FORMATION OF NANOEMULSION - BASED DELIVERY SYSTEMS FOR LIPOSOLUBLE VITAMINS: STABILITY ASSESSMENT AND DIGESTION STUDIES

by

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To my dear grandparents,

my parents and my sister…

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ABSTRACT

FORMATION OF NANOEMULSION - BASED DELIVERY SYSTEMS FOR LIPOSOLUBLE VITAMINS: STABILITY ASSESSMENT AND DIGESTION STUDIES

Liposoluble vitamins are the biological active micronutrients which have health promoting effects. They may possess poor chemical stability, poor water solubility and low bioavailability. Therefore it is required to design effective delivery systems to incorporate these bioactives into functional food and beverage products. Nanoemulsion-based vitamin delivery systems were aimed to be produced using natural emulsifiers: lecithin, quillaja saponin, whey protein isolate (WPI) and gum arabic (GA) in a high pressure homogenizer. The effect of nanoemulsion composition and environmental stresses (pH, ionic strength and temperature) on the mean particle diameter and zeta-potential of the delivery systems were investigated and the stability was assessed by referring to theoretical phenomena. Simulated gastrointesinal system was used to evaluate the influence of carrier oil type on the digestion and bioaccessability of the vitamin D_3.

The mean particle diameter had a minimum value at an intermediate vitamin E-to-orange oil ratio in the oil phase: d_{32}= 0.13μm for lecithin and 0.12μm for quillaja saponin at 50% vitamin E. WPI (d_{32} = 0.11 μm, 1% emulsifier) was better than GA (d_{32} = 0.38 μm, 10% emulsifier) at producing small droplets at low emulsifier concentrations. Quillaja saponin and lecithin formed stable nanoemulsions at pH 3-8, however the loss of electrostatic repulsion caused instability at pH 2. Higher salt concentrations >100 mM NaCl for lecithin and ≥ 400 mM NaCl for quillaja saponin caused instability due to the electrostatic screening. All the sytems were stable at temperatures from 30 to 90 °C. WPI-stabilized nanoemulsions were unstable to flocculation at pH 5, at high ionic strength (>100 mM), and at elevated temperatures (>60 °C), whereas GA-stabilized emulsions were stable. The rate of free fatty acid release during lipid digestion decreased in the following order: medium chain triglycerides (MCT) > corn oil ≈ fish oil > orange oil > mineral oil. Long chain triglycerides were the most effective at increasing vitamin D_3 bioaccessibility. These results provide useful information about the emulsifying and stabilizing capacities of natural emulsifiers for forming food-grade vitamin-enriched delivery systems.

ÖZET

YAĞDA ÇÖZÜNEN VİTAMİNLER İÇİN NANOEMÜLSİYON BAZLI TAŞIMA SİSTEMLERİNİN OLUŞTURULMASI - STABİLİTE VE SİNDİRİM ÇALIŞMALARI

Yağda çözünen vitaminler sağlığa faydalı etkileri olan biyoaktif mikronütrientlerdir. Bu vitaminler, zayıf kimyasal stabiliteye, düşük suda çözünürlüğe ve dolayısıyla düşük biyoyararlılığa sahip olabilmektedir. Bu nedenle, bu bileşikleri fonksiyonel gıda ve içecek ürünlerine ekleyebilmek için etkin taşıma sistemlerinin tasarlanması gerekmektedir. Lesitin, quillaja saponin, peyniraltı suyu izolatı (PSI), arap gamı (GA) gibi doğal emülgatörler kullanılarak, nanoemülsiyon bazlı vitamin taşıma sistemlerinin yüksek basınçlı homojenizatör ile üretilmesi amaçlanmıştır. Nanoemülsiyon kompozisyonu ve çevresel faktörlerin (pH, iyonik güç ve sıcaklık) taşıma sistemlerinin ortalama partikül boyutu ve zeta potansiyel değerleri üzerine etkisi araştırılmış olup, teorik kavramlara dayandırılarak stabiliteleri değerlendirilmiştir. Simüle mide-bağırsak sistemi kullanılarak, taşıyıcı yağ tipinin, sindirim ve D_3 vitamini biyoerişilebilirliği üzerine etkisi incelenmiştir.

Yağ fazında orta düzeyde (50%) E vitamini/portakal yağı içeren emülsiyonlarda minimum ortalama partikül boyutu elde edilmiştir: d_{32}= 0.13µm (lesitin) ve 0.12µm (quillaja saponin). Düşük emülgatör konsantrasyonunda küçük partikül oluşturmada, PSI (d_{32}= 0.11 µm, 1% PSI), GA'dan (d_{32} = 0.38 µm, 10% GA) daha iyidir. Quillaja saponini ve lesitin pH 3-8 aralığında stabil nanoemülsiyon oluştururken, elektrostatik itme kuvveti kaybından dolayı pH 2'de stabiliteleri bozulmuştur. Yüksek tuz konsantrasyonu (>100 mM NaCl (lesitin); ≥400 mM NaCl (saponin) elektrostatik perdelemeden dolayı stabilitenin bozulmasına neden olmuştur. Bütün sistemler sıcaklık 30 ila 90°C aralığında stabildir. PSI-bazlı nanoemülsiyonlar pH 5, NaCl >100 mM ve yüksek sıcaklıkta (>60 °C) floküle olmuşken, GA-bazlı emulsiyonlar stabil kalmıştır. Sindirim sırasında, serbest yağ asidi salınımı azalan oranla; orta zincirli trigliserit> mısır yağı ≈ balık yağı> portakal yağı> mineral yağ şeklindedir. D_3 vitamini biyoerişilebilirliğinde en etkin uzun zincirli trigliseritlerdir. Bu tez sonuçları, gıda bazlı stabil vitamin taşıma sistemlerinin oluşturulması için, doğal emülgatörlerin emülsifikasyon ve stabilizasyon kapasitelerinin belirlenmesinde faydalı olacaktır.

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c_{sat}	Concentration of adsorbed emulsifier (kg m^{-3})
d_{32}	Surface-weighted mean particle diameter
e	Elementary charge
g	Standart gravity
GIT	Gastrointestinal tract
ΔG	Gibbs free energy change
ΔH	Enthalpy change
I	Ionic strength
k_B	Boltzmann constant
M_i	Molar concentration of ions
N_A	Avogadro's number
pK_a	Dissociation constant of a weak acid
r	Particle radius
R_g	Radius of gyration
S	Stern layer
ΔS	Entropy change
T	Temperature
U	Velocity of creaming
x	Distance from the particle surface
z_i	Valency of the ion
β-lg	Beta-lactoglobulin
κ^{-1}	Debye length
ρ	Density of the phases
ε	Dielectric constant of the solvent
ψ	Electrostatic potential (mV)
ψ_0	Electrostatic potential at surface of the droplet (mV)
π	Pi number
Γ	Surface load (mg m^{-2})
η	Viscosity of continuous phase

ø	Volume fraction of oil phase
ζ	Zeta potential (mV)

Ala	Alanine
Arg	Arginin
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
CO	Corn oil
DDL	Double diffuse layer
DLVO	Deryaguin, Landau, Verwey and Overbeek
EOR	Emulsifier to oil phase ratio
FFA	Free fatty acids
FO	Fish oil
GA	Gum arabic
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
HCl	Hydrochloric acid
His	Histidine
Ile	Isoleucine
LCT	Long chain triglycerides
Leu	Leucine
Lys	Lysine
MCT	Medium chain triglycerides
Met	Methionine
MO	Mineral oil
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OO	Orange oil
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
Phe	Phenylalanine

PI	Phosphatidylinositol
Pro	Proline
QN	Q-Naturale
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
vdW	van der Waals
VE	Vitamin E acetate
WPI	Whey protein isolate

1. INTRODUCTION

1.1. SIGNIFICANCE OF THIS THESIS

There is considerable interest in fortifying food and beverages with oil-soluble vitamins so as to improve human health and wellness. Bioactive compounds (antioxidants, vitamins, probiotics, enzymes, etc.) that have health promoting effects are mostly sensitive molecules which can be degraded when exposed to high temperature conditions, light, pH, moisture and oxygen.

Vitamin E refers to a group of oil-soluble bioactive compounds that includes various tocopherols and tocotrienols [1]. The most nutritionally important form of vitamin E is α-tocopherol, and so there has been great interest in incorporating this compound into food products. Nevertheless, numerous challenges must be eliminated before α-tocopherol can be successfully incorporated into functional food and beverage products, due to its poor water-solubility, chemical instability (to oxygen, light, and heat), and variable oral bioavailability. The poor chemical stability of α-tocopherol can be largely overcome by using the esterified form, i.e. α-tocopherol acetate [1,2]. Many of the other challenges associated with using α-tocopherol can be accomplished by incorporating it into colloidal delivery systems that can be dispersed into aqueous-based food and beverage products [3].

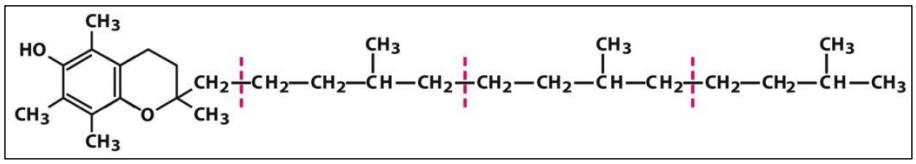


Figure 1.1. Molecular structure of vitamin E.

Vitamin D is a lipid soluble vitamin which is essential in human diet. It has two major chemical forms, vitamin D2 (ergocalciferol) and D3 (cholecalciferol) [4]. Vitamin D3 has more potency than vitamin D2 in humans and it is synthesized in the skin after light exposure and it displays different chemical structures, e.g. calciol, calcidiol, calcitriol.

Among them calcitriol (25-dihydroxyvitamin D_3) is the active form which controls calcium and phosphorus homeostatis, intestinal transport, bone metabolism and renal calcium reabsorption, blood pressure and insulin secretion [5]. Vitamin D is also very sensitive to environmental factors (light, heat, oxygen) and can easily be oxidized which lead to loss of its functionality and physiological benefits [4].

Vitamin E and D_3 are in the class of oil soluble vitamins which have low water and body fluid-solubility due to their apolar molecular structures (Figure 1.1 and 1.2). The challenge here was to design a delivery system to encapsulate liposoluble vitamins in order to increase water solubility and get efficient delivery in gastrointestinal sytem and absorbed in the mucosal cells of small intestine (permeation efficiency) reaching the systemic circulation without losing significant amount of its bioactivity. Therefore, encapsulation of liposoluble vitamins is required to make them stabilized in the food systems during processing and also increase their bioavailability in the gastrointestinal system. In order to use the food bioactives effectively in the food production, nanoencapsulation techniques help to improve the stability of the bioactives and enhance the bioavailability by the controlled release of these compounds in gastrointestinal system [6].

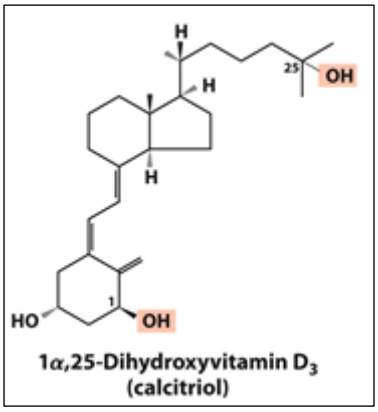


Figure 1.2. Molecular structure of vitamin D3.

One of the key factors for getting benefit from the functional foods is the rate of bioaccessability/bioavailability of the bioactive compound. Oral bioavailability of the nutrients depends on bioaccessability from food matrix, absorption by the intestinal cells and metabolism. Bioaccessability is known as the ratio of the amount of bioactive in mixed

micelle to the amount in the digested intestinal fluid. As the amount of liposoluble vitamin incorporated into mixed micelles increases, the bioaccessability also increases [7, 8].

Lipid digestion is carried out mainly in small intestine of human gastrointesinal system. The transfer of the lipid soluble vitamins from food matrix to the mixed micelles is generated by lipolysis of dietary fat during digestion (Figure 1.3). Emulsions may undergo a series of destabilization mechanisms during digestion steps in human gastrointestinal system, where the lipid products are exposed to various physiological conditions (saliva, mucinuous compounds, enyzmes, pH, bile salts) [8,9]. They may reassemble into a variety of structures, and mixed micelles are formed upon the digestion of the emulsion droplets by the action of lipase in the presence of bile salts. Mixed micelles form spontaneously and consist of phospholipids, free fatty acids, monoacylglycerols, cholesterol, and bile salts [10].

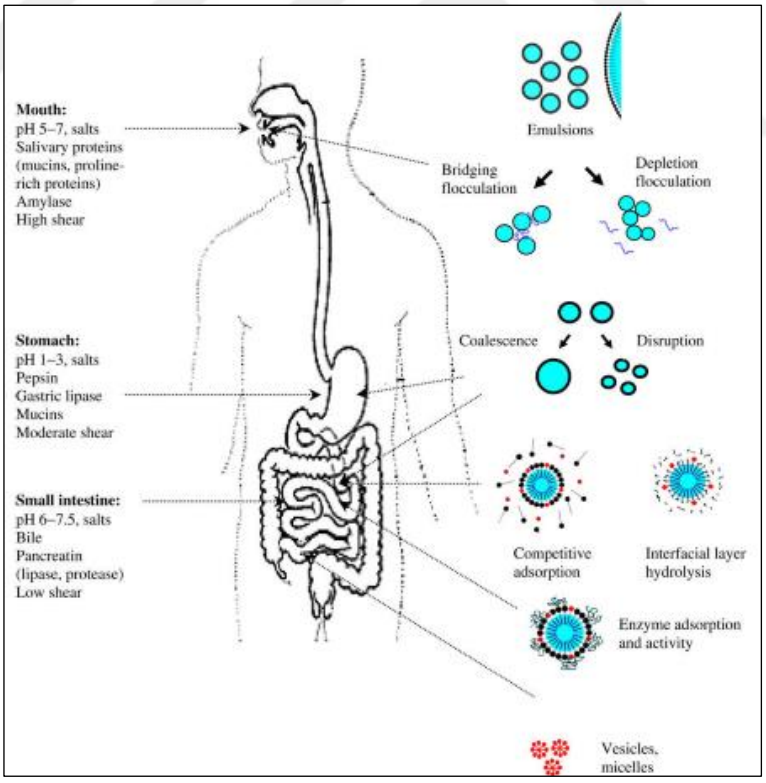


Figure 1.3. Representative diagram of human gastrointestinal system and possible changes in physical and interfacial properties of emulsion structure during lipid digestion [9].

Lipid soluble vitamins have low water solubility, therefore sustainability of delivery of functional bioactive compounds to target site is very low. Nanoemulsion-based delivery systems help to increase the solubilization of incorporated lipid soluble vitamins in the mixed micelles, thus enhancing the absorption through the mucosal membrane to the enterocytes (bioavailability). Several studies have been performed in order to investigate the bioavailability of various bioactive compounds using the simulated digestive system of human [11,12,13,14].

1.2. EMULSION-BASED DELIVERY SYSTEMS

One of the most important application of nanotechnology in food science and technology and human nutrition is to design and develop novel functional food formulations with value added properties, such as improved water solubility, thermal stability, enhanced bioavailability and physiological performance. This application area of nanotechnology has great potential due to the increasing demand on the functional foods every coming year. Bioactive compounds (i.e. probiotics, catechins, curcumin, omega-3, vitamins, etc.) are generally sensitive materials when they are exposed to light, oxygen and heat during the food processing and storage. Therefore, there is an increasing need and interest on designing effective delivery systems for these compounds.

Lipid-based colloidal delivery systems, such as microemulsions or nanoemulsions are promising systems which are utilized mostly in the food and pharmaceutical applications to encapsulate, protect, and deliver lipophilic bioactive compounds [15]. These delivery systems with their small particle sizes ($r < 100$ nm) provide a number of advantages of increased bioavailability of the bioactives, enhanced physical stability of the emulsions and higher optical clarity for some applications [16]. Understanding of the emulsion structure, parameters for its formation, and stability mechanisms is very important to design an efficient delivery system to encapsulate bioactive compounds.

1.2.1. Emulsions

Emulsions are the systems in which one liquid dispersed as droplets in a continuous phase of another immiscible liquid and it is generally stabilized by amphiphilic molecules to prevent phase separation and form a metastable dispersion with enhanced stability [16, 17, 18]. Conventional emulsions typically have particles,i.e. mean radii ranging from 100 nm to 100 μm. Emulsions with smaller droplet sizes can be grouped as nanoemulsions and microemulsions. A nanoemulsion, is a kinetically stable, but thermodynamically unstable system that can be considered to be a conventional emulsion which contains very small particles, i.e., mean radii between about 10 to 100 nm. A micro-emulsion, sometimes referred to as a swollen micelle, that typically contains particles with radii in the range of 2 to 100 nm [19]. Microemulsions are thermodynamically stable systems when a particular range of compositions and environmental conditions favor. Their stability may depend on the variations of these conditions due to dilution effects, ingredient type and concentration and temperature changes. Since nanoemulsions are thermodynamically unstable systems they may tend to breakdown during storage or transportation [20].

Oil-in-water and water-in-oil emulsions are among the two classes of emulsions of which oil-in-water types are the most commonly used types in the food industry. Oil-in-water nanoemulsions and microemulsions are both suitable for use in encapsulation and delivery of lipophilic compounds in the food and beverage industry. Both microemulsions and nanoemulsions may have same components (oil, water, and surfactant) but in different ratios [21]. In oil-in-water nanoemulsions, the non-polar segments of the emulsifier molecules extend into the oil phase (lipophilic core), while the polar segments (hydrophilic head groups or chains) of the emulsifiers protrude into the surrounding aqueous phase (Figure 1.4).

Many foods are generally aqueous-based products and some of them naturally contain o/w emulsions. Oil-in-water nanoemulsion-based delivery systems are among the best applications of encapsulating lipophilic compounds in order to be used in food fortification and enrichment. Food products and emulsions may undergo destabilization during food process, storage and transport. These destabilization mechanisms, such as ostwald ripening, sedimentation, flocculation leading to coalescence and then phase separation cause undesirable attributes in the food systems. Nanosized emulsion systems provide

better kinetic stability during food product formulation and storage which prevent the formation of these undesirable attributes.

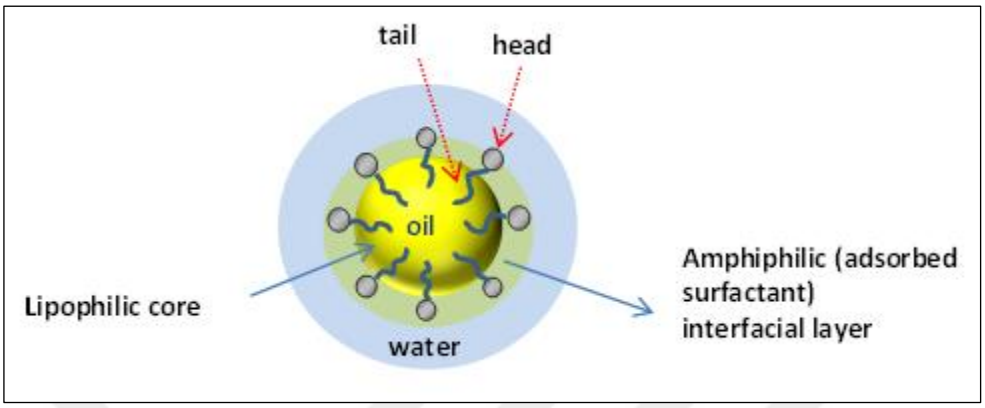


Figure 1.4. Structure of oil-in-water nanoemulsion stabilized by a surfactant. Lipophilic core (tail) and hydrophilic head group of surfactant.

1.2.2. Emulsion Stability

Emulsion stability can be basically described as the capability of emulsion to resist the changes in its properties over time. Stability mostly depends on the balance between the particle interaction forces, such as repulsive (steric, electrostatic) and attractive (van der Waals, hydrophobic) forces and physicochemical properties of the components of the emulsions. Emulsions may undergo several destabilization reactions when the compositional and environmental conditions favor [22]. These undesirable changes are resulted from various physiochemical mechanisms that occur within the food and beverage emulsions, including creaming, flocculation, coalescence, and Ostwald ripening (Figure 1.5).

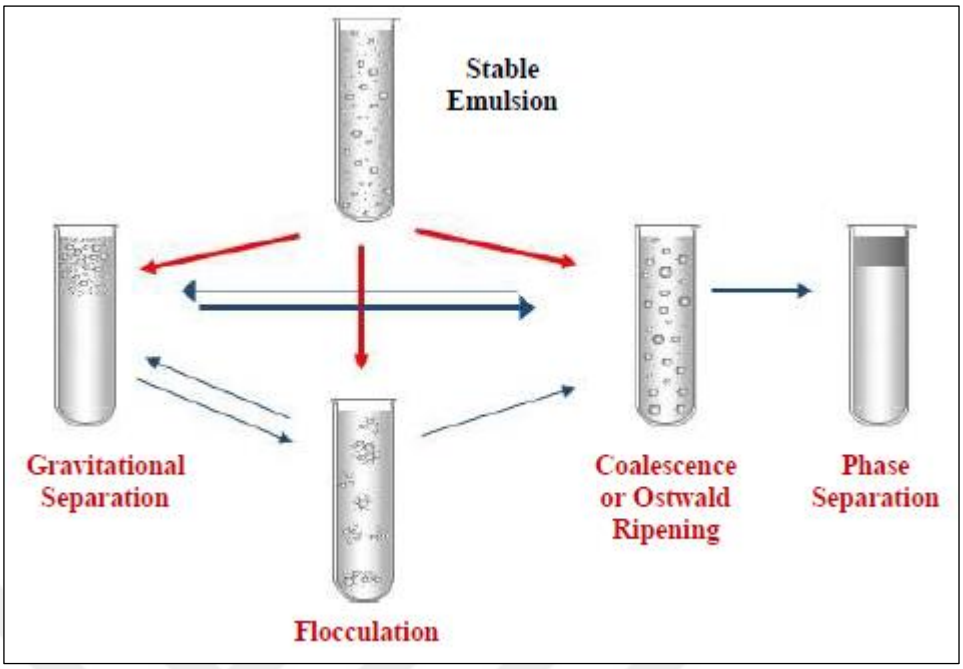


Figure 1.5. Emulsion instability mechanisms [23].

The droplets in an emulsion and the liquid that surrounds them in the emulsion have different densities which results in a net gravitational force acting on the droplets. In oil-in-water nanoemulsions, droplets tend to cream because the less dense structure of the oil droplets dispersed in water phase have a propensity to move upward, which is referred to as *creaming.* The creaming rate of small spherical particles in a newtonian fluid is given by Stoke's Law Equation [24].

$$U = -2r^2(\rho_2 - \rho_1)g / 9\eta_1 \qquad (1.1)$$

Where, 1: continuous (water phase), 2: oil phase, ρ is density of the phases, η is the viscosity of phase 1, *g* is the gravity, r is the radius of the particles, U is the velocity of creaming. Positive (+)U refers to *creaming* which may occur when the density of continuous phase is larger than the dispersed phase and negative (-)U refers to the reverse physical mechanism called as *sedimentation*.

The emulsion stability in terms of aggregation is a measure of the tendency for droplets to become flocculated or coalesced under the designated environmental conditions, such as pH, ionic strength, temperature, shearing rate. *Flocculation* is the aggregation of two or more droplets forming floc structures in which individual droplets entities are retained [22, 24]. Flocculation depends on the balance of colloidal interactions and occurs when *attractive* forces (i.e. van der Waals, hydrophobic) outweigh *repulsive* forces (i.e. electrostatic, steric), and can therefore be prevented by increasing repulsive interactions which are described in *Theory* section of this thesis.

Coalescence is the aggregation of two or more individual droplets through fusing together to form a bigger droplet. Increase in size of the droplets through the coalescence may cause more rapid creaming or sedimentation. In oil-in-water emulsions, coalescence eventually leads to the formation of *oiling off* (a layer of oil) on top of the material. The rate coalescence can be decreased by prevention of droplets contact and rupture of interfacial membranes [22, 25].

Another instability mechanism that the droplets may experience is the *Ostwald ripening*. It is explained as the growth of large droplets by the molecular diffusion of oil molecules through the aqueous phase at the expense of small droplets. Laplace pressure difference in the droplets may drive this mechanism. The rate of Ostwald ripening increases when dispersed phase has a larger equilibrium solubility in the continuous phase. For example, the use of flavor oils which are more polar than the triaclglycerols (poorly water soluble) may facilitate the existence of Ostwald ripening in the emulsions. Ostwald ripening can be reduced by using an appropriate emulsifier which has higher surface activity properties or by decreasing the diffusion coefficient of the dispersed phase in the membrane, or by increasing the thickness of the interfacial membrane [22, 25].

1.2.3. Emulsifier Characteristics

Emulsifier characteristics is a determinative factor in the stability of a nanoemulsion to environmental stresses such as pH, ionic strength, heating, or long-term storage. Emulsifiers are the surface-active molecules which have the capability of adsorbing to oil-water interface, and making the droplet disruption easier by reducing interfacial tension at interface and prevent the droplets from aggregation. In high-energy approaches (e.g., high

pressure/shear homogenizers), the emulsifier facilitates droplet disruption within the homogenizer through lowering the interfacial tension and favoring the production of small droplets. To enhance the formation and stabilization of nanoemulsions, emulsifiers must rapidly adsorb to the surface of the freshly produced emulsion droplets during homogenization, and reduce the interfacial tension by a significant amount, form an interfacial amphiphilic layer that prevents the droplets from aggregating under the environmental conditions pertaining to the emulsion [19, 20].

Some examples of the physical expressions are described previously to characterize an emulsifier such as; surface load, adsorption kinetics, interfacial membrane layer [21]. The *surface load* at saturation (Γsat) is the mass of emulsifier adsorbed per unit surface area of interface when the interface is saturated with emulsifier, and is given as mg m^{-2} (Eq. 1.2). The surface load indicates the minimum amount of emulsifier required to produce an emulsion with a given droplet size or surface area. When the value of Γ is high, then the required amount of emulsifier for complete coverage of the same surface area increases [22]. This relation is expressed as follows:

$$d_{32\min} = \frac{6 \cdot \Gamma_{sat} \cdot ø}{c_{sat}} \tag{1.2}$$

where d_{32min} is the minimum mean particle diameter, Γsat is the excess surface concentration of the emulsifier at saturation (in kgm^{-2}), Ø is the volume fraction of disperse phase, and c_{sat} is the concentration of emulsifier in the emulsion (in kgm^{-3}). This equation represents that the minimum droplet size can be decreased by increasing the emulsifier concentration, decreasing the dispersed phase concentration, or using an emulsifier with a smaller Γ_{sat}.

The other parameter which can show the efficacy of an emulsifier is the *interfacial tension* which is the amount of the free energy required to increase the interfacial area by a unit amount (J m^{-2} or N m^{-1}). Emulsifiers can reduce the interfacial tension resulting in the minimization of the thermodynamically unfavorable contact area between the different

molecules at the interface. It is an important parameter because it affects the droplet size during homogenization and the stability of droplets to coalescence and Ostwald ripening.

In high energy method of emulsion production, droplet break up is determined by the balance of interfacial forces and disruptive forces generated within the homogenizer. An emulsion droplet tends to be spherical because this shape minimizes the thermodynamically unfavorable contact area between the oil and aqueous phases. The droplet shapes may change upon the disruption in the homogenizer which may lead to increase in contact area and therefore increased amount input of free energy is required. Laplace pressure acting across the oil-water interface facilitate the droplets to retain their spherical shapes [22, 25].

Adsorption of emulsifiers on the droplet surface follows two main steps; diffusion of emulsifier molecules from the bulk liquid to the vicinity of the interface and attaching to the interface including reorganization. *Adsorption rate*, how fast it adsorbs to an interface, is a very important factor determining the emulsifying ability of the emulsifier [18]. Molecular characteristics of the emulsifier such as size, conformation, interfacial membrane flexibility, and interactions, the physicochemical properties of the surrounding liquid (e.g., viscosity, polarity), and the prevailing environmental conditions (e.g., temperature and flow profile) have effects on the adsorption rate [22].

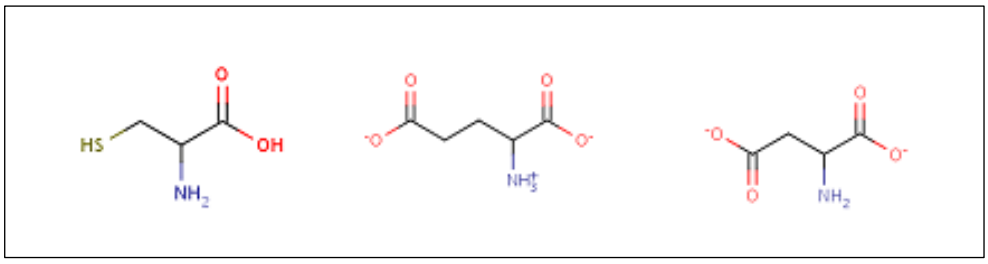
Interfacial membrane is an important factor in the emulsion stability, since the emulsion droplets have a tendency to coalesce during homogenization due to the frequent collisions caused from Brownian motion, gravity and mechanical agitation. They should be protected by a strong interfacial membrane in order to prevent coalescence before they have time to collide with the neighbour droplets. The resistance of an interfacial membrane to coalescence depends on the concentration of emulsifier molecules present, as well as their structural and physicochemical properties (i.e. dimensions, electrical charge, packing, and interactions) [22].

1.2.4. Emulsifier Type

Emulsifier type has a major effect in determining the emulsion production method, the characteristics of the produced particle and also the stability of the final emulsion. The

most important types of emulsifiers in the food applications are small molecule surfactants, phospholipids, proteins, and polysaccharides. The small molecule surfactant stabilized nanoemulsions can be effectively produced using both high- and low-energy methods. On the other hand, proteins and polysaccharides are suitable for producing nanoemulsions using high-energy methods, however they are not usually as effective as surfactants at forming nanoemulsions with small droplet sizes [22]. There has been a high demand on using natural emulsifiers in the production of nanoemulsions for the food and beverage industry due to their "label-friendly" properties. Molecular structures and compositions of the emulsifiers determine their emulsifying behaviours.Therefore, in this thesis, four types of natural emulsifiers with different molecular structures were used to produce stable vitamin E nanoemulsion-based delivery systems. The molecular compositions and structural properties of whey protein isolate (WPI), gum arabic (GA), Quillaja saponins, and soy lecithin are described as follows.

Whey protein isolate contains almost 90% of protein and more than 60% of total WPI is beta-lactoglobulin (β-lg). β-lg is a globular protein and has two intramolecular disulphide bonds and a thiol group. It is generally found in dimer form in aqueous environment, however it can form oligomeric conformations by non-covalent and reversible interactions [26]. The surface activity of the proteins depends on the hydrophobic and hydrophilic aminoacid balance in their structure. β-lg has aminoacid composition that consists of Asp, Asn, Thr, Ser, Glu, Gln, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, Lys**,** His, Trp, and Arg. Among these, suphur-containing aminoacids (5 Cys residues) with two intermolecular disulphide bonds and one unreacted sulphydryl (SH) group residue trigger polymerization by covalent intermolecular disulphide bonds during heat treatments and storage. Moreover, β-lg molecule has glutamic acid and aspartic acid aminoacids containing ionizable groups in their side chains which have also important roles in the stability of emulsions in different pH ranges (Figure 1.6) [27].



(a) (b) (c)

Figure 1.6. Structures of the WPI amino acids which play key roles in the stabilization of the nanoemulsion. (a) Cysteine (b) Glutamic acid (c) Aspartic acid.

Once the globular proteins adsorbed on the oil-water interface, they may undergo conformational changes due to the tendency of protruding of the hydrophobic inner regions of protein molecule towards the oil core of the emulsion droplets. Therefore globular proteins may be found as in folded, unfolded or partial unfolded conformations at the interface (Figure 1.7).

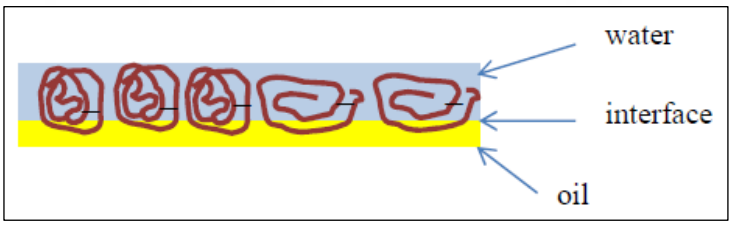
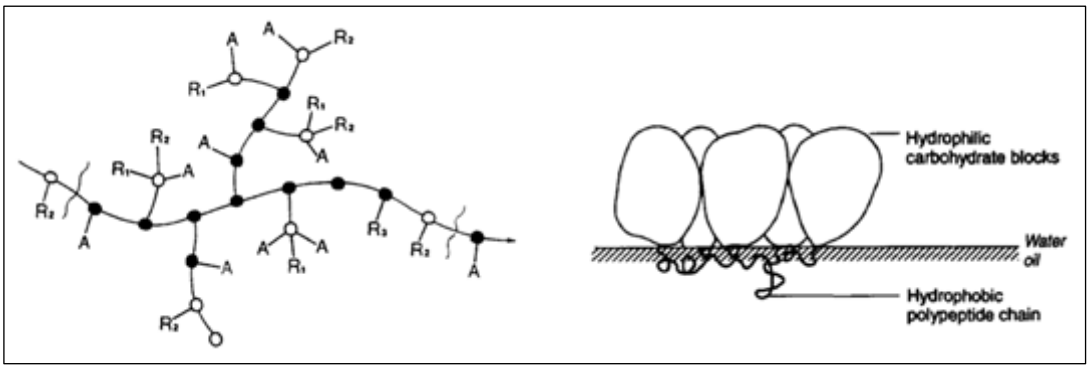


Figure 1.7. Conformations of the adsorbed globular proteins at oil-water interface.

Gum arabic (GA) is a proteinaceous polysaccharide, where branched arabinogalactan blocks attached to a polypeptide backbone. Highly branched polysaccharide consists of b-(1-3) galactose backbone with linked branches of arabinose and rhamnose, which terminate in glucuronic acid and this arabinogalactan chain is covalently linked to a protein chain through serine and hydroxyproline groups forming the hydrophobic proteinaceous region [28]. GA carries a net negative charge at highly acidic pH ranges conferred by its ionizable glucuronic acid residues (pK_a 3-3.5). Hydrophobic polypeptide chain is believed to be responsible for the emulsifying properties of GA by anchoring the molecules to the droplet surface, while the highly branched hydrophilic arabinogalactan parts extend into the

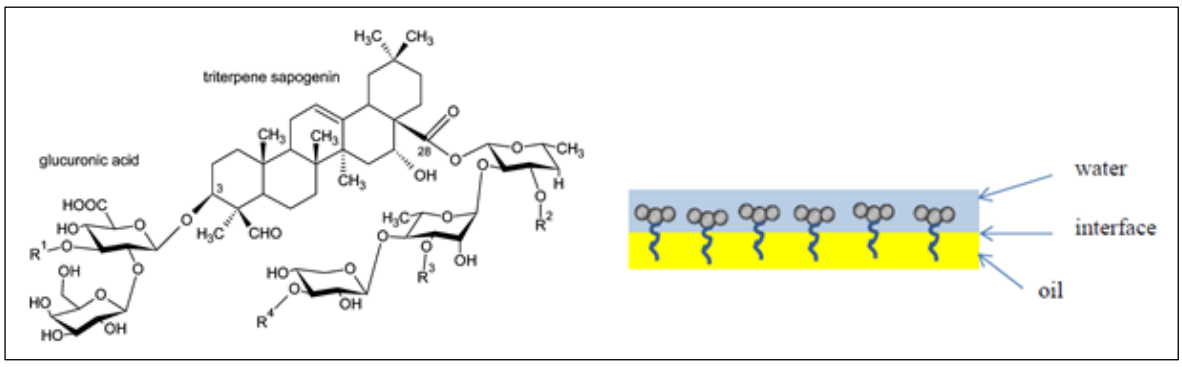
solution, providing stability against droplet aggregation through steric and electrostatic repulsion [29].



(a) (b)

Figure 1.8. (a) Molecular structure and (b) adsorption model of gum arabic [30].

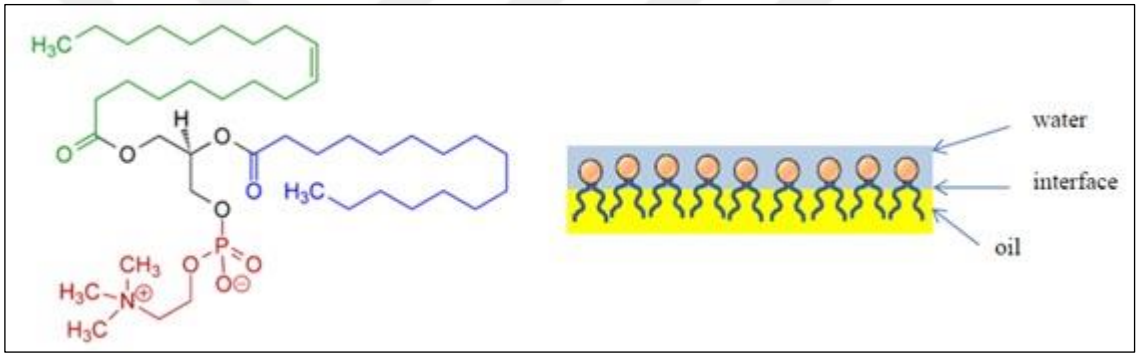
Quillaja saponin is a secondary metabolite comprising sugar moieties glycosidically linked to a hydrophobic agylcone (sapogenin), which may be a triterpene or steroidal extracted from *Quillaja saponaria* tree bark. The main aglycone moeity is quillaic acid, a triterpene consisted of mainly 30-Carbon atoms (hydrophobic) of the Δ-oleanane type. It is linked to glucuronic acid and also to various sugar chains (hydrophilic) including glucose, fucose, rhamnose [31, 32].



(a) (b)

Figure 1.9. (a) Molecular structure of Quillaja saponin [33] and (b) adsorbed monolayer model.

Soy lecithin is mainly derived from soy phospholipids and consisted primarily of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Phospholipids have a surface active amphiphilic structure which is composed of hydrophobic esterified fatty acid tail groups and hydrophilic head groups including phosphoric acid esterified with glycerol and/or choline. The ionization of phosphate and base groups makes them strong amphiphilic. Lecithins are not overwhelmingly soluble in either water or oil [25]. The building block of the phospholipids is phosphatidic acid which has a pKa value around 1.5 [34]. When the phosphoric acid is esterified with choline it is called phosphatidylcholine (lecithin).



(a) (b)

Figure 1.10. (a) Molecular structure of lecithin (phosphotidylcholine) and (b) adsorbed monolayer model.

1.3. OBJECTIVES

This research was based on the understanding of the production of nanoemulsion-based liposoluble vitamin delivery systems using four natural emulsifiers with different molecular structures, and of their emulsifying and stabilizing capacities related to a number of theoretical intermolecular and surface phenomena. Four objectives were determined as follows;

First objective was to examine the major factors (i.e. vitamin E loading capacity, oil phase composition, emulsifier type, and concentration) influencing the initial size of the droplets in oil-in-water nanoemulsions produced by high pressure homogenization using four different types of natural emulsifiers (quillaja saponin, lecithin, WPI, GA).

Second objective was to examine the influence of various environmental stresses (pH, ionic strength and temperature) on the stability of nanoemulsions by particle size and charge measurements.

Third objective was to assess the stabilizing mechanisms of different types of emulsifiers by comparing the experimental results with theoretical model explanations.

Fourth objective was to examine the influence of nanoemulsion composition in terms of carrier oil type (i.e. MCT, LCT, fish oil, orange oil, mineral oil) on the lipid digestion and bioaccessability of vitamin D3.

2. THEORY

Destabilization mechanisms of emulsion-based delivery systems under various environmental stresses may rely on theoretical basis. Therefore, a few of these theories were mentioned in this chapter of this thesis.

2.1. HENDERSON-HASSELBACH EQUATION

Dispersed particles may acquire an electric charge through ionization of the charged groups on the surface. In food colloids the important ionizing polyelectrolytes -CO2- and -NH3+ groups of the proteins and -CO2- and -SO4- groups the acidic polysaccharides, for which degree of ionization is very sensitive to pH (25). When the dissociation of a weak acid is expressed as HA <=> H+ + A- and its equilibrium constant as K = [H+] [A-] / [HA] the Henderson-Hasselbach equation expresses the effect of the pH on the dissociation constant as

$$pK = pH - \log [A^-] / [HA] \quad (2.1)$$

where A- is the proton acceptor and HA is a proton donor. Stronger acids donate their protons, faster and have higher dissociation constants when compared to the weaker acids. Proteins contain many ionizable groups on the side chains of their amino acids as well as on their amino- and carboxyl- termini. The pH of the solution, the pK of the side chain and the side chain's environment influence the charge on each side chain [35].

2.2. DLVO THEORY

Flocculation or colloidal stability was explained by DLVO (Derjaguin, Landau and Verwey, Overbeek) theory which is derived from the balance between double layer electrostatic repulsive forces which increase exponentially with decreasing distance from the surface of the particle, and van der Waals attractive forces between the particles. It was assumed in this theory that the more long range interparticle interactions mainly governs

the stability. Long range van der Waals (vdW) forces between the particles are not sensitive to the changes in electrolyte concentration or pH changes. Van der Waals forces are originated from the dipole-dipole, dipole-induced dipole or dispersion forces between the molecular entities or the intramolecular groups. If the particles are similar in nature, the force is attractive [34].

The colloidal particles dispersed in an aqueous electrolyte environment generally assume a net electric charge which affects the distribution of ions in the neighboring solution. These colloidal particles have a charge either from their surface charge groups or by specific ion adsorption from the solution [25]. Similar charges of the particles lead to a repulsive double-layer force [36]. The combined effect of vdW attraction curve and the electrostatic repulsion curve was represented as the net interaction energy curve by the DLVO theory to explain the flocculation stability of the colloids. Repulsive part of the net curve designates the energy barrier which indicates the amount of resistance of the system to effective coagulation. Attractive region of the curve is called energy trap since the colloids are trapped together by vdW forces [37].

The ionic cloud around the surface of the particles governs the net electrostatic interactions between the particles. Oppositely charged counter ions will move very close to the surface and the co-ions will repel from the surface of the charged particle. The region electrical double layer refers to the sum of the stern layer where the counter ions dominate near the surface and diffuse layer where co-ions diffuse. Debye length contributes the distance from the surface where the surface potential decreased to its 37% of value and it is expressed as the order of $1/\kappa$ ((Figure 2.1).

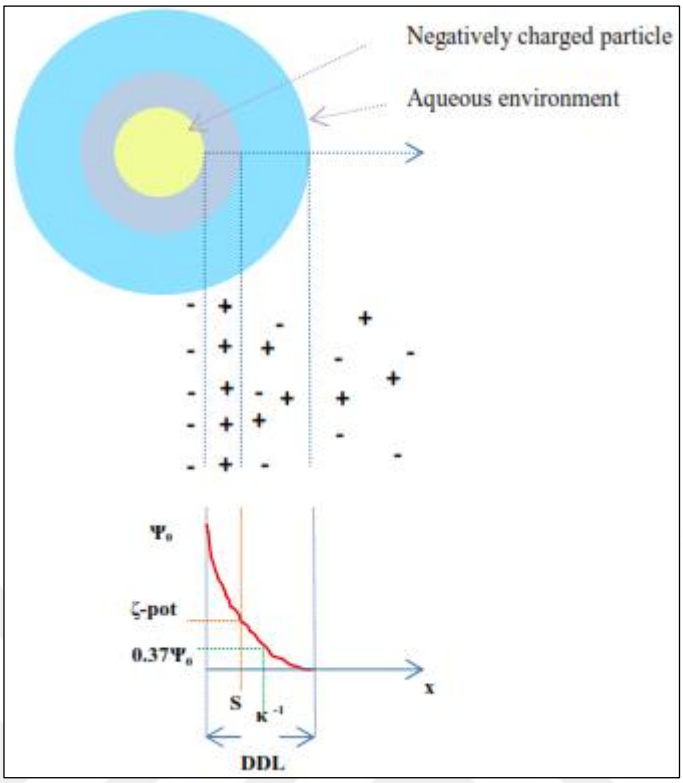


Figure 2.1. Double diffuse layer around a charged particle. DDL: double diffuse layer, S: stern layer, κ^{-1}:Debye length, ψ_0: surface charge, ζ-pot: electrical potential in shear plane.

Ion distribution in an electrolyte solution outside a charged particle is expressed by Poisson-Boltzmann equation (Eq. 2.2).

$$\frac{d^2\,\psi\,(x)}{d\,x^2} = \frac{-4\,\pi e^2 \psi}{\varepsilon k_B T} \sum_i z_i^{\,2}\; n_{i0} \qquad (2.2)$$

where ψ is electrostatic potential (mV), x is the distance from the particle surface, e is elementary charge ($1.6\text{x}10^{-19}$ coulomb), ε is dielectric constant of the solvent, k_B is the Boltzmann constant, T is absolute temperature, z_i is valency of ion, and n_{i0} is concentration of ion in the bulk.

Linearizing Poisson-Boltzmann equation leads to exponentially decaying potentials with distance around a spherical ion which was described in Debye-Hückel theory [36]. The

assumption of low potentials at distances far from the surface leads to Debye-Hückel approximation [37].

$$\frac{d^2\psi(x)}{dx^2} = \kappa^2\psi \tag{2.3}$$

and through the linearization, this equation becomes

$$\psi(x) = \psi_0 \exp(-\kappa x) \tag{2.4}$$

where x is the distance from the charged surface, κ is the Debye-Hückel parameter expressed as

$$\kappa = \sqrt{\left(\frac{8\pi e^2 N_A}{1000\,\varepsilon\, k_B T}\right) I} \tag{2.5}$$

where, N_A is the Avogadro's number, and z_i is valency of the ion, M_i is molar concentration of ions, the term $I = \sqrt{\left(\frac{1}{2}\sum_i z_i^2 M_i\right)}$ is the ionic strength of the solution.

As it is seen in equation (2.5), the Debye length (1/κ) depends on the ionic strength of the solution in which ionic strength is determined by the concentration and valency of ions in the medium. The ratio between surface potential and zeta potential depends on the double layer thickness which may be changed by the ion concentration in the medium. Increasing the concentration of the ions (M_i) or their valence (z_i) are both triggering factors to cause the double layer compression which facilitates flocculation of the particles. Following the addition of higher amounts of salt, the concentration of ions increase and this may cause double layer compression which result in flocculation of the droplets. During this salting out process, the double layer and repulsive curves are compressed until the energy barrier

diminishes so that the particles can easily jump in to the van der Waals trap and thus agglomeration occurs [38].

2.3. STERIC STABILIZATION

Steric stabilization is generally pursued by macromolecules such as gum arabic and milk casein [25, 39]. They have amphiphilic structures and at the time of adsorption to a surface they undergo reorganization, unfold and reorient to form an interfacial film [40]. Partially unfold and denatured and native fractions of the protein molecule may coexist upon adsorption to an interface, the hydrophobic parts anchor the oil droplet surface, and provide the attachment for hydrophilic part. Macromolecules may have differents segments, such as train, loop and tail as a result of various configurations on the surface. The groups of the molecule forming the train segment affect the stern layer, and the others in loops and tails may affect the charges of diffuse layer [25, 40]. The hydrophilic regions of the polymers move towards the water phase to achieve steric stabilization [39].

Steric interaction energy between two particles is strongly repulsive in short ranges. When two droplets with adsorbed layer of molecules approach each other in the aqueous environment, the flexible hairy molecular chains protruding the continuous phase may interpenetrate or compress each other and reduce entropy and restrict conformation. These hairy segments (e.g. tail, loops) become concentrated and thereby osmotic pressure increases. This triggers the water to enter into the overlapped region leading to repulsion [41]. The loss of entropy due to the volume restriction also affects and osmotic pressure increases the free energy and causes repulsion [40]. This steric force may depend on the amount of polymer and surface coverage. In case of low surface coverage, each chain may interact with the chain at opposite surface, they do not interact with each other on the same surface. However, in high surface coverage, the chains extend away and entanglement of the polymer chains both on the same surface and opposite surface may form which induce the formation of a brush thickness layer (L) that is larger than radius of gyration (R_g) the molecule. The thickness of brush layer is proportional to the length of polymer chain, M, (i.e. $L \alpha M$ for high surface coverage and $L \alpha M^{0.5}$ for low coverage) [34].

When the two colloids approach each other at very short distances (< 2nm), DLVO forces may fail to explain the interaction between them. There is another additional force which

may be stronger than DLVO forces at small separations. Surface-solvent interactions may induce positional order in adjacent liquid and form a solvation force. This short range interaction force is called as hydration force when the solvent is water. It depends on the chemical and physical properties of the surfaces. Surfactant soap films, lipid bilayers or biological membranes may swell and repel each other in aqueous environment. The additional hydration repulsive force formed between hydrophilic surfaces is exponentially repulsive. Repulsive hydration may occur when the water molecules strongly bind to the hydrophilic surfaces. One type of this forces, steric hydration force may occur between fluid like amphiphilic surfaces, such as soap films or zwitterionic or sugar head groups. These forces may arise from the overlaping of head groups protruding from these surfaces when they aproach each other or of the thermally excited chains in phospholipids [34, 36].

2.4. PROTEIN STRUCTURE AND FOLDING

Amino acids Ala, Cys, Leu Met, Glu, Gln, His and Lys tend to form the α-helix; Val, Ser, Asp, Asn, Pro and Arg tend to form the β-sheet sections of the secondary structures hold together by hydrogen bonds along the backbone of the polypeptite chain. The secondary structures bond together with stronger covalent cross links to give rise to the tertiary structure. The folded native structure of a protein is derived from the balance of various attractive and repulsive forces within the polypeptide itself and between the polypeptide and the solvent molecules [42]. The hydrophobic groups of the nonpolar amino acids are packed in the interior of the protein molecule and polar amino acid groups are distributed on the outer surface [43]. Tertiary structure of a protein in a given environment as determined by the solvent, pH, ionic strength and temperature is assumed to be the thermodynamically most stable form in which the Gibbs free energy of the whole system is the lowest [41, 42]:

$$-RT \ln K = \Delta G_{total} = \Delta H_{chain} + \Delta H_{solvent} - T.\Delta S_{chain} - T.\Delta S_{solvent} \qquad (2.6)$$

In equation (2.6) the term ΔS_{chain} (S_N-S_R) is negative for all parts of the chain, because random state of the protein is always less ordered and has higher entropy than the native state of the protein. Hydrogen bonding and van der Waals attractions are included in the

term ΔH_{chain}. The terms ΔHchain and ΔHsolvent may be evaluated separately for non-polar and polar parts of the protein structure. For non-polar parts, ΔH_{chain} and $\Delta H_{solvent}$ have small magnitudes where ΔH_{chain} is (+), but $\Delta H_{solvent}$ has a negative value, because hydrophobic part does not provide binding energy, however water as a solvent is capable of doing hydrogen bonds. $\Delta S_{solvent}$ is positive and large for non-polar parts due to the disordering of water molecules around the nonpolar groups (hydrophobic effect) and thereby favoring the protein folding. Therefore in overall, the highest contribution for the protein stability is provided by $\Delta S_{solvent}$ coming from non-polar parts which again proves that the stability of protein folding is triggered mainly by hydrophobic effect. Thermodynamic properties, which are given in equation (2.6) are sensitive to temperature, ionic strength and pH of the environment as discussed by Alberty (2003) [44].

3. FORMATION AND STABILIZATION OF NANOEMULSION-BASED VITAMIN E DELIVERY SYSTEMS USING NATURAL EMULSIFIERS

3.1. INTRODUCTION

There is considerable interest in fortifying foods and beverages with oil-soluble vitamins so as to improve human health and wellness [45]. Vitamin E refers to a group of oil-soluble bioactive compounds that includes various tocopherols and (α-, β-, γ-, and δ-tocopherol) and tocotrienols (α-, β-, γ-, and δ-tocotrienol) [1]. Vitamin E has been used for the fortification of various food and beverage products due to its potential disease-preventive effects [46, 47, 48, 49, 50]. The nutritionally and biologically important compund α-tocopherol, is the most preferentially used form of vitamin E in fortifying foods. Nevertheless, this compound has a number of challenges due to its low water-solubility, poor chemical stability (to oxygen, light, and heat), and variable bioavailability. The fortification of the foods with the esterified form of α-tocopherol (α-tocopherol acetate) is an effective way of preventing the poor chemical stability of vitamin E [1, 2].

The other challenges on the poor solubility properties and low bioavailability can be overcome by encapsulating vitamin E in the colloidal delivery systems that can be dispersed into food and beverage products [3, 45, 51]. Emulsions and nanoemulsions, are good candidate delivery systems to encapsulate the lipid-soluble bioactive compounds. They have the advantages of being produced by simple production methods and formed using natural food ingredients, and being designed to increase water-dispersion and bioavailability [52]. Food-grade emulsifiers (protein, polysaccharides, phospholipids and surfactants) preferably natural ones are required to stabilize the emulsion-based delivery systems for the food applications [53, 54, 55, 56]. Proteins, polysaccharides, phospholipids, or surfactants being the examples of food grade emulsifiers, have their own advantages and disadvantages for particular applications, and therefore it is important to determine the most appropriate emulsifier for specific products.

Consumers are increasingly demanding “clean” labels on food and beverage products, and therefore it is important to develop emulsion-based delivery systems using natural emulsifiers. In this part of the thesis, four different types of emulsifiers (two natural small-molecule surfactants, soybean lecithin and quillaja saponin, and two natural biopolymers, GA and WPI) were aimed to be used in formation and stabilizing the vitamin E-enriched nanoemulsions. Soybean lecithin is a natural surfactant derived from the cell membranes of the soybeans [57]. Quillaja saponin (marketed commercially as Q-Naturale®) is a natural surfactant extracted from the bark of the *Quillaja saponaria* tree which has previously been reported to be effective at forming stable nanoemulsions with achieved small droplet sizes [58, 59].

GA is the most widely used amphiphilic polysaccharide to stabilize beverage emulsions. It is derived from the acacia Senegal tree and contains at least three high molecular weight fractions. The emulsifying ability of GA at oil-water interfaces can be attributed to the hydrophobic protein fraction that is covalently linked to highly branched hydrophilic polysaccharide backbone. The hydrophobic part anchors the GA molecules to the droplet surfaces, while the hydrophilic part provides stability against droplet aggregation through steric and electrostatic repulsion [60, 61]. WPI is frequently used as a natural emulsifier in foods and beverages to facilitate the formation and stabilization of oil-in-water emulsions. WPI is a mixture of different globular proteins, with β-lactoglobulin being the most abundant followed by α-lactalbumin. WPI forms relatively thin interfacial layers that mainly stabilize emulsions against flocculation through electrostatic repulsion, and is therefore particularly sensitive to pH and ionic strength. WPI-stabilized emulsions are also highly sensitive to thermal processing due to interfacial protein denaturation at elevated temperatures. WPI is therefore only suitable for use in products where the composition and environmental conditions favor a stable product [60, 62, 63].

Two of the most important types of emulsion-based delivery system are nanoemulsions (d < 200 nm) and emulsions (d > 200 nm), which can be distinguished in terms of their particle diameters [16]. For certain applications there are advantages of using nanoemulsions over emulsions. Nanoemulsions tend to have high optical clarity, greater physical stability, and better oral bioavailability [52, 64, 65]. Nanoemulsions can be produced using either high-energy (*e.g.* high pressure homogenization) or low-energy (*e.g.* spontaneous emulsification) methods [16]. High-energy methods are more advantageous

than low energy methods in terms of the requirement of lower emulsifier-to-oil ratios to produce very small droplets which provides the benefit of low toxicity, better flavor in the nanoemulsions [66]. Therefore, in this thesis high pressure homogenization was chosen as to produce nanoemulsions.

Droplet size of the emulsion-based delivery system has an important role in determining the stability to phase separation, flocculation, or coalescence [3]. Decrease in droplet size has also an effective role in enhancing the bioavailability of the encapsulated bioactives [52, 64, 65]. Food biopolymers and small molecule surfactants have different emulsifying abilities due to differences in their molecular structures that affect their surface adsorption characteristics during homogenization, and their ability to stabilize droplets once formed. Therefore the influence of the major factors, *i.e.*, oil phase composition, emulsifier type, and emulsifier concentration, on the initial size of the droplets were examined in the production of oil-in-water nanoemulsions using high pressure homogenization. Moreover, the influence of various environmental factors, *i.e.*, pH, ionic strength, and temperature on the emulsion stability was also examined by trying to be consistent to the harsh conditions during food processing, storage, transportation, and also physiological digestion conditions.

Finally, the emulsifying and stabilizing properties of the two biopolymers (GA and WPI) and two small molecule surfactants (Lecithin and Q-Naturale) were compared in order to obtain representative information which should be important for the the utilization of natural emulsifiers in the development of label-friendly emulsion-based delivery systems for oil-soluble vitamins and other lipophilic nutraceuticals.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Quillaja saponin (Q-Naturale®100) was kindly provided by Ingredion Inc. (Westchester, IL). Soy lecithin was provided by Archer Daniels Midland Co. (Decatur, IL). Whey protein isolate (WPI) was kindly provided by Davisco Foods International Inc. (Le Sueur MN). Gum arabic (GA) was provided by TIC Gums (Belcamp, MD). Orange oil (10×) was provided from International Flavors and Fragrances (Union Beach, NJ). Vitamin E acetate

(VE) was obtained from BASF (Florham Park, NJ). Sodium phosphate monobasic, sodium phosphate dibasic, and sodium chloride were purchased from Sigma-Aldrich Co. (St. Louis, MO). Double distilled water was used to prepare all solutions and emulsions.

3.2.2. Emulsion Preparation

Oil-in-water nanoemulsions were prepared by homogenizing 10% (w/w) of lipid phase (orange oil and vitamin E-acetate) with 90% (w/w) of aqueous phase. The aqueous phase consisted of emulsifiers (lecithin or Q-Naturale (QN)) at the concentrations 0.0005-5% (w/w), WPI or GA at the concentrations 0.1-10% (w/w) and buffer solution (10 mM sodium phosphate, pH 7.0). QN contained 14 wt% active saponins in the extract, so that the concentration of this surfactant is reported on an active ingredient basis (rather than total mass basis), and 0.0035-35.71% (w/w) of QN extracts were weighed in order to achieve 0.0005-5% (w/w) of active saponins. The coarse emulsions were prepared by blending both lipid and aqueous phase together using a high-speed blender (Bamix, Switzerland) for 2 min at room temperature and then they were passed through a high pressure homogenizer (Microfluidics M110L, Newton, MA, USA) for 3 cycles at 12,000 psi in order to obtain fine emulsions.

3.2.3. Emulsion Stability Testing

The stability of the resulting emulsion-based delivery systems to environmental stresses that might occur during industrial utilization was tested.

3.2.3.1. Influence of pH

Emulsions containing 10% (w/w) oil phase (5% VE and 5% orange oil) and 90% (w/w) aqueous phase (2% lecithin, QN, WPI or 10% GA in 10 mM Na-phosphate buffer, pH 7.0) were prepared as described in Section 3.2.2. Freshly produced emulsions were then placed in 20 ml beakers and each sample was adjusted to a different pH value (2-8) using HCl or NaOH solutions at various concentrations. Finally, the samples were transferred to small

test tubes and stored at ambient temperature for 24 h prior to particle size, charge, and creaming stability.

3.2.3.2. Influence of Ionic Strength

Freshly produced emulsion-based delivery systems containing 2% lecithin, 2% QN, 2% WPI or 10% GA (pH 7) as emulsifier were transferred into glass test tubes and appropriate amounts of 1M NaCl solution or buffer solution were added to each tube to obtain samples with a range of different final salt concentrations (100, 200, 300, 400 and 500 mM). Another sample was prepared without adding additional salt and it was expressed as the sample '0 mM'. The samples were then vortexed and stored for 24 h at ambient temperature prior to particle size, charge, and creaming stability.

3.2.3.3. Influence of Temperature

Freshly produced emulsion-based delivery systems containing 2% lecithin, 2% QN, 2% WPI or 10% GA (pH 7) as emulsifier were transferred into glass test tubes that were then placed into water baths set at different temperatures (30-90 ˚C) for 30 min, and then cooled to room temperature. They were then mixed and stored for 24 h at ambient temperature prior to particle size measurements and creaming stability observations. WPI and GA-based emulsions containing salt (150 mM NaCl) were also treated at the same temperatures to observe the effect of salt addition on the thermal stability of the emulsions.

3.2.3.4. Interrelations of Binary Effects of Environmental Stresses

The influence of pH, temperature and ionic strength and their binary effects were evaluated according to the theories of Henderson-Hasselbach, DLVO, steric effects and protein unfolding mechanisms. These effects are illustrated in a matrix (Table 3.1).

Table 3.1. Interaction matrix of the temperature, ionic strength, hydrophobic and steric effects

Binary effects	**Temperature effects**	**Ionic strength effects**	**Hydrophobic effects**	**Steric effects**
pH effects	temperature and pH interactions	pH and ionic strength interactions	pH and hydrophobic interactions	pH and steric interactions
Temperature effects		temperature and ionic strength interactions	temperature and hydrophobic interactions	temperature and steric interactions
Ionic strength effects			ionic strength and hydrophobic interactions	ionic strength and steric interactions
Hydrophobic effects				hydrophobic and steric interactions

3.2.4. Determination of Particle Size

The particle size of the Vitamin E emulsion-based delivery systems was measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Malvern, UK). The particle size of each sample was represented as the surface-area-weighted mean diameter (d_{32}), which was calculated as

$$d_{32} = \frac{\sum d_i . n_i^{\ 3}}{\sum d_i . n_i^{\ 2}} \tag{3.1}$$

where n_i is the number of droplets of diameter d_i. The refractive index of the droplets were taken as, 1.473 for orange oil and 1.333 for the aqueous phase.

3.2.5. Determination of Zeta (ζ)-potential

The electrical charge (ζ-potential) on the emulsion droplets in the delivery systems was measured using particle microelectrophoresis (Zetasizer Nano ZS-90, Malvern Instruments, Worcestershire, UK). Samples were diluted with buffer solutions at appropriate pH prior to measurements in order to avoid multiple scattering effects.

3.2.6. Statistical Analysis

All results of each experiment are given as the average and standard deviation of the six measurements obtained from at least twice replicated experiments.

3.3. RESULTS AND DISCUSSION

3.3.1. Influence of Emulsifier Type and Concentration on Particle Size

Industrial manufacturers of emulsion-based products often need to select the most appropriate emulsifier for a particular application. Therefore, the influence of emulsifier type and concentration on the formation of nanoemulsion-based delivery systems for two small molecule surfactants: Q-Naturale and lecithin, and two natural biopolymers: GA and WPI were examined in this part of the study. Initially, a series of aqueous phases were prepared by dissolving different amounts (0.0005 to 5 % (w/w)) of either lecithin or Q-Naturale (on the basis of active saponins) and (0.1 to 10 % (w/w)) of either GA or WPI in aqueous buffer solutions (10 mM sodium phosphate buffer, pH 7.0). Emulsions were then formed by homogenizing 90% (w/w) aqueous phase with 10% (w/w) oil phase (5% VE and 5% orange oil) using a high pressure homogenizer (12,000 psi, 3 passes). The mean particle diameter and particle size distributions were then measured after the samples were stored for 1 week at ambient temperature.

Mean particle diameter (d_{32}) decreased with increasing emulsifier concentration for the emulsion stabilized by all types of studied emulsifiers (Figure 3.1). The amount of emulsifier needed to create small droplets was higher for lecithin than for Q-Naturale, *e.g.*,

to achieve d_{32} < 150 nm, about 2% lecithin was needed, but only 0.5% Q-Naturale. At the highest emulsifier levels, mean droplet diameters were fairly similar for both types of emulsifiers, which suggests that the droplet size was then limited by the maximum disruptive energy generated by the homogenizer under the operating conditions used, rather than by emulsifier type. On the other hand, the amount of emulsifier needed to create small droplets was considerably higher for GA than for WPI, e.g., about 10% GA was needed to achieve d_{32} = 384 nm, but only 1% WPI was needed to achieve d_{32} = 110 nm (Figure 3.1). WPI- and GA-stabilized emulsions formed at the same homogenization conditions (12,000 psi, 3 passes) have different particle sizes at all emulsifier concentrations, which suggests that the droplet size was limited by emulsifier type (rather than homogenization conditions).

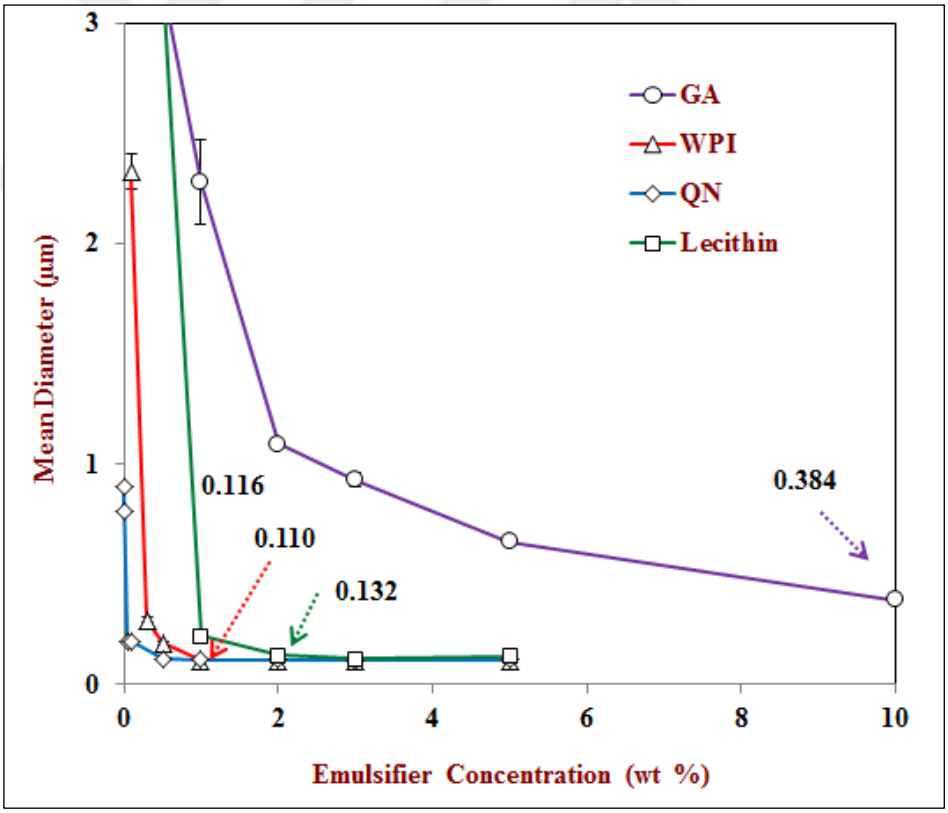


Figure 3.1. Influence of emulsifier type and concentration on the mean particle diameter of emulsions (10% w/w of oil phase (5% VE + 5% orange oil) and 90% w/w aqueous phase (emulsifiers in 10 mM Na-phosphate buffer, pH 7.0)) produced using standardized homogenization conditions (12,000psi, 3 passes).

The observed differences in the minimum amount of emulsifier required to attain small droplets (c_{min}), and in the minimum diameter of the droplets produced (d_{min}) can be attributed to differences in the nature of the two emulsifiers. GA leads to relatively high c_{min} and d_{min} values because (i) it contains large molecules that adsorb slowly to the droplet surfaces; (ii) it has a relatively low surface-activity; and (iii) it forms thick interfacial layers [29, 63]. In contrast, WPI gives relatively low c_{min} and d_{min} values because (i) it contains small molecules that adsorb rapidly to the droplet surfaces; (ii) it has a relatively high surface-activity; and (iii) it forms thin interfacial layers [62].

Multimodal particle size distributions were observed below 2% lecithin, below 0.5% Q-Naturale, below 1% WPI and 1% GA, but monomodal ones were observed at higher emulsifier concentrations (Appendix A1, A2, A3 and A4). The observed decrease in droplet diameter with increasing emulsifier concentration can be attributed to various factors: faster emulsifier adsorption to oil droplet surfaces during homogenization leading to lower interfacial tensions which facilitate droplet breakup; more emulsifier available to cover the droplet surfaces formed during homogenization [67, 68]. From a practical point of view, it is usually advantageous to use the least amount of emulsifier required to form stable emulsions, since this reduces costs, off-flavors, and toxicity. Besides this, high concentrations of non-adsorbed emulsifier may promote the destabilizing mechanisms Ostwald ripening or droplet flocculation in the emulsions [57, 69, 70].

At low emulsifier concentrations, visible phase separation of the emulsions was observed after storage, which can indicate the formation of droplet aggregation and creaming. A distinct cream layer was observed on top of emulsions containing <0.5 % (w/w) Q-Naturale and <2 % (w/w) lecithin and <5 % (w/w) GA and <0.5 % (w/w) WPI, which is due to the instability related to the formation of large droplets in these systems. However, negligible or no creaming was observed at higher emulsifier concentrations in all emulsions, which can be attributed to the small droplet size in these systems. The creaming rate is proportional to the square of the droplet diameter (Eq. 1.1), and so a reduction in droplet size decreases the susceptibility of an emulsion to gravitational separation. In addition, the gravitational forces become comparable to Brownian motion effects for sufficiently small droplets [16]. Brownian forces favor an even distribution of droplets throughout the system, and therefore can counteract the gravitational forces that favor an uneven vertical distribution of droplets.

It was found that a lower amount of Q-Naturale (≈ 0.5%) was required to form small droplets (d_{32} < 130 nm) than for lecithin (≈ 2%). A lower amount of WPI (c_{min} ≈ 1%) was required to form small droplets (d_{32} < 110 nm) than for GA (c_{min} ≈ 10%) (Figure 3.1). When the size of the droplets is limited by the amount of emulsifier present (rather than homogenization conditions), the minimum emulsifier concentration required to cover all the droplets produced is given by [71]:

$$c_{sat} = \frac{6 \cdot \Gamma \cdot \phi}{d_{32min}} \tag{3.2}$$

Here, c_{sat} is the concentration of surfactant in the emulsion (kg m^{-3}), Γ is the surface load of the surfactant at saturation (kg m^{-2}), ϕ is the disperse phase volume fraction and d_{32min} is minimum droplet diameter that can be obtained under the specific homogenizer conditions. It can be explained from the equation that the emulsifier concentration required to produce small droplets increases as the surface load of the emulsifier increases. In this part of the study, the equation was used to calculate the effective surface load of the emulsifiers based on the known droplet concentration (ϕ ≈ 0.1 for 10% w/w oil phase), and measured mean diameters d_{32min} ≈ 116 and 132 nm at 0.5% Q-Naturale and 2% lecithin, respectively. These calculations suggest that the surface load of Q-Naturale (Γ ≈ 1.0 mg m^{-2}) is about four times smaller than that of lecithin (Γ ≈ 4.4 mg m^{-2}), which may be due to differences in packing of the molecules at the oil-water interface. However it was assumed in the equation that all of the emulsifier present in the system is adsorbed to the droplet surfaces, and that adsorption occurs very rapidly in the homogenizer. In practice, there may also be differences in the adsorption kinetics between emulsifiers leading to varying droplet size obtained during homogenization, or not all of the emulsifier adsorbs to the droplet surfaces. Moreover, effective surface load of WPI and GA was also calculated based on the known droplet concentration (ϕ ≈ 0.1), and measured mean particle diameters d_{32min} ≈ 110 and 384 nm at 1% WPI and 10% GA, respectively. These calculations suggest that the surface load of WPI (Γ ≈ 1.83 mg m^{-2}) is about 35 times smaller than that of GA (Γ ≈ 64 mg m^{-2}), which may be due to differences in interfacial thickness and packing of the molecules at the oil-water interface. GA forms much thicker layers than WPI at oil-water

interfaces. It should also be noted that this equation assumes that all of the emulsifier adsorbs to the droplet surfaces during homogenization, and that adsorption occurs extremely quickly so re-coalescence is avoided. In the case of GA, it is likely that an appreciable fraction of the emulsifier did not adsorb to the droplet surfaces due to its low surface activity, and that adsorption was not fast enough to prevent re-coalescence within the homogenizer. Thus, the calculated surface load for GA should be treated with some caution. These experiments indicated that WPI could be used to form food-grade nanoemulsions (d < 200 nm), whereas GA could only be used to form emulsions.

3.3.2. Influence of Oil Composition on Particle Size

The influence of oil phase composition on the droplet size and vitamin E acetate (VE) loading capacity of the nanoemulsions were examined using the four natural emulsifiers; soy lecithin, Q-Naturale, WPI and GA. Oil phase composition was varied by mixing different ratios of VE (0-100% w/w) and orange oil (0-100% w/w) prior to homogenization. This procedure was carried out because VE is a highly viscous liquid that is difficult to homogenize alone, and so it was blended with a low viscosity oil (orange oil) to facilitate homogenization. Emulsions containing 10% (w/w) oil phase and 90% (w/w) aqueous phase (2% w/w Q-Naturale, lecithin, WPI or 10% w/w GA, 10 mM sodium phosphate buffer, pH 7.0) were produced by high pressure homogenization (12,000 psi, 3 passes).

Mean particle diameters (Figure 3.2) and particle size distributions (Appendix B) were measured after 1 week storage of the emulsions at ambient temperature. In general, the mean particle diameter (d_{32}) initially decreased with increasing VE, reached a minimum value around 40-50% VE, and then increased upon further VE addition. However, at high VE levels the mean droplet diameters were much greater for lecithin-stabilized emulsions than for Q-Naturale-stabilized ones. At intermediate VE levels, both types of small molecule surfactant were able to form nanoemulsions, *i.e.*, d_{32} < 200 nm. In WPI-stabilized emulsions, the mean particle diameter (d_{32}) initially decreased with increasing VE, reached a minimum value around 50% VE, and then increased upon further VE addition. In GA-stabilized emulsions, d_{32} initially decreased with increasing VE, reached a minimum value around 80% VE, but then increased for pure VE (Figure 3.2). In addition,

a distinct cream layer was observed on top of the emulsions containing 100% VE after storage, whereas the other emulsions appeared homogeneous.

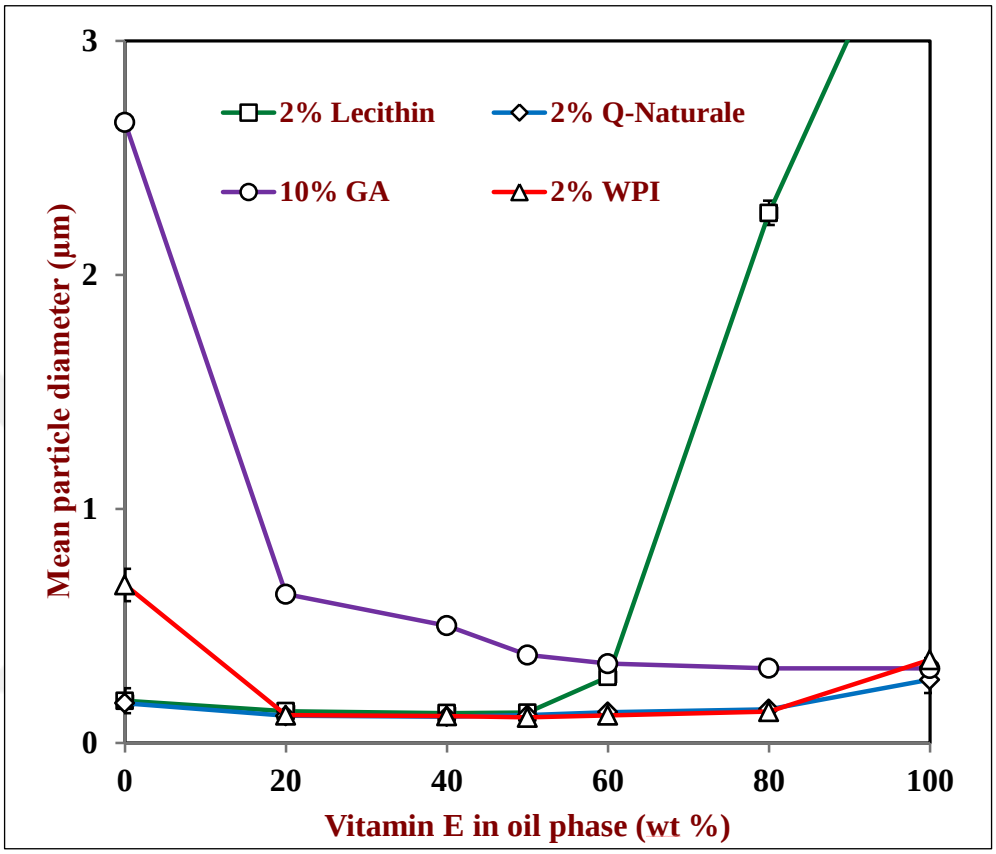


Figure 3.2. Influence of oil composition on the mean particle diameter of emulsions (10% w/w of oil phase (VE and orange oil) and 90% w/w aqueous phase (emulsifiers in 10 mM Na-phosphate buffer, pH 7.0)) produced using standardized homogenization conditions (12,000psi, 3 passes).

The minimum droplet diameter at a particular vitamin E acetate level can be attributed to two effects: Ostwald ripening and droplet disruption within the homogenization. At low VE levels (*i.e.*, high orange oil levels), the emulsions are highly susceptible to Ostwald ripening due to the appreciable water-solubility of orange oil and there is rapid droplet growth after droplet formation in the homogenizer [72]. Adding a highly water-insoluble substance, such as VE, to the oil phase prior to homogenization inhibits Ostwald ripening

due to an entropy of mixing effect (“compositional ripening”) that opposes droplet growth [68]. Thus, adding intermediate levels of VE to the oil phase leads to the production of smaller droplets. However, if the VE level is increased too much, then the viscosity of the oil phase becomes so large that droplet disruption within the high pressure homogenizer becomes inefficient, leading to larger droplets that are susceptible to creaming [67]. Consequently, an intermediate oil phase composition is required to form small and stable oil droplets.

Monomodal particle size distributions were observed when the oil phase contained 40 and 50 % (w/w) vitamin E acetate for the lecithin-stabilized emulsions (Appendix B1) and around 20 to 80 % (w/w) VE for the Q-Naturale-stabilized emulsions (Appendix B2) whereas broad bimodal distributions were observed at other oil phase compositions. Monomodal particle size distributions were observed when the oil phase contained ≤ 80 % (w/w) vitamin E acetate for the GA-stabilized emulsions and from 20 to 60 % (w/w) VE for the WPI-stabilized emulsions (Appendix B3 and B4) and small stable droplets can be produced at relatively high vitamin E acetate levels (60-80%), which means that a high vitamin loading capacity can be achieved. The other emulsions at the other oil phase compositions showed broad bimodal distributions. The emulsions that contained relatively large droplets were highly susceptible to creaming and undergone phase separation after 1 week storage. In summary, oil phase composition had an important effect on the formation and stability of the VE enriched emulsion-based delivery systems and had to be optimized to create small droplets containing relatively high vitamin E acetate levels (50-80%) in the oil phase.

3.3.3. Influence of Environmental Conditions on Emulsion Stability

Emulsion-based delivery systems may experience a variety of environmental stresses (e.g. pH, ionic strength, or temperature changes) during food processing, storage, transportation, and utilization. It is therefore important to establish the influence of these environmental stresses on emulsion stability. In this section, the influence of pH, ionic strength, and temperature on the physical stabilities of 2% lecithin- and 2% QN-stabilized, 2% WPI-stabilized nanoemulsions and 10% GA-stabilized emulsions was examined. All the

systems contained 5% VE and 5% orange oil in the lipid phase, which was a level where small stable droplets could initially be fabricated.

These variables are interrelated through Henderson-Hasselbach, DLVO (Debye-Hückel and double layer compression) theories and influential on generating of the steric effects and the thermodynamic state of the chemical structures. In this thesis, the destabilization phenomena was assessed in terms of the binary interactions of these variables by referring to the existing theories to understand the mechanisms causing the destabilization better.

3.3.3.1. The pH and Steric Effect Interactions

In oil-in-water emulsion structures, the hydrophilic part of the emulsifiers extend to the water phase of the interface and the lipophilic parts protrude into the oil phase (Figure 1.3). Some emulsifiers may contain hydrolysable groups in hydrophilic part of their structure. According to Henderson-Hasselbach equation (Eq. 2.1), the pH of the solution, the pKa of the hydrolysable groups and the concentration of the acid and the conjugate base are in a relating environment. As the pH becomes higher than the pKa value, the hydrolysable groups are ionized. The pH changes of the prevailing solution also affect the surface charge of the droplets. For example, as the pH decreases, H+ ions concentrate more and protonation of the negatively charged groups on the surface of the emulsifier layer may occur in the emulsions. Increasing the number of the electrons and κ decreases the Debye length (κ^{-1}) around the surface and thus decreases the zeta potential at shear plane (ψ_0) (Eq. 2.4). The decrease in ζ-potential may cause loss of electrostatic repulsion and facilitate the instability of the emulsion.

In the scope of this thesis, emulsions stabilized by four types of emulsifiers were subjected to different pH ranges in order to investigate the effect of pH on mean particle diameter, ζ-potential, and physical stability of the emulsions. QN- and lecithin-stabilized emulsions had relatively small particle diameters from pH 3 to 8 and each emulsifier could stabilize the nanoemulsions when the pH of the emulsion environment was ranged between 3 to 8. However, they became highly unstable to phase separation when stored at pH 2, a transparent serum layer was observed at the bottom of the test tubes. High aggregation occurred in both type of the nanoemulsions and therefore particle size increased dramatically (Figure 3.3). The WPI-stabilized nanoemulsions contained relatively small

droplets that were visibly stable to creaming at pH values from 2 to 5 and from 6 to 8 (Figure 3.3). The mean particle diameter increased appreciably at pH 5 and the nanoemulsion became highly unstable to phase separation with a transparent serum layer being observed at the bottom of the test tubes after storage. The 10% GA-stabilized emulsions were stable to droplet aggregation and creaming across the entire pH range.

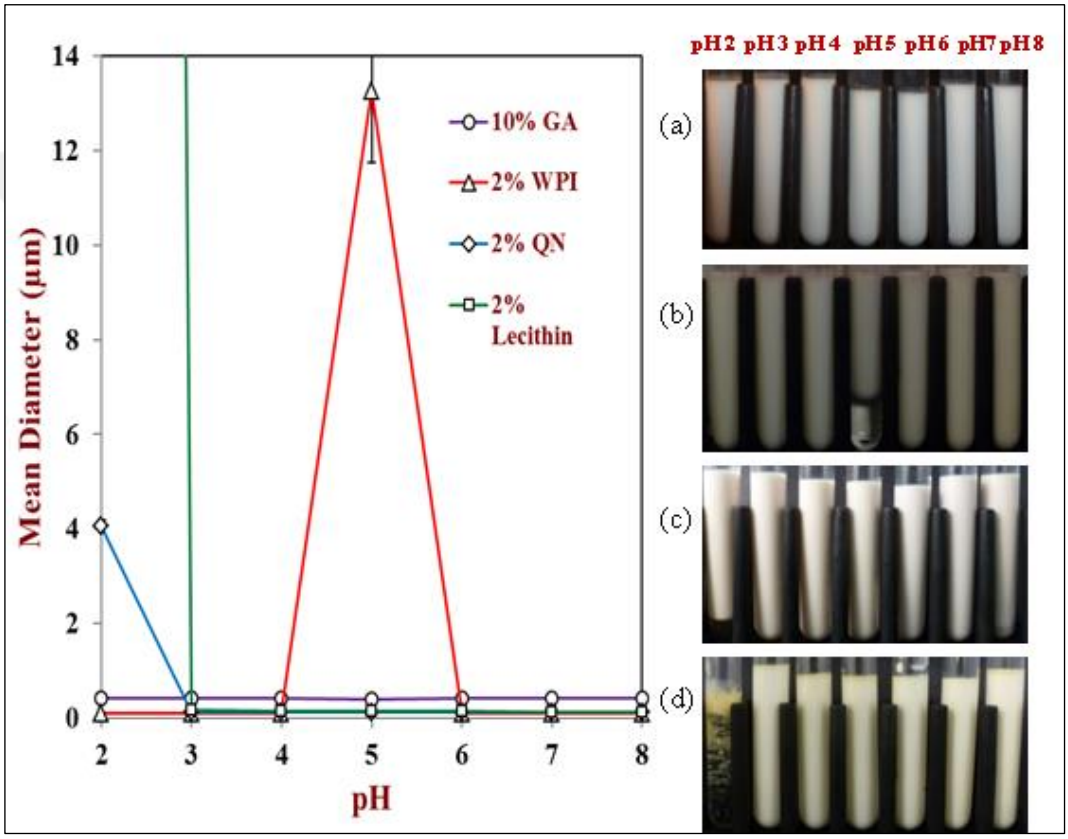


Figure 3.3. Influence of pH on the mean particle diameter of emulsions stabilized by (a) GA, (b) WPI, (c) QN, (d) lecithin.

To provide some insight into the origin of emulsion instability the electrical characteristics of the oil droplets were measured as a function of pH (Figure 3.4). For both surfactants, the electrical charge went from highly negative (≈ -60 mV) at pH 8 to slightly negative at pH 2 (≈ -5 to -10 mV). The good stability of the nanoemulsions at high pH values may therefore be at least partly atttributed to a strong electrostatic repulsion between the highly charged droplets. When the acid was added in the solution, the concentration of (+) ions start to

increase and thus pH decreases. The concentration of the positive and the negative charges around the group becomes close to each other at a point, thus the stability of the droplets might be destroyed because of the loss of electrostatic repulsive interactions between the droplets. Therefore, at pH 2 the electrostatic repulsion may not have been strong enough to overcome the attractive interactions (e.g., vdW and hydrophobic) acting between the droplets, which may lead to droplet aggregation.

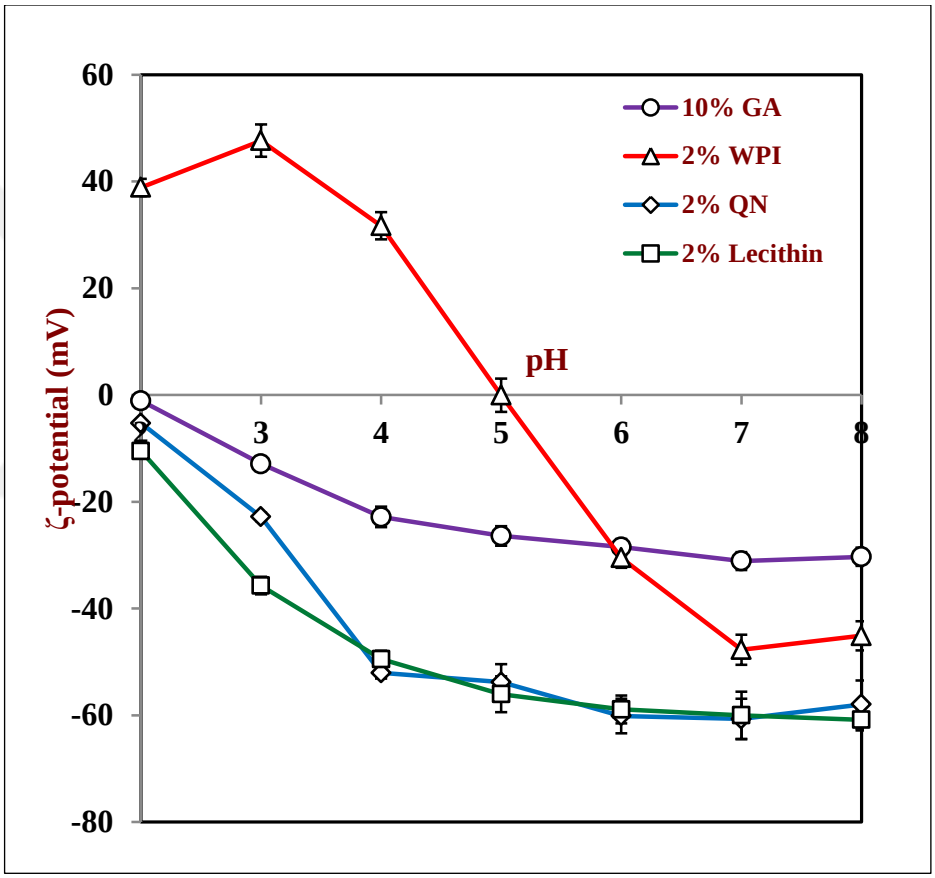


Figure 3.4. Influence of pH on zeta-potential of emulsions stabilized by WPI, GA, QN and lecithin.

The steep decrease in ζ-potential when the emulsion was reduced from pH 4 to 2 can be related to the pK_a values of the surfactants. Q-Naturale contains surface active saponins, which have hydrophilic head groups containing glucuronic acid, which has a pK_a value around pH 3 to 3.5 [31, 58]. The decrease in charge in this system can therefore be attributed to protonation of the carboxylic acid groups around and below this pH value

(i.e., -COOH). According to Henderson-Hasselbach equation (Eq. 2.1), the extent of ionization and electrical charge may be calculated when the pH of the solution changes. As the pH of the solution becomes closer to the pKa value of the ionizable group, the opposite sign of the ions around this group becomes equal leading the loss of the charge and causing aggregation. Phospholipid headgroups in lecithin also contain ionizable anionic groups that have pK_a values in the acidic pH range [34, 73], and may lose their charge at lower pH values. This might happen due to the protonation of the glucuronic acid in quillaja saponin and phosphatidic acid (pKa ≈1.5) in lecithin with the increase in H^+ ions at pH 2 (Figure 3.4).

Proteins may contain ionizable groups on their side chains, and amino - and carboxyl-termini in their structure. The extent of the ionization and the electrical charge on the side chains are affected by the ion concentration in the solution. When the pH of the solution becomes close or equal to the isoelectric point of the proteins (pH≈ pI), the net charge on the protein surface becomes zero and cause aggregation of the protein, thereby instability of the emulsion. In this thesis, it was observed that for WPI-stabilized emulsions, the electrical charge was highly positive well below the isoelectric point (pI ≈ 5) and highly negative well above it. The WPI stabilized nanoemulsions were undergone aggregation at its isoelectric point (pH 5.0) due to the screening of electrostatic repulsion around the droplets (Figure 3.3). The flocculation stability of the emulsions depended on the balance of the electrostatic repulsive and attractive van der Waals forces according to the DLVO theory. At the isoelectric point of the protein, van der Waals attraction forces dominate electrostatic repulsion, leading to the droplet flocculation.

When the pH > pI, the proteins are negatively charged and when the pH < pI, they are positively charged [74, 75]. WPI stabilized emulsion droplets produced in this thesis study had high negative charges (-47 mV) at pH 7.0 (Figure 3.4). Electrostatic repulsive forces were higher due to high amount of negative ions and therefore dominated vdW forces preventing the aggregation or flocculation. The surface charge of the droplets in WPI stabilized emulsions is governed by the ionization degree of amino groups (–NH) and carboxyl groups (–COOH) and that of the ionizable side chain (glutamic acid and aspartic acid) in protein molecules. The ionization degree might change depending on the pH (Eq. 2.1) and ionic strength of the surrounding aqueous phase [74]. Proteins stabilize the

emulsions both by electrostatic repulsion and steric repulsion, however the electrostatic repulsion dominates due to the thin layer of adsorbed protein on the surface.

There was no creaming or instability observed in gum arabic coated emulsions and mean particle diameter did not change (Figure 3.3), although the magnitude of the zeta potential values decreased when pH is lower than 5.0 (Figure 3.4). Zeta potential value decreased at pH values close to pKa value of glucuronic acid (pH 3.0 -3.5) which is the hydrolyzable group in GA structure. The reduction of negative charge from pH 5 to 2, can be attributed to protonation of the glucuronic acid group on the polysaccharide (-COOH). Although electrostatic potential decreased (screening) around the droplet surface (Eq. 2.2), steric repulsion caused by highly branched arabinogalactan tails was high enough to keep the droplets far from each other to prevent the droplet to droplet aggregation. Even though droplet charge may not be critical for the stability of the GA-coated oil droplets, it may be important for their chemical stability, as anionic droplets can attract cationic transition metals (e.g., Fe^{2+}) to their surfaces, which can promote oxidation.

In total, steric repulsion played dominant role in the stabilization of GA-based emulsions, whereas electrostatic repulsion was dominant force in WPI-based nanoemulsions. This may occur because high molecular weight and highly branched structure of gum arabic could form thicker interfacial layer than that the smaller biopolymer, WPI could form [76]. This can be seen from the surface load calculations of these emulsions in the previous study. When the full coverage of the surface by the emulsifier was assumed, the amounts of the adsorbed emulsifier concentrations were found as 1.83 mg/m^2 and 64 mg/m^2 for WPI and GA, respectively [77].

Both Quillaja saponin and lecithin are small molecule surfactants which can rapidly adsorb on the droplet interface to form very small droplets. Quillaja saponins have hydrophobic aglycone part linked by branched sugar moieties forming the head groups which may provide repulsion effects during emulsification. Lecithin may be capable of generating sufficient steric repulsive forces due to the hydrophilic head group in its structure. However, the repulsion is mostly generated by electrostatic forces. In the highly acidic conditions (pH 2), due to the loss of the electrostatic repulsion, van der Waals attractive forces might overcome the electrostatic repulsion and cause aggregation (Figure 3.4).

3.3.3.2. The Ionic Strength and Steric Effect Interactions

Food and beverages may include different concentrations of salt, therefore it was required to examine the influence of ionic strength on the stability of vitamin E nanoemulsions formed using Q-Naturale, Lecithin, WPI and GA as emulsifiers. The influence of ionic strength of the prevailing environment may be governed by the valency and concentration of the added salt. Increase of the valency (z_i) and concentration (M_i) of the salt ions causes an increase of the ionic strength of the solution as described in *section 2.2*. Increasing the ionic strength decreases the Debye length (Eq. 2.5), and the electrostatic potential and cause instability due to the loss of the strength of the electrostatic repulsions.

Influence of salt concentration (ionic strength) on the mean particle diameter and zeta potentials of the emulsions stabilized by WPI, GA, Q-Naturale and lecithin is shown in Figure 3.5 and 3.6. Visible observation of the nanoemulsions indicated that extensive phase separation occurred at > 100 mM NaCl for the lecithin-stabilized systems, with an yellowish oil layer being observed at their surfaces. On the other hand, the formation of a white cream layer at the top of the Q-Naturale-stabilized emulsions was observed at ≥ 400 mM NaCl. Interestingly, the particle size measurements indicated that appreciable droplet growth occurred in the lecithin nanoemulsions at ≥ 100 mM NaCl, but there was no increase in droplet size in the Q-Naturale nanoemulsions across the entire range of salt concentrations studied (Figure 3.5a). These results suggest that the droplets in the Q-Naturale nanoemulsions were weakly flocculated (rather than coalesced) due to the electrostatic screening of the ions around the droplet surface, and droplets could easily be dispersed upon dilution and stirring within the chamber of the light scattering instrument (Figure 3.6). Presumably, the quillaja saponin was able to generate a strong short-range steric repulsion that prevented the droplets from coming close enough to coalesce [78].

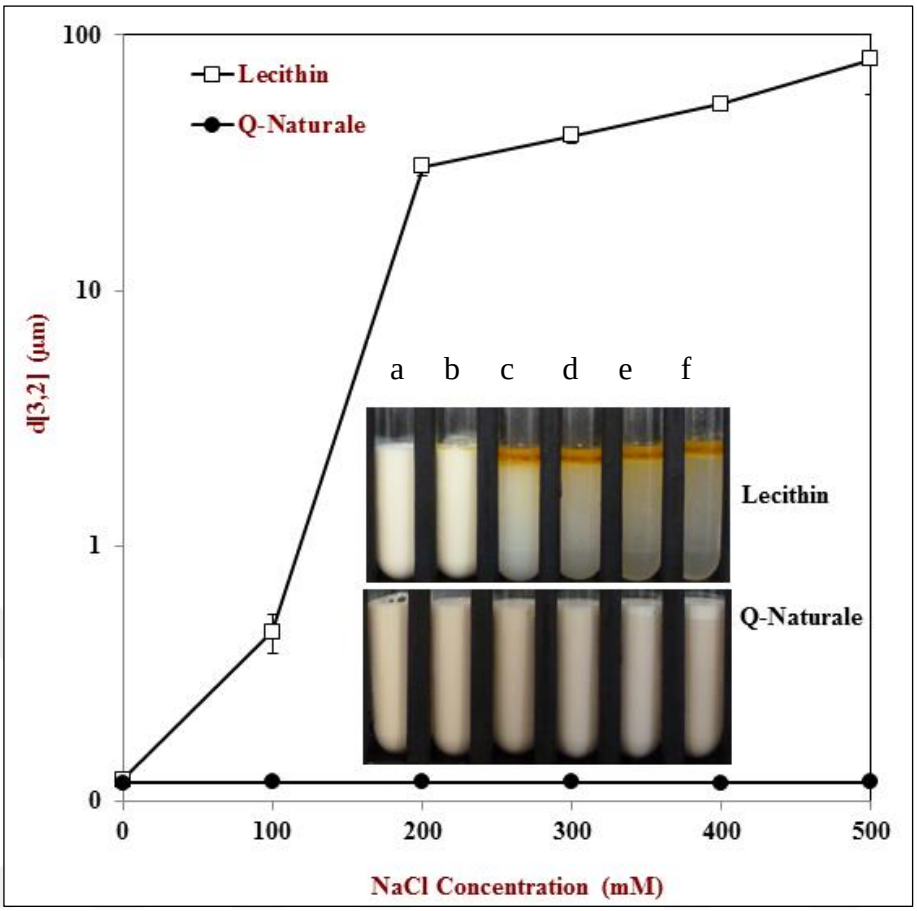


Figure 3.5 (a) Influence of ionic strength on mean particle diameter (d_{32}) of emulsions stabilized by Q-Naturale and lecithin. (a) 0, (b) 100, (c) 200, (d) 300, (e) 400, (f) 500 mM NaCl concentrations.

Visible observation of the WPI-stabilized nanoemulsions indicated that a thin cream layer was present at the top of the samples at >200 mM NaCl. On the other hand, no cream layer was observed on top of the 10% GA-stabilized emulsions. Surprisingly, particle sizes determined by static light scattering indicated that there was little particle aggregation in WPI-stabilized nanoemulsions or GA-stabilized emulsions across the entire range of salt concentrations studied (Figure 3.5b). Nevertheless, there was a slight increase in droplet diameter (d_{32}) for the WPI-stabilized emulsions, from around 0.110 µm at 0 mM NaCl to 0.118 µm at 500 mM NaCl. The fact that some creaming of the droplets was observed at high salt concentrations in this system, suggests that the droplets may have been weakly flocculated and that these flocs were disrupted when the emulsions were diluted and stirred during the particle size measurements.

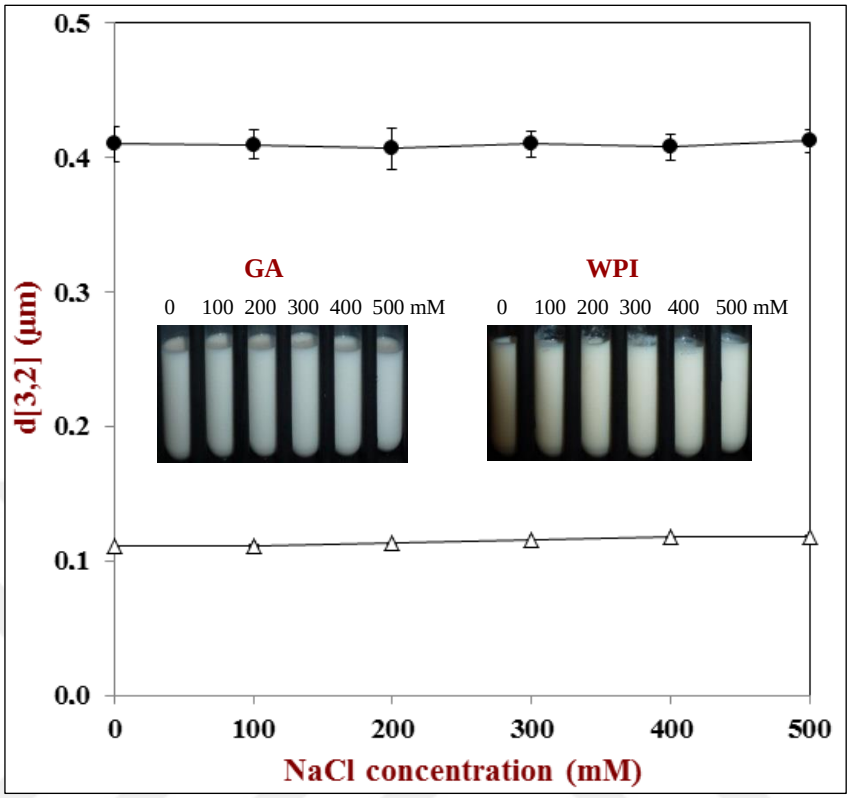


Figure 3.5 (b) Influence of ionic strength on mean particle diameter (d_{32}) of emulsions stabilized by GA (black circles) and WPI (white triangles).

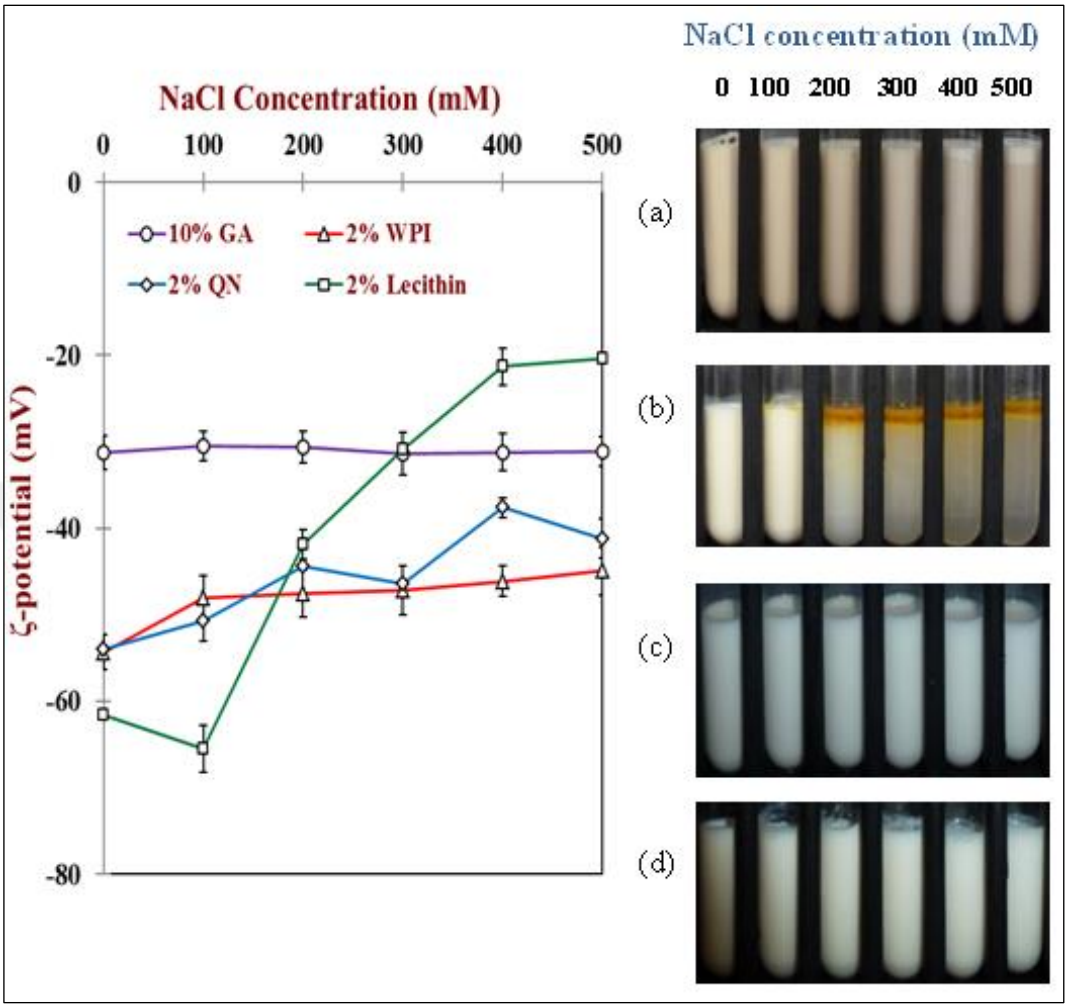


Figure 3.6. Influence of ionic strength on zeta-potential of emulsions stabilized by WPI, GA, Q-Naturale and lecithin. Pictures represent (a) QN, (b) lecithin, (c) GA and (d) WPI-based emulsions.

Lecithin-based nanoemulsions had high magnitude of (-) charge at pH 7.0 when salt was not added, however magnitude of particle charge decreased drastically as the salt concentration increased (NaCl > 100 mM) (Figure 3.6). When the electrostatic repulsion forces diminished due to the electrostatic screening effects of counter-ions, lecithin-based droplets might collapse and therefore particle diameter increased dramatically. Eventually, the emulsions were coalesced and oil-off occurred on top of the surface. This result may be related to the flexible and easy to disrupt interfacial layer that is formed upon the adsorption of phospholipids at oil-water interface [79, 80]. It is mentioned that chaotrophic

ions (i.e. Ca^{2+}, N^{+} and K^{+}) may cause loss of membrane integrity in phospholipid structure and the electrolytes may enhance the vibration of phospholipid groups at the interfaces, thereby causing the poor stability in the emulsions [80]. The presence of the monovalent cations in the prevailing environment decreases the fluid spaces between the charged polar head groups causing the screening of the charge [81]. Accumulation of the counter-ions around the droplet surfaces cause electrostatic screening and may lead a decrease in the Debye length (κ^{-1}) thereby reduce the electrostatic repulsion (Eq. 2.4). Upon the loss of the electrostatic repulsion and the insufficient steric repulsive effect in the lecithin-based nanoemulsions, the attractive forces dominate the repulsion forces and lead to aggregation as described by the DLVO theory (Section 2.2).

The addition of salt (NaCl ≥ 100 mM) caused a slight decrease in the magnitude of the negative charge on the WPI-coated droplets (Figure 3.6), which may be attributed to electrostatic screening effects [78]. The salting out effect is observed when the salts exceed the critical concentration in the solution. Both the valence and concentration of chaotropic salts have effect on disruption of the protein stabilized emulsions by compressing the diffuse double layer and reducing the energy barrier of repulsive forces [75, 82]. According to the Debye-Hückel approximation, the stability of the particles increases with the concentration and the valence of the ions. The diffuse double layer compression occurs and the Debye length (reciprocal of Debye-Hückel parameter, κ) decreases and becomes closer to the particle surface (Eq. 2.3). When the Debye length around the particles decreases, they approach each other rapidly and thus aggregation may occur.

However, salt addition had little impact on the magnitude of the electrical charge on the GA-coated droplets (Figure 3.6), which suggests that there may have been a charge regulation process occurring. For example, the addition of salt may have changed the thickness and internal structure of the adsorbed gum arabic layer, as well as promoting electrostatic screening effects [78]. The salt addition did not cause any charge reduction since the polysaccharides generally stabilize the emulsion droplets by the steric forces. Chanamani and McClements, 2002 studied the effects of the environmental conditions on the stability of the GA-based emulsions and found that GA formed very stable emulsions in a wide range of environmental stresses (pH, T and salt) due to the strong steric repulsive interactions [60].

3.3.3.3. The pH and Ionic Strength Effect Interactions

The pH and ionic strength of the solutions affect the electrostatic and hydrophobic interactions and alter the ability of the protein molecules to interact via the attractive and repulsive forces [27]. The emulsions were prepared using Na-phosphate buffer at pH 7.0 and the produced droplets contained negative ions on their surface (Figure 3.4). Upon addition of HCl or NaCl, positively charged (H^+ and Na^+) ions act as counterions and move towards the negatively charged groups on the surface of GA-, Q-Naturale, lecithin and the WPI stabilized emulsions. Consequently, this may cause protonation of the negatively charged groups allowing the double layer of the counter ions and diffused co-ions to become thinner and thereby decreasing the electrostatic repulsion (Eq. 2.4). When the concentration of the ions increases by addition of higher amounts of salt or acid, the double layer compression may play dominant role in terms of interactions and cause a decrease in the zeta potentials and forming electrostatic screening effect in the emulsion which were stabilized mainly by electrostatic forces (Figure 3.6).

3.3.3.4. The pH and Temperature Effect Interactions

Globular proteins usually unfold and become insoluble upon heating, exposure to extremes of pH, or treatment with certain reagents and lose their biological activity without damage to the polypeptide covalent backbone structure. Denaturation by heat processing ($T \geq 65$ °C) involves the unfolding of the polypeptide chain which may lead to the exposure of hydrophobic groups to the solution and activate the sulphydryl groups promoting the covalent protein-protein interaction via disulphide bonding [27].

The ability of proteins to form gels may depend on the balance of attractive and repulsive forces between the protein molecules. As the temperature increases, internal energy increases due to the breaking up of the hydrogen bonds in protein molecule and unfolding of the protein structure occurs. Upon unfolding hydrophobic surfaces expose to the environment with further increase in temperature, the protein - protein interaction increases due to the linkage of the hydrophobic groups and disulphide bonding as explained in Monahan et al., 1995 [83].

As the pH of the solution increases, surface charge of the droplets may change due to the increasing negative ions in the environment, as explained in Henderson-Hasselbach equation (Eq. 2.1**)**. Monahan et al (1995) [83] studied the effects of the pH (3-11) and temperature on the surface hydrophobicity and they observed that as the pH increased further from 7 to 9 and 11, the amount of surface hydrophobicity increased in solutions containing 13% WPI (higher amount of protein). Therefore, at extremes of pH the rate of unfolding increases and possibility of protein-protein interactions increased due to the exposure of higher amounts of hydrophobic groups. In the WPI emulsions at pH 11 the protein molecules had strong electrostatic repulsion, but gel formation occurred even at low temperatures due to the extensive unfolding and protein-protein interactions, including the disulphide bond formation. However at pH 7, electrostatic repulsion was not as high as it was at pH 11, there was insufficient unfolding to be able to form gels at high temperatures. Monahan et al (1995) studied the effects of the pH with the 1% WPI solutions under the same conditions, and found that the SH bond formation was less likely to happen due to the lower frequency of the interaction between the molecules. The surface hydrophobicity increased when pH increased between 9 and 11 and it was increased with temperatures above 70 °C, at pH 7.0 indicating unfolding of the chain. With the dilute WPI solutions unfolding was not enough to form gels. Therefore, protein unfolding and gelation may depend on different factors, such as protein concentration, pH and temperatures.

Emulsion-based delivery systems may be exposed to temperature variations during food processing, storage, transport, and utilization. The freshly prepared emulsions (pH 7.0) stabilized by 10% GA, 2% WPI, 2% QN and 2% lecithin had high magnitude of zeta potential values which provided strong electrostatic repulsion between the droplets (Figure 3.4). They were exposed to different temperatures (30-90 °C) for 30 minutes and finally they did not show any destabilization reactions, there was no creaming and increase in mean particle diameter in any of the samples (Figure 3.7). Electrostatic and steric repulsions overcame hydrophobic and van der Waals attractions, thereby maintain the stability against aggregation at temperatures between 30-90 °C. They had good thermal stability, presumably the electrostatic and steric repulsion between the droplets was high enough to inhibit droplet aggregation at all temperatures.

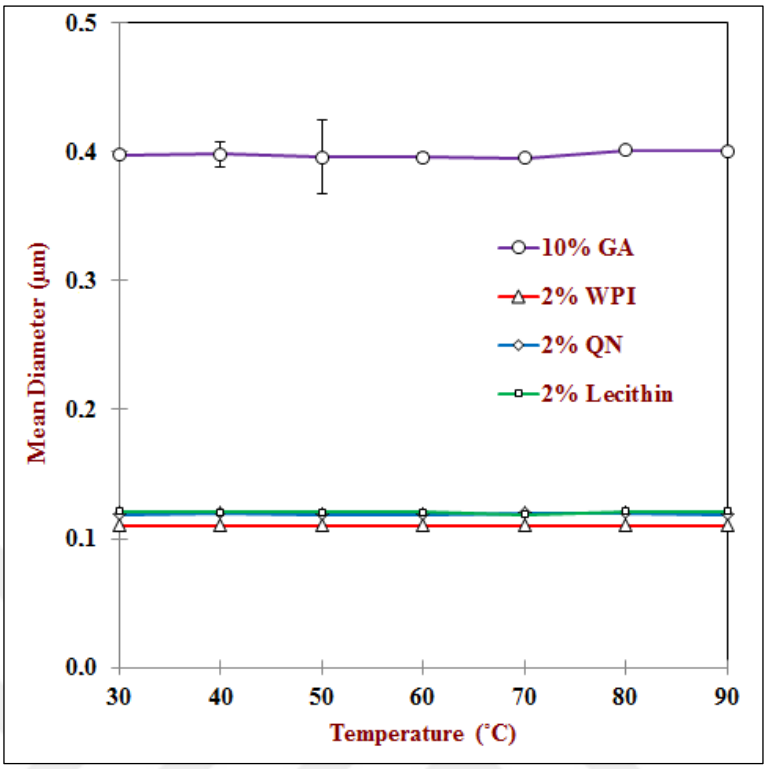


Figure 3.7. Influence of temperature on mean particle diameter of emulsions stabilized by WPI, GA, QN and lecithin.

Relkin (1996) made a graph relating the denaturation temperature of beta-lactoglobulin to pH and observed that decrease in the pH increased the denaturation of the protein, for example when the denaturation temperature of beta-lactoglobulin was around 65-70 °C at pH 7.0, it became 80-85 °C, when the pH> 4.0 [43]. These results were well correlated with the results of this thesis where denaturation might have occurred above 60 °C. Because of denaturation particle growth may be expected in the WPI nanoemulsions when T > 65°C. However, there was no appreciable particle growth at all range of temperatures in the WPI stabilized emulsion droplets due to electrostatic repulsion (- 47 mV, at pH 7.0) (Figure 3.7). As described by the DLVO theory of particle interactions, instability can be overcome when higher repulsive electrostatic forces dominate the attractive van der Waals forces. During the temperature increase denaturation might have occurred above 65 °C. The reason that the particle growth was not observed at these high temperatures may be the dispersion of the weak aggregates upon unfolding by stirring of the emulsions before measuring the particle size. These results may be due to the higher electrostatic and steric

repulsions overcoming the hydrophobic and van der Waals attractions, thereby maintaining the stability against aggregation at temperatures between 30-90 °C.

It was previously mentioned that once adsorbed at the interface, there is a structural reorganization of the protein in order to partially unfold and expose hydrophobic residues toward the lipophilic phase [84]. Partially unfold, denatured or native conformations of the proteins can be already found at the same time when they are adsorbed on the oil-water interface. This partial unfolding was caused from the affinity of aminoacids with different polarity to the two phases of the emulsion. Therefore upon high temperature, there might not be enough additional conformational distribution due to unfolding at the surface unless other environmental conditions (salt addition, exposure time increase, pH changes) are favored. There was no gel formation at this condition since gel formation by β-Lg is greatly influenced by salt addition or pH changes [27, 85].

3.3.3.5. The pH and Hydrophobic Effect Interactions

The hydrophobic groups have main role of anchoring the emulsifier molecules to the emulsion droplets surface and they tend to protrude into the lipophilic phase of emulsion droplet. Proteins may undergo reorganization and rearrangements upon adsorption and may expose their hydrophobic groups at high pH and temperature changes [83]. Surface hydrophobicity increases by the detachment of hydrophobic groups in protein molecule upon unfolding. The unfolding of the proteins may also be triggered upon extreme pH changes, unfolding becomes easier when the pH of the environment is far from that of the isoelectric point due to the weakening of the van der Waals forces which are dominant at the isoelectric points [43, 83]. Altering of the net charge upon the change of the electrolyte concentration may determine the mechanism of the precipitation and gelation in β-lactoglobulin. The gelation or aggregation of proteins not only depends on pH but also protein concentration and the amount of hydrophobic surfaces. Monahan et al., 1995 [83] reported that with 13 % WPI, which imply a high concentration of the protein, gel formation occurred at pH 11, even at the ambient temperature and the surface hydrophobicity increased. Indeed, at the pH extremes, the protein may undergo extensive unfolding allowing the large hydrophobic surfaces to attract the others and increase the protein-protein interactions. However, in this thesis, high repulsive forces might have

protected the emulsion droplets from aggregation by dominating the weaker hydrophobic attraction forces in the pH range above 6 may be because of the prevailing low temperatures and the lower concentration of WPI emulsions (Figure 3.4 and 3.7).

3.3.3.6. The Temperature and Hydrophobic Effect Interactions

Protein folding is mainly governed by the hydrophobic effect occurring due to the movement of the hydrophobic groups toward the interior of the protein, which are trying to go away from the water and decrease their free energy. However, there are also weak non-covalent bonds which hold the protein structure in native form. The hydrogen bonds start breaking and the internal energy increases upon the temperature increase, which increase the free energy and open the way to unfolding. Further increase in temperature leads to extensive unfolding and expose the hydrophobic surface groups to the environment.

Electrostatic and steric repulsions were high enough at temperatures between 30-90 °C to maintain the stability in four types of emulsifier stabilized emulsions. However it is well known that proteins (i.e. WPI) denature at higher temperatures and due to the unfolding of the protein chain, hydrophobic and disulphydryl groups expose to the bulk environment which may then interact each other and form aggregates and network based structures. The folded native protein is thermodynamically favoured ($\Delta G < 0$) because of the outstanding contribution of the positive entropy term for cavity reduction. However, upon increase in temperature higher than denaturation temperatures, the water network (highly ordered) around the protein molecules mey be broken up due to the breaking of hydrogen bonds between the ordered water molecules. Free water molecules which are less ordered upon the unfolding of the 3-dimensional protein structure may generate larger rooms and large cavity between the unfolded protein and free water molecules; thereby encourage the negative entropy production in the system $\Delta S << 0$ which may cause increase in free energy favoring the unfolded state (Eq. 2.6). This cavity formation is believed to disrupt the entropy driven hydrophobic bonds which have driving role in favoring the native folded structure of the protein [86]. The surface hydrophobicity also increases with the exposed hydrophobic groups upon unfolding at high temperatures [87].

The weak aggregation due to the unfolding of the WPI might also be encouraged in our study when temperature was more than 65 °C. Since there was no additional stress such as

salt addition or pH change to encourage the proteins to polymerize and form strong aggregates and gel, the weak fractal aggregates were easily dispersed upon the stirring [88] and the droplet growth was prevented due to insufficient van der Waals and hydrophobic attractions (Figure 3.7).

Buffo et al. (2001) found that pasteurized GA stabilized beverage emulsions maintained their stability, probably due to unfolding of the proteinacieus regions of gum arabic and exposure of the hydrophobic aminoacids which facilitate faster adsorption [89]. However, harsher conditions such as heat treatment for 6 hours of at 100 °C may be required to break the molecular structure of GA [90]; therefore particle growth was not observed upon heating of the gum arabic stabilized emulsions under the effect of the dominating steric repulsive forces.

3.3.3.7. The Temperature and Ionic Strength Effect Interactions

Whey proteins denature at high temperatures in aqueous solution and lead to irreversible aggregation, which may lead to gel formation under the favorable environmental conditions. Gel formation depends on pH, protein concentration, temperature, amount and type of salt in the solution [91]. Protein gelation consisted of sequential steps including denaturation and polymerization. The first step is denaturation which is triggered by the high temperature processing facilitates the weakening and breaking of the H- and S-S bonds to disrupt the native protein conformational structures and disperse the fractal protein aggregates. The second step is the polymerization of the denatured and dissociated protein molecules to produce a 3-dimensional-network of gel when the environmental conditions are favorable, such as the availability of high concentration of salt [92]. Salts containing chaotrophic ions mainly affect the stability of proteins. The stability of the emulsions depends on the balance of repulsive and attractive interaction forces between the droplets. Increase in the valency and concentration of the salt cause double layer compression and decrease the Debye length, κ^{-1}, as expressed in (Eq. 2.5), which may than decrease the electrostatic potential (Eq. 2.4) and decrease the energy barrier allowing the attractive forces become dominant and thereby cause aggregations.

The effect of salt addition (150 mM NaCl) on the thermal stability of the 2% WPI-stabilized nanoemulsions and the 10% GA-stabilized emulsions was examined within the

scope of this thesis. For WPI-stabilized nanoemulsions, there was an appreciable increase in mean particle diameter for samples held at T ≥ 60˚C, and visible gelation was observed at T ≥ 70˚C (Figure 3.8).

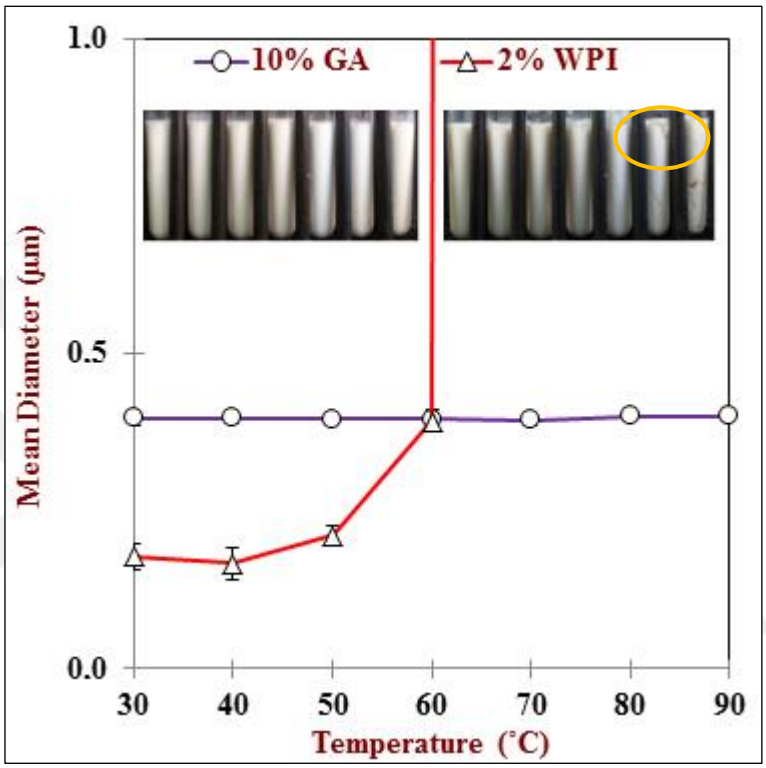


Figure 3.8. Mean diameter (d_{32}) graph. Influence of salt addition on thermal stability of emulsions stabilized by 2% WPI and 10% GA.

Presumably, the instability of WPI-stabilized systems to heating in the presence of salt was related to thermal denaturation of adsorbed proteins on the surface of the oil droplets [93]. Hydrophobic and sulfhydryl groups are exposed when denaturation occurs which favors droplet-droplet aggregation through hydrophobic attractions and covalent disulfide bonds [62]. When salt is added to protein stabilized emulsions, it may compress the double layer thereby diminish the repulsive forces (Eq. 2.5). In the absence of salt, the electrostatic repulsion is strong enough to overcome these attractive interactions, but in the presence of salt the electrostatic repulsion is reduced through the double layer compression and the attractive interactions (vdW and hydrophobic) dominate, leading to aggregation [93]. Gel

formation by β-Lg is greatly influenced by salt addition or pH changes [27, 85]. The main component of the WPI, β-lactoglobulin, contains two cysteine residues and one sulphydryl group; upon unfolding these groups may be exposed and crosslinked and exchange reactions between the protein chains might occur leading to further gel formation (Figure 3.9).

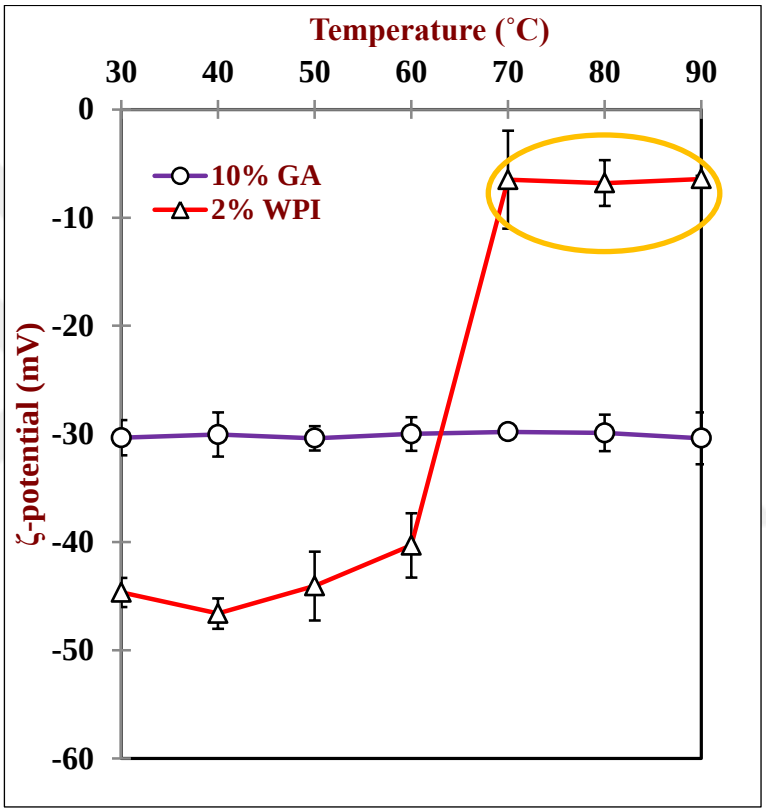


Figure 3.9. Zeta-potential (mV) graph. Influence of salt addition on thermal stability of emulsions stabilized by 2% WPI and 10% GA.

Mulhivill and Kinsella (1988) observed no gel formation in the β-lactoglobulin solutions at pH 8.0 upon heating when the no salt was added, where the higher magnitude of negative ions caused electrostatic repulsion and prevented the gel formation [85]. Nevertheless, salt (>100 mM NaCl) addition to the solution prior to heating supported the gel formation.

The mean particle diameter did not change and aggregation was not observed when GA-stabilized emulsions containing 150 mM NaCl were exposed to high temperatures (30-90

°C for 30 min) since polysaccharides generally stabilizes the emulsion droplet by steric forces (Figure 3.8). Neither the high salt concentrations (Figure 3.6) nor the high temperature (Figure 3.8) causes a decrease in the zeta-potentials of the GA-stabilized emulsions. The strong steric forces generated by the hydrophilic parts of GA and also by the negative ions distributed on the tails prevent the particles from approaching to each other and avoid the emulsions from aggregating. The origin of stability at higher temperatures may also be attributed to the insufficient exposed hydrophobic groups and lack of sulfhydryl groups in protenacious region of GA.

3.3.3.8. The Ionic Strength and Hydrophobic Effect Interactions

The surface hydrophobicity triggers the gel formation, as hydrophobic groups increase at the surface and the frequency of hydrophobic interactions between the protein molecules increase. The gel formations may occur under the favorable environmental conditions. Thermal denaturation may increase the hydrophobicity by implementing conformational changes and enhencing the hydrophobic interactions in the protein structure [27, 43].

Fisicaro et al. 2011 reported increased hydrophobicity via thermal denaturation and chemical denaturation [86]. Chemical denaturation by increase in valency or concentration of ions (i.e. salts, or H^+) triggers the disorganization of the water molecules and cause large cavity around the protein stabilized droplets which than leads to a negative entropy production (Eq. 2.6). Thus protein molecule may open up its structure upon exposing the hydrophobic groups to the solvent. The salting-in effect formed by the salt addition declined with the hydration when both the dissociation of the free water molecules from the bulk of the water clusters with the formation of a larger cavity, and the binding of the salt ions to the protein structure with the hydrogen bonds or preferentially to free water molecules result in negative entropy. The negative entropy contribution has great impact in the increase of the free energy and favor the unfolding of the protein molecule as referred in Eq. 2.6.

3.3.3.9. The Temperature and Steric Effect Interactions

At 30-90 °C emulsions were not destabilized for 30 minutes when evaluated both by the particle size measurements and the visual observation. All emulsion had high electrostatic potential when they were prepared at pH 7.0 (Figure 3.4). The GA-, Q-Naturale, lecithin- and WPI-based nanoemulsions maintained their stability at temperatures between 30-90 °C. This may be explained by the co-existence of steric stabilization and the electrostatic repulsion and their high magnitutes over the van der Waals and hydrophobic attraction forces (Figure 3.6).

3.3.3.10. The Hydrophobic and Steric Effect Interactions

The hydrophobic attraction forces are created upon the exposure of the hydrophobic groups to the solvent phase from the oil-water interface at either high temperatures or with the addition of a chemical agent, such as salts. The double layer compression may easily occur especially in the emulsion droplets stabilized elecrostatically by the emulsifiers when salt is added to the system. Besides this, in the study of Dickinson and Davies (1999), increasing the divalent cations (i.e. addition of $CaCl_2$) was shown to reduce the steric repulsion in the sodium caseinate stabilized emulsions [94]. The loss of the electrostatic potential upon addition of higher amounts of NaCl >200 mM may lead to flocculation in WPI stabilized emulsions obtained in this thesis. The higher steric repulsive effects due to the highly branched tail groups of gum arabic dominate the screening effects of the electrostatic repulsion and the attraction of the hydrophobic groups and thus prevent the emulsion droplet from aggregation (Figure 3.4 and 3.6).

3.4. CONCLUSION

In this part of this thesis, some of the major factors influencing the formation and stability of Vitamin E-enriched nanoemulsions produced using two natural food-grade small molecule surfactants (lecithin and QN) and two natural biopolymers (WPI and GA) were examined. The emulsion formation and stabilizing capabilities of these emulsifiers were compared in Table 3.2.

Table 3.2. Comparison of influence of natural emulsifier type on the formation and stability of vitamin E-acetate emulsions.

Emulsifier	**Formation**		**Stability**		
	$d[3,2]_{min}$	EOR_{min} [a]	pH	NaCl (mM)	T (˚C)
QN	0.116	0.5:10	3 - 8	≤300	≤90
L	0.132	2.0:10	3 – 8	≤100	≤90
WPI	0.110	1.0:10	2-4; 6-8	<100	<60 [b]
GA	0.384	1.0:1.0	2 - 8	≤500	≤90

[a] EOR_{min} is the minimum emulsifier-to-oil ratio to produce small droplets.
[b] Particle size increased in salt added (150 mM NaCl) WPI-based emulsions treated at T ≥ 60˚C.

The biopolymer WPI could form droplets that were as small as those produced by the small molecule surfactants (lecithin and QN). On the other hand, the biopolymer GA could not form very fine droplets (d> 300 nm), and was therefore unsuitable for fabrication of nanoemulsions. In addition, the amount of WPI required to form stable nanoemulsions was fairly similar to that of the small molecule surfactants, with the emulsifier-to-oil ratio (EOR) required to form small droplets being around 1:10. Conversely, the EOR was much higher for GA than any of the other natural emulsifiers studied, being around 10:10. The reason that the amount of WPI required to produce smaller droplets is lower than that of GA could be attributed three major differences between the protein and polysaccharide emulsifiers: (i) the faster adsorption of the small protein molecules to the droplet surfaces within the homogenizer; (ii) the higher surface activity of the protein; and, (iii) the lower surface load (thinner interfacial layers) of the protein.

There were also appreciable differences between the ability of the four natural emulsifiers to stabilize the emulsion-based delivery systems under different environmental conditions. Despite being the least effective emulsifier at forming small droplets, GA was the most effective emulsifier at stabilizing against environmental stresses. It produced emulsions that were stable to a wide range of pH values (2 – 8), ionic strengths (0 to 500 mM), and temperatures (30 to 90 ºC). Conversely, the WPI was the emulsifier that was most sensitive to changes in environmental conditions, exhibiting instability at intermediate pH values

(pH 5), elevated salt concentrations (> 100 mM), and higher temperatures (> 60 ºC). Whereas lecithin and Q-Naturale-based nanoemulsions were stable at wide range of pH. All the systems except WPI-stabilized ones showed great stability against the entire range of temperatures (30-90 ˚C). The natural small molecule surfactants had relatively good stability to pH and temperature, but exhibited some aggregation at high salt levels, which can be attributed to electrostatic screening.

The instability of the emulsions was related to a variety of intermolecular and surface forces which rely on theoretical basis. For example, WPI nanoemulsions became unstable to heating above 60 ºC in the presence of salt (150 mM NaCl), which can be attributed to the compression of double layer, thereby decreasing of the electrostatic repulsion along with the hydrophobic attractions between the droplets when the adsorbed protein molecules become thermally denatured. GA-stabilized emulsions were not affected by thermal treatment in the absence or presence of salt because of the strong steric repulsion resulted from the overlapping of the highly branched tail regions of between the droplets.

Although GA seemed to be the most effective stabilizing emulsifier, it could not produce nanoemulsions, Quillaja saponin was a very good alternative that was chosen as the most effective emulsifier to form nanoemulsions. Therefore, Q-Naturale (QN) was used for the next study on the digestion and bioaccessability studies of vitamin D3. The results of this part of the thesis should provide useful information about the potential application of natural emulsifiers to encapsulate lipophilic compounds to be incorporated in commercial food and beverage products.

4. NANOEMULSION DELIVERY SYSTEMS FOR OIL-SOLUBLE VITAMINS: INFLUENCE OF CARRIER OIL TYPE ON LIPID DIGESTION AND VITAMIN D3 BIOACCESSIBILITY

4.1. INTRODUCTION

Vitamin D_3 deficiency often occurs in people who are not exposed to sufficient sunlight, and in individuals with metabolic (*e.g.* obesity or hyperparathyroidism) or gastrointestinal (*e.g.* celiac or inflammatory bowel disease) disorders [95, 96]. Malfunction of the regulation of calcium and phosphorus absorption and bone metabolism can lead to osteoporosis and osteomalacia (pore formation and softening of bones) in adults, while rickets may occur in children in developing countries [97]. Vitamin D sources are fairly limited in foods, and include products such as beef liver, dairy products, egg yolk, and fish [4, 98]. Therefore, there is a high demand to enrich food and beverages with vitamin D_3.

Vitamin D_3 is highly sensitive to environmental stresses (such as light, heat, and oxygen) and can easily be oxidized leading to loss of its functionality and physiological benefits [4]. Oil-soluble vitamins are typically absorbed at particular locations in the small intestine along with the digestion products of ingested dietary fats and oils [99]. They may be absorbed by both passive and active transport mechanisms [10, 100]. The oil-soluble vitamins are incorporated into chylomicrons (consisting of bile salts, phospholipids, and lipid digestion products), which are released into the systemic circulation *via* the lymphatic system, and then activated in the liver [101]. Due to its poor water-solubility and low oral bioavailability, vitamin D is often encapsulated within lipid-based delivery systems that can improve its bioaccessibility.

A considerable amount of research has been carried out to identify lipid-based delivery systems to encapsulate, protect, and release lipophilic bioactive agents. Nanoemulsions are particularly good candidates for delivery of lipid-soluble bioactives (such as vitamin D) because they can be produced with natural food ingredients using simple production methods, and can be designed to increase both water-dispersibility and oral bioavailability [4, 52, 102, 103, 104, 105].

Vitamin D_3 is crystalline at ambient temperature and therefore needs to be dissolved within a suitable carrier oil before it can be incorporated into nanoemulsion-based delivery systems. Previous studies have shown that the nanoemulsion composition (*i.e.,* emulsifier and carrier oil type) effects lipid digestion rate and bioactive bioavailability [12, 13, 14, 106, 107, 108, 109, 110]. In particular, these studies have shown that carrier oil type has a major impact on the bioaccessibility of lipophilic bioactives. Consequently, it is important to optimize the nature of the carrier oil used to formulate nanoemulsions in order to ensure good bioavailability of any encapsulated lipophilic bioactive components. Therefore, in this part of the thesis, the influence of carrier oil type on lipid digestion and vitamin bioaccessibility was investigated. Carrier oils were selected that had different susceptibilities to lipase digestion and different molecular characteristics: medium chain triglycerides (MCT), corn oil, fish oil, orange oil, and mineral oil. MCT, corn oil and fish oil are all triglyceride oils that are digestible by lipase. Corn oil and fish oil are both examples of long chain triglycerides (LCT), but corn oil contains a high proportion of monounsaturated fatty acids, whereas fish oil contains a high proportion of polyunsaturated fatty acids. Orange oil and mineral oil are both examples of indigestible oils. This study should have important implications for designing effective nanoemulsion-based delivery systems to increase the bioaccessibility of vitamin D_3.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Quillaja saponin (Q-Naturale®100) was kindly provided by Ingredion Inc. (Westchester, IL). It is actually a mixture of various saponin components dispersed in water with the major fraction being reported to have a molecular weight around 1650 g mol^{-1} (31). Vitamin D_3 and mineral oil were purchased from Sigma-Aldrich (St. Louis, MO). Orange oil (10×) was purchased from International Flavors and Fragrances (Union Beach, NJ). Corn oil (Mazola, ACH Food Companies, Inc., Memphis, TN) was purchased from a local supermarket. The triglycerides in corn oil have been reported to be about 11.2% $C_{16:0}$, 2.2% $C_{18:0}$, 28.9% $C_{18:1}$, 55.5% $C_{18:2}$, 1.1% $C_{18:3}$, and 0.4% $C_{20:1}$ (111). Medium chain triglyceride (MCT) oil (Miglyol 812) was purchased from Coletica (Northport, NY). The

triglycerides in MCT were reported to contain 60% octanoic acid ($C_{8:0}$) and around 40% capric acid ($C_{10:0}$). Fish oil was provided by DSM Nutritional Products Ltd (Basel, Switzerland). Lipase, bile salts, mucin and pepsin were purchased from the Sigma Chemical Company (St. Louis, MO). All other chemicals used were analytical grade. Double distilled water was used to prepare all solutions and emulsions.

4.2.2. Nanoemulsion Preparation

Oil-in-water nanoemulsions were prepared by homogenizing 10% (w/w) oil phase with 90% (w/w) aqueous phase using a well-established two-step procedure [112]. The oil phase consisted of 0.1% w/w vitamin D_3 dissolved within 99.9 % w/w carrier oil (MCT, corn oil, fish oil, mineral oil, or orange oil). The aqueous phase consisted of 2% w/w surfactant (Q-Naturale) dispersed within 98% w/w buffer solution (10 mM sodium phosphate, pH 7.0). Q-Naturale contained 14 wt% active saponins (with the remainder being mainly water), and that percentage was taken into account while reporting the concentration of this surfactant. Coarse emulsions were prepared by blending both oil and aqueous phase together using a high-speed blender (Bamix, Switzerland) for 2 min at room temperature. Fine emulsions were prepared by passing the coarse emulsion through a high pressure homogenizer (Microfluidics M110L, Newton, MA, USA) for 3 cycles at 12,000 psi.

4.2.3. Particle Characterization

The particle size of the vitamin D_3 nanoemulsions was measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Malvern, UK). The particle size of each sample was represented as the surface-weighted mean diameter (d_{32}), which was calculated from the full particle size distribution.

The droplet charge (ζ-potential) of the nanoemulsions was measured using particle microelectrophoresis (Zetasizer Nano ZS-90, Malvern Instruments, Worcestershire, UK). Prior to measurements dilution was carried out using buffer solutions with the same pH as the samples being tested to avoid multiple scattering effects, i.e., they had the same pH as the appropriate gastrointestinal region (initial, mouth, stomach, or small intestine).

4.2.4. *In Vitro* Digestion

The original nanoemulsions, containing 10% (w/w) oil phase (0.01 % vitamin D_3 and 9.99 % carrier oil) and 90% (w/w) aqueous phase (2% Q-Naturale and 98% buffer solution), were diluted five-times in buffer solution so that the samples used in the *in vitro* digestion studies initially contained 2% oil and 0.002% vitamin D3.

Mouth stage: Simulated artificial saliva solution (SASS) was prepared according to a previous study [113, 114]. 20 ml of emulsion (2% w/w oil phase) was placed in a 125 ml flask and then 20 ml of SASS containing 0.6 g mucin was added into the flask. This mixture was adjusted to pH 6.8 and then shaken continuously at the rate of 100 rpm in a temperature controlled incubator (37 ˚C) for 10 min (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA).

Stomach stage: Simulated gastric fluid (SGF) was prepared by dissolving 2 g of NaCl and 7 ml of HCl in water (1 L total volume) and the pH of the solution was adjusted to pH 1.2 using 1M HCl. 20 ml of the sample from the mouth stage was mixed with 20 ml of SGF containing 0.064 g pepsin, and the mixture was adjusted to pH 2.5. The resulting mixture was then shaken for 2 hours at 37 ˚C at 100 rpm.

Intestine stage: 30 ml of digesta sample from the stomach stage was added into a clean beaker and placed into the water bath (37˚C) connected to an automatic titration unit used as a pH-STAT (Metrohm, USA Inc., Riverview, FL, USA). The sample was adjusted to pH 7.00 using NaOH solutions (range from 0.01 to 6 M), then simulated intestinal fluid containing 1.5 ml salt solutions (10 mM $CaCl_2.2H_2O$ and 150 mM NaCl) and 3.5 ml bile salts (5 mg ml^{-1}) were added respectively and pH was adjusted to 7.00 using HCl and NaOH solutions. Afterwards, freshly prepared 2.5 ml lipase solution (1.6 mg ml^{-1}) was added to the sample and the automatic titration unit was started. The volume of 0.1 N NaOH solution required to maintain the pH of the sample at 7.00 was recorded using the software program. A control study was performed using the buffer solution as the sample, and the amount of alkali solution titrated into the reaction chamber for the control was subtracted from that for the test samples. Free fatty acids (FFA) release was calculated according to Li and McClements (2010) [113]. In vitro digestion in the small intestine

stage lasted for 2 h and then physicochemical and structural characterization of the samples at each stage were performed.

4.2.5. Bioaccessibility Determination

The bioaccessibility of lipophilic components is normally defined as the fraction that is solubilized within the mixed micelle phase after lipid digestion [115, 116]. After the full digestion, two portions of 10 ml of samples were collected and centrifuged (4000 rpm, Thermo Scientific, CL10 centrifuge) at 25 ˚C 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 creamed phase at the top or on the wall of the centrifuge tube. An aliquot (1 ml) of micelle phase or raw digesta sample was vortexed after adding an organic solvent mixture (1:3 isooctane: ethyl alcohol) at 1:5 to extract vitamin D_3 and then centrifuged at 1750 rpm for another 10 min. The supernatant phases were used as samples for determination of vitamin D_3 using UV-Vis Spectrophotometer at 265 nm wavelength (Ultrospec 3000 pro Pharmacia Biotech, Biochrom Ltd., Cambridge, UK).

Absorbance's of different concentrations of vitamin D_3 standard were measured at 265 nm wavelength to obtain a calibration curve for vitamin D_3 concentration *versus* absorbance (r^2= 0.9996).

4.2.6. Microstructural Analysis

A Nikon Confocal Fluorescent Microscope (C1 Digital Eclipse, Tokyo, Japan) with a 60× oil immersion objective lens was used to capture images of the emulsions and of samples taken after each stage of digestion. Nile red (a fat soluble fluorescent dye) was excited with a 488 nm argon laser line.

4.2.7. Statistical Analysis

Each experiment was performed at least twice from the beginning, and all results were reported as the calculated average and standard deviation of these measurements using Microsoft Office Excel 2010.

4.3. RESULTS AND DISCUSSION

Previous studies have highlighted the potential impact of carrier oil type on lipid digestion and bioactive bioaccessibility [12, 14, 110]. In particular, it was found that the bioaccessibility of β-carotene was much higher when a long chain triglyceride (corn oil) was used as a carrier oil, rather than a medium chain triglyceride (MCT) or indigestible oil (flavor oil). In this study, it was aimed to examine whether a similar effect was observed for another important type of lipophilic bioactive molecule, *i.e.*, vitamin D_3.

4.3.1. Influence of Carrier Oil Type on Physical Stability of Nanoemulsions

Nanoemulsions were prepared using both digestible (MCT, corn oil, and fish oil) and non-digestible (orange oil and mineral oil) carrier oils. The mean particle diameter (d_{32}) and microstructure of the initial nanoemulsions and of samples collected after each digestion stage were measured (Figures 4.1 and 4.2). With the exception of orange oil, all of the other nanoemulsions had relatively small initial mean particle diameters (0.14 – 0.19 μm) and monomodal particle size distributions. The mean particle diameter of the orange oil nanoemulsions was appreciably higher (0.29 μm) than the other nanoemulsions, which can probably be attributed to Ostwald ripening effects due to the relatively high water-solubility of flavor oils [117].

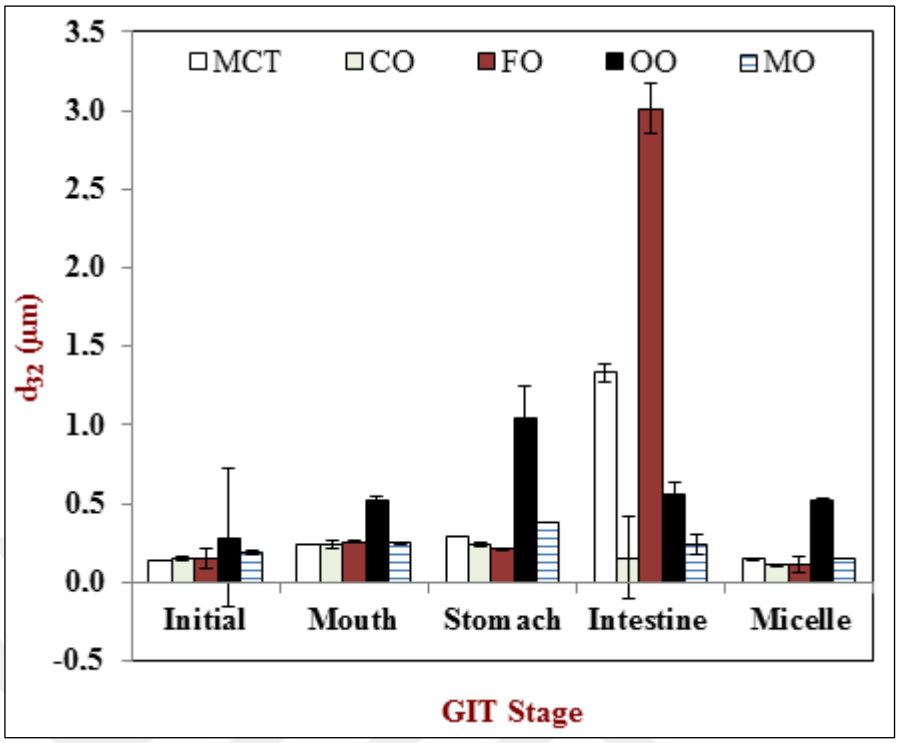


Figure 4.1. Influence of gastrointestinal tract (GIT) stage and oil type on (d_{32}) of oil-in-water emulsions. MCT: medium chain triglycerides, CO: corn oil, FO: fish oil, OO: orange oil, MO: mineral oil.

Again, with the exception of orange oil, there was only a slight increase in the mean particle diameters of the nanoemulsions after incubation in artificial saliva and gastric solutions (Figure 4.1), and the particle size distributions remained monomodal, which suggested that these nanoemulsions were stable against coalescence under simulated mouth (pH 6.8, 10 min) and stomach (pH 2.5, 2 h) conditions. This protection can be attributed to the strong steric stabilizing effect of the natural surfactant (Q-Naturale) used. In our previous study, it was reported that Q-Naturale coated lipid droplets were stable against droplet coalescence over a wide range of pH conditions *i.e.,* pH 3 to 8 [119]. Moreover, there were no highly surface active components present within the simulated saliva or gastric environment that might have caused emulsion instability by adsorbing to the surfaces of the Q-Naturale coated droplets. Finally, Q-Naturale would be expected to be resistant to pepsin digestion within the gastric fluids, which may increase the stability of the emulsions to coalescence in the stomach phase. Having said this, the confocal

microscopy images indicated that appreciable droplet flocculation actually occurred under mouth and stomach conditions (Figure 4.2). Droplet flocculation may have occurred due to electrostatic screening effects (due to mineral ions), loss of droplet charge (under acidic conditions), or bridging or depletion attraction (due to mucin). Presumably, these flocs were relatively weak and broke down when the emulsions were diluted for the light scattering experiments. In contrast, there was a relatively large increase in mean particle diameter of the orange oil emulsions as they passed from the initial to mouth to stomach stages, which may be due to Ostwald ripening or coalescence leading to droplet growth. Indeed, large spherical droplets were observed in this sample in the stomach phase (whereas irregular shaped particles were observed in the other nanoemulsions), which suggests that the individual oil droplets had grown in size rather than become flocculated.

There were appreciable changes in the particle size and microstructure of the nanoemulsion samples after they were incubated in the small intestine phases, which depended on carrier oil type (Figures 4.1 and 4.2). These changes may have occurred for a number of different reasons, such as lipid digestion, droplet aggregation, mixed micelle formation, or generation of insoluble sediments (such as calcium soaps). Lipid digestion might be expected to reduce the size of the individual lipid droplets since some of the oil phase would be removed. However, lipid digestion may also promote droplet aggregation (flocculation or coalescence) due to changes in interfacial and core characteristics. In addition, aggregation may be promoted due to droplet interactions with other components within the small intestinal fluids, such as mineral ions, bile salts, or proteins. The mixed micelles formed from lipid digestion products actually consist of a complex mixture of different types of colloidal particles, such as micelles, vesicles, bilayers, and liquid crystals. Finally, any long chain fatty acids generated during lipid digestion may form insoluble calcium soaps. All of these different types of colloidal particles contribute to the light scattering pattern measured in a particle size analyzer, which makes it difficult to accurately interpret particle size measurements made on intestinal digesta [9, 118]. With the exception of orange oil, the micelle phases all contained relatively small particles (< 200 nm), which can be attributed to the fact that large particles either creamed or sedimented during centrifugation. The micelle phase obtained after the digestion of orange oil-based nanoemulsions contained relatively large particles, which may have been due to some droplet coalescence or Ostwald ripening after centrifugation.

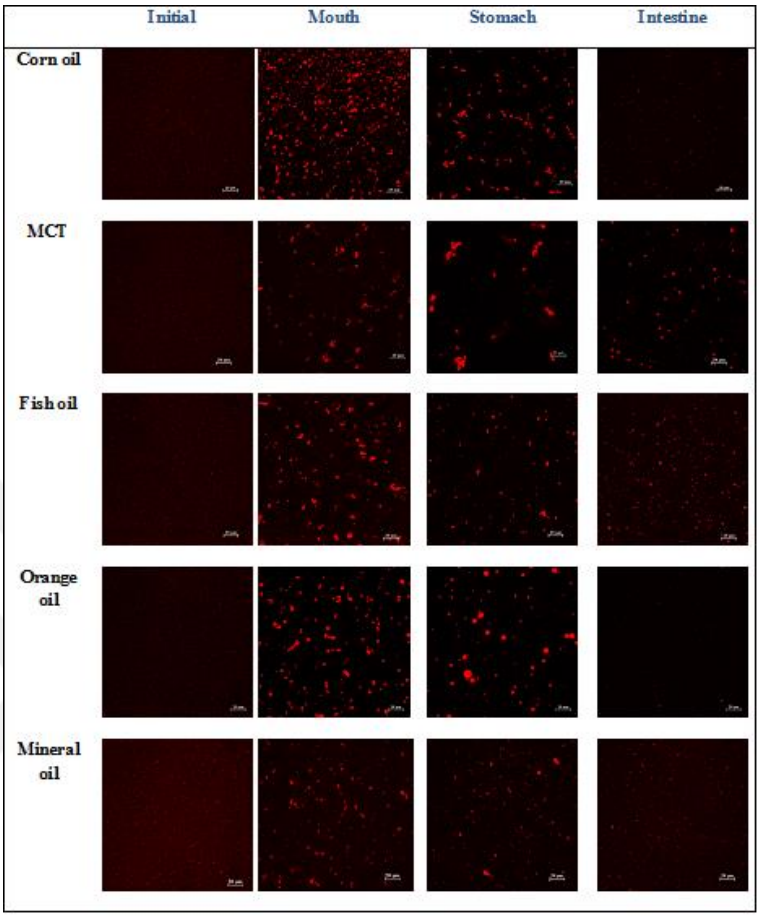


Figure 4.2. Influence of oil type and gastrointestinal tract stage on microstructure (confocal fluorescence) images of the initial and digested Vitamin D3 encapsulated oil-in-water emulsions.

The electrical charge on the particles in the various samples was measured after each stage of the model GIT to provide some information about changes in interfacial characteristics (Figure 4.3). Initially, freshly prepared nanoemulsion droplets were highly negatively charged (between -65 and -70 mV) independent of carrier oil type. This result can be attributed to the fact that the electrical characteristics of the droplets were dominated by the presence of the adsorbed surfactant layer. Previous studies have shown that lipid droplets coated with Q-Naturale have a high negative charged at neutral pH [58, 119]. An appreciable decrease in the magnitude of the negative charge on the droplets was observed

after the mouth stage in all of the samples, which may have been due to electrostatic screening caused by salts in the simulated saliva, or due to adsorption of mucin molecules to the droplet surfaces [120, 121].

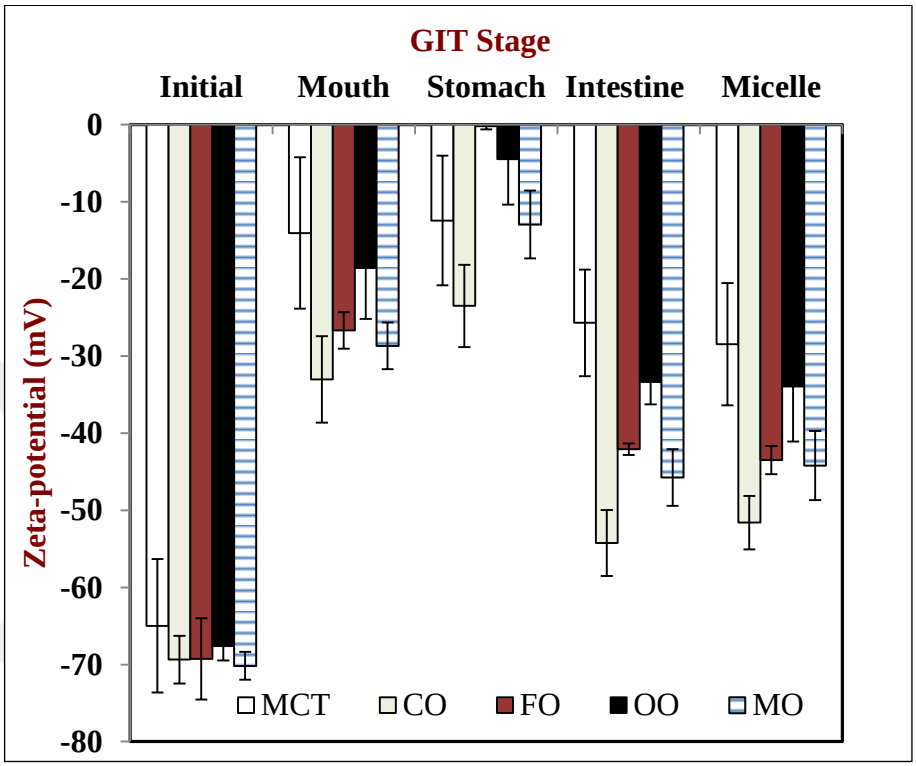


Figure 4.3. Influence of gastrointestinal tract (GIT) stage and oil type on zeta-potential of oil-in-water emulsions. MCT: medium chain triglycerides, CO: corn oil, FO: fish oil, OO: orange oil, MO: mineral oil.

The magnitude of the negative charges decreased further after exposure to simulated gastric conditions, which can be mainly attributed to changes in the electrical characteristics of the Q-Naturale at low pH values. Previous studies have shown that this surfactant loses its negative charge under highly acid conditions due to protonation of the carboxyl groups on the quillaja saponin molecules [58, 119]. There were some differences in the magnitude of the electrical charge on droplets containing different types of carrier oil (Figure 4.3). This may have been due to differences in the susceptibility of the emulsions to competitive adsorption effects.

After exposure to the simulated small intestine stage, the magnitude of the negative charge on all the nanoemulsions increased appreciably. This change in droplet electrical characteristics can be attributed to various factors [12, 110]. First, the increase in pH would cause the surfactant molecules to become more negatively charged. Second, the adsorption of anionic phospholipids and bile salts to the droplet surfaces would lead to a negative charge. Third, the generation of anionic free fatty acids during lipid digestion of triglyceride oils would contribute to the negative surface charge. The particles in the micelle phase were also strongly negatively charged, which can be attributed to the fact that they consisted of particles coated by anionic phospholipids, bile salts, and possibly free fatty acids.

4.3.2. Influence of Carrier Oil Type on *In Vitro* Digestion

The influence of carrier oil type on the rate and extent of lipid digestion of vitamin-loaded nanoemulsions under simulated small intestinal conditions was investigated using the pH-STAT method. The percentage of free fatty acids (FFA) released was calculated according to the following equation [113]:

$$FFA(\%) = 100 \times (V_{NaOH} \times m_{NaOH} \times M_{Lipid}) / (w_{Lipid} \times 2) \quad (4.1)$$

Where V_{NaOH} is the volume of sodium hydroxide required to neutralize the FFAs produced (L), m_{NaOH} is the molarity of sodium hydroxide solution used (in M), M_{Lipid} is the molecular weight of the triacylglycerol oil (in g/mol), and w_{Lipid} is the total mass of triacylglycerol oil initially present in the digestion cell (in g). Blanks were performed using solutions with the same composition as the samples, except that they contained no oil, and these values were subtracted from the values measured on the samples. The lipid digestion profiles are reported as FFA (%) values *versus* digestion time (min).

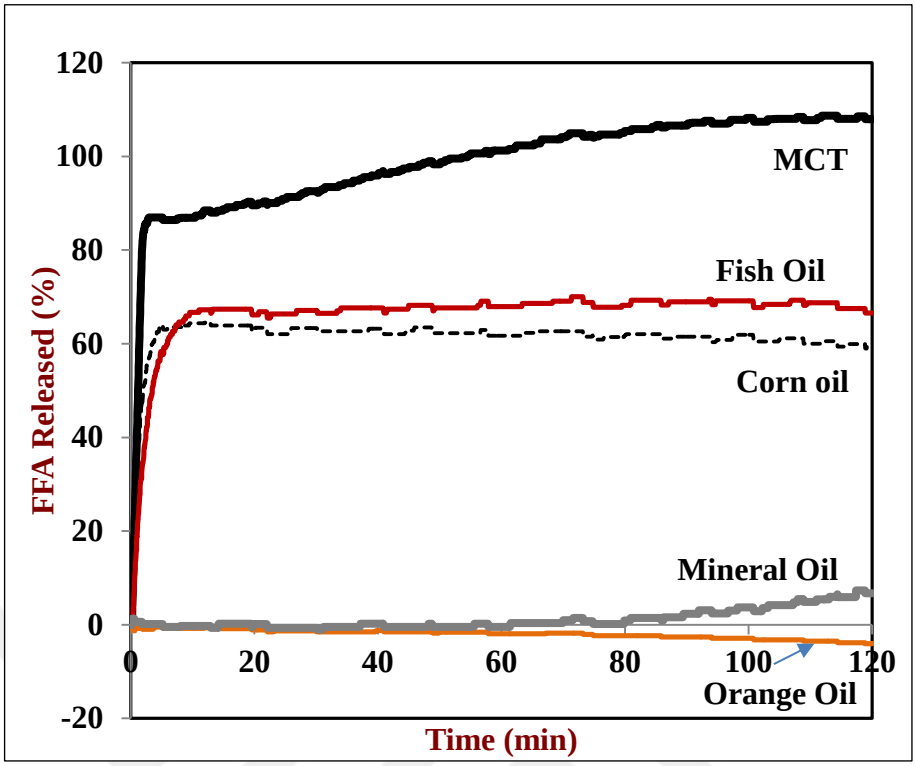


Figure 4.4. Free fatty acid release (digestion rate).

The digestion profiles could be divided into two groups: (i) digestible oils (corn oil, fish oil, MCT); (ii) indigestible oils (mineral oil and orange oil). For all the digestible oils, there was initially a rapid increase in FFAs produced during the first 10 minutes after lipase addition, which suggested that the lipase rapidly adsorbed to the droplet surfaces and converted the triglycerides to free fatty acids and monoacylglycerols. At longer times (10 to 120 minutes), the amount of FFAs increased only slowly or remained relatively constant, suggesting that the lipid digestion process was complete. The final extent of lipid digestion was appreciably higher for the MCT nanoemulsions than the ones containing LCT (corn oil or fish oil). This effect can be attributed to the fact that long chain FFAs released from corn oil or fish oil may have accumulated at the lipid droplet surfaces and therefore inhibited further lipase action [122]. On the other hand, medium chain FFAs rapidly moved into the aqueous phase after formation making it easier for the lipase to continue working [123]. The lipid digestion profiles of fish oil and corn oil nanoemulsions were fairly similar, which is probably because they both contained long chain triglycerides. Orange oil and mineral oil would not be expected to be digested by lipase, which would

account for the fact that there was little change in the calculated FFAs released over time (Figure 4.4).

4.3.3. Influence of Carrier Oil Type on Vitamin D3 Bioaccessibility

In this series of experiments, the influence of carrier oil type on vitamin D_3 bioaccessibility after full digestion by the simulated GIT system was studied. The bioaccessibility of vitamin D_3 was determined by measuring its concentrations in the micelle phase and the total digesta using solvent extraction and UV-Vis spectrophotometry. The bioaccessibility was then calculated using the equation below:

$$\text{Bioaccessibility} = 100 \times (C_{\text{Micelle}} / C_{\text{Raw Digesta}}) \quad (4.2)$$

Where C_{Micelle} and $C_{\text{Raw Digesta}}$ are the concentrations of vitamin D_3 in the micelle and total digesta samples after the small intestine stage (end of pH-STAT experiment). The bioaccessibility was also measured after filtration of the micelle phase (with 0.45μm pore size). Prior to absorption, the mixed micelles and other colloidal particles must pass through the mucus layer that lines the GIT prior to absorption, which has been reported to have a pore size around 0.4 μm [124]. Consequently, filtration step in this study may have removed some of the larger particles that would not be expected to pass through the mucus layer.

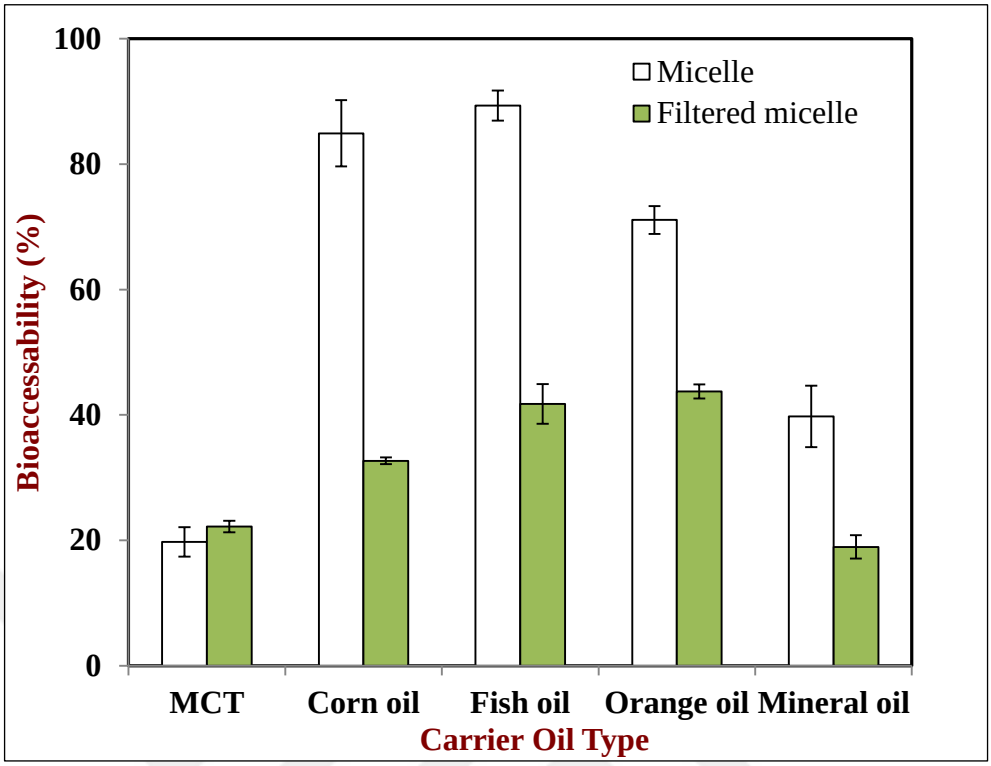
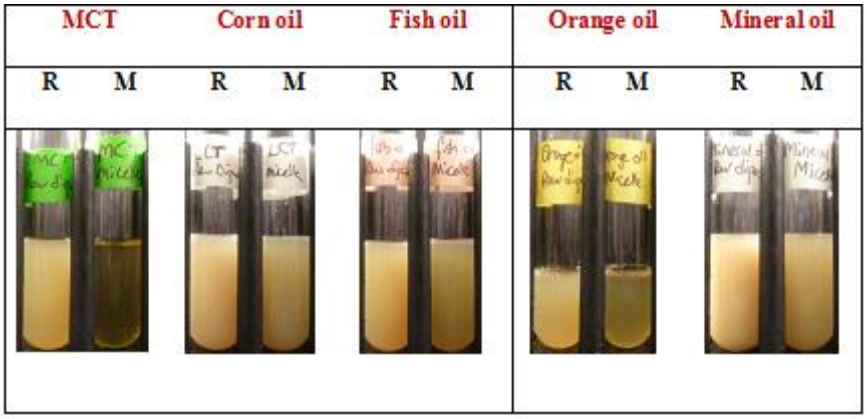


Figure 4.5. Influence of carrier oil type on the bioaccessibility of vitamin D_3.

The bioaccessibility was highly dependent on the type of carrier oil present in the nanoemulsions (Figure 4.5). For the digestible oils, the bioaccessibility of vitamin D_3 was appreciably higher from the nanoemulsions containing LCTs (corn oil and fish oil) than from those containing MCTs, orange oil and mineral oil, with the magnitude of the effect depending on filtration. Before filtration there were much larger differences in bioaccessibility than after filtration. It is possible that filtration removed relatively large vitamin-loaded vesicles or undigested lipid droplets from the LCT nanoemulsions. Indeed, the micelle phase collected from these nanoemulsions was relatively turbid prior to filtration, suggesting that it did contain some large particles (but not large and/or dense enough to be removed by centrifugation) (Table 4.1). Conversely, there was little change in vitamin bioaccessibility when the micelle phase collected from the MCT nanoemulsions was filtered. The reason for this effect is that the micelle phase in these systems was transparent, which suggested that it did not contain any large particles that would be removed by filtration.

Table 4.1. Digital photographs of raw digesta (R) and the micelle phase (M), which was collected after centrifugation (4000 rpm, 40 min, 25˚ C).



The higher bioaccessibility of vitamin D_3 in the LCT nanoemulsions can be attributed to the solubilization capacity of the micelles formed after digestion. Corn oil and fish oil contain long chain fatty acids (*e.g.* C_{16} to C_{18}) whereas MCT contains medium chain fatty acids (*e.g.* C_8 to C_{10}). Long chain fatty acids form mixed micelles (micelles and vesicles) that have larger non-polar regimes capable of accommodating large lipophilic bioactive molecules [110]. Conversely, large lipophilic bioactives cannot easily be accommodated into the smaller non-polar regimes found in mixed micelles formed by medium chain fatty acids. Consequently, the mixed micelles formed from long chain FFAs tend to have higher solubilization capacities than those formed by medium chain FFAs.

Indigestible oils (such as mineral oil and orange oil) do not form free fatty acids in the presence of lipase. As mentioned earlier, free fatty acids can combine with bile salts and phospholipids to form mixed micelles that can solubilize hydrophobic molecules. Consequently, one would expect the solubilization capacity of the intestinal fluids formed from nanoemulsions containing indigestible oils to be relatively low. Surprisingly, it was found that the bioaccessibility of the vitamin D_3 was relatively high in the micelle phase collected from the orange oil nanoemulsion (Figure 4.5). Indeed, the bioaccessibility appeared to be higher for both orange oil and mineral oil than for MCT, despite the fact that MCT was fully digestible. This effect may be an artefact of the method used to measure vitamin bioaccessibility. There may have been relatively small non-digested lipid

droplets containing vitamin D_3 in the micelle phases collected from the orange oil and mineral oil nanoemulsions. These droplets may have been so small that they were not removed by centrifugation or filtration. This result raises an interesting question: would these lipid droplets pass through the mucus layer and be adsorbed by the epithelium cells in the human body? Further studies are clearly needed to establish the influence of lipid digestibility on the biological fate of oil-soluble vitamins encapsulated in nanoemulsions.

4.4. CONCLUSION

In this study, the influence of the type of carrier oil used to formulate nanoemulsion-based delivery systems on the bioaccessibility of an important oil-soluble bioactive (vitamin D_3) was examined. As observed with other highly lipophilic bioactive agents, in this study the nature of the carrier oil had a major influence on the bioaccessibility of vitamin D_3 measured using a simulated gastrointestinal model.

The rate and extent of lipid digestion was higher for MCT nanoemulsions than LCT nanoemulsions (corn oil and fish oil), which was attributed to accumulation of long chain FFAs at the lipid droplet surfaces inhibiting lipase activity. As expected, indigestible lipids (orange oil and mineral oil) did not produce FFAs when exposed to lipase. Vitamin bioaccessibility was higher in LCT nanoemulsions than in MCT nanoemulsions, presumably due to the higher solubilization capacity for vitamin D_3 of mixed micelles formed by long chain FFAs. Surprisingly, a relatively high bioaccessibility was found for the nanoemulsions prepared from indigestible oils, which may have been an experimental artefact associated with the presence of vitamin-containing small lipid droplets in the micelle phase. Alternatively, it may be possible for these small lipid droplets to be adsorbed by the human body, and therefore increase vitamin bioavailability. In summary, LCT nanoemulsions were found to be the most suitable for increasing the bioaccessibility of vitamin D_3. These results are important for formulating nanoemulsion-based delivery systems for oil-soluble vitamins and other lipophilic nutraceuticals.

5. CONCLUSION

In this thesis, small molecule natural surfactants (lecithin and Quillaja saponin) and two natural biopolymers (WPI and GA) were compared in terms of forming nanoemulsion-based delivery systems for liposoluble vitamins and their stabilizing abilities under various environmental conditions. Afterwards, Quillaja saponin, being the most effective emulsifier to produce nanoemulsions was used to examine the influence of the type of carrier oil used to formulate nanoemulsion-based delivery systems on the bioaccessibility of an important oil-soluble bioactive (vitamin D_3).

The biopolymer WPI could form droplets that were as small as those produced by the small molecule surfactants (lecithin and QN). On the other hand, the biopolymer GA could not form very fine droplets (d> 300 nm), and was therefore unsuitable for fabrication of nanoemulsions. In addition, the amount of WPI required to form stable nanoemulsions was fairly similar to that of the small molecule surfactants, with the emulsifier-to-oil ratio (EOR) required to form small droplets being around 1:10. Conversely, the EOR was much higher for GA than any of the other natural emulsifiers studied, being around 10:10. GA was effective in loading more vitamin E acetate (i.e. 80% in oil phase) than the others, but produced larger droplets. However, relatively higher amount of vitamin E acetate (i.e. 50-60% in oil phase) could be loaded into the oil phase of the nanoemulsions stabilized by WPI, QN and lecithin.

There were also appreciable differences between the ability of the four natural emulsifiers to stabilize the emulsion-based delivery systems under different environmental conditions. Despite being the least effective emulsifier at forming small droplets, GA was the most effective emulsifier at stabilizing against environmental stresses. It produced emulsions that were stable to a wide range of pH values (2 – 8), ionic strengths (0 to 500 mM), and temperatures (30 to 90 ºC). Conversely, the WPI was the emulsifier that was most sensitive to changes in environmental conditions, exhibiting instability at intermediate pH values (pH 5), elevated salt concentrations (> 100 mM), and higher temperatures (> 60 ºC). Whereas lecithin and Q-Naturale-based nanoemulsions were stable at wide range of pH. All the systems except WPI-stabilized ones showed great stability against the entire range of temperatures (30-90 ˚C). The natural small molecule surfactants had relatively good

stability to pH and temperature, but exhibited some aggregation at high salt levels, which can be attributed to electrostatic screening. Although gum arabic seemed to be the most effective stabilizing emulsifier, it could not produce nanoemulsions. Quillaja saponin was a very good alternative that was chosen as the most effective emulsifier to form nanoemulsions which are stable over a wide range of pH, salt and temperature.

It was concluded that the nature of the carrier oil had a major influence on the bioaccessibility of vitamin D3 measured using a simulated gastrointestinal model. In vitro studies showed that the rate and extent of lipid digestion was higher for MCT nanoemulsions than LCT nanoemulsions (corn oil and fish oil), which was attributed to accumulation of long chain FFAs at the lipid droplet surfaces inhibiting lipase activity. As expected, indigestible lipids (orange oil and mineral oil) did not produce FFAs when exposed to lipase. As a conclusion, LCT nanoemulsions were found to be the most suitable for increasing the bioaccessibility of vitamin D_3, presumably due to the higher solubilization capacity for vitamin D3 of mixed micelles formed by long chain FFAs.

The results of the thesis are important for formulating nanoemulsion-based delivery systems for oil-soluble vitamins and other lipophilic nutraceuticals, since they provide useful information about the potential applications of natural emulsifiers to encapsulate lipophilic compounds to be incorporated in commercial food and beverage products in a stabilized form.

6. FUTURE WORK

In the future studies, it is aimed to investigate the storage stability of the nanoemulsions and emulsions stabilized by these four natural emulsifiers at different temperatures (5, 25, 37, 50 °C). Characterization of the emulsion droplets will be performed periodically (i.e. once or twice a month) for one year. Therefore, the stabilizing effects of these emulsifiers will be determined for long term stability of the emulsion products.

Powdered forms of these nanoemulsions are aimed to be produced using drying processes (i.e. spray drying, fluidized bed drying) at low relative humidity conditions. The influence of the drying process parameters on the chemical stability of the bioactive compounds and microstructure of the emulsions will be investigated. The emulsions enriched with liposoluble vitamins can be incorporated into the beverages at different pHs and powdered food products either in liquid or powder forms, and therefore the influence of addition of emulsion on the rheological properties and sensory characteristics of the food products will be investigated. Moreover, physical and chemical stability assessment is aimed to be done using particle characterization and effective bioactive determination methods.

The *in vivo* bioavailability studies will be performed using Caco2-cell culture experiments to evaluate the vitamin uptake by the human intestinal cells. Animal studies may also be utilized to simulate the uptake by the human intestinal system. Release kinetics of the bioactive liposoluble vitamins either *in vitro* and *in vivo* will be performed using mathematical drug release models and the results of *in vivo* and *in vitro* studies will be compared.

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APPENDIX A: PARTICLE SIZE DISTRIBUTION GRAPHS

Figure A.1 to Figure A.4 illustrate particle size distributions of the nanoemulsions produced using Q-naturale, lecithin, whey protein isolate and gum arabic, respectively.

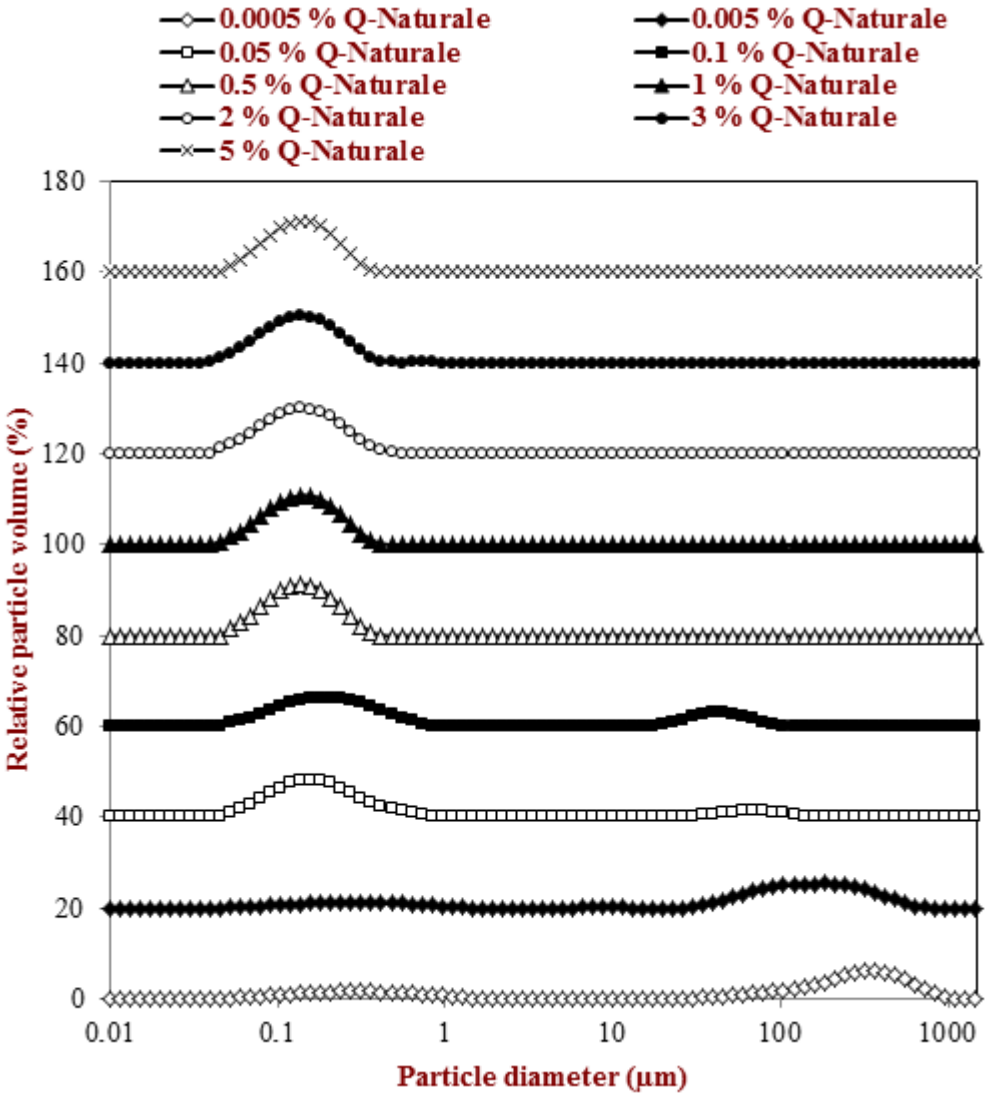

Figure A.1. Particle size distribution graph of nanoemulsions produced using different concentrations of Q-Naturale.

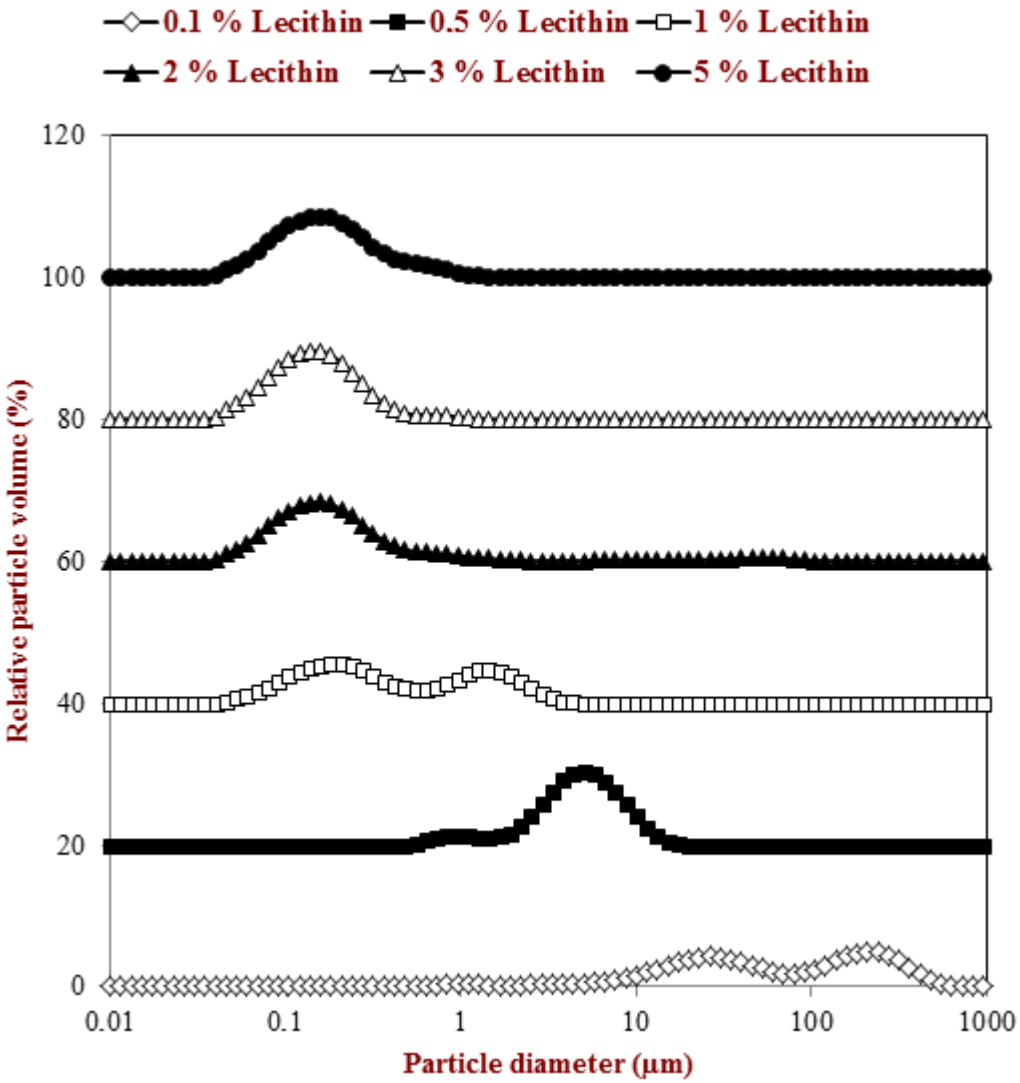

Figure A.2. Particle size distribution graph of nanoemulsions produced using different concentrations of Lecithin.

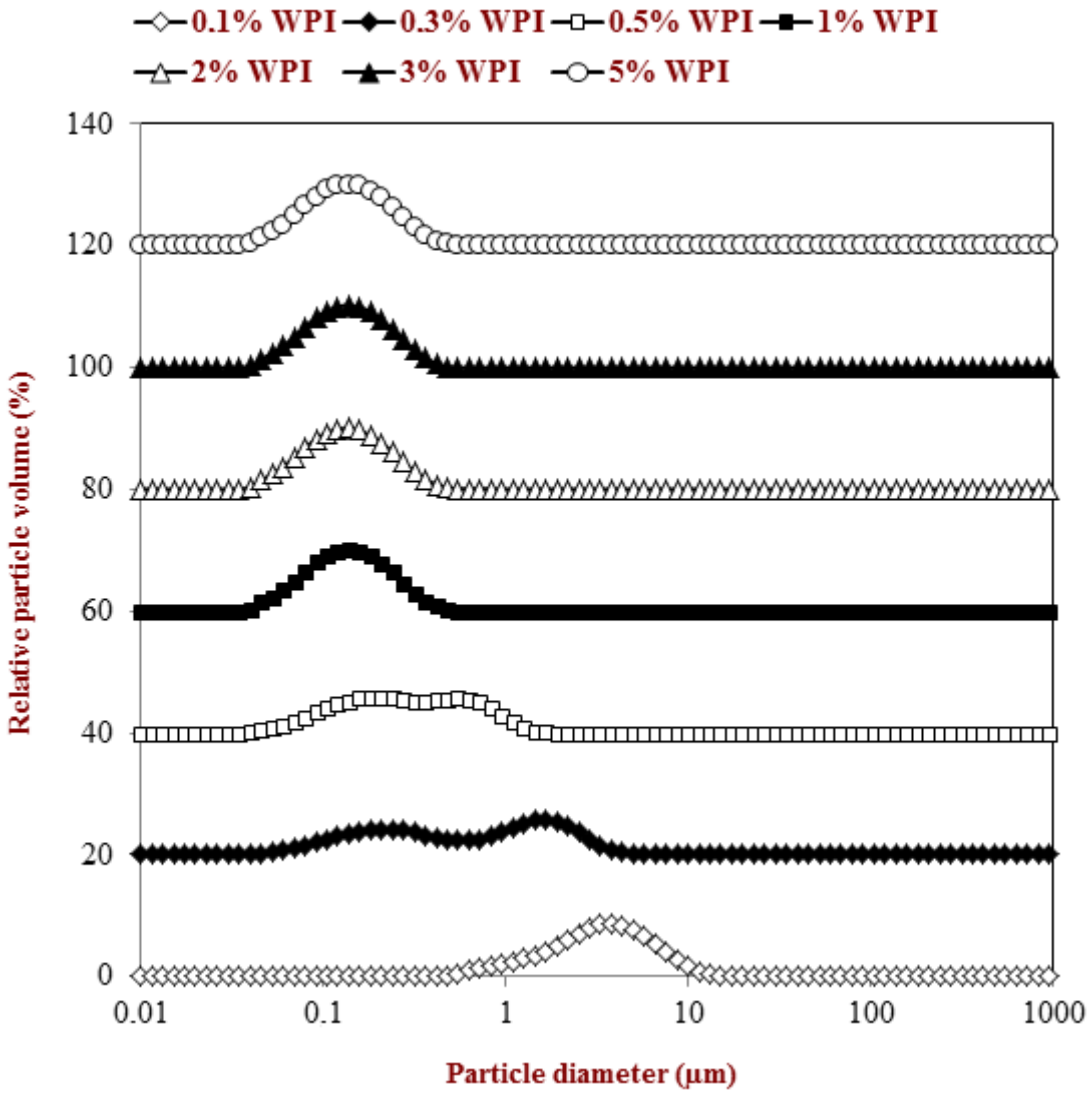

Figure A.3. Particle size distribution graph of nanoemulsions produced using different concentrations of whey protein isolate (WPI).

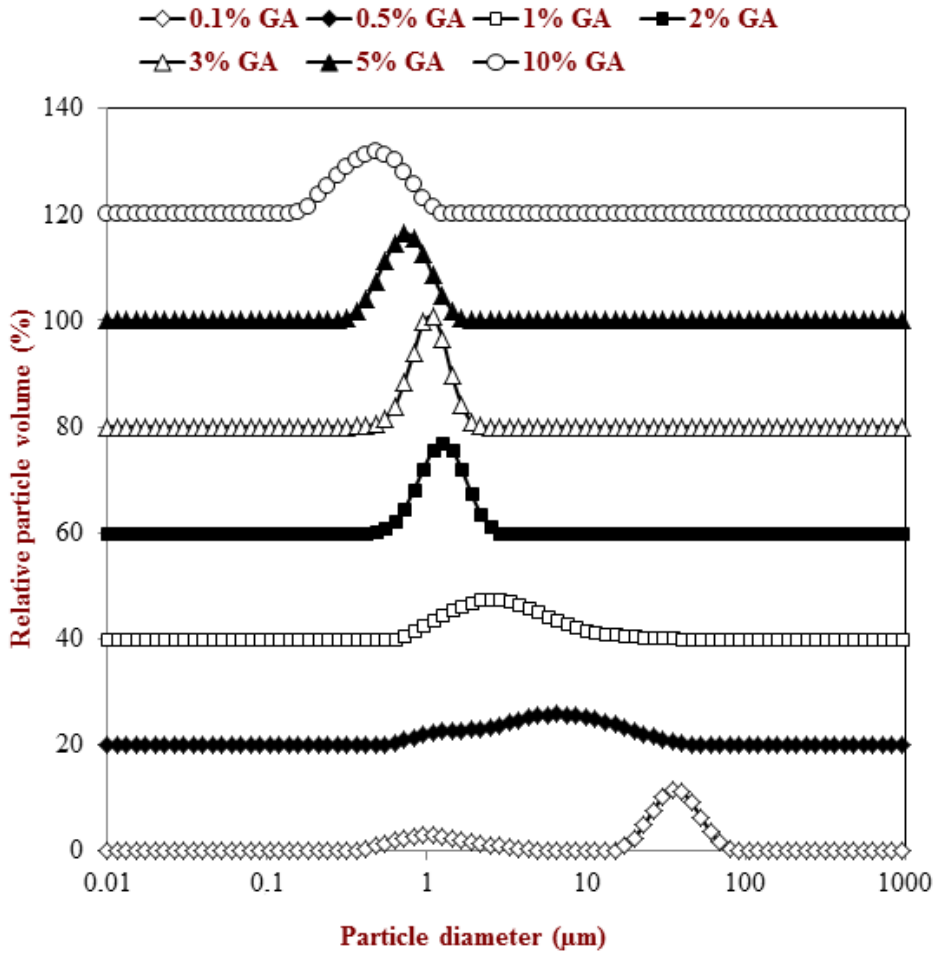

Figure A.4. Particle size distribution graph of nanoemulsions produced using different concentrations of gum arabic (GA).

APPENDIX B: PARTICLE SIZE DISTRIBUTION GRAPHS

Figure B.1 to Figure B.4 illustrate particle size distributions of the nanoemulsions produced using different concentrations of Vitamin E acetate in the oil phase. Number of the figures refer to lecithin, Q-naturale, whey protein isolate and gum arabic, respectively.

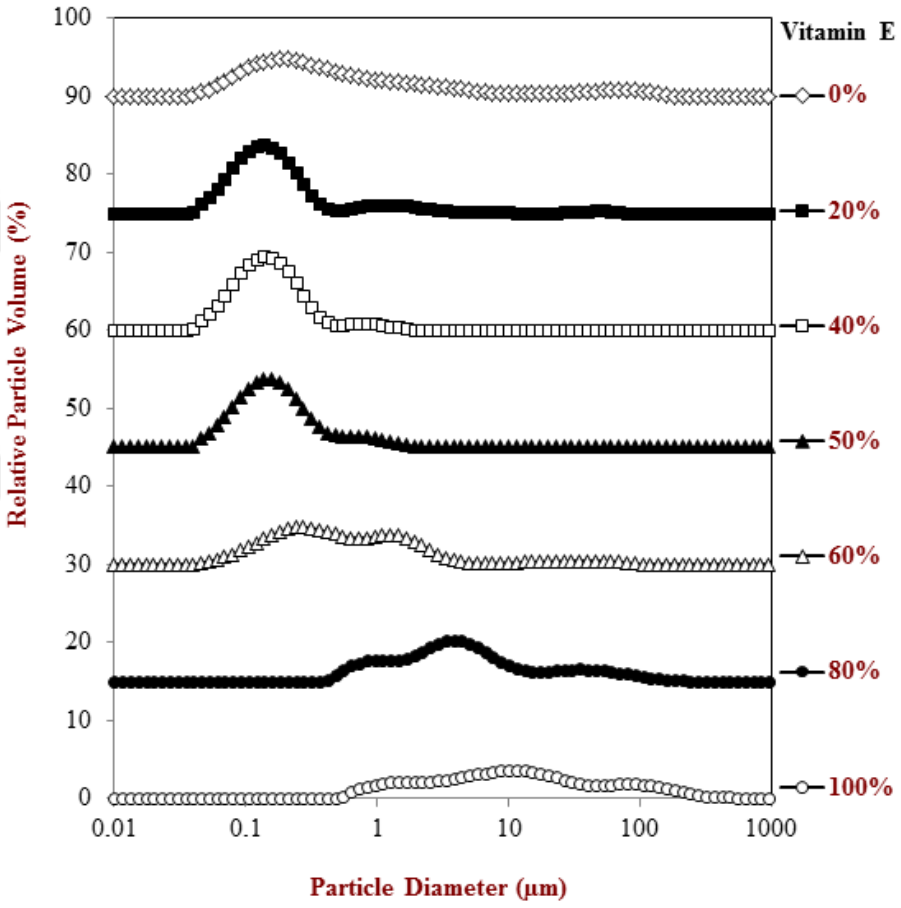

Figure B.1. Particle size distribution graph of nanoemulsions produced using different concentrations VE in oil phase stabilized by 2 wt% lecithin in the nanoemulsion.

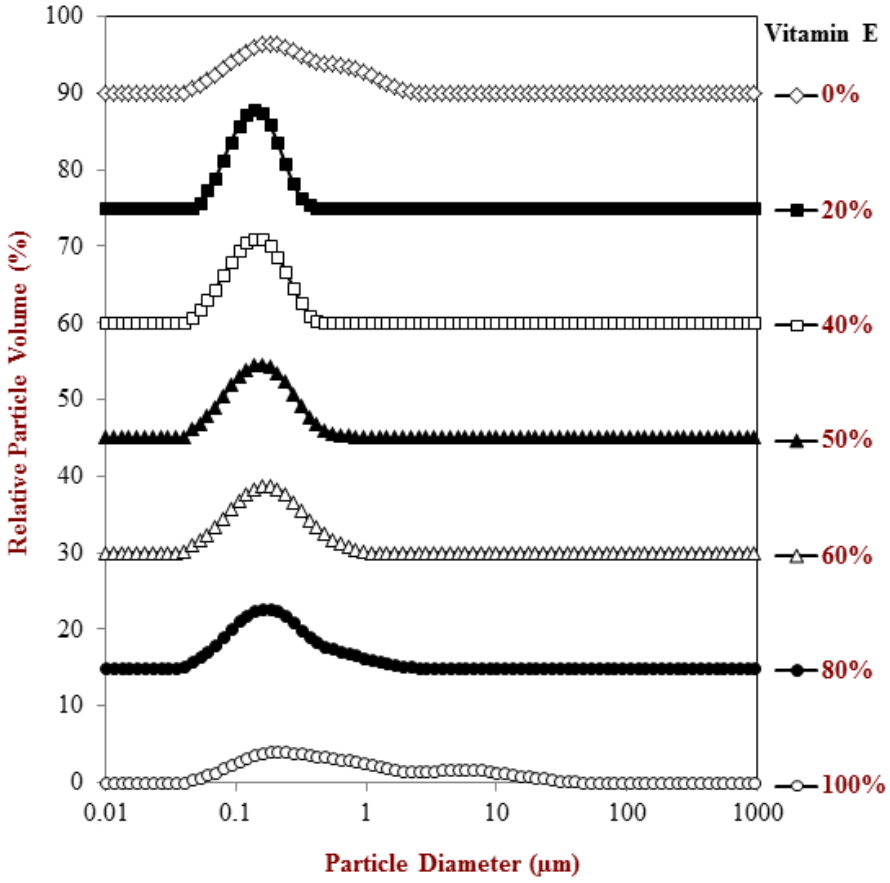

Figure B.2. Particle size distribution graph of nanoemulsions produced using different concentrations VE in oil phase stabilized by 2 wt% Q-Naturale in the nanoemulsion.

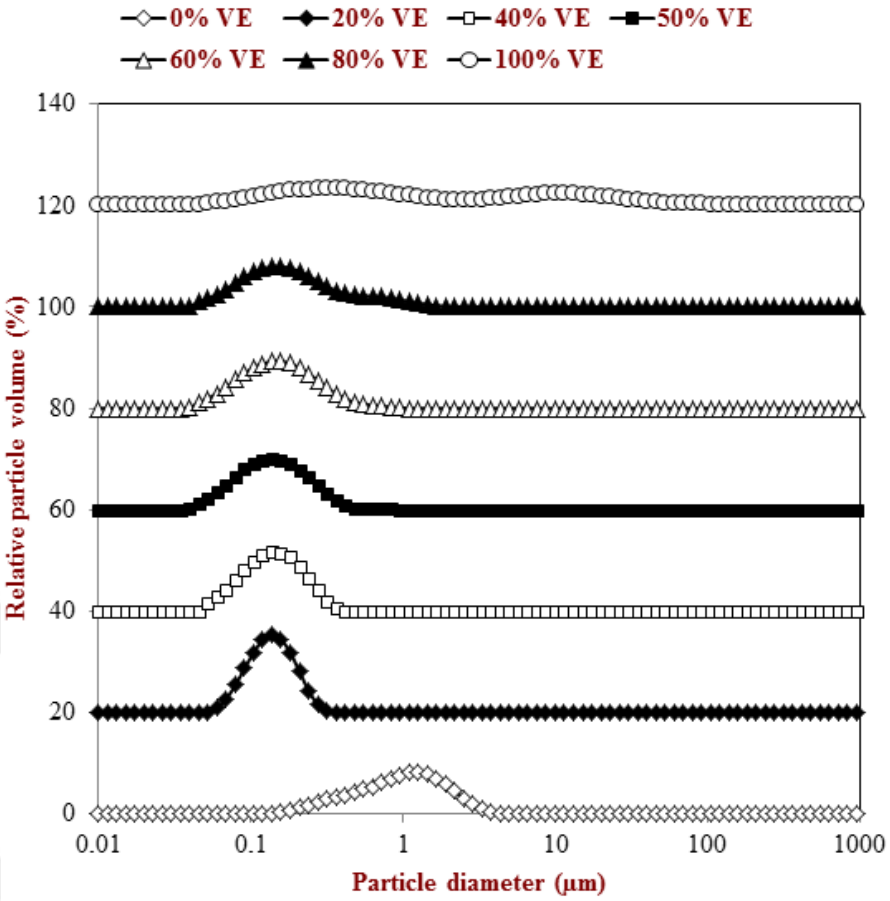

Figure B.3. Particle size distribution graph of nanoemulsions produced using different concentrations VE in oil phase stabilized by 2 wt% WPI in the nanoemulsion.

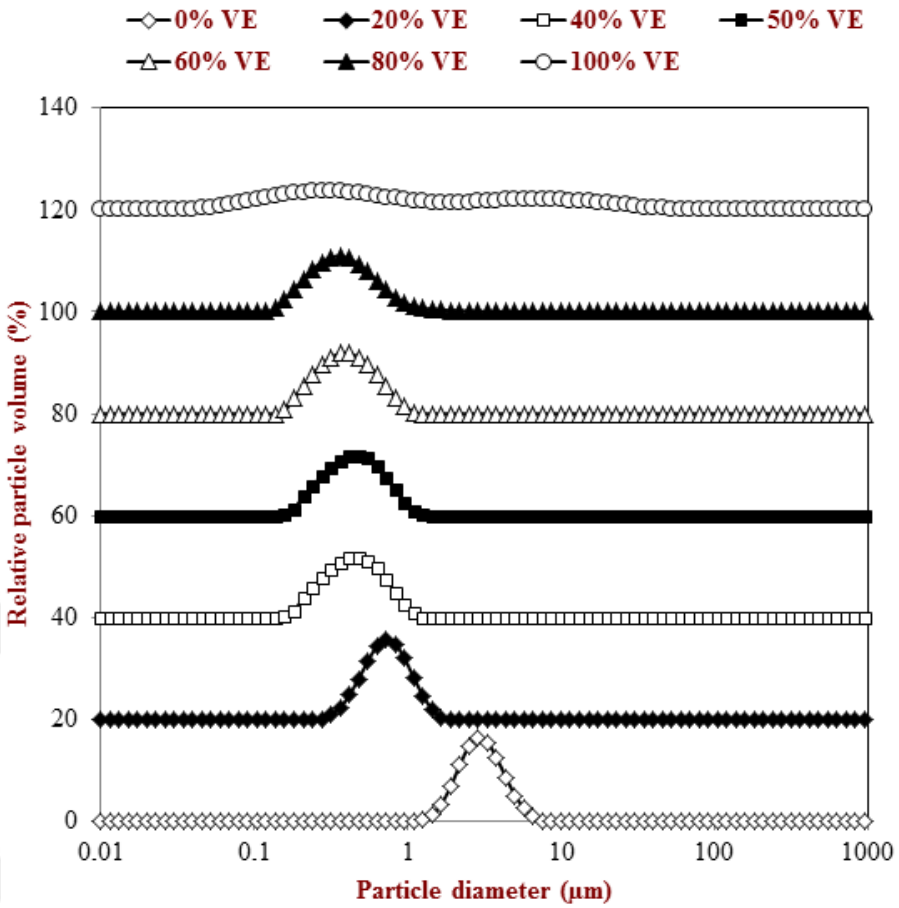

Figure B.4. Particle size distribution graph of nanoemulsions produced using different concentrations VE in oil phase stabilized by 10 wt% gum arabic in the emulsion.