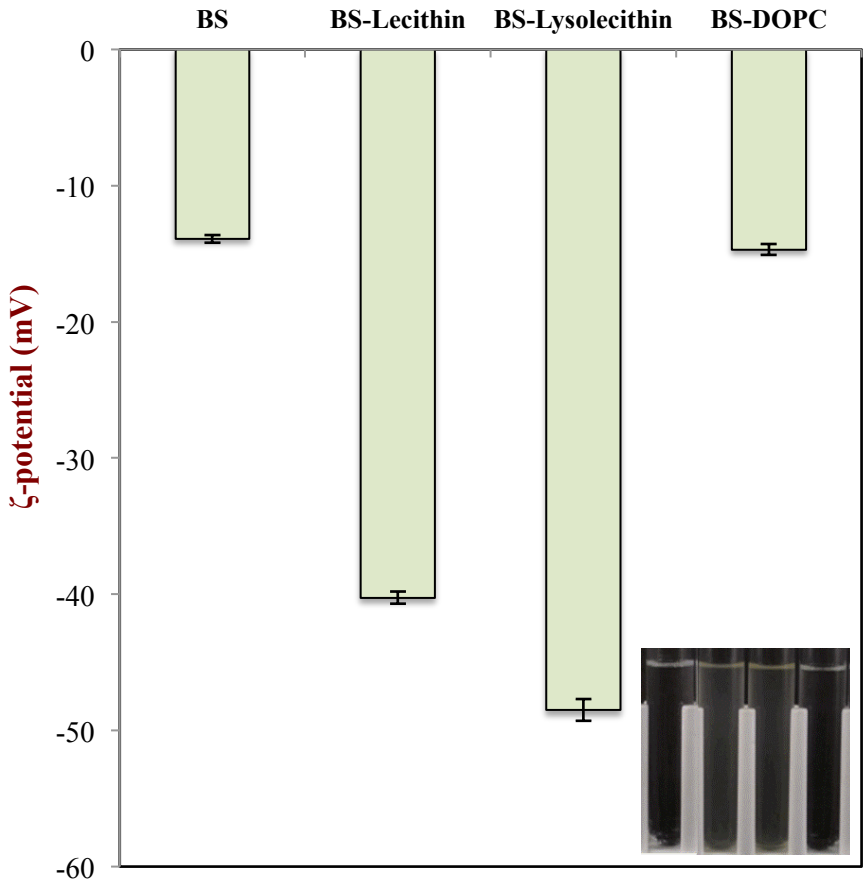


Figure 7.4a Influence of phospholipid type on electrical characteristics and appearance of mixed micelle solutions formed by mixing bile salts and phospholipids (lecithin, lysolecithin, or DOPC) before solvent evaporation.

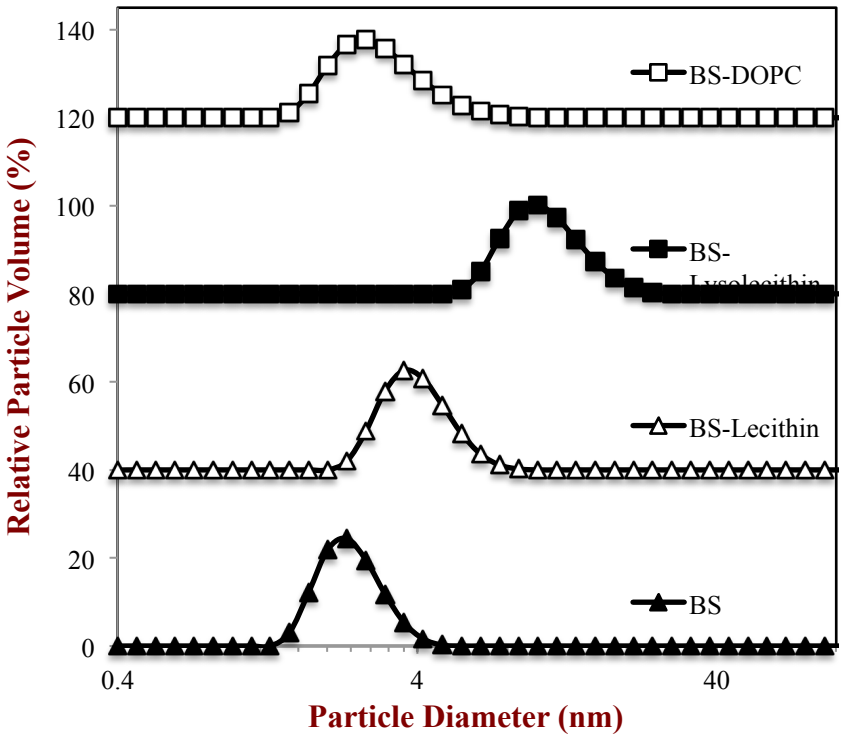


Figure 7.4b Influence of phospholipid type on particle size distributions of mixed micelle solutions formed by mixing bile salts and phospholipids (lecithin, lysolecithin, or DOPC) before solvent evaporation.

The electrical characteristics of the colloidal structures formed when bile salts were added before solvent evaporation also depended on phospholipid type (**Figure 7.4a**). Mixed micelles containing lecithin or lysolecithin had relatively high negative charges (≈ -40 to -50 mV), whereas those containing DOPC had much smaller negative charges (≈ -14 mV). As mentioned earlier, DOPC has a net neutral charge whereas lecithin and lysolecithin contain anionic phospholipids (such as phosphatidic acid).

7.3.2 Influence of fatty acid type on mixed micelle properties

In a previous study using a simulated gastrointestinal tract (GIT) model, we found

that the bioaccessibility of vitamin E was considerably higher when it was encapsulated in emulsions prepared using corn oil (LCT) than in medium chain triglyceride (MCT) oil (Ying and McClements, 2013). This difference was attributed to the fact that the mixed micelles containing long chain fatty acids (LCFA) generated by digestion of corn oil had a higher solubilization capacity than those formed from the medium chain fatty acids (MCFA) resulting from digestion of MCT. One of the main purposes of the present study was therefore to examine the influence of fatty acid type on the solubilization properties of mixed micelles. The dominant fatty acids in corn oil are 18-carbon unsaturated fatty acids and therefore we used linoleic acid ($C_{18:1}$) to represent them [304, 305], whereas the dominant fatty acids in MCT are 8- and 10-carbon saturated fatty acids and therefore we used octanoic acid ($C_{8:0}$) to represent them [306]. Mixed micelles were prepared by mixing bile salts, DOPC, and fatty acids in organic solvent, evaporating the solvent, and then rehydrating them using an aqueous bile salt solution. All mixed micelle solutions were passed through a 440 nm filter prior to analysis to mimic passage through the mucus layer that coats the epithelium cells, since the pore size of the mucus layer has been determined to be around this dimension [280].

The mean particle diameter and full particle size distribution of mixed micelle solutions with different compositions were measured (**Figures 7.5a and 7.5b**). All the mixed micelle solutions had a monomodal particle size distribution and contained relatively small particles ($d \approx 5$ nm) suggesting that simple micelles were present (rather

than vesicles or other colloidal structures). There was an appreciable increase in the mean particle diameter of the mixed micelles when both types of free fatty acids were added to the bile-phospholipid system, with the increase being larger for the long chain fatty acids ($C_{18:1}$) than for the medium chain fatty acids ($C_{8:0}$). This increase in the diameter of the micelles may account for the increase in solubilization capacity of mixed micelles in the presence of free fatty acid digestion products observed using simulated gastrointestinal models (Yang and McClements, 2013). Nevertheless, the overall increase in the mixed micelle size due to the presence of the free fatty acids was relatively small. The influence of fatty acid type on the electrical characteristics of the particles in the mixed micelle solutions were also measured (**Figure 7.5c**). The negative charge on the mixed micelles was appreciably higher in the systems containing fatty acids (-36 to -40 mV) than in their absence (-15 mV), which can be attributed to the anionic nature of the free fatty acid head groups ($-COO^-$).

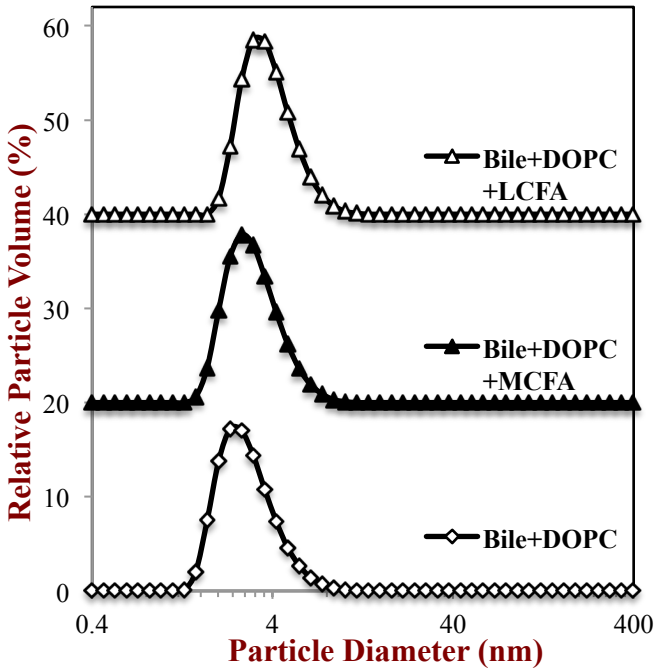


Figure 7.5a Influence of mixed micelle composition on volume-weighted particle size distribution measured by dynamic light scattering. **Key**: BS = bile salts (NaC and NaDC); PL = phospholipid (DOPC); MCFA = medium chain fatty acid ($C_{8:0}$); LCFA = large chain fatty acid ($C_{18:1}$).

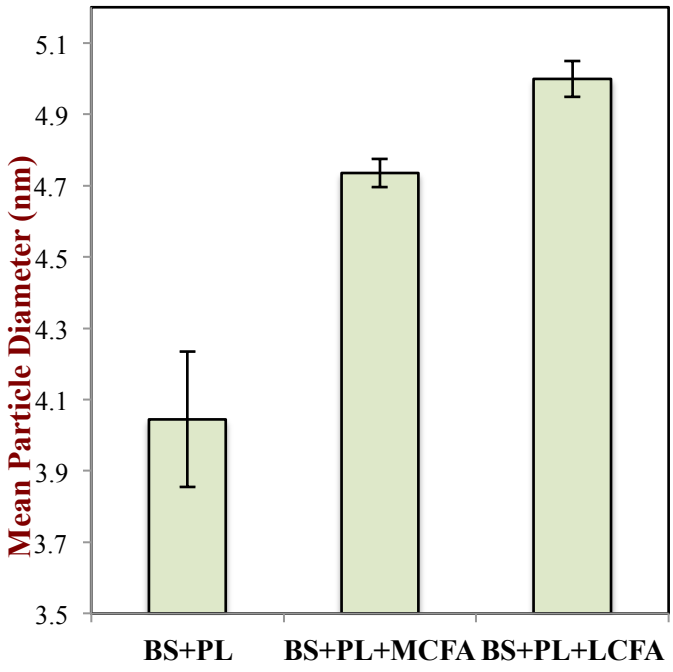


Figure 7.5b Influence of mixed micelle composition on the mean particle diameter measured using dynamic light scattering. **Key**: BS = bile salts (NaC and NaDC); PL = phospholipid (DOPC); MCFA = medium chain fatty acid ($C_{8:0}$); LCFA = large chain fatty acid ($C_{18:1}$).

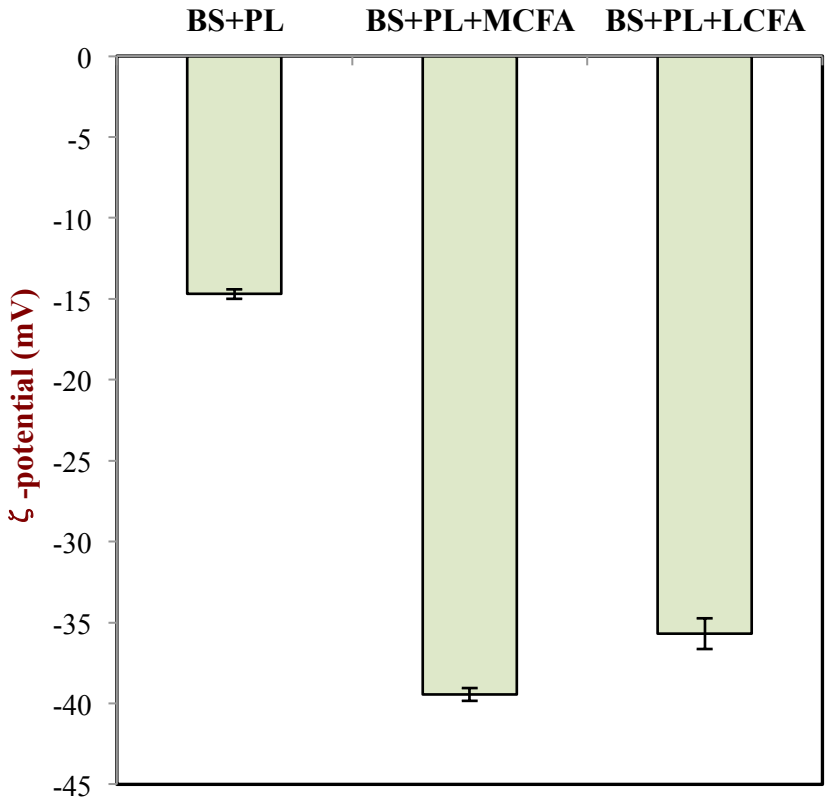


Figure 7.5c Influence of composition on the electrical characteristics of mixed micelles. **Key**: BS = bile salts (NaC and NaDC); PL = phospholipid (DOPC); MCFA = medium chain fatty acid ($C_{8:0}$); LCFA = large chain fatty acid ($C_{18:1}$).

Figure 7.5d Appearances of mixed micelle solutions with different compositions before and after one day storage

The mixed micelle solutions containing free fatty acids remained transparent for a few hours after preparation, but they did become turbid after they were stored overnight at refrigerated or ambient conditions (**Figure 7.5d**). Examination of these samples using an optical microscope showed that they contained some large liquid crystalline

structures, suggesting that some self-association of the lipid molecules had occurred during storage. Thus, the emulsion titration assay should be carried out within a few hours of preparing the mixed micelle solutions containing fatty acids to ensure they remain transparent.

7.3.3 Influence of structure and composition on solubilization capacity of mixed micelles

Previous research has reported that the presence of mixed micelles in the intestinal lumen improves the solubility and adsorption of lipophilic bioactive components [307, 308]. As mentioned earlier, we previously found that vitamin E (α-tocopherol) and vitamin E acetate (α-tocopheryl acetate) had a higher solubility in mixed micelles formed from long chain fatty acids than medium chain fatty acids (Yang and McClements, 2013). In this section, we examined the influence of mixed micelle structure and compositions on the solubilization of vitamin E and vitamin E acetate in mixed micelles using the emulsion titration assay. This method involves titrating aliquots of vitamin emulsions into an aqueous mixed micelle solution (**Figure 7.2**), and then measuring the change in turbidity.

Immediately, after an aliquot of vitamin emulsion was added to the mixed micelle solution the turbidity was relatively high due to light scattering from the vitamin droplets (**Figure 7.6**a). When the vitamin emulsion was added to a buffer solution (containing no mixed micelles) the turbidity remained high over time, indicating that no

solubilization occurred. However, when the vitamin emulsion was added to a mixed micelle solution the turbidity decreased over time (**Figure 7.6a**), indicating that solubilization occurred, *i.e.,* vitamin molecules moved from the droplets into the mixed micelles causing a reduction in the number and/or size of the droplets thereby reducing the degree of light scattering. The solubilization process was relatively slow, taking over an hour for the turbidity to reach a final constant level for the vitamin E systems. The rate and extent of solubilization was slower for the vitamin E acetate systems (data not shown). The solubilization process was also confirmed using optical microscopy measurements. Immediately after a vitamin emulsion was added to a mixed micelle solution large fat droplets could be observed, but after 3 hours of solubilization they had disappeared (**Figure 7.6b**).

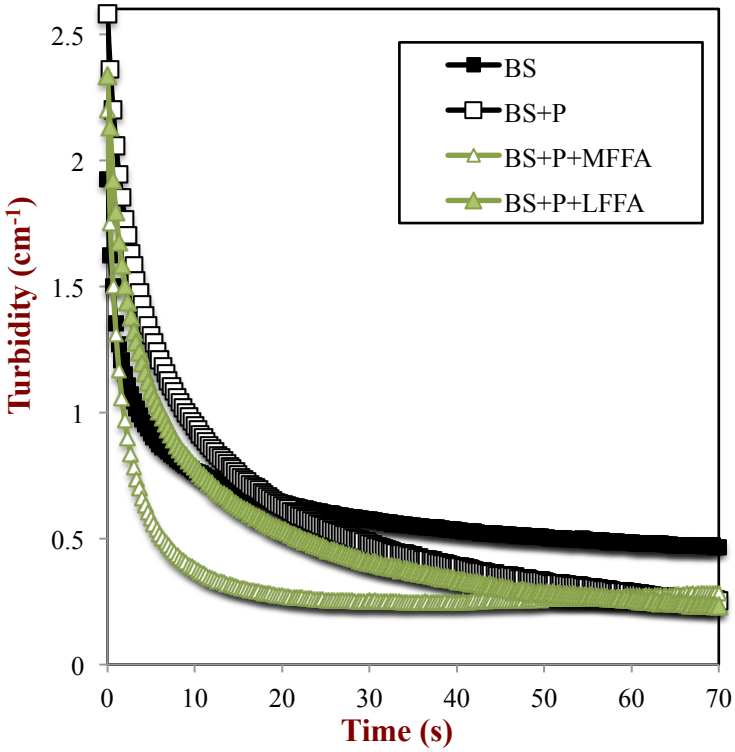


Figure 7.6a. Change in turbidity over time after 25 µl of vitamin E emulsion was injected into 3 mL of mixed micelle solutions with different compositions (pH 7.4).

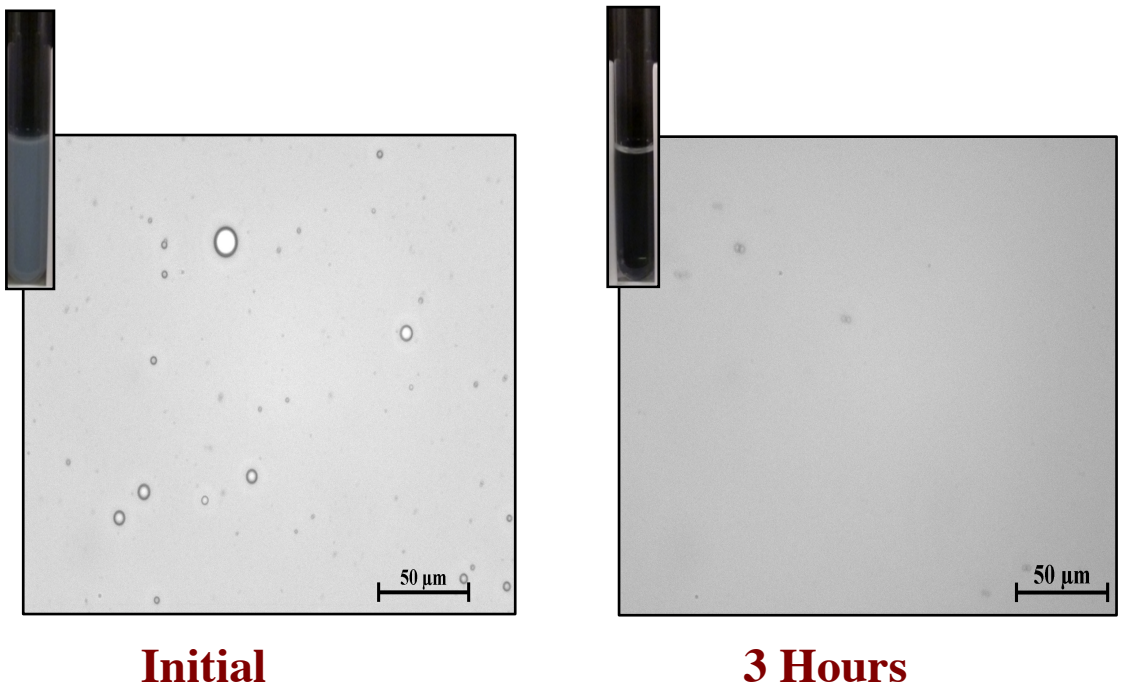


Figure 7.6b Optical microscopy images of vitamin droplets immediately after injection into mixed micelle solutions (BS+PL) and after 3 hours.

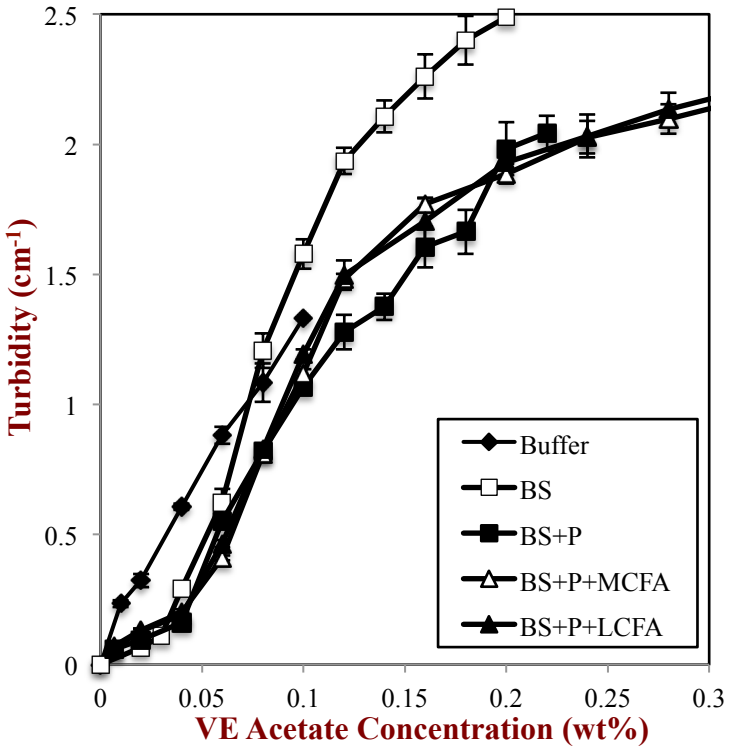


Figure 7.6c Change in turbidity with increasing vitamin E acetate concentration in mixed micelle solutions with different compositions.

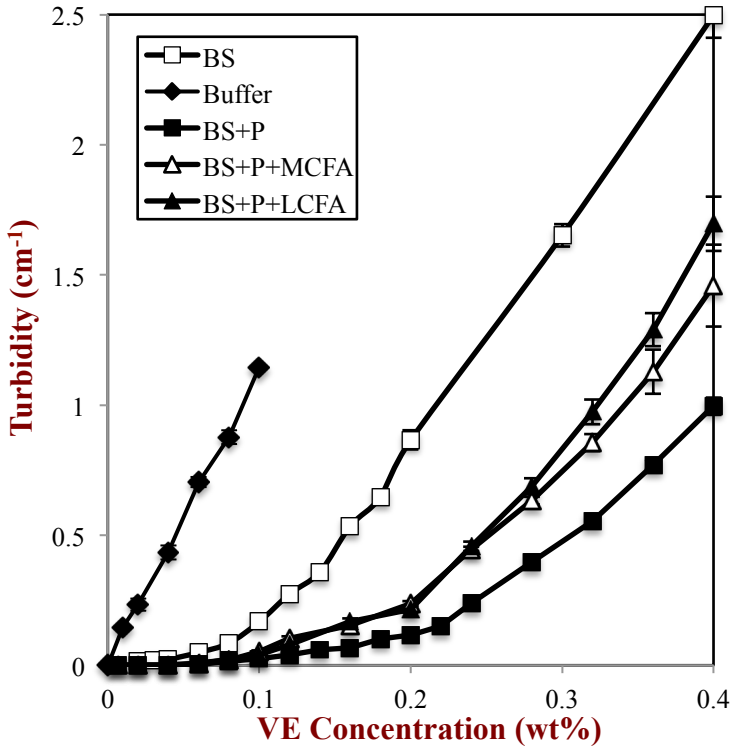


Figure 7.6d Change in turbidity with increasing vitamin E acetate concentration in mixed micelle solutions with different compositions.

The solubilization capacity of the mixed micelle solutions was determined by measuring the turbidity of solutions containing different types and amounts of vitamin after overnight storage to ensure that full solubilization had occurred (**Figure 7.6c,d**). The solubilization capacity depended on the composition of the mixed micelles as well as on the molecular form of the vitamin. For both vitamin E and vitamin E acetate, the turbidity increased linearly when the fat droplets were added to pure buffer solution (no mixed micelles), which can be attributed to the fact that no solubilization occurred. Hence, the amount of light scattering by the mixed systems increased as the fat droplet concentration increased. When vitamin E acetate was titrated into any of the mixed

micelle solutions the increase in turbidity with vitamin concentration was somewhat less than for titration into pure buffer solution, which suggested that a small amount of the vitamin was solubilized within the micelles (**Figure 7.6c**). On the other hand, when vitamin E was titrated into the mixed micelle solutions they initially remained transparent over a range of vitamin concentrations suggesting that all of the vitamin droplets had dissolved. Once a certain vitamin concentration was exceeded the turbidity of the mixed micelle solutions increased, which suggested that the micelles had become saturated with vitamin and the vitamin droplets no longer dissolved. The vitamin E concentration where this increase in turbidity was first observed depended on mixed micelle composition: BS + PL > BS + PL + MCFA ≈ bile + BS + PL + LCFA > BS (**Figure 7.6d**).

The maximum solubilization capacity (C_{sat}) of the mixed micelles was estimated by finding the intersection point of two straight lines drawn through the data at relatively low and at high vitamin concentrations [295]. The low concentration range included those solutions where there was little change in turbidity with increasing vitamin, whereas the high concentration range included those solutions where there was an approximately linear increase in turbidity with increasing vitamin. In general, we found that the solubilization capacity of the mixed micelles was higher for vitamin E than that for vitamin E acetate (**Figure 7.7**), which is consistent with our previous experiments using a simulated GIT model (Yang and McClements, 2013). The origin of this effect

can be attributed to the smaller molecular dimensions of the non-esterified form of α-tocopherol making it easier to be accommodated within the hydrophobic core of the mixed micelles. The solubilization of vitamin E acetate in mixed micelles containing fatty acids was slightly higher than in those containing no fatty acids, which is again in agreement with our previous studies. On the other hand, the influence of mixed micelle composition on the solubilization capacity of vitamin E did not follow the expected trend. The solubilization capacity was actually higher in the mixed micelles containing bile and phospholipids than in those containing bile, phospholipids, and fatty acids. In addition, the solubilization capacity of the mixed micelles containing LCFAs was fairly similar to those containing MCFAs, whereas in the simulated GIT experiments we found a considerably higher bioaccessibility of vitamin E in emulsions prepared using LCFAs rather than MCFAs (Yang and McClements, 2013). One reason for this effect may have been because the mixed micelles containing MCFAs or LCFAS prepared in this study had fairly similar dimensions (**Figure 7.5**), and might therefore be expected to accommodate fairly similar sized lipophilic molecules. Previous studies have also reported that the solubilization of α-tocopherol was fairly similar in mixed micelles in the absence and presence of free fatty acids (MCFAs or LCFAS) [300].

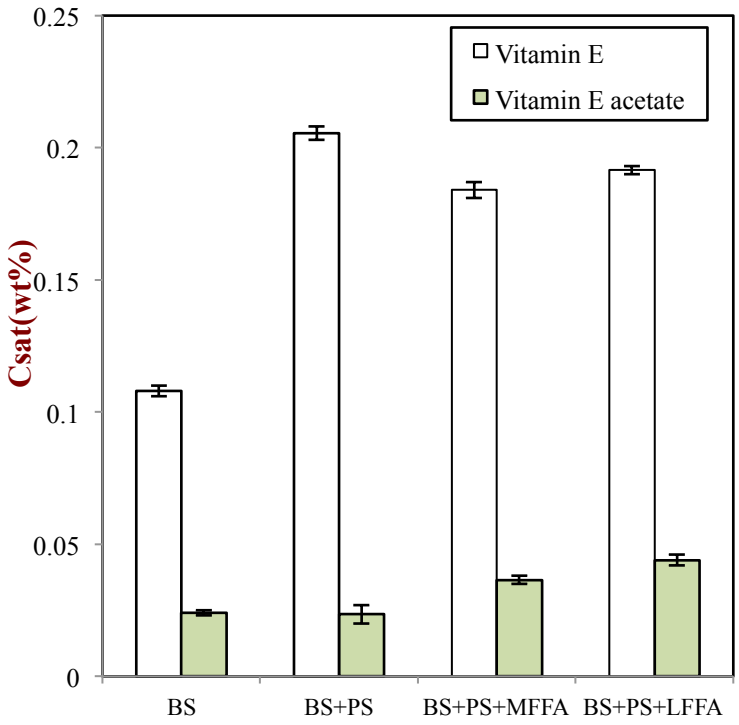


Figure 7.7 Solubilization capacity of mixed micelle solutions with different compositions for vitamin E and vitamin E acetate.

There are a number of possible physicochemical mechanisms that might account for the difference between the results of the emulsion titration assay and experiments carried out using GIT models. First, the mixed micelles used in this study contained free fatty acids (FFA), but they did not contain monoacylglycerols (MAG), which are also digestion products of triacylglycerols. Studies in the pharmaceutical industry have shown that the solubilization of α-tocopherol in mixed micelles containing MAGs was appreciably higher than mixed micelles containing FFAs [300]. Second, when a GIT model is used to study the bioaccessibility of lipophilic bioactive components there will be various other types of colloidal structures formed during lipid digestion, such as

vesicles and liquid crystals that may play an important role in the solubilization of oil-soluble vitamins. Third, in the emulsion titration assay the oil-soluble vitamins must move from vitamin droplets into micelles, but in the simulated gastrointestinal model the vitamins are trapped within fat droplets that also contain triacylglycerols that are hydrolyzed by lipase during the lipid digestion process. The continuous formation of FFAs and MAGs at the fat droplets during digestion, and their subsequent movement into the surrounding aqueous phase, may also play an important role in the solubilization of oil-soluble vitamins. Finally, the simulated intestinal fluids used within GIT models contain various other components that may impact the solubilization process, such as calcium ions that form insoluble calcium soaps with long chain FFAs [309]. Clearly, further research is needed to establish the molecular basis for differences in the solubilization capacities of mixed micelle phases with different compositions and structures.

7.4 Conclusions

The purpose of this study was to utilize a well-defined model system to determine the influence of mixed micelle composition and structure on the solubilization of oil-soluble vitamins. Initially, we establish an appropriate procedure for preparing transparent mixed micelle solutions containing bile salts, phospholipids, and free fatty acids. Mixed micelle solutions were formed that contained relatively small particles ($d \approx 5$ nm) whose precise size depended on system composition. An emulsion titration

assay was then used to study the solubilization of vitamin E and vitamin E acetate emulsions in the mixed micelle solutions. The solubilization capacity of the mixed micelle solutions was higher for vitamin E than for vitamin E acetate, which was attributed to differences in the ability of the vitamin molecules to be incorporated into the micelle structures. We did not find a strong influence of free fatty acid type ($C_{8:0}$ *versus* $C_{18:1}$) on the solubilization capacity of the mixed micelles, which was different from results obtained using a simulated gastrointestinal model. This study provides an improved understanding of the influence of mixed micelle composition and structure on the solubilization of α-tocopherol and α-tocopheryl acetate, which may be useful for the design of emulsion-based delivery systems for oil-soluble vitamins.

CHAPTER 8

ENHANCING VITAMIN E BIOACCESSIBILITY: FACTORS IMPACTING SOLUBILIZATION AND HYDROLYSIS OF A-TOCOPHEROL ACETATE ENCAPSULATED IN EMULSION-BASED DELIVERY SYSTEMS

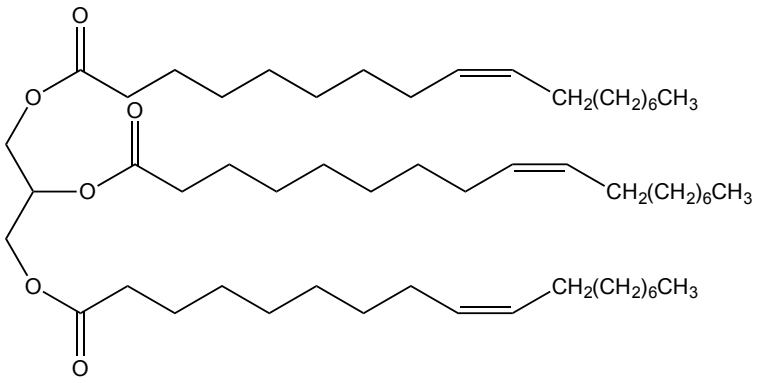
8.1. Introduction

The term "vitamin E" refers to a group of naturally occurring compounds that have common molecular, physicochemical, and biological features, with α-tocopherol being the most biological active form [310]. As well as its important role as an essential nutrient, vitamin E may also provide additional health benefits, such as reducing cardiovascular disease, diabetes, and cancer due to its antioxidant and non-antioxidant biological activities [244, 311, 312]. Vitamin E can also protect lipids in foods against oxidation due to the ability of α-tocopherol to trap peroxyl radicals, which are responsible for the initiation of lipid oxidation [87, 88]. The food industry is therefore interested in fortifying functional foods and beverages with vitamin E due to its antioxidant activity and potential health benefits [22]. However, the incorporation of α-tocopherol into many commercial products is a challenge because of its relatively low chemical stability, water-solubility, and bioavailability [244, 311, 312].

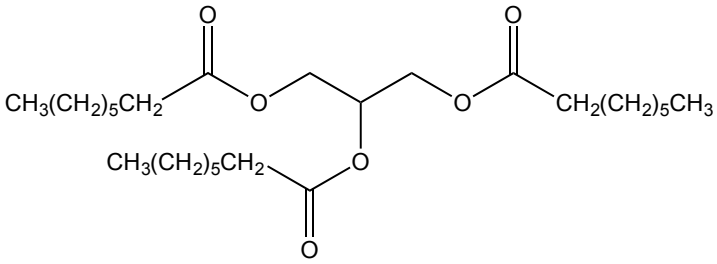
Emulsion-based delivery systems are especially suitable for encapsulating, protecting and delivering lipophilic bioactive components, such as ω-3 fatty acids, carotenoids, curcuminoids phytosterols, and oil-soluble vitamins [3, 22, 163]. A

considerable amount of research has already been carried out to identify the major factors affecting the bioavailability of lipophilic bioactive molecules encapsulated within emulsion-based delivery systems, such as particle size, physical state, and interfacial properties [313-316]. It is important that any encapsulated bioactive component has a high oral bioavailability so that it can effectively deliver its health benefits after ingestion. However, a number of physicochemical and physiological processes occur within the human gastrointestinal tract that impact the oral bioavailability of lipophilic vitamins [275, 276]. After ingestion, vitamin E is usually released from the food matrix, solubilized within mixed micelles in the small intestine, and then transported to the epithelium cells where it is absorbed [256, 317]. Mixed micelles are complex colloidal structures formed from bile salts and phospholipids present in the intestinal fluids, as well as free fatty acids and monoacylglycerols generated by lipid hydrolysis. The bioaccessibility of oil-soluble nutraceuticals, vitamins and drugs has previously been shown to increase when the amount of mixed micelles present within the intestinal fluids increases, which typically occurs as the amount of co-ingested digestible lipids (triacylglycerols) increases [257, 296]. The bioaccessibility also depends on the nature of the mixed micelles formed after lipid digestion, *i.e.,* the composition and nature of the colloidal structures formed [257-259]. Indeed, highly lipophilic components encapsulated using delivery systems containing long chain triglycerides (LCT) have been reported to have a higher bioaccessibility than those containing medium chain

triglycerides (MCT) (**Figure 8.1**), which can be attributed to the ability of the mixed micelles formed by LCT to incorporate larger lipophilic molecules [318, 319].



Glyceryl trioleate (C18:1)



Glyceryl trioctanoate (C8:0)

Figure 8.1 Structures of long chain triglyceride (glyceryl trioleate, C18:1) and medium chain triglyceride (glyceryl trioctanoate, C8:0) used in this study

The chemical form of the vitamin E present within a food or beverage product also influences its bioavailability. Vitamin E is often incorporated into foods in an esterified form (α-tocopherol acetate) because it has a higher chemical stability than the non-esterified form (α-tocopherol) [250]. However, the esterified form of vitamin E has a lower bioaccessibility than the free form, presumably because it is more difficult to incorporate into mixed micelles [260-263]. Consequently, the bioavailability of vitamin

E would be increased if there were greater conversion of α-tocopherol acetate to α-tocopherol in the gastrointestinal tract due to the presence of digestive enzymes.

Numerous factors influence the digestion of emulsified triglycerides by pancreatic lipase and the subsequent formation of mixed micelles, including droplet surface area, interfacial composition, carrier oil type, calcium ions, bile salts, and phospholipids [271, 279, 320]. Calcium ions have been identified as playing a particularly important role in the digestion of emulsified LCTs. In the absence of calcium, long-chain free fatty acids (FFAs) accumulate at the oil-water interface and inhibit further lipid digestion, presumably by preventing lipase from reaching non-digested lipids at the core of the emulsion droplets [321]. The presence of calcium ions facilitates lipid digestion due to the formation of insoluble calcium soaps that remove long-chain FFAs from the emulsion droplet surfaces, thereby allowing the lipase to remain in close contact with the non-digested lipids [322-327]. One might expect the bioaccessibility of oil-soluble vitamins to increase in the presence of calcium ions since then more LCTs would be digested, leading to the release of more vitamin molecules from the lipid droplets and to the formation of more mixed micelles capable of solubilizing them. On the other hand, calcium ions may interact with mixed micelles and form insoluble complexes that actually reduce the bioaccessibility of oil-soluble vitamins by preventing them from being absorbed [328]. The rate and extent of lipid digestion in emulsion-based delivery systems comprised of MCT (rather than LCT) have

been shown to be much less sensitive to calcium ions [271]. Research is therefore needed to determine the potential effect of carrier oil type and calcium ions on the bioaccessibility of emulsified oil-soluble vitamins.

In a recent study using a simulated gastrointestinal tract (GIT) model, we found that the bioaccessibility of vitamin E was higher in emulsions prepared using LCTs than in those prepared using MCTs [318]. The LCT (corn oil) and MCT used in that study were food-grade oils containing a mixture of different triacylglycerols. In a follow up study, we used model mixed micelles assembled from well-defined fatty acids (C8:0 or C18:1) to provide further insights into the impact of MCT and LCT digestion products on vitamin E solubilization [329]. However, we did not find an appreciable difference between the vitamin E solubilization capacity of mixed micelles prepared from C8:0 or C18:1. The apparent discrepancy between these two studies may have been due to differences in the nature of the lipids used or due to differences in the simulated gastrointestinal conditions used.

The purpose of the current study was therefore to use a simulated GIT model to establish the influence of carrier oil type (C8:0 *versus* C18:1) and small intestinal composition (bile, calcium, and phospholipids) on the bioaccessibility of emulsified vitamin E. The information gained from this study will be useful for designing more effective emulsion-based delivery systems for these important lipophilic bioactive components.

8.2 Materials and methods

8.2.1 Materials

Sodium cholate (NaC), sodium deoxycholate (NaDC), glyceryl trioleate, and glyceryl trioctanoate were purchased from the Sigma Chemical Company (St. Louis, MO). 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Vitamin E acetate was kindly supplied by BASF (Florham Park, NJ). Lipases from porcine pancreas pancreatin and bile extract (porcine) were also obtained from the Sigma Chemical Company (St. Louis, MO). All other chemicals used were of analytical grade. Double distilled water was used for the preparation of all solutions and emulsions.

8.2.2 Vitamin E emulsion preparation

Vitamin E emulsions were prepared by homogenizing 2.5 wt% lipid phase (Vitamin E acetate : triacylglycerol = 1:1) with 97.5 wt% aqueous phase. The aqueous phase was comprised of surfactant (0.5 wt% bile salt) and buffer solution (10 mM sodium phosphate buffer, pH 7.0). A coarse emulsion premix was prepared by blending the lipid and aqueous phases together using a high-speed mixer (Bamix, Biospec Products, Bartlesville, OK) for 2 min at room temperature. Fine vitamin E emulsions were formed by passing the coarse emulsions through an air-driven microfluidizer (Microfluidics, Newton, MA, USA). The coarse emulsions were passed through the homogenizer for 4 passes at 9,000 psi.

8.2.3 Particle characterization

Mean particle sizes and particle size distributions of initial emulsions and samples exposed to GIT conditions were measured using static light scattering (Mastersizer 2000, Malvern Instruments, Malvern, UK), while their electrical charge (ζ-potential) was measuring by electrophoretic mobility (Nano-ZS, Malvern Instruments, Worcestershire, UK). The mean particle diameter, particle size distribution, and electrical charge of mixed micelles were determined by dynamic light scattering and electrophoretic mobility (Nano-ZS, Malvern Instruments, Malvern, UK). Samples were equilibrated for 1 min inside the instrument before data were collected over at least 10 sequential readings and analyzed using the Smoluchowski model.

8.2.4 *In vitro* small intestine digestion

Each emulsion sample was passed through a simulated small intestine model. Samples (10 ml) were added to a clean beaker, mixed with 20 mL pH 7.0 PBS buffer and then incubated in a water bath (37 ºC) for 10 min, and then adjusted to pH 7 using NaOH solution (range from 0.05 to 1 M). The mixture was then incubated for 2 h at 37 ºC with simulated small intestinal fluids (SSIF) of different compositions (**Table 8.1**). SSIFs with five different compositions were prepared: bile extract without $CaCl_2$; bile extract with $CaCl_2$; bile salts (NaC and NaDC) without $CaCl_2$; bile salts (NaC and NaDC) with $CaCl_2$; bile salts (NaC and NaDC) with $CaCl_2$ and DOPC. A pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at pH 7.0) of the digestion solution after the sample

and SSIF were mixed [271]. The amount of alkali solution (0.25 M NaOH) that had to be added to the reaction chamber to maintain the pH at 7.0 was recorded, and used to determine the percentage of free fatty acids (FFA) released from the system (McClements & Li, 2010a). A control (containing bile salts but no oil) was run under the same conditions as the samples, and the amount of alkali titrated into the reaction chamber for the control was subtracted from that for the samples before calculating the FFA released. Samples were also taken for physicochemical and structural characterization after 2 h incubation in the small intestinal stage.

Table 8.1 Compositions of the simulated small intestinal fluids (SSIFs) used in the study: NaC = sodium cholate; NaDC = sodium deoxycholate; DOPC = 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine (phospholipid).

SSIF	Bile Extract	NaC	NaDC	DOPC	$CaCl_2$	Lipase
Bile Extract	4 mL (187.5 mg)	**0**	**0**	**0**	**0**	2.5 mL (60 mg)
Bile Extract & Ca^{2+}	4 mL (187.5 mg)	**0**	**0**	**0**	1m L (110 mg)	2.5 mL (60 mg)
Bile Salts	**0**	4 mL (73.22 mg NaC and 114.06 mg NaDC)		**0**	**0**	2.5 mL (60 mg)
Bile Salts & Ca^{2+}	**0**	4 mL (73.22 mg NaC and 114.06 mg NaDC)		**0**	1m L (110 mg)	2.5 mL (60 mg)
Bile Salts & Ca^{2+} & DOPC	**0**	4 mL (73.22 mg NaC, 114.06 mg NaDC and 36 mg DOPC)			1m L (110 mg)	2.5 mL (60 mg)

8.2.5. Bioaccessibility determination

Vitamin E is solubilized within mixed micelles consisting present in the intestinal lumen before uptake into intestinal epithelial cells [330]. The fraction of lipophilic bioactive compounds solubilized within the mixed micelle phase is usually regarded as the bioaccessibility [119, 331]. The bioaccessibility of vitamin E was determined using a method described previously [318]. Briefly, the digesta resulting from small intestine digestion of the samples was collected and then centrifuged (4000 rpm; CL10 centrifuge, Thermo Scientific, Waltham, MA, USA) at 25 ºC for 40 min. Samples after centrifugation separated into an optically opaque sediment phase at the bottom, a relatively clear aqueous phase in the middle, and sometimes a thin oily or creamed phase at the top. The middle phase was assumed to be the "micelle" phase that solubilized the vitamin E. Vitamin E was extracted from the middle phase using an organic solvent mixture (1:3 isopropanol and isooctane) at 1:5 and then centrifuged at 1750 rpm for another 10 min. 1 mL of the top layer was removed and dried using nitrogen evaporation and stored in the -80 ºC refrigerator prior to further analysis. Before detection by HPLC, samples were dissolved in 200 μL methanol. The vitamin E concentrations in the samples were determined using HPLC (Shimadzu, Kyoto, Japan). A C_{18} reverse phase column (150 - 4.6 mm, 5 μm, Beckman Coulter) was used for the chromatographic separation of α-tocopherol acetate and α-tocopherol. The flow rate of the mobile phase was 1.0 ml/min. An isocratic elution was carried out using

HPLC-grade solvent (95% methanol and 5% double distilled water containing 0.5 % phosphoric acid). The α-tocopherol acetate and α-tocopherol contents were determined using a PDA detector at 295 nm. Tocopherol quantification was determined using external standards. The overall bioaccessibility of vitamin E was estimated using the following expression:

$$Bioaccessibility = \frac{C_{micelle}}{C_{Total}} \times 100\%$$

Here $C_{micelle}$ and C_{Total} represent the total concentration of vitamin E (α-tocopherol acetate + α-tocopherol) in the micelle phase and in the overall system after digestion, respectively. The percentage of specific forms of vitamin E solubilized within the micelle phase was also calculated using the same expression, but for α-tocopherol acetate and for a-tocopherol separately. The conversion of α-tocopherol acetate to α-tocopherol in the overall system after digestion was calculated from the following expression:

$$Conversion = \frac{C_{VE}}{C_{Total}} \times 100\%$$

Here C_{VE} and C_{Total} represent the concentration of α-tocopherol and the total concentration of vitamin E (α-tocopherol acetate + α-tocopherol) in the overall system after digestion, respectively. A preliminary experiment was carried out to estimate the recovery of the total tocopherols using the solvent extraction and HPLC analysis method described above. The recovery of the total tocopherols (VE + VE acetate) was always > 90%, which indicates that the methods used were appropriate.

8.2.8 Statistical analysis

All measurements were performed on at least two freshly prepared samples (*i.e.,* new samples were prepared for each series of experiments) and were reported as means and standard deviations.

8.3 Results and discussion

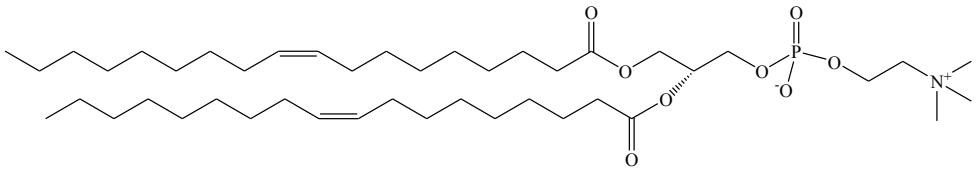
8.3.1 Impact of SSIF composition and carrier oil on gastrointestinal fate of emulsions

Initially, we studied the influence of SSIF composition and carrier oil type on the potential gastrointestinal fate of emulsion-based delivery systems using a simulated small intestine model (pH-stat). Vitamin-fortified emulsions containing 2.5 wt% lipid phase were produced using either long chain triglycerides (C18:1) or medium chain triglycerides (C8:0) as carrier oil. The emulsions were then mixed with SSIFs with different compositions: bile extract (with and without $CaCl_2$); pure bile salts (with and without $CaCl_2$); and, pure bile salts with $CaCl_2$ and phospholipids (DOPC). The influence of SSIF composition and carrier oil type on particle characteristics after digestion were then measured.

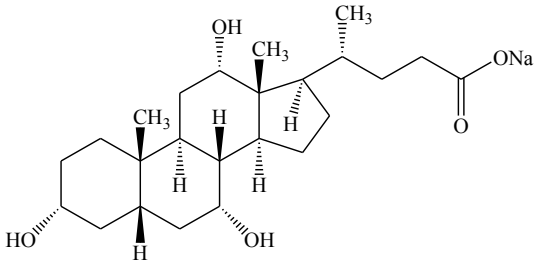
8.3.1.1 Influence on particle size

For both MCT-VE and LCT-emulsions, the initial systems (before digestion) had monomodal particle size distributions (PSDs) and relatively small mean particle diameters (d = 140-150 nm) (**Figures 8.3a-c**). The properties of the vitamin-fortified

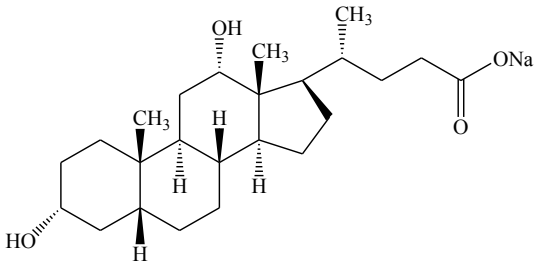
emulsions were also measured after they were exposed to the simulated small intestinal model: mean particle diameters (**Figure 8.3a**) and PSDs (**Figures 8.3b and 8.3c**).



DOPC



NaC



NaDC

Figure 8.2 Structures of the various molecules used for the simulated small intestine fluid (SIF) in this study: Sodium cholate (NaC); Sodium deoxycholate (NaDC) and phospholipid (DOPC).

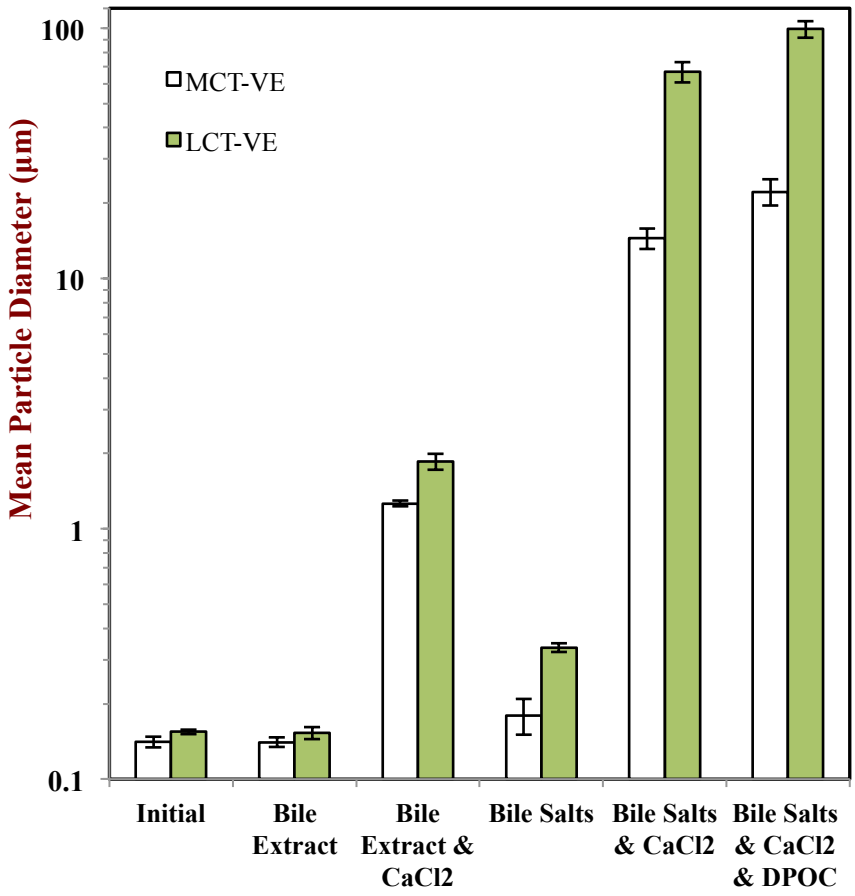


Figure 8.3a Influence of carrier oil type and simulated small intestine fluid composition on the mean particle diameter (d_{32}) of oil-in-water emulsions after passing through a simulated small intestine tract.

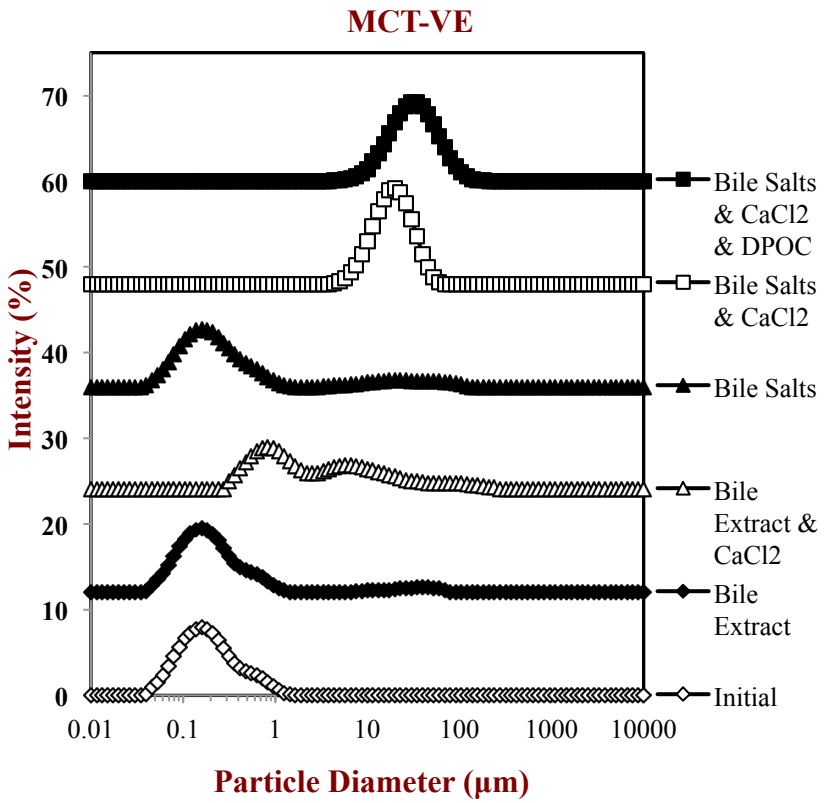


Figure 8.3b Influence of carrier oil type and simulated small intestine fluid composition on the particle size distribution of MCT-VE emulsions passing through the simulated small intestine tract.

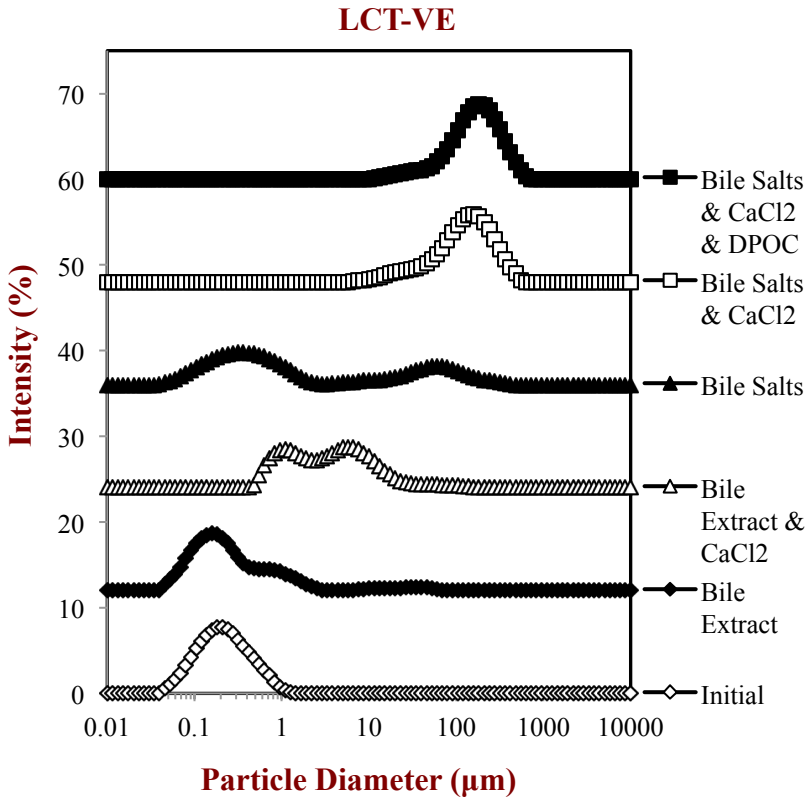


Figure 8.3c Influence of carrier oil type and simulated small intestine fluid composition on the particle size distribution of LCT-VE emulsions passing through the simulated small intestine tract.

After passage through the simulated small intestinal stages, the mean particle diameters of all samples increased and there was evidence of large particles in the PSDs (**Figures 8.3a-c**). The composition of the simulated small intestinal fluids (SSIFs) had a pronounced influence on the particle size of the emulsions after digestion. The presence of bile extract in the SSIF caused little change in particle size, but the presence of pure bile salts (NaC and NaDC) caused an appreciable increase in particle size. The presence of calcium ions in the SSIFs caused a large increase in mean particle diameter (**Figure**

8.2a) and there was evidence of large particles in the PSDs for both MCT- and LCT-emulsions (**Figures 8.2b and 8.2c**). The presence of these large particles indicates a marked change in the structure of the systems after exposure to small intestinal conditions, which may be due to several physicochemical phenomena. The pancreatic lipase in the SSIFs will adsorb to the lipid droplet surfaces and convert the triacylglycerols (TAGs) into free fatty acids (FFAs) and monoacylglycerols (MAGs). The products of lipid hydrolysis may move into the surrounding aqueous phase or remain at the droplet surfaces depending on their water-dispersibility, which is related to their chain length [257, 259]. Long chain FFAs tend to remain at the droplet surface (in the absence of bile salts or calcium), whereas short and medium chain FFAs tend to move into the aqueous phase. Lipid digestion may therefore reduce the size of the initial lipid droplets due to removal of FFA and MAG digestion products from their surfaces [275, 276]. On the other hand, partially digested lipid droplets may be more prone to droplet coalescence due to the change in their interfacial properties, which would lead to an increase in particle size [275, 276]. It should also be noted that the light scattering instrument is sensitive to all kinds of particles that scatter light within the sample, which includes any mixed micelles or insoluble calcium complexes formed after digestion [327].

The measured particle size did not change much after MCT-VE or LCT-emulsions were exposed to SSIFs in the absence of calcium, regardless of whether

bile extract or pure bile salts were used (**Figures 8.3a-c**). This may have occurred because some of the lipid droplets were not digested and retained their original size, but this is unlikely since the pH-stat measurements (described below) indicated that lipid digestion had occurred. It is therefore possible that the mixed micelles formed by lipid digestion were of a similar size to the original lipid droplets in the system.

In general, the droplet sizes were appreciably larger when the SSIFs contained bile salts than when they contain bile extract (**Figure 8.3a**). Bile extract from porcine is a complex mixture that contains various bile salts, phospholipids and other components, whereas the bile salts only contained pure NaC and NaDC. Hence, the concentration of actual bile salts in the system would be higher for the pure bile salts than for the bile extract, which may have led to the formation of more insoluble complexes with calcium. In addition, the mixed micelles formed by pure bile salts may have been larger than those formed by bile extract.

Another complication associated with interpreting the results of light scattering measurements in complex colloidal dispersions is associated with data analysis. The software used to calculate the particle size distribution of a colloidal dispersion from its light scattering pattern usually assumes that the particles are spherical, dilute, and have well-defined refractive indices. However, the colloidal dispersions resulting from lipid digestion contain a complex mixture of particles with different compositions and structures, such as undigested lipid droplets, partially digested lipid droplets, micelles,

vesicles, and various other colloidal structures. Light scattering results should therefore be treated with some caution for this type of complex colloidal dispersion.

8.3.1.2 Influence on particle charge characteristics

In this section, changes in the electrical charge on the particles in the samples after digestion were measured to provide some information about possible changes in interfacial composition (**Figure 8.4**). Initially, all of the oil droplets coated by bile salts were highly negatively charged (-61.6 mV for MCT emulsions and -63.7 mV for LCT emulsions), which can be attributed to ionization of the bile salts. At neutral pH, bile salts have an appreciable negative charge due to the presence of anionic carboxyl groups, *i.e.,* $-COO^-$ [301]. The particles in all the samples were negative after exposure to the simulated small intestine stage, although there was some reduction in the magnitude of their negative charge (**Figure 8.4**). The negative charge on the particles may be a result of some of the initial bile salts remaining at the droplet surfaces, as well as due to the absorption of other anionic surface active species from the digestion medium. The reduction in droplet charge may have been because some of the bile salts were displaced by FFAs or because of the increase in ionic strength in the system [332].

The charge on the particles present in the digesta was less negative in the presence of calcium ions (-21 to -32 mV) than in their absence (-44 to -52 mV) for both carrier oil types, which can be attributed to binding of cationic Ca^{2+} ions to the surfaces of the anionic droplets and mixed micelles, as well as some electrostatic screening effects

[332]. The negative charge was higher on the particles present in LCT-emulsions than those present in MCT-emulsions after digestion (**Figure 8.4**) which may be due to the fact that glyceryl trioleate contains long chain fatty acids (C18:1) that accumulate at oil-water interfaces, whereas glyceryl trioctanoate contains medium chain fatty acids (C8:0) that tend to move into the surrounding aqueous phase [333].

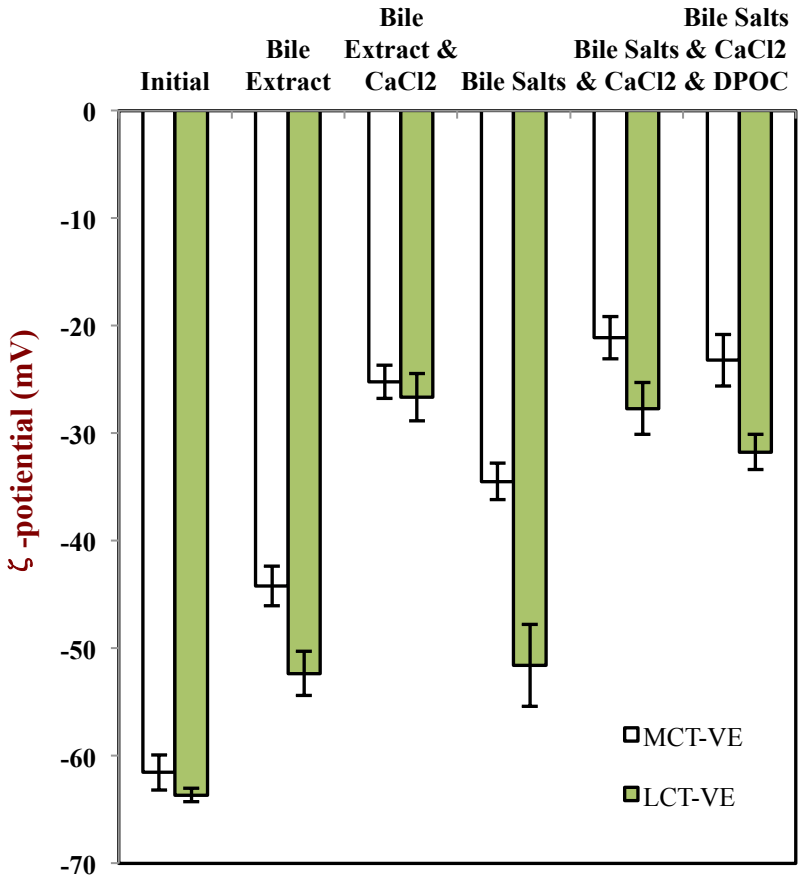


Figure 8.4 Influence of carrier oil type and SSIF composition on the electrical characteristics (ζ-potential) of the particles in oil-in-water emulsions passed through simulated small intestine.

It should be noted that it is not clear exactly what types of particles are detected by an electrophoresis instrument in a complex colloidal dispersion that contains different

types of charged particles that scatter light. Moreover, since measurements were made on diluted and stirred samples, the nature of the particles in the measurement cells might be different from the particles in the original undiluted samples. One should therefore be cautious when interpreting the results from electrophoresis measurements on this type of complex colloidal dispersion.

8.3.2 Impact of SSIF composition and carrier oil type on lipid digestion

In this part of the study, the influence of carrier oil type and SSIF composition on the rate and extent of lipid digestion was measured using the pH-stat method, which is widely used in pharmaceutical and food research for this purpose [271, 277, 322, 334-337]. The principle of the pH-stat method is to measure the volume of alkaline solution (0.25 M NaOH) required to neutralize the free fatty acids (FFAs) released from a sample when incubated in SSIFs containing lipase. This information is then used to calculate the percentage of FFAs released from the sample, assuming that a maximum of two FFAs are released per triglyceride molecule.

Generally, there was a rapid initial increase in the volume of NaOH added to the emulsions during the first few minutes of incubation in SSIFs, followed by a more gradual increase at longer times (**Figure 8.5**). This result suggests that lipases in the SSIFs were able to quickly adsorb to the lipid droplet surfaces and convert encapsulated TAGs into FFAs and MAGs. Nevertheless, vitamin-fortified emulsions prepared using different types of carrier oil exhibited quite different behavior. In the absence of

calcium, the rate and extent of lipid digestion was appreciably higher for MCT-emulsions than for LCT-emulsions (**Figure 8.5e**), which is in good agreement with previous studies [257, 259, 279]. As mentioned earlier, long-chain FFAs accumulate at lipid droplet surfaces in the absence of calcium ions [333], which inhibits digestion by preventing lipase molecules from reaching non-digested TAGs at the core of the lipid droplets [321].

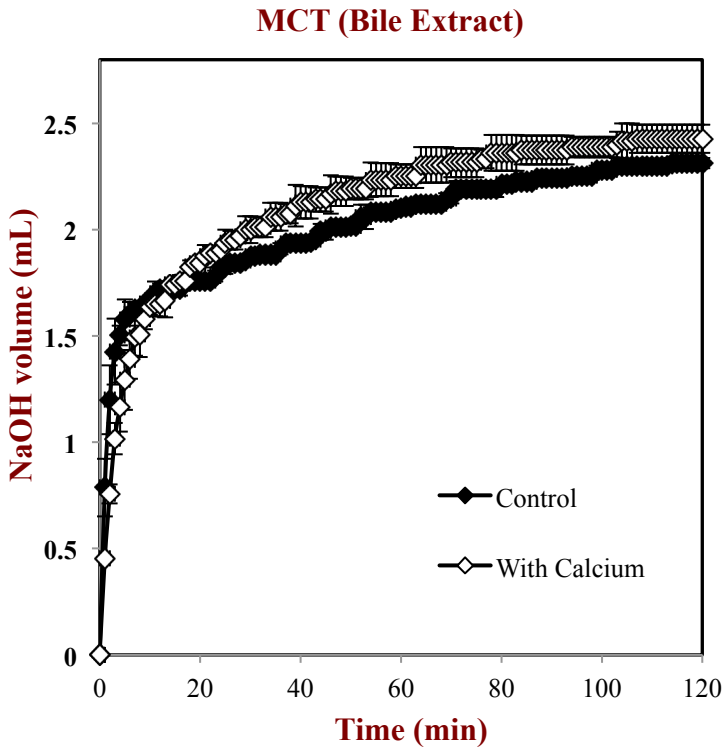


Figure 8.5a Effect of calcium ($CaCl_2$) on the rate and extent of lipid digestion in MCT-VE emulsions determined using a pH-stat method with SSIF containing bile extract

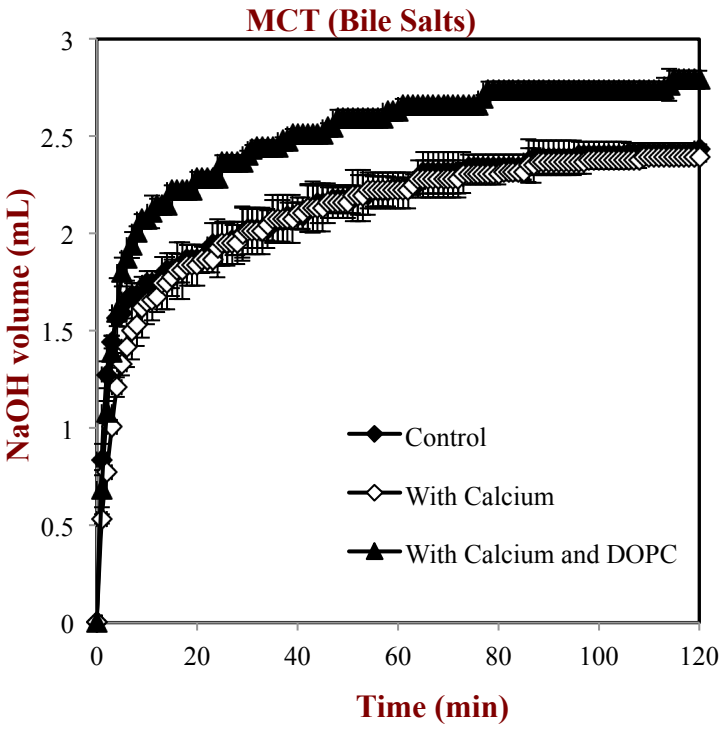


Figure 8.5b Effect of calcium ($CaCl_2$) and phospholipids (DOPC) on the rate and extent of lipid digestion in MCT-VE emulsions determined using a pH-stat method with SSIF containing pure bile salts (NaC and NaDC).

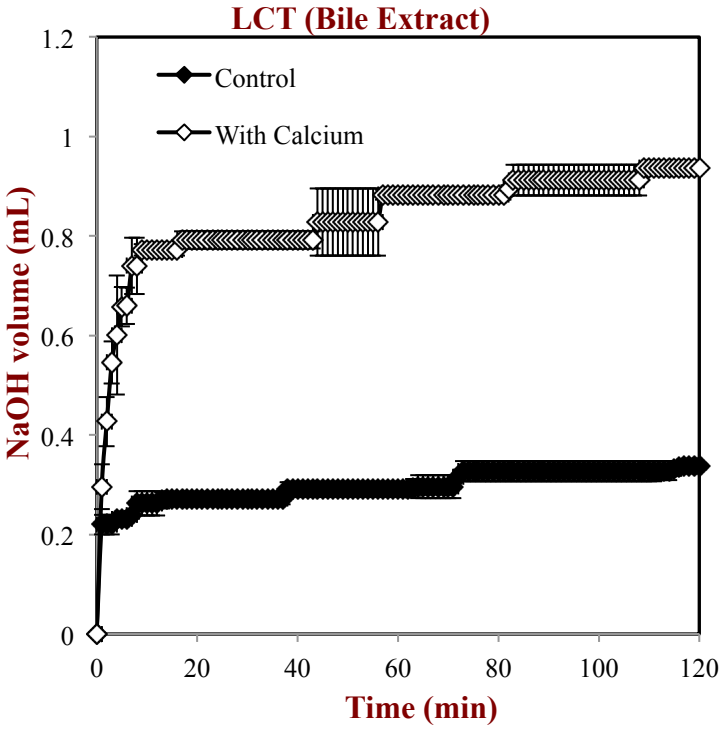


Figure 8.5c Effect of calcium ($CaCl_2$) on the rate and extent of lipid digestion in LCT-VE emulsions determined using a pH-stat method with SSIF containing bile extract.

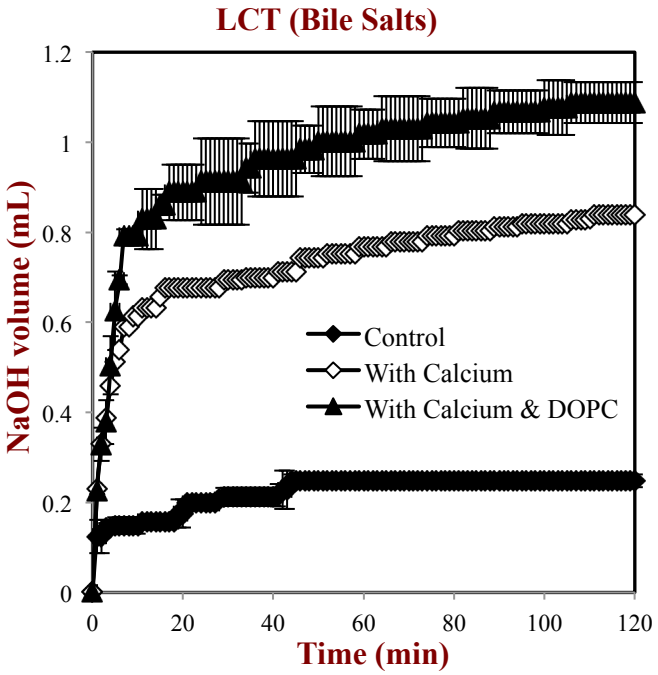


Figure 8.5d Effect of calcium ($CaCl_2$) and phospholipids (DOPC) on the rate and extent of lipid digestion in LCT-VE emulsions determined using a pH-stat method with SSIF containing pure bile salts (NaC and NaDC).

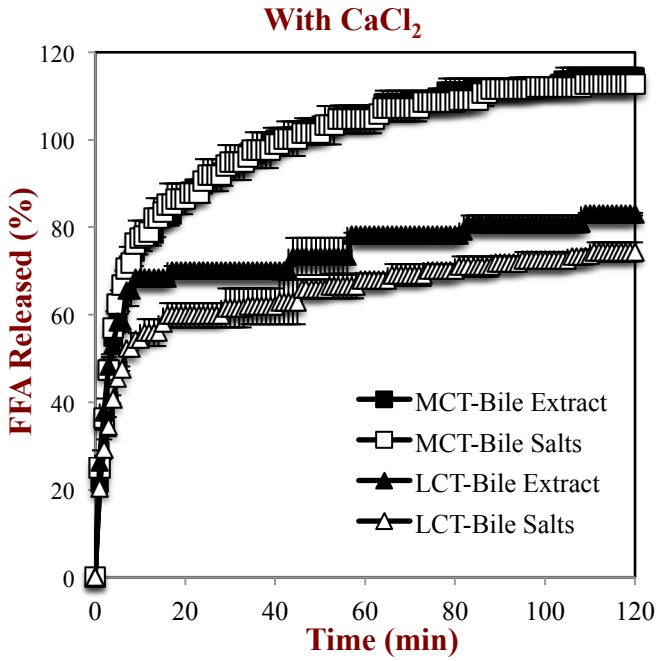


Figure 8.5e Influence of carrier oil type and bile salt type on the rate and extent of lipid digestion in vitamin E fortified emulsions measured using a pH-stat method. The SSIFs all contained calcium.

The influence of calcium and phospholipid addition on the titration of FFAs during *in vitro* digestion was also studied (**Figure 8.5**). For MCT-emulsions, the presence of calcium ions in the SSIF did not cause an appreciable alteration in lipid digestion (**Figures 8.5a and 8.5b**). However, for LCT-emulsions, the addition of calcium ions led to an appreciable increase in the final amount of lipid digestion products generated (**Figures 8.5c and 8.5d**). These measurements clearly show that calcium ions

have a major impact on lipid digestion in LCT-emulsions but not in MCT-emulsions, which is in agreement with previous research [327]. This result may be explained by a number of physicochemical mechanisms. First, a certain amount of calcium is required as a co-factor to activate pancreatic lipase [338, 339]. Thus, the lower extent of FFA production in the absence of calcium may be partly due to the fact that the enzyme was not in its most active form. However, this is unlikely to be important because lipid digestion still occurred in the MCT-emulsions in the absence of calcium. Second, calcium ions bind to long-chain FFAs generated during the digestion of emulsified LCT and form insoluble calcium soaps that remove them from the droplet surfaces [322, 323, 326, 327]. The precipitation of these long-chain fatty acids enables lipase molecules to come into close contact with the remaining non-digested lipids and facilitate their digestion [340]. The digestion of emulsified MCT is less dependent on calcium ions because the lipid digestion products (medium-chain FFAs) are more water-dispersible and rapidly move into the surrounding aqueous phase, thereby enabling lipase to continue operating at the droplet surfaces. Calcium ions may also impact lipid digestion by affecting other characteristics of emulsions, such as droplet aggregation [341, 342]. Anionic lipid droplets may become flocculated in the presence of cationic calcium ions due to ion binding and electrostatic scattering screening effects, which may reduce the ability of lipase to interact with the lipid droplet surfaces [145, 343].

For both MCT-VE and LCT-emulsions, the addition of phospholipids (DOPC)

into the SSIFs increased the final extent of lipid digestion. This may have occurred because phospholipids facilitated the ability of the lipase to interact with the emulsified TAGs, or because the phospholipids were themselves hydrolyzed and released FFAs. Based on the amount of DOPC (36 mg) present in the SSIF, and the assumption that one FFA is released per phospholipid molecule, we calculated that about 1.8 mL of 0.25 M NaOH would be required to neutralize any fatty acids produced due to phospholipid hydrolysis. This value is quite close to the difference between the volumes of NaOH required to neutralize calcium-containing samples in the presence and absence of DOPC (**Figures 8.5b and 8.5d**). We therefore conclude that the increased amount of alkali required for the samples containing DOPC is mainly due to the hydrolysis of the phospholipid by digestive enzymes in the SSIFs.

8.3.3 Impact of SSIF composition and carrier oil type on vitamin E bioaccessibility

The impact of SSIF composition and carrier oil type on the bioaccessibility of vitamin E after passage through the simulated small intestine was also examined. The bioaccessibility was determined by incubating the emulsions in SSIFs for 2 hours, centrifuging the resulting digesta, and then determining the concentration of vitamin E in the micelle phase and overall digesta using HPLC.

The overall appearance of the digesta after exposure to SSIFs depended on calcium content (**Figure 8.6**). In the absence of calcium, samples separated into a thick white layer at the top ("cream"), a clear or slightly turbid layer in the middle ("micelle

phase"), and a thin white layer at the bottom ("sediment"). The white layer at the top probably consisted of non-digested fat droplets and possibly some large mixed micelle structures (which are less dense than water), while the white layer at the bottom probably contained insoluble matter such as bile salt or protein complexes (which are denser than water). In the presence of calcium, we only observed a single white layer ("sediment") at the bottom of the samples with a clear or slightly turbid layer above ("micelle phase"). The fact that a cream layer was not observed in the samples containing calcium can be attributed to two factors. First, calcium promoted digestion of the lipid phase (**Figure 8.5**), and so there would be less non-digested lipid droplets present. Second, cationic calcium ions (Ca^{2+}) formed dense insoluble aggregates with anionic species, such as bile salts and free fatty acids, which sedimented to the bottom of the tubes.

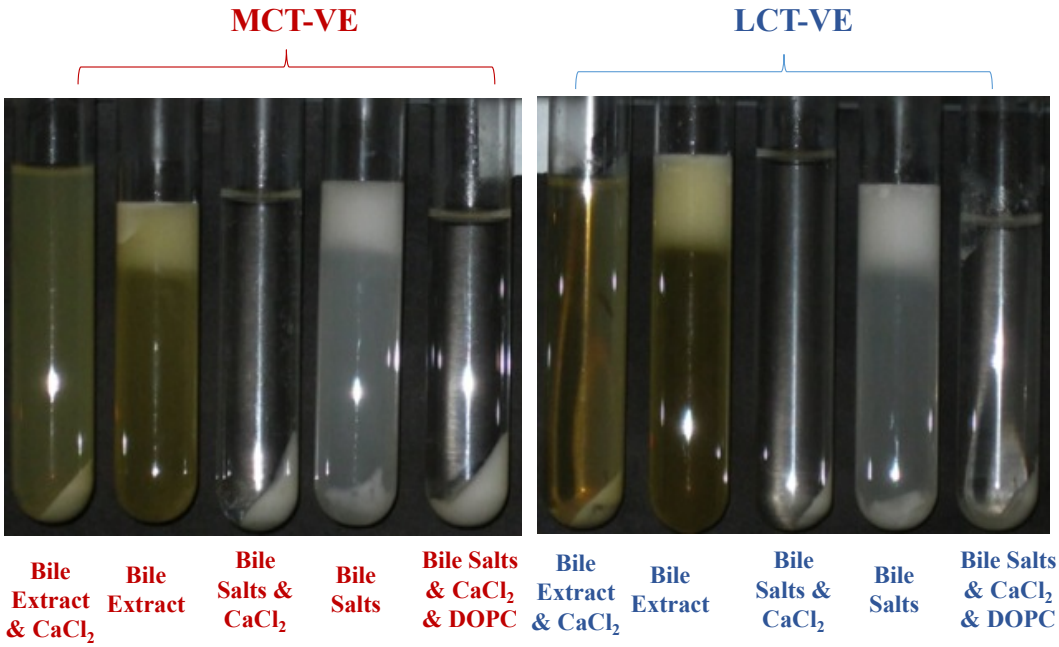


Figure 8.6 Influence of carrier oil type and SSIF composition on the appearance of the micelle phase after *in vitro* digestion of vitamin E fortified emulsions.

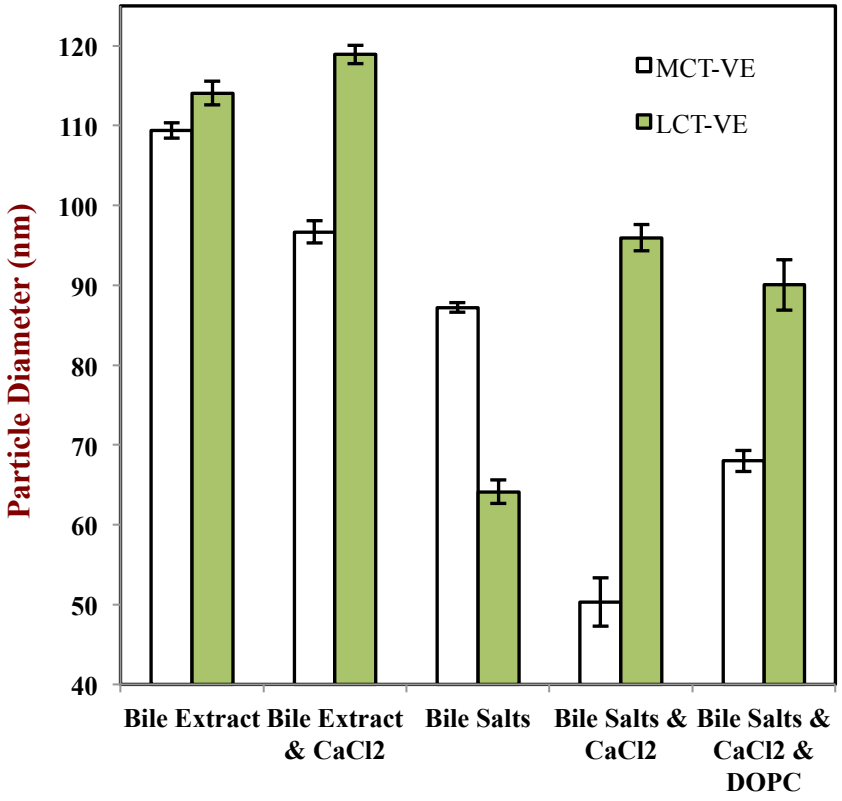


Figure 8.7 Influence of carrier oil type and SSIF composition on the mean particle diameter of the micelle phase after *in vitro* digestion of vitamin E fortified emulsions. The samples were filtered using a 450 nm filter prior to analysis to simulate passage through the mucus layer.

The mixed micelles formed in the human body are compositionally and structurally complex colloidal dispersions whose properties depend on the nature of any co-ingested lipids [257, 296]. Mixed micelles contain bile salts, phospholipids, and cholesterol from the small intestinal fluids, as well as monoacylglycerols (MAG) and free

fatty acids (FFAs) from any lipid digestion products. These surface-active lipids self-assemble into the mixed micelle phase, which may contain micelles, vesicles, and liquid crystalline phases that vary in composition, dimensions, and structure [297, 298]. The micelle phase was therefore passed through a 450 nm pore size filter before measuring the bioaccessibility to more closely simulate gastrointestinal conditions [280, 344]. Lipophilic bioactive components solubilized within mixed micelles must pass through the mucus layer before they can be absorbed by the human body. The mucus layer acts as a biological filter that only allows particles smaller than about 400 nm to pass through [280]. Filtering the micelle layer prior to analysis may therefore provide a more accurate representation of the potential bioavailability of a lipophilic compound that needs to be transported by mixed micelles through the mucus layer.

In the presence of calcium ions, the size of the particles in the micelle phases collected from the LCT-emulsions were slightly larger than those collected from the MCT-emulsions (**Figures 8.7**), which suggests that that the colloidal structures in the micelle phase formed by LCT digestion products were larger than these formed by MCT digestion products.

The nature of the bile salts present in the SSIFs also had an influence on the size of the structures formed in the mixed micelle phase. The particle size was larger when the SSIF contained bile extract, than when it contained pure bile salts (**Figure 8.7**). Bile extract contains a mixture of bile salts, phospholipids, and other components [327, 345],

which may have led to the formation of larger mixed micelles. Indeed, when phospholipids (DOPC) were added to the SSIF containing bile salts and calcium ions there was an appreciable increase in the size of the colloidal structures present in the micelle phase (**Figure 8.7**). In addition, bile extract may have contained some insoluble matter that also contributed to the light scattering signal.

The bioaccessibility of the vitamin E was determined by measuring the concentrations of α-tocopherol acetate and α-tocopherol in the micelle phase and within the total digesta after *in vitro* digestion (**Figure 8.8**). Calcium ions had a major impact on the bioaccessibility of vitamin E, which depended strongly on the nature of the carrier oil used. For the MCT-emulsions, the addition of calcium ions to the SSIF led to an appreciable decrease in the bioaccessibility of vitamin E, regardless of the nature of bile salts used. For example, the vitamin bioaccessibility decreased from around 24% to 9% when calcium was added to SSIFs containing bile extract, whereas it decreased from around 31% to 1% when calcium was to SSIFs containing pure bile salts. It is possible that cationic calcium ions formed insoluble precipitates with mixed micelles containing solubilized vitamin E, thereby reducing the amount of vitamin E present within the micelle phase. Conversely, for the LCT-emulsions, the addition of calcium ions to the SSIF led to an appreciable increase in the bioaccessibility of vitamin E. For example, the vitamin bioaccessibility increased from around 22% to 32% when calcium was added to SSIFs containing bile extract, whereas it increased from around 11% to 37% when

calcium was to SSIFs containing pure bile salts. In addition, there was a further increase (to around 66%) when phospholipids (DOPC) were incorporated into the SSIFs for the LCT-emulsions. Previous researchers have also reported that phospholipids can increase the bioavailability of lipophilic bioactive components [346-349]. The solubilization of lipophilic components in the micelle phase usually depends on the total amount of mixed micelles available for transporting them across the mucus layer. Our pH-stat experiments show that a higher amount of lipid digestion products (FFAs and MAGs) are formed when calcium is present during the digestion of LCT-emulsions, and so there should be a greater amount of mixed micelles present to solubilize the vitamin E. In addition, a greater amount of vitamin E should have been released from the lipid droplets when more TAGs were digested. The addition of calcium ions to the LCT-emulsions may therefore increase the amount of vitamin E in the mixed micelle phase. Nevertheless, one might still expect the calcium ions to cause some precipitation of the mixed micelles (as with the MCT-emulsions), which would reduce the amount of vitamin E in the micelle phase. Our results suggest that the greater release of vitamin E from the lipid droplets and the higher amount of mixed micelles formed in the presence of calcium, outweigh the precipitation effect for the LCT-emulsions. These results may have important implications for the design of effective emulsion-based delivery systems for vitamin E. Calcium is normally present within the fluids secreted by the human gastrointestinal tract. This calcium may reduce the bioavailability of vitamin E

delivered in MCT-emulsions, which might be overcome by incorporating some calcium chelating agents in the delivery system (such as EDTA or alginate). On the other hand, calcium may increase the bioavailability of LCT-emulsions, and therefore it may be advantageous to avoid the presence of calcium chelating agents in these delivery systems or to supplement them with additional calcium.

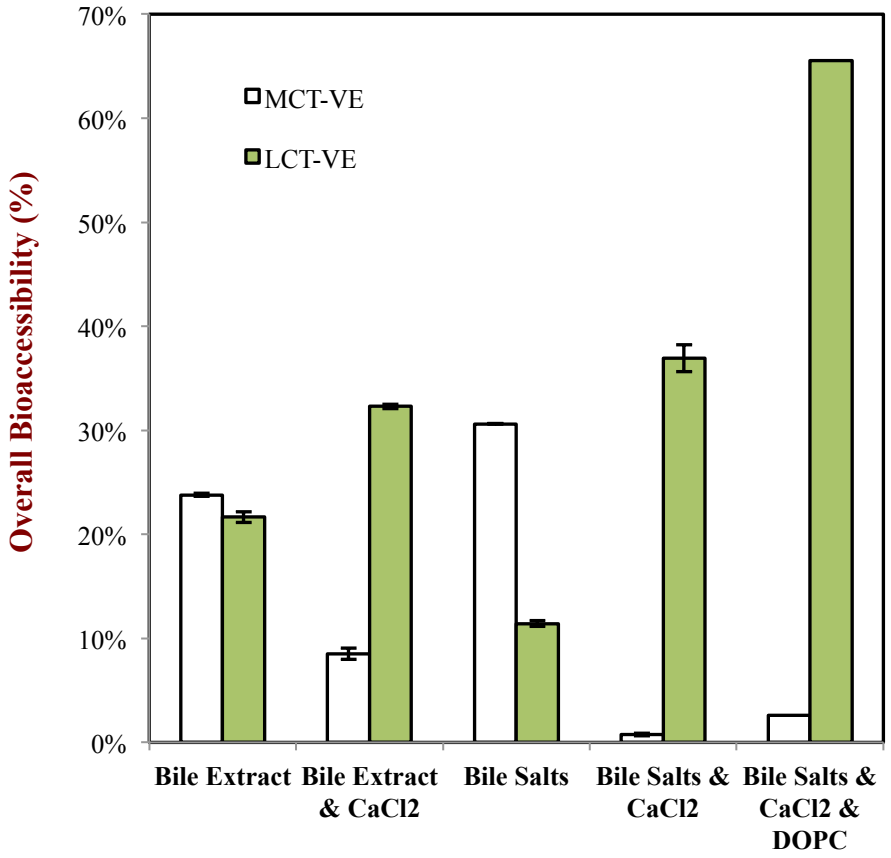


Figure 8.8 Influence of carrier oil type and simulated small intestine fluid composition on the overall bioaccessibility of Vitamin E (α-tocopherol + α-tocopherol acetate) in emulsion-based delivery systems after digestion.

Under realistic digestion conditions (*i.e.,* samples containing bile salts, calcium and phospholipids), the bioaccessibility of vitamin E was appreciably higher when it was

encapsulated in LCT-emulsions than in MCT-emulsions (**Figure 8.8**). The long-chain free fatty acids ($C_{18:1}$) arising from the lipolysis of glycerol trioleate (LCT) can presumably form larger colloid structures with a larger solubilization capacity than the medium chain fatty acids ($C_{8:0}$) generated from the hydrolysis of glycerol trioctanoate (MCT). The α-tocopherol molecule has a non-polar chain with 14 carbon atoms (C_{14}), which is presumably too long to be accommodated into the micelles or vesicles formed by medium-chain fatty acids, but short enough to be incorporated into those formed by long chain fatty acids. Researchers in the pharmaceutical area have also reported that the bioaccessibility of some highly oil-soluble drugs is greater when LCT was used as a carrier oil rather than MCT [281, 282].

8.3.4. Impact of SSIF composition and carrier oil type on vitamin E conversion

The esterified form of α-tocopherol is often used in foods and other commercial products rather than the free form because it is more stable to oxidation during processing, transportation and storage [223, 224]. Previous research has shown that the molecular form of vitamin E has a major impact on the bioaccessibility of vitamin E, *i.e.,* α-tocopherol *versus* α-tocopherol acetate [260-263]. Thus, we determined the amount of α-tocopherol acetate converted to α-tocopherol after *in vitro* digestion (**Figure 8.9**). Our previous research using food-grade oils showed that carrier oil type had an appreciable impact on the hydrolysis of α-tocopherol acetate to α-tocopherol, with the extent of conversion being about 29% for LCT-emulsions and 17% for MCT-emulsions

[318]. This result suggests that the conversion of α-tocopherol acetate to α-tocopherol occurred more readily when LCT was used as the carrier oil than when MCT was used [318]. In the current study, we used purified LCT and MCT carrier oils to provide further insights into this important effect.

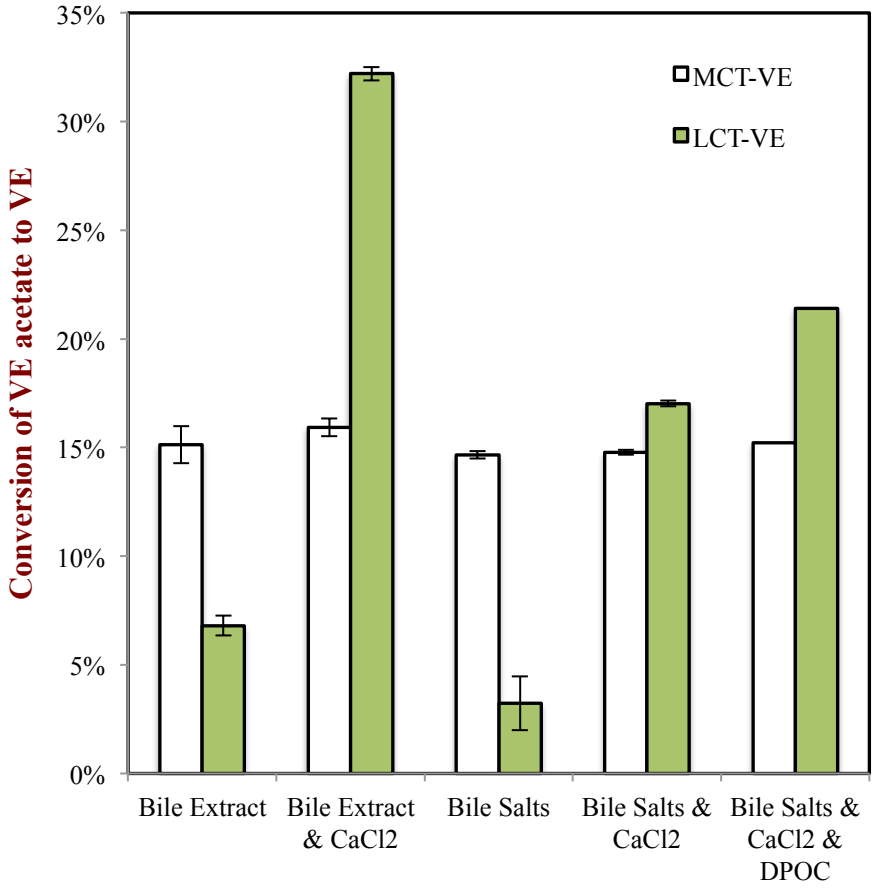


Figure 8.9 Influence of carrier oil type and SSIF composition on the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion of vitamin E fortified emulsions

For MCT-emulsions, the SSIF composition did not have an appreciable impact on the conversion of α-tocopherol acetate to α-tocopherol (**Figure 8.9**). Conversely, for LCT-emulsions, the SSIF composition had a major impact on α-tocopherol acetate

hydrolysis. For the LCT-emulsions, the conversion of α-tocopherol acetate to α-tocopherol increased when calcium and phospholipid were incorporated into the SSIFs (**Figure 8.9**). There therefore appeared to be a correlation between the bioaccessibility of vitamin E and the hydrolysis of α-tocopherol acetate. It is likely that α-tocopherol acetate can only be hydrolyzed by digestive enzymes after it is released from the interior of the fat droplets. Hydrolysis may occur at the lipid droplet surfaces or after the α-tocopherol acetate is incorporated into mixed micelles, which would account for the increase in hydrolysis with increasing bioaccessibility. This effect may also account for that fact that the extent of hydrolysis was greater for the LCT-emulsions than the MCT-emulsions in the presence of calcium ions (**Figure 8.9**).

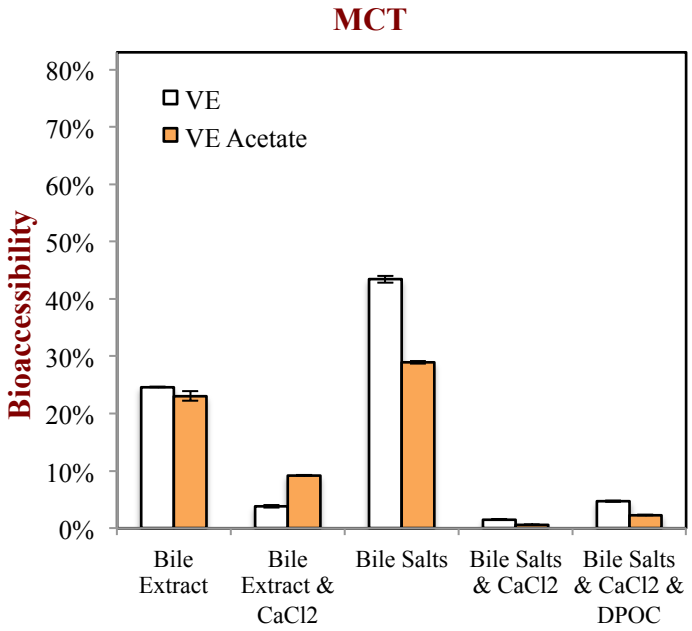


Figure 8.10a Influence of carrier oil type and simulated small intestine fluid composition on the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion of MCT-VE emulsions.

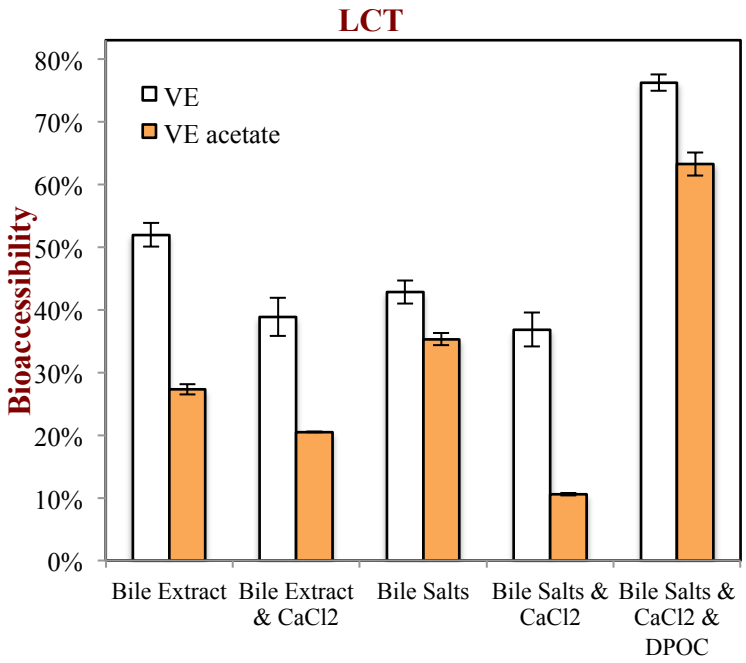


Figure 8.10b Influence of carrier oil type and simulated small intestine fluid composition on the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion of LCT-VE emulsions.

Finally, we calculated the bioaccessibility of the different forms of vitamin E (**Figure 8.10**). The fraction of the non-esterified form (α-tocopherol) in the micelle phase was higher than the esterified form (α-tocopherol acetate) for all LCT-VE samples (**Figure 8.10b**) and most MCT-VE samples (**Figure 8.10a**), which suggests that the non-esterified form can be incorporated into the mixed micelles more easily than the esterified form. The reason for the higher bioaccessibility of α-tocopherol acetate for

the MCT-emulsions when the SSIF contained bile extract and calcium is currently unknown. A schematic representation of the important physicochemical events occurring with the gastrointestinal tract based on our results is shown in **Figure 8.11**.

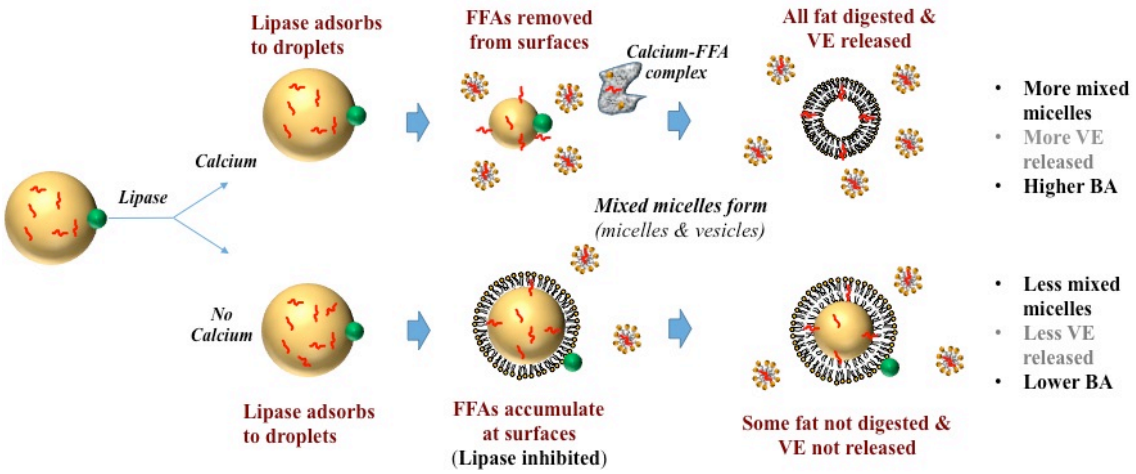


Figure 8.11a Schematic representation of influence of calcium on physicochemical phenomena occurring within gastrointestinal tract during lipid digestion, vitamin release, and solubilization.

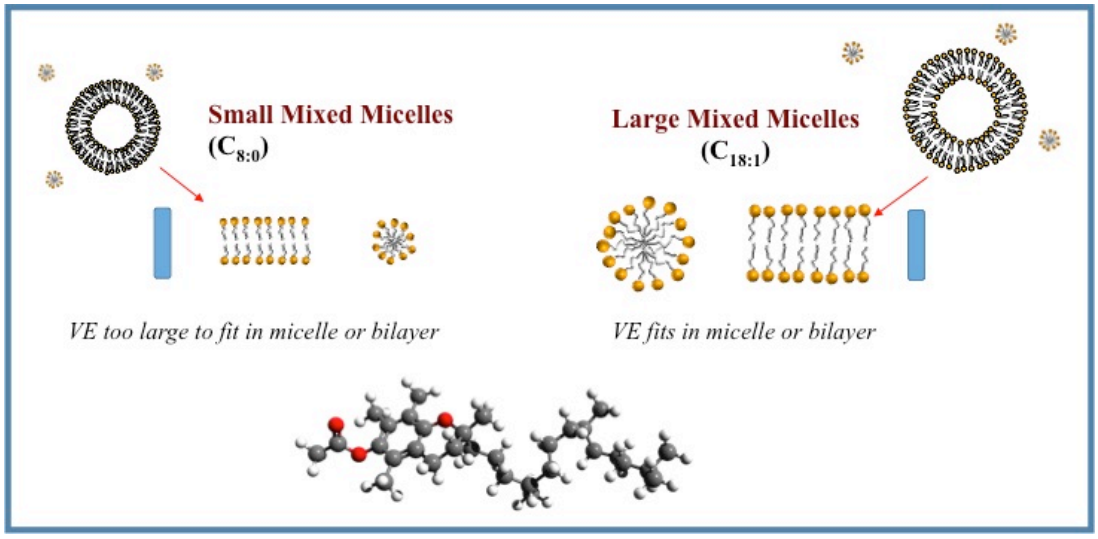


Figure 8.11b Schematic illustration of the influence of free fatty acid chain length on the bioaccessibility of vitamin E. Long chain FFAs form mixed micelles that can easily accommodate large VE molecules, whereas mediuc chain FFAs do not.

8.4 Conclusions

The purpose of this study was to identify the key factors impacting the bioaccessibility of emulsified α-tocopherol acetate using a simulated small intestine model. We have shown that the rate and extent of lipid digestion was higher for MCT-emulsions than for LCT-emulsions, which was attributed to differences in the water-dispersibility of the medium and long chain fatty acids formed during lipolysis. The addition of calcium ions to the SSIFs greatly increased the extent of lipid digestion for LCT-emulsions, but had little effect on MCT-emulsions, which was attributed to the ability of calcium ions to remove long-chain fatty acids from droplet surfaces. The addition of calcium ions and phospholipids into the SSIFS also had a major impact on the bioaccessibility of vitamin E depending on carrier oil type. The addition of calcium ions greatly improved the bioaccessibility of vitamin E in LCT-emulsions, but reduced it in MCT-emulsions. Finally, calcium addition increased the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion of LCT-emulsions, but had little effect on α-tocopherol acetate hydrolysis in MCT-emulsions.

In summary, our results suggest that the bioaccessibility of vitamin E encapsulated in emulsion-based delivery systems is strongly influence by carrier oil type,

bile salt type, calcium ions, and phospholipids. This information is important for developing effective emulsion-based delivery systems for oil-soluble vitamins and testing their potential efficacy.

CHAPTER 9

CONCLUSION

The overall aim of this work was to create food grade nanoemulsion-based delivery systems for encapsulating lipophilic bioactive components, such as vitamin E, β-carotene *etc.*, which can be applied into function foods and beverage products. Stable nanoemulsion-based delivery system could be successfully prepared using both low energy and high energy methods. The high energy method (microfluidization) was able to produce emulsions with small droplets (r < 100 nm) using low surfactant-to-oil ratios (< 1:10), but it required relatively expensive specialized equipment and has relatively high operating costs. Conversely, the low energy method (spontaneous emulsification) is inexpensive and can be implemented using simple equipment (stirring), but it requires high surfactant-to-oil ratios (> 5:10) to produce small droplets. High concentrations of surfactant levels may lead to problems in some food and beverage products, such as high ingredient costs, off flavors, and safety concerns. Moreover, high surfactant levels may also cause depletion flocculation or Ostwald ripening effects.

Q-Naturale® is a natural food-grade surfactant isolated from the bark of the *Quillaja saponaria* Molina tree. Q-Naturale® was found to be highly surface active, and had similar interfacial properties as Tween 80， a synthetic non-ionic surfactant widely used in the food industry. Q-Naturale® was able to form oil-in-water emulsions with relatively small droplet sizes (d < 200 nm) at low surfactant-to-oil ratios (SOR ≈ 1:10).

These emulsions formed by Q-Naturale® were stable to droplet coalescence over a range of pH values (2 to 8), salt concentrations (0 to 500 mM NaCl) and temperatures (20 to 90 ºC). In addition, they had good long-term stability (one month) when stored at various holding temperatures (5, 37, and 55 °C). However, some droplet flocculation was observed under highly acidic (pH 2) and high ionic strength (≥ 400 mM NaCl) conditions, which was attributed to screening of electrostatic repulsion. As Q-Naturale® is an effective surfactant, it was then used to form "all natural" oil-in-water emulsions containing α-tocopherol acetate. Tween 80 was more effective at producing small droplets at relatively low vitamin loadings (≤ 40%), whereas Q-Naturale® was more effective at relatively high vitamin loadings (60% - 80%). The size of the droplets in the emulsions could be reduced by decreasing the vitamin concentration in the oil phase, or increasing the amount of glycerol in the aqueous phase.

The carrier lipid type had great impacts on the bioaccessibility and hydrolysis of emulsified α-tocopherol acetate during the *in vitro* digestion study. LCT was a more effective carrier lipid than MCT for increasing the overall bioaccessibility of emulsified vitamin E. The total bioaccessibility of vitamin E after digestion was higher for LCT-emulsions than for MCT-emulsions, which was attributed to the greater solubilization capacity of mixed micelles formed from long chain fatty acids due to their ability to better accommodate lipophilic vitamin E molecules. Moreover, the conversion of α-tocopherol acetate to α-tocopherol after *in vitro* digestion was also

considerably higher for LCT-emulsions than for MCT-emulsions. The influence of mixed micelle composition and structure on the solubilization of oil-soluble vitamins was further studied by utilizing a well-defined model system. Mixed micelle solutions were formed that contained relatively small particles ($d \approx 5$ nm) whose precise size depended on system composition. An emulsion titration assay was then used to study the solubilization of α-tocopherol and α-tocopherol acetate emulsions in the mixed micelle solutions. The solubilization capacity of the mixed micelle solutions was higher for α-tocopherol than for α-tocopherol acetate, which was attributed to differences in the ability of the vitamin molecules to be incorporated into the micelle structures. However, there was no strong influence of free fatty acid type ($C_{8:0}$ *versus* $C_{18:1}$) on the solubilization capacity of the mixed micelles, which was different from results obtained using a simulated gastrointestinal model.

Finally, overall study of all the key factors impacting the bioaccessibility of emulsified α-tocopherol acetate, such as calcium ions, phospholipids, carrier oil type *etc.,* was taken by using a simulated small intestine model. Calcium addition to the SSIFs greatly improved the extent of lipid digestion for LCT-emulsions, but had little effect on MCT-emulsions, which could be attributed to the ability of calcium ions to remove long-chain fatty acids from interface of the oil phase and the water phase. The addition of calcium and phospholipids (DOPC) increased vitamin E bioaccessibility in LCT-emulsions, but decreased vitamin E bioaccessibility in MCT-emulsions. The

addition of calcium also increased the conversion ratio of α-tocopherol acetate to α-tocopherol during *in vitro* digestion of LCT-emulsions, but had little impact on α-tocopherol acetate hydrolysis in MCT-emulsions.

In summary, nanoemulsions may be a highly effective delivery system for encapsulating, transporting and protecting lipophilic bioactive components. The knowledge gained from this research will guide the rational design of food-grade nanoemulsion that can be used in the future fortified foods.

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