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Synergistic Interfacial Properties of Casein and Small Molecule Surfactants for Fabrication of Essential Oil Nanoemulsions

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To the Graduate Council:

I am submitting herewith a thesis written by Dan Su entitled "Synergistic Interfacial Properties of Casein and Small Molecule Surfactants for Fabrication of Essential Oil Nanoemulsions." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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Synergistic Interfacial Properties of Casein and Small Molecule Surfactants for Fabrication of Essential Oil Nanoemulsions

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ABSTRACT

Nanoemulsions of essential oils are important for delivery of flavors and antimicrobial preservatives in food systems. The overall goal of this work was to study the formation and properties of essential oil nanoemulsions fabricated with sodium caseinate (NaCas) or its hydrolysates and small molecule surfactants (SMSs). The first group of lemon oil nanoemulsion was prepared with NaCas and Tween 20 using a phase inversion temperature (PIT) method. The combination of NaCas and Tween 20 reduced the turbidity and droplet dimension of emulsions than using them individually. Heating at 90 °C for >1 h resulted in transparent nanoemulsions for samples with 1.5% lemon oil. Negative and positive effects on nanoemulsion formation were observed with 0.2-0.4 and 0.6-0.8mM NaCl, respectively. The nanoemulsion prepared with 2% NaCas, 0.4-1.2% Tween 20 and 1.5% lemon oil had a volume-area mean diameter of around 100 nm that was optically stable during 15-day storage at room temperature, while creaming occurred after longer time storage.

Protein hydrolysates have a higher diffusion rate and flexibility than proteins. In order to improve the emulsification efficiency and emulsion stability, the second group of thymol nanoemulsions fabricated with combinations of casein hydrolysates (CH) and sucrose stearate (SS) was studied. For NaCas hydrolyzed by pancreatin for different durations, the product hydrolyzed for 10 min resulted in the most transparent and stable emulsions due to limited reduction of casein molecular weight. Thermal treatment further improved the emulsion stability because of the improved solubility of SS and the strengthened interactions between CH and SS during heating. Addition of 0.25-1% SS improved the emulsification capacity of 2% CH and the emulsion clarity, while excess (2%) SS significantly increased turbidity and particle dimension of emulsions. With the increased thymol concentration (0-3%), stability of emulsions improved. Emulsions prepared with 0.25% SS and 1.5-3% thymol, or 1% SS and 3% thymol, had stable droplet dimensions during ambient storage for over two months. The combination of SS and CH also enabled stable emulsions at pH 5. Therefore, combinations of SMSs with NaCas and its hydrolysate can be used as novel approaches to prepare essential oil nanoemulsions for various applications.

Keywords: sodium caseinate, casein hydrolysates, small molecule surfactant, nanoemulsion, essential oils

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CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

This thesis is about nanoemulsion fabrication using casein and small molecule surfactants (SMSs). In Chapter 2, nanoemulsion prepared with sodium caseinate and polyoxyethylene (20) sorbitan monolaurate (Tween 20) using PIT method was studied. In Chapter 3, nanoemulsion fabrication with casein hydrolysates (CHs) and sucrose stearate (SS) was studied. Chapter 1 gives review of nanoemulsion preparation methods, properties of essential oils, emulsification properties of different emulsifiers as well as interactions between SMSs and proteins.

With improved life quality and increased attention to health, functional ingredients in food are increasingly needed in the food industry.[1] Adding bioactive ingredients to manufactured functional foods is usually aimed to improve flavor,[2] extend shelf-life,[3] and increase health benefits.[4] However, many bioactive compounds are lipophilic, which leads to poor solubility in water and thus limits bioavailability.[5] Moreover, many of these compounds are extracted from plants or animal tissues, but easy to be destroyed by environment, such as light, heat, oxygen.[6]

Essential oils are functional oils extracted from plant materials, such as, flowers, seeds, leaves, bark, fruits and roots.[7] In nature, they play a very important role in protection of plants from diseases caused by bacteria, fungi and virus.[8] And they can attract some insects to favor the distribution of pollens and seeds. [8] Taking advantages of their antimicrobial activities,[9] antioxidative activities,[10] and attractive flavors,[11] essential oils have been widely used as food preservatives and flavourings. However, essential oils are sensitive and vulnerable to heat, light and oxygen, resulting in unpleasant flavor, unfavorable color and lose of functionality.[12]

Delivery systems have been used in the pharmaceutical science for a long time to improve drug efficiency and protect drug from destruction before getting to the targeting site,[5] which are also good options to protect and deliver essential oils.[13] In food science study, surface-active biopolymers and emulsion-based delivery systems are commonly used.[14,15] Emulsions with a mean droplet radius smaller than <100 nm are nanoemulsions.[16] Compared with conventional emulsions, nanoemulsions are more stable because of relatively small mean droplet size.[5] Also, because of the droplets being significantly smaller than wavelength of visible light, nanoemulsions are optically transparent or translucent [17], which can be used to incorporate lipophilic ingredients in transparent products.[18]

In this work, essential oil nanoemulsions are fabricated with proteins and small molecule surfactants (SMSs) were studied. This chapter reviews of methods of nanoemulsion preparation, stability of nanoemulsions and interactions between proteins and SMSs.

1.2. Preparation of nanoemulsions

There are two common approaches for nanoemulsion preparation, namely "top-down" and "bottom-up" methods (Figure 1.1). The "top-down" method is to use mechanical forces to break oil particles into nanometer size range.[19] In comparison, "bottom-up" method uses thermodynamic properties to self-assemble molecules to produce emulsions with ultrafine particle size droplets.[20]

In the food industry, "top-down" methods are usually used in unit operation with high energy,[18] such as high-pressure homogenization,[21] microfluidization,[22] and sonication,[23] and microporous membrane.[24] Low-energy method such as phase inversion temperature (PIT),[6] phase inversion composition (PIC),[25] and anti-solvent precipitation method (ASP) methods,[26] work more effectively than high-energy method in making ultrafine droplets.[18]

1.2.1. High-energy emulsification methods

Intense energy levels are needed for the disruptive forces to exceed the restorative forces holding the droplets into spherical shapes.[27] The reduction of droplet size in high-energy method is based on the homogenizer type, the temperature conditions, homogenization duration and energy intensity and also the composition and physicochemical properties of component phases.[28,29]

High-pressure valve homogenizers are the most commonly used devices in food industry to create fine emulsions.[30] Coarse emulsions are produced by a high shear mixer before being fed into a high-pressure valve homogenizer. Intense disruptive forces are applied on the coarse emulsion droplets when they are passed through the valve. These devices can be used to produce nanoemulsions when they are able to generate intense disruptive forces and the disperse-to-continuous phase viscosity ratio falls between 0.05 and 5.[31]

Microfluidizers are similar to high-pressure valve homogenizers in design. The coarse emulsion is entered into a channel, by high pressure and be splits into two streams that impinge on each other at a high speed in an interaction chamber,[29] producing fine emulsion droplets. The

homogenization pressure and number of passes are the major factors that influence mean droplet size in addition of emulsifier used.[21] SMSs, such as SDS, Tweens, and Spans are more effective at making small droplets under similar homogenization conditions than surface active biopolymers, as they can adsorb on the interface more quickly than biopolymers.[32] For SMSs, the larger disruptive forces used, the finer emulsions can be obtained. Usually the limitation for reducing droplet size emulsions is the operating pressure rather than the amount of emulsifiers used.[21] Conversely, particle size may increase with increase of pressure for emulsions formed by biopolymers.[33]

In fabricating nanoemulsions, the energy consumption in the sonication method was considerably lower than the two devices discussed above.[34] Ultrasound waves have a wavelength of 20-100 kHz. When ultrasound travels through a medium, it brings physical and chemical changes.[35] The ultrasonic emulsification process is described as two-phase phenomena.[36] Firstly, the dispersed phase is incorporated into an aqueous phase by ultrasonic waves as a coarse emulsion. Secondly, larger droplets are disrupted into smaller ones by cavitation.[37] Cavitation is the formation and collapse of microscopic cavities as a result of ultrasound propagation in a medium.[38] The propagation of ultrasound depends on the viscosity and conductivity of the medium and the ultrasonic energy is consumed and transferred to thermo energy. Besides the continuous phase, dispersed phase and emulsifiers used, to obtain relatively small droplets, parameters that affecting cavitation, such as ultrasonic frequency and ultrasonic power are critical influential.[22]

Membrane emulsification is another method using mechanical processes to fabricate nanoemulsions. In this method, the dispersed phase is pressed to pass through a porous membrane while the continuous phase flows along the membrane surface on the other side.[24] The method is able to control droplet size and distribution, however the emulsifying rate relatively low and the application is limited to fluid with low viscosity.[39]

1.2.2. Low-energy emulsification methods

Different from high-energy methods, low-energy methods do not require intense mechanical energy. They are used to prepare nanoemulsions utilizing changes of interfacial film curvatures as a function of composition and environmental conditions.[40] They have lower

equipment and energy costs. However, relatively high surfactant-to-oil ratios are needed and limited types of surfactants can be used.[18]

1.2.2.1. Phase inversion temperature method (PIT)

PIT method relies on changes in the optimum curvature or solubility of surfactant during temperature change.[41] Figure 1.2 is the schematic description of how SMSs, such as Tweens and Spans perform during heating and cooling. The driving force is the temperature dependent physicochemical properties of surfactants and can be described using a packing parameter (p)[42]:

$$p = v/la_0 \quad (1.1)$$

where v and l represent the volume and length of the hydrophobic tail, while a_0 is the cutting-across area of hydrophilic head. Due to amphiphilic properties of surfactants, they tend to associate with each other on the interface and form a monolayer. To have efficient packing of the molecules, the optimum curvature of the monolayer has the lowest free energy and depends on the p of surfactants. At $p<1$, the hydrophilic head is bigger, and it is more likely to form oil/water emulsions; conversely, at $p>1$, because of the dehydration of head groups, hydrophobic tail is bigger than the hydrophilic head, and water/oil emulsions are more likely formed; and when p equals 1, the curvature of monolayer is zero, so a bicontinuous system forms.[42] The dehydration of the head groups increases with increasing temperature, so the affinity to the non-polar phase also increases.[43] With the increase of hydrophobicity of surfactant, emulsion system turns from O/W emulsion to bicontinuous system and finally goes to W/O emulsion.[43] When the system is rapidly cooled from a temperature higher than PIT, surfactants quickly moved from the oil phase to the water phase, forming smaller droplets.

Nonionic surfactants were widely investigated to make nanoemulsions using PIT method, such as, water/tetraethylene glycol monododecyl ether ($C_{12}E_4$)/isohexadecane system,[90] milk fat/Polyoxyethylene (20) sorbitan monooleate (Tween 80)/water system.[6] However, nature surface-active biopolymers alone were not able to form nanoemulsion using PIT method, [44] since temperature cannot change the curvature interface film formed by ionic surfactants and emulsifiers with large molecule dimension.[25]

1.2.2.2. Phase inversion concentration method (PIC)

Nonionic surfactants, ionic surfactants and zwitterionic surfactants all can be used in PIC method based on changing optimum curvature of the interface film by altering the composition of the system.[45] For example, adjustment of ionic strength[25] and pH values[46] can be used in emulsions with ionic surfactant. When stabilized by ionic surfactant, an O/W emulsion can be inverted to a W/O emulsion by adding salt, as salt ions were able to screen the electrical charges on surfactant head group.[25] It is also reported that when alkaline is added during emulsification process, fatty acids in the system can be neutralized, resulting in fatty carboxylate acts as ionic surfactant stabilizing the emulsion system.[46]

Moreover, droplet volume fraction and surfactant-to-oil ratio can be used as additional parameter to facilitate phase inversion.[47] A W/O emulsion prepared with nonionic surfactants can be inverted to an O/W emulsion by diluting it in water (Figure 1.3)[48].

1.2.2.3. Anti-solvent precipitation method

Anti-solvent precipitation method is another method with no need for specialized equipment and complex operating conditions. The driving force for precipitation is rapid and high supersaturation when rapidly transfer both the surfactant and core material solutions to an anti-solvent.[49] For example, thymol and zein can be dissolved in ethanol/water solution (80:20 v/v), and by adding more water and ethanol evaporation, thymol and zein can self-assembled into small particles.[26] This supersaturation can be obtained by pH adjustment,[50,51] spray drying,[52] and by using solvents with different solubilities.[26] Nanoemulsions with droplet size from 100 to 900 nm can be obtained using this method, and summarized in Table 1.1.[53]

1.3. Factors affect stability of nanoemulsions

Conventional emulsions are thermodynamically unstable systems and tend to break down over time due to a variety of mechanisms, such as gravitational separation, coagulation, flocculation, and Ostwald ripening (Figure 1.4).[54]

The gravitational separation phenomena including “ringing”, “creaming”, and “oiling-off” in an oil/water emulsion, when the relative density of droplets is lower than density of water.[55] The density of shell is usually higher than that of oil phase and water phase.[29] When the density of interfacial shell is high enough, the density of droplets can be higher than water, and

droplets tend to precipitate. Besides choosing suitable core material and shell material for emulsion fabrication, weighing agents can be used to decrease the density difference between the dispersed and continuous phases, thus to prevent instability caused by gravity.[56] For nanoemulsions with a droplet radius below about 10 nm, gravitational separation is not expected to see due to the domination of Brownian motion. Even with larger droplet size, separation may not be observed visually in a short time scale for large droplets.[29]

Flocculation and coagulation are another two different kinds of destabilization mechanisms. Reversible aggregation is called flocculation and irreversible aggregation is called coagulation, which depend on the interactions between emulsion droplets.[57] Colloidal interactions between two droplets can be summed up with four types---van der Waals, electrostatic, steric, and hydrophobic interactions.[54] The van der Waals and hydrophobic interactions are attractive forces, while steric and electrostatic interactions are repulsive forces. When the attractive interactions are stronger, droplets tend to get together and aggregation and sedimentation occur.[58] However, when the repulsive interactions are stronger, droplets tend to get away from each other. Flocculation is caused by very weak interactions between emulsion droplets, while coagulation is caused by strong short-range interactions.[59]

Ostwald ripening is usually a problem for emulsions with oil of relatively high water solubility, such as flavor oils and essential oils,[28] which is caused by molecular diffusion of oil between oil droplets through the continuous phase.[60] The rate of Ostwald ripening is based on the oil solubility in continuous phase.[61] In oil/water emulsions, adding long chain triglyceride oils (e.g., corn oil, soybean oil) into high-water-solubility oils can prevent Ostwald ripening,[28] as these polar oils have a higher coefficient towards triglycerides than water in partitioning.

1.4. Emulsions prepared with SMSs and proteins

1.4.1. Emulsions prepared with SMSs

SMSs can be classified into three categories based on their charge characteristics---ionic, non-ionic, and zwitterionic surfactants.[29] Most of them are synthesized and their usage level is limited in food products.[62]

Dodecyl trimethyl ammoniu bromide (DTAB) and sodium dodecyl sulfate (SDS) are commonly used cationic surfactant and anionic surfactant that were approved to be used in the

food industry.[63] Cationic SMSs can be used to decrease oil oxidation by electrostatically repelling metal cations away from the lipid core.[64] Ionic SMSs are not frequently used because their regulated levels are typically lower than requirements in forming W/O emulsions.[65] And cationic SMSs have even higher toxicity than anionic SMSs.[62]

Non-ionic SMSs have been widely used to form nanoemulsions due to their high efficiency in emulsification. They can be used in both high-energy and low-energy methods. The most commonly used surfactants are sugar fatty acid esters,[2] alkyl ethoxyldes[66] and ethoxylated sorbitan esters[67]. Nonionic SMSs can enhance oil solubility in water by incorporating lipids into surfactant micelles.[68]

Different from ionic SMSs, zwitterionic surfactants have oppositely charged groups in one molecule. Phospholipids, such as lecithin or phosphatidylethanolamine.[69] Zwitterionic surfactants also can be used in both high-energy and low-energy methods. Usually, these surfactants cannot stabilize nanoemulsions individually, but they may perform well when used with cosurfactants.[15]

1.4.2. Emulsions prepared with proteins

Proteins are an important class of surface-active biopolymers with wide sources. The most commonly used proteins in the food industry are caseins, whey proteins, and soybean proteins.[29] They are natural emulsifying agent of excellent emulsification activities.[70] They plays a very important role in many emulsion systems, such as ice cream, cream liqueur, and milk beverages. Formation of nanoemulsions using proteins were widely studies these years, such as, corn oil nanoemulsions prepared with β-lactoglobulin using high-pressure homogenization,[21] peppermint oil nanoparticle dispersions prepared with zein using anti-solvent precipitation method,[71] thymol nanoparticle dispersions prepared with sodium caseinate.[3]

Emulsions prepared with protein performed good stability.[70] The hydrophobic part of protein is embedded into oil phase, while the hydrophilic part still remains in water phase.[62] Protein conformation changes upon adsorption, which stronger the hydrophobic interaction between proteins and the interface.[72] Intermolecular interactions between the neighboring adsorbed proteins make the interface film very viscoelastic, which significantly prevent oil droplets from aggregation.[73] At the same time, the hydrophilic group remaining in water phase

provide both steric and electrostatic repulsion to stabilized the emulsion.[74] The amount of charges and the strength of electrostatic repulsion are dependent on pH conditions.[9]

1.4.3. Emulsions prepared with SMSs and proteins

In food systems, SMSs usually are co-present with proteins, and their interactions significantly affect the properties and stability of products.[75] On one hand, synergistic effects may be achieved by utilizing SMSs in emulsions prepared with proteins, as SMSs are more effective in lowering the interfacial tension to a great extent than proteins and proteins can provide large electrostatic and steric repulsion to prevent emulsions from aggregation.[15,76] On the other hand, addition of water soluble SMSs and oil soluble SMSs may result in surface protein concentration reduction by competitive adsorption, which may destabilize emulsions.[77] These two aspects are to be studied with care.

Synergistic effects of proteins and SMSs can be enabled by co-absorption on the oil/water interface (Figure 1.5). The emulsion prepared with Tween 20 and *β*-lactoglobulin using a membrane homogenizer was reported to have both surfactants on the interface and have droplets significantly smaller than emulsions prepared using each surfactant individually[78].

Another mechanism is by the binding of SMSs and proteins. SMSs can bind with proteins on droplet surface and thus change the protein conformation.[79] For ionic surfactants, the ionic head groups may bind to the oppositely charges groups on protein to form protein-surfactant complexes.[80] For nonionic surfactants, they may interact with proteins though hydrophobic interactions.[81] The interactions between ionic surfactant and proteins may result in a thicker and stronger interfacial layer to improve the emulsion stability.[82]

In contrast, coexistence of both proteins and SMSs can also destabilize emulsions due to the complicated interactions between them. In most cases, SMSs tend to displace proteins because SMSs are more efficient in lowering interface tension at high SMSs concentration.[83] The adsorption status of protein and SMSs on the interface depend on SMS-to-protein molar ratio ($R_{s\text{-}p}$). At a high $R_{s\text{-}p}$, the protein on droplet surface is completely displaced from the surface by more surface-active SMSs. At the air-water interface in a thin liquid film, *β*-lactoglobulin molecules are effectively immobilized up to $R_{s\text{-}p}$=0.9, before transition to a mobile protein layer of higher $R_{s\text{-}p}$, however, at $R_{s\text{-}p}$=5, protein are completely displaced by added Tween 20 (polyoxyethyene

sorbitan monolaurate).[84] For emulsions made with β-lactoglobulin or α-lactalbumin and β-lactoglobulin mixtures, protein coats were completely displaced by Tween 20 at $R_{s\text{-}p}$=17.[85] For the emulsions prepared with NaCas using high pressure homogenization, complete protein displacement by added Tween 20 was reported at $R_{s\text{-}p}\approx$ 5.5.[86] Emulsions prepared with β-casein were reported to have the complete displacement of β-casein by additional $C_{12}E_8$ (Octaethylene glycol monododecyl ether) at $R_{s\text{-}p}$=17.[87] In contrast, when both SMSs and proteins were both added before emulsification, only partial protein displacement by Tween 60 (polyoxyethylene sorbitan monostearate) at event $R_{s\text{-}p}$>100.[88]

Interactions between SMSs and proteins are complicated, which may depend on oil type,[83] surface charge of SMSs,[89] molecular structure of SMSs,[90] molecular structure of protein, [91,86] and the SMSs/protein molar ratio[92] used in emulsion system. By studying these parameters, SMSs and proteins may be used together as a combination to make nanoemulsions.

Studies on nanoemulsions prepared with casein hydrolysates using high-energy method were reported and most of the samples are not stable. This was shown that extensive hydrolysis of milk proteins resulted in the reduced emulsion stability, suggesting a minimum peptide length is needed to prepare good emulsions with desirable properties.[93] Therefore, cosurfactants may be needed for nanoemulsion formation using protein hydrolysates.

If these combinations can be used, proteins can be involved in phase inversion method. Since proteins were not able to change the curvature with change of temperature or with change of system composition,[16] they cannot be used alone in nanoemulsion fabrication using phase inversion method. However, when proteins co-adsorb on the interface with SMSs whose interface film curvature can be controlled, proteins thus can be involved. And at the same time, the concentration requirement of SMSs may be decreased in phase inversion method with addition of another natural emulsifier. Besides, nanoemulsion fabrication using both SMSs may result in a more stable emulsion system, as the head group of SMSs can provide steric repulsion to help protect the nanoemulsion from aggregation.

1.5. Conclusions for literature review

Nanoemulsions can be fabricated using high-energy method and low-energy method. Low-energy method causes more interest because of its low equipment and energy cost. Natural

surface-active biopolymers are not able to change the curvature of interface film with the change of temperature or composition, so their use in phase inversion method is limited. To involve biopolymers in phase inversion method, SMSs and protein combinations can be used, which can also result in lowering the dosage requirement of SMSs in phase inversion method.

1.6. Hypotheses and scope of work

The objective of this thesis is to fabricate nanoemulsions of essential oils with casein and SMSs and study their interactions in order to decrease the dosage required of SMSs in nanoemulsion preparation. Lemon oil nanoemulsions prepared with NaCas and Tween 20 and thymol nanoemulsions prepared with CH and SS are studied in Chapter 2 and Chapter 2, respectively. Lemon oil and thymol are studied as representative flavor and antimicrobial essential oils. Compared with lemon oil, thymol also has a higher purity and higher antimicrobial activity.

Sodium caseinate (NaCas) is derived from caseins, which comprises of 80% of milk proteins.[94] Comprised with α_{s1}-casein β-casein, NaCas is an excellent emulsifier that has been widely investigated.[95,96] Different from other proteins, the structure of NaCas is very disordered,[73] and it is not easy to become denatured when extreme processing conditions, such as heating and high pressure homogenization are used.[21] Similar to other proteins, NaCas cannot be used alone in the phase inversion method, due to its large molecule dimension and negative charge. Tween 20 was selected for nanoemulsion fabrication in combination with NaCas because of its suitable HLB (16.7). Lemon oil as a widely-used flavoring agent was selected as the oil phase.[97] The hypothesis of Chapter 2 is that both Tween 20 and NaCas will adsorb on the O/W interface and the curvature of interface film formed by the emulsifier combinations can be controlled by temperature.

Another way to improve emulsifying properties of proteins is to decrease the dimension of NaCas by enzymatic hydrolysis. Hydrolysates are more flexible and reduce interfacial tension more effectively than proteins.[98,99] They can diffuse to and become attached on the interface quicker and rearrange on the interface easier than proteins.[100] Since the stability of emulsions prepared with hydrolysates is not very good, due to the weakened electrostatic and steric repulsion after hydrolysis,[101] SS was selected to form nanoemulsions in combination with

hydrolysates. Because the solubility of SS in water can be controlled by temperature, heating method was used to prepare nanoemulsions using both SS and CH.

Besides, similar to NaCas, CHs have an isoelectric point (pI), which is close to pH 4.6. At pI, the net charge of CH is zero, and it tends to precipitate due to lack of electrostatic repulsion.[102] Therefore, the stability of emulsions prepared with CH will also be impaired at pH 4.6, which may be improved by SS, particularly at increased temperatures. Therefore, the hypothesis of Chapter 3 is that improved solubility and emulsifying activity of SS with the increase of temperature can improve the stability of nanoemulsion prepared with SS and CH.

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Appendix

Table 1.1. Publications using anti-solvent precipitation method to prepare nanoemulsions (Reproduced from Ref. 53**).**

Biopolymer	Enclosed compound	Solvent/anti-solvent	Particle size	Morphology	Reference
Gliadin	All-trans-retinoic acid	Binary alcohol-water system/water	Around 500 nm	Spherical	[103]
Zein	Curcumin	Binary alcohol-water system/water	100-150 nm	Spherical	[104]
Zein	Thymol	Binary ethanol-water system/water	Around 200 nm	Spherical	[105]
Gelatin	FITC-dextran	Water/ethanol	200-300 nm	Spherical	[106]
Silk Fibroin/albumin	Methotrexate	Water/acetone	Around 200 nm	Spherical	[107]

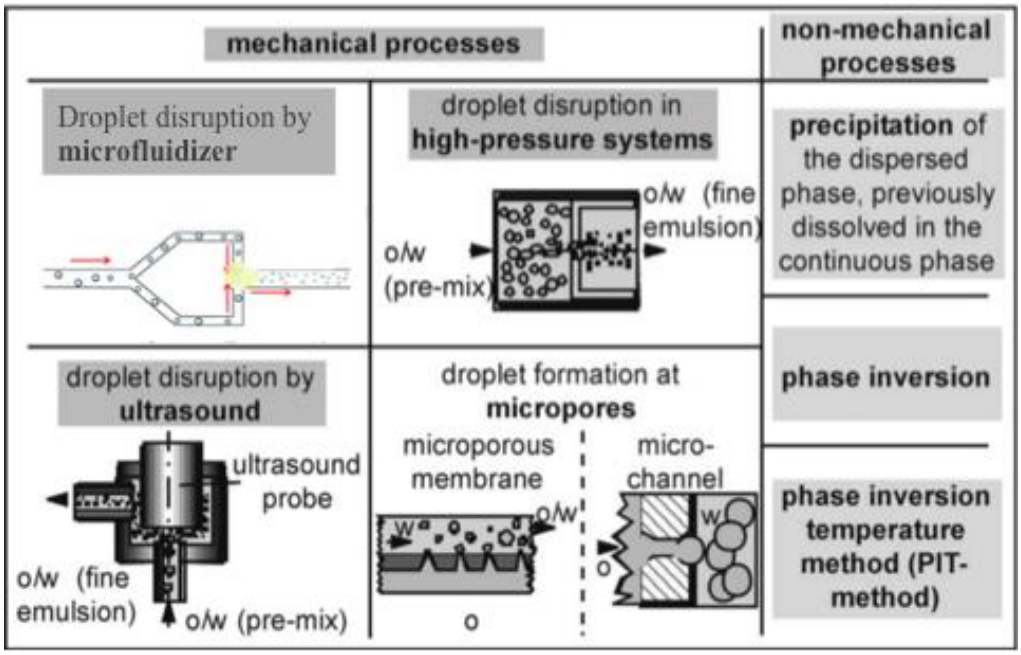

Figure 1.1. High-energy and low-energy methods in emulsification (Reproduced from Ref. 5 and 30).

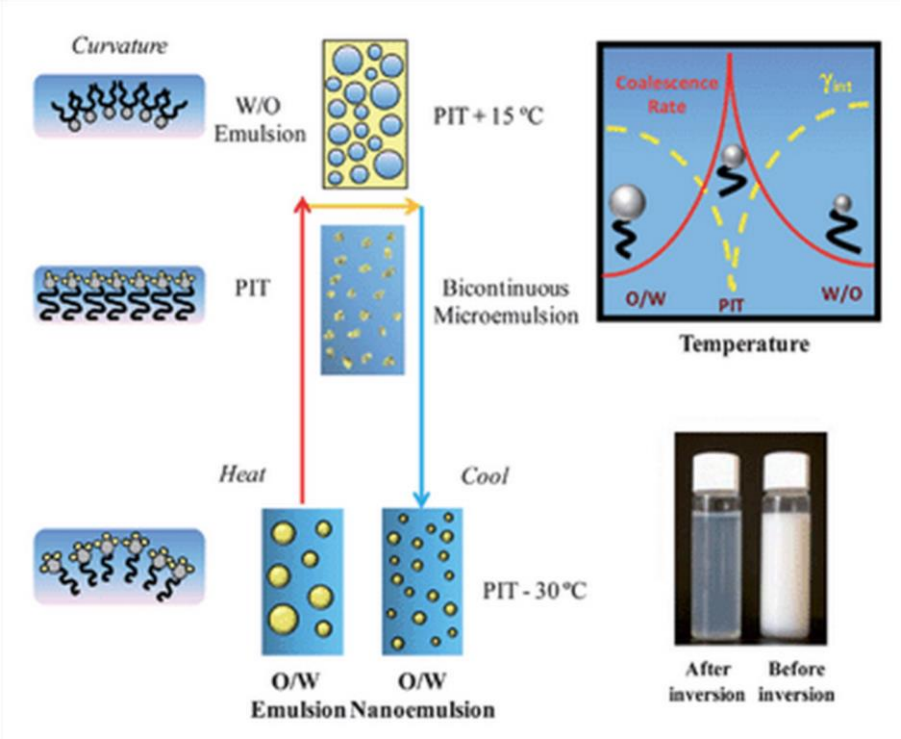

Figure 1.2. Nanoemulsion formation using PIT method (Reprinted from Ref. 29).

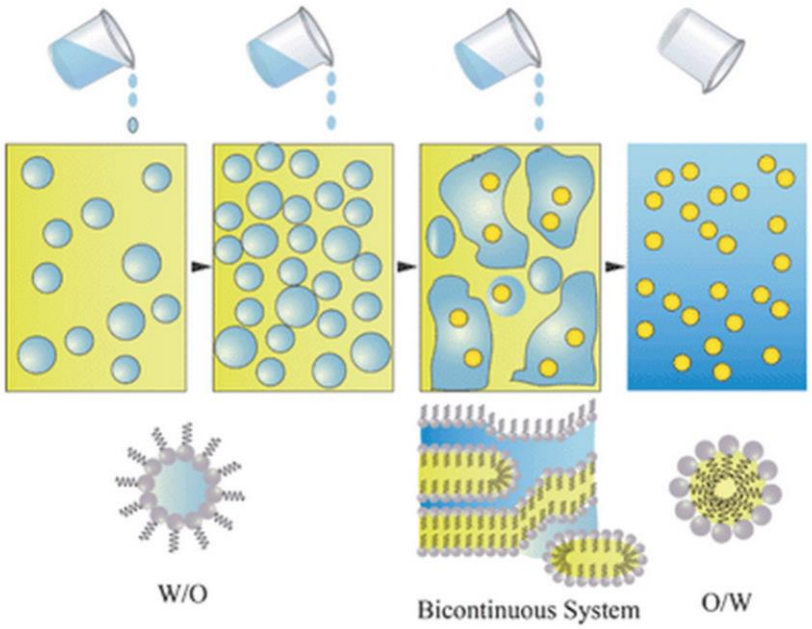

Figure 1.3. Principle of phase inversion composition method (Reprinted from Ref. 29).

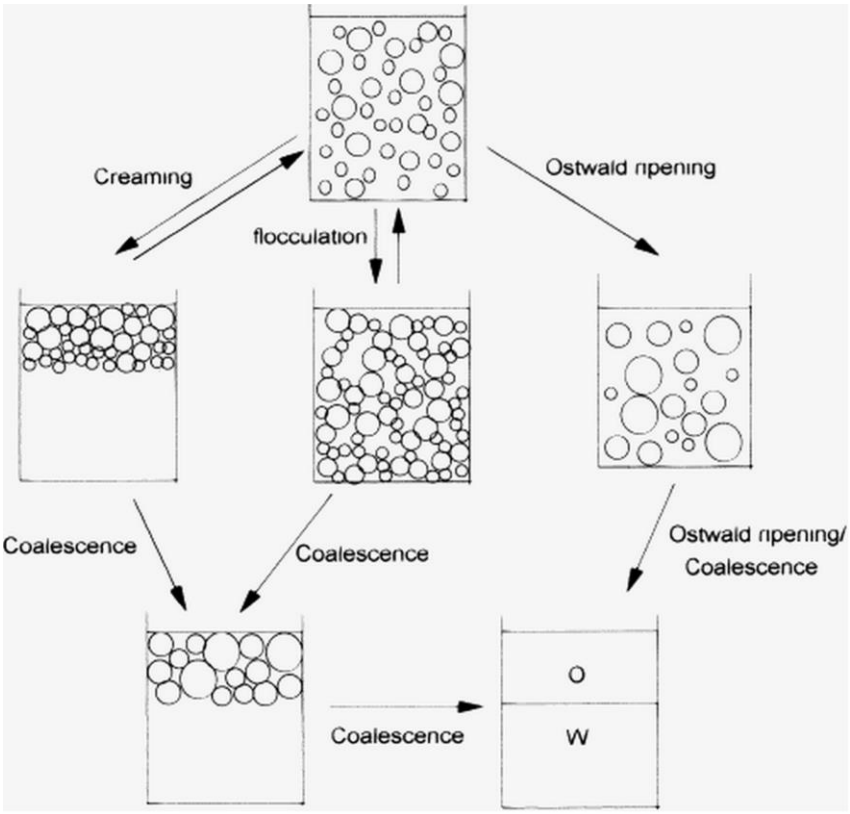

Figure 1.4. Different underlying mechanisms leading to emulsion breakdown (Reprinted from Ref. 90).

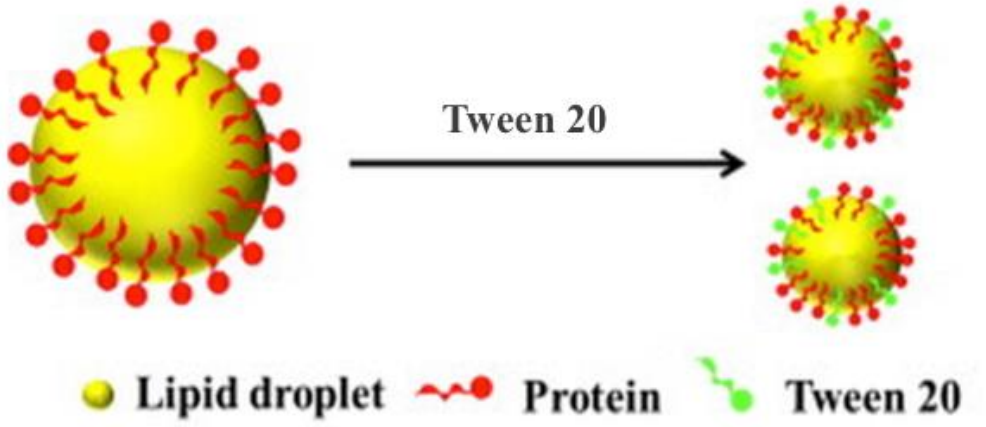

Figure 1.5. Co-adsorption of *β*-lactoglobulin and Tween 20 on the interface (Reproduced from Ref. 82).

CHAPTER 2 LEMON OIL NANOEMULSIONS FABRICATED WITH SODIUM CASEINATE AND TWEEN 20 USING PHASE INVERSION TEMPERATURE METHOD

Abstract

The phase inversion temperature (PIT) method has been studied to fabricate nanoemulsions with small droplets by heating oil-water mixtures with non-ionic synthetic surfactants, but not for food biopolymers. In this study, the objective was to investigate lemon oil nanoemulsion formation by combining of Tween 20 and sodium caseinate (NaCas) in the PIT method. For mixtures with 2% NaCas, 1% Tween 20 and 1.5% lemon oil, the combination of NaCas and Tween 20 reduced the turbidity and droplet dimension of emulsions than using them individually, and the co-adsorption of NaCas on oil droplets decreased with increasing Tween 20 concentration. Addition of 0.2 and 0.4 mM NaCl negatively affected the formation of nanoemulsion, while nanoemulsion formation was favored at 0.6 and 0.8 mM NaCl. Turbidity and rheology results showed the PIT was between 80 and 90 °C. The nanoemulsions prepared with 2% w/v NaCas, 0.4-1.2% w/v Tween 20 and 1.5% w/v lemon oil had a volume-area mean diameter of around 100 nm and the emulsion was stable during 15-day storage at room temperature followed by creaming after longer storage. Therefore, NaCas can be used to partially replace synthetic surfactants to prepare nanoemulsions of flavor oils for short-time storage.

Keywords: sodium caseinate, PIT method, nanoemulsions, Tween 20, lemon oil

2.1. Introduction

Nanoemulsions are dispersions with droplets smaller than 200 nm in diameter.[1] The reduced droplet dimension in nanoemulsions makes the thermal energy of droplets significant when comparing to the gravitational energy and possibly attractive colloidal forces. Therefore, nanoemulsions are able to stabilize oil droplets against instability mechanisms of gravitational sedimentation and droplet aggregation.[2] The reduced droplet dimension also weakens the scattering of visible light and thus improves optical clarity of emulsions,[3] which makes it possible to use nanoemulsions in transparent products.[4]

Oil-in-water nanoemulsions can be prepared by two groups of methods that differ in the amount of mechanical energy input.[5] In high-energy methods such as high shear homogenization, high pressure valve homogenization, microfluidization, and sonication,[6] substantial mechanical energy is used to break up oil droplets,[6] which increases capital and operating costs.[7] It is difficult to produce fine oil droplets when the dispersed-to-continuous phase viscosity ratio is not suitable and disruptive forces are weaker than restoring interfacial forces.[2] Moreover, "over-processing" caused by high pressures and long emulsification times can take place when proteins used as emulsifiers are denatured, resulting in increased particle size.[8] Conversely, low-energy methods do not require intense mechanical energy. They are used to prepare nanoemulsions utilizing curvatures of interfacial films being a function of compositions and environmental conditions, which makes it possible for the inversion of dispersed and continuous phases.[9] Controlling interfacial properties enables the prepared of emulsions with fine droplets using low energy methods can have smaller droplets.[2] High concentrations of synthetic nonionic small molecule surfactants (SMSs) such as Tween[10] and Span[11] family surfactants are commonly used to form nanoemulsions using low-energy methods that usually involve a phase inversion upon changes in composition (PIC)[12] or temperature (PIT).[13] On the other hand, proteins and polysaccharides are considered to be infeasible to form nanoemulsions in PIC and PIT methods, because they are not able to change the curvature of interfacial films due to their surface charge and relatively large molecular weight.[14] To reduce the amount of synthetic SMSs used in nanoemulsion fabrication, combinations of synthetic and natural emulsifiers may be an option, which has not been studied for SMSs and biopolymers.

Sodium caseinate (NaCas), produced by precipitation of caseins in milk at pH 4.6 and neutralization the precipitate using sodium hydroxide,[15] is a well-known protein emulsifier.[16] The emulsifying property of NaCas is derived from its high contents of hydrophobic amino acids such as proline, tyrosine, and tryptophan, which makes caseins naturally occurring amphiphilic block copolymers.[17] Main surface-active components of NaCas are α_{s1}- and β-caseins.[18] α_{s1}-casein has a hydrophobic blocks at both ends and one in the middle, while β-casein has one phosphoseryl cluster (hydrophilic region) and one hydrophobic region.[33] Like synthetic block copolymers, NaCas adsorbed on oil droplets can take the "train-loop-tail" conformation, which provides steric repulsion, together with electrostatic repulsion due to protein charges,[19] to stabilize oil droplets against aggregation.[20] The excellent emulsifying and stabilizing properties of NaCas make it a popular emulsifier in the food industry.[20]

Utilizing distinct properties of SMSs and proteins to prepare emulsions has been studied using high-energy methods.[21] SMSs with a better packing efficiency, can lower the surface free energy, thereby reduce droplet dimension more effectively than proteins.[22] This has been demonstrated for smaller droplets in emulsions prepared with the combination of polyoxyethylene (20) sorbitan monolaurate (Tween 20) and *β*-lactoglobulin than those using *β*-lactoglobulin alone.[23] The competitive adsorption of proteins and SMSs has been studied extensively.[20, 24, 25] Because only one-third of the available interfacial area is taken up by the train segments of NaCas at the monolayer saturation coverage,[22] it is possible for SMSs to adsorb at the interface and displace the adsorbed NaCas.[21] It is also possible for the two groups of emulsifiers to co-exist on the interface to form a stable film.[26] The exact physical event is decided by many factors, such as oil type,[27] surface charge of SMSs,[28] molecular structures of both SMSs and protein, [29, 30] and SMS-to-protein molar ratio ($R_{s\text{-}p}$).[31] It is reported that when NaCas and Tween 60 were used to prepare soybean oil nanoemulsion, NaCas was present on the interface even at a $R_{s\text{-}p}$ value of 100.[20] In another study using dioxyethylene glycol *n*-dodecylether ($C_{12}E_2$) and *β*-casein to prepare *n*-hexadecane nanoemulsions, 60% of *β*-casein adsorbed at the interface at a $R_{s\text{-}p}$ =100.[31] Therefore, it is possible to prepare nanoemulsions co-emulsified and stabilized by Tween 20 and NaCas. Because synthetic block copolymers have been studied in phase inversion method,[34, 35] it may be possible to prepare nanoemulsions using NaCas-SMS combination in the PIT method.

In the present study, the objective was to fabricate lemon oil nanoemulsions with NaCas and Tween 20 or polyoxyethylene (20) sorbitan monooleate (Tween 80) using the PIT method. Lemon oil was selected because it is a widely-used flavoring agent. Tween family surfactants with a polyethylene head group and different hydrophobic fatty acid tails have been used to prepare nanoemulsions and microemulsions using the PIT method.[10] Tween 20 has been studied to form microemulsions of peppermint oil with lecithin using the PIT method.[32] Our hypothesis is that the interfaces can be co-adsorbed with Tween 20/80 and NaCas, and phase inversions during thermal treatments will enable the formation of nanoemulsions with both surfactants at the interface. In addition to identifying nanoemulsion preparation conditions, interactions between NaCas and Tween and the properties of nanoemulsions were studied.

2.2. Materials and methods

2.2.1. Materials

Lemon oil produced by cold pressing was a product from Now Foods Company (Bloomingdale, IL, USA). The oil contains approximately 70% limonene and 2.2-3.8% aldehydes, according to the supplier. NaCas and sodium chloride (purity >99.5%) were products of Sigma-Aldrich Corp. (St. Louis, MO, USA). Tween 20, Tween 80, hexane, and acetone were purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was used in sample preparation.

2.2.2. Preparation of nanoemulsions

NaCas and Tween 20 or Tween 80 were dissolved in deionized water at concentrations of 2% w/v and 0-1.2% w/v, respectively, by stirring overnight to ensure complete hydration. The pH of emulsifier solution was about 6.8. To prepare coarse emulsions, different amounts of lemon oil (1.0-3.5% w/v) were added into the surfactant solution and vortexed for 2 min. The mixtures were then homogenized at 10,000 rpm for 2 min using a Cyclone I.Q. microprocessor homogenizer (The VirTis Co., Inc., Gardiner, NY, USA). The coarse emulsions were heated at a constant temperature for 0-3 h in a water bath, followed by immediately quenching in ice/water with hand shaking.

2.2.3. Turbidity measurements

Sample turbidity was measured for absorbance at 600 nm (Abs_{600}) as an indicator using a UV-Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA, USA). Deionized water was used as a blank. To determine PIT using turbidity measurement, 1 mL of a sample was transferred into a cuvette when a sample in a 50 mL centrifuge tube heated in a water bath reach a required temperature, and Abs_{600} was measured immediately.

2.2.4. Determination of droplet size

Size distributions of emulsion droplets were measured using a Delas Nano particle analyzer (Beckman Coulter, Fullerton, CA, USA). Samples were diluted to an appropriate concentration in deionized water to fit sensitivity requirement of the instrument. The volume-surface area ($d_{3,2}$) mean diameter was calculated from the number of droplets (n_i) with the corresponding diameter (d_i) based the following equation:

$$d_{3,2} = \sum_{i=1}^{N} n_i \, d_i^3 / \sum_{i=1}^{N} n_i \, d_i^2 \qquad (2.1)$$

2.2.5. Viscosity measurement

Rheological properties of emulsions were studied with an AR 2000 rheometer (TA Instrument, New Castle, DE, USA) using a Searle setup (bob outer diameter of 28 mm and cup inner diameter of 30 mm). The samples were heated from 25 to 90 °C at 1 °C/min while being sheared at 200 s^{-1}.

2.2.6. Atomic force microscopy (AFM)

The shape and dimension of droplets were characterized using a Multimode VIII microscope (Bruker Corporation, Billerica, MA, USA). A 2% w/v lemon oil emulsion prepared with 2% w/v NaCas, 1.2% w/v Tween 20, or 2% NaCas and 0.2% Tween 20 were diluted to a lemon oil concentration of 100 ppm using deionized water. 5 μL of a diluted sample was dropped on a freshly cleaved mica disk and dried under ambient temperature (21 °C) in a chemical fume hood overnight. Images were collected at the tapping mode and analyzed using the instrument software.

2.2.7. Quantification of protein adsorbed on the droplet surface

Nanoemulsions were prepared with 2% w/v NaCas, 0.2-1.2% w/v Tween 20, and 1.5% w/v lemon oil by heating at 90 °C for 1 h. Emulsion with 1.5% w/v lemon oil and 2% w/v NaCas only was prepared without heating. Samples were centrifuged at 30,000 *g* for 30 min at 25 °C using a Sorvall Legend 26R centrifuge (Thermo Scientific, Waltham, MA, USA) to obtain the supernatant for protein assay. Lemon oil precipitated with NaCas for emulsion prepared with Tween 20 and NaCas, with an exception for the emulsion prepared with NaCas only showing oil and NaCas on the top. Because residual Tween 20 and lemon oil interfered with protein assay, a binary mixture with equal volumes of acetone and hexane was used to extract Tween 20 and lemon oil and precipitate protein in the serum. 100 μL of the serum was mixed with 1.5 mL acetone-hexane (1:1, v/v) mixture. After 30-s vortexing, the mixtures were centrifuged at 6700 *g* for 3 min using an Eppendorf MiniSpin plus centrifuge (Eppendorf AG, Hamburg, Germany). The upper organic phase was discarded and the bottom protein precipitate was washed 3 times using 1.5 mL acetone-hexane mixture. The final precipitate was re-dissolved in 100 μL of 0.1 M NaOH and diluted 25 folds. The diluted protein sample was quantified using the bicinchoninic acid (BCA) method with reagents from Bioworld Technology Inc. (Minneapolis, MN, USA), using NaCas as a reference protein. The surface load of NaCas (Γ_s) was determined using the equation below:

$$\Gamma_s = M_s \, d_{3,2} / 6V_{oil} \tag{2.2}$$

where M_s is the mass of NaCas adsorbed on oil droplets, and V_{oil} is the volume of lemon oil. The lemon oil density used in calculation was 0.8656 g/mL, determined based on gravimetry at 21 °C.

2.2.8. Statistical analysis

Statistical analyses were performed using the SAS software (version 9.4, SAS Institute, Cary, NC, USA). One-way analysis of variance was carried out. Differences between pairs of means were compared using a Tukey's Test. The significance level was set to be 0.05.

2.3. Results and discussion

2.3.1. Emulsion formation conditions

2.3.1.1. Difference between Tween 20 and Tween 80

The first group of samples with 2% w/v lemon oil was prepared with 2% w/v NaCas alone, 0.2% w/v Tween 80 or Tween 20 alone, and their combinations by heating coarse emulsions at 90 °C for 1 h. Samples prepared with NaCas-Tween combinations showed better clarity than those prepared with an individual emulsifier (Figure 2.1A). The sample prepared with the NaCas-Tween 20 combination was clearer than that with the NaCas-Tween 80 combination. Emulsions prepared with an individual surfactant were all highly unstable to creaming, which may be due to the relatively large droplets.

The improved clarity of emulsion prepared with NaCas and Tween 20 than that with NaCas and Tween 80 may be due to the different HLB value of Tween 20 and Tween 80. Lemon oil nanoemulsions were prepared by Tween 80 alone using the PIT method in a previous study,[10] which suggests the appropriateness of the HLB value of Tween 80 (15.0) for emulsifying lemon oil.[32] The respective HLB of Tween 20 and NaCas are 16.7 and 14.0.[36] The combination of Tween 20 and NaCas may have an overall HLB value closer (around 15.0) to that required to emulsify lemon oil in the PIT than the combination of Tween 80 and NaCas.[37] Therefore, Tween 20 was selected for further studies.

The effects of Tween 20 concentration on the turbidity of emulsions prepared 2% w/v NaCas and 1.5% w/v lemon oil were further studied. With an increase of Tween 20 concentration, Abs_{600} of samples prepared with and without 2% NaCas both decreased (Figure 2.1B). Samples prepared with both NaCas and Tween 20 showed significantly lower Abs_{600} than those with NaCas or Tween 20 alone ($P < 0.05$). There was no significant difference in Abs_{600} of emulsions prepared with NaCas and different Tween 20 concentrations ($P > 0.05$).

A greater reduction of O/W interfacial tension and droplet size of emulsions were reported for $C_{12}E_2$ and NaCas mixture being surfactants and n-tetradecane being the oil phase, and the reductions were more significant with above ~ 0.1% w/v of $C_{12}E_2$.[37] This study showed the significance of SMSs in reducing droplet during high pressure homogenization with NaCas. Like wise, the improved clarity of emulsion with both Tween 20 and NaCas should be attributed

to the role of Tween 20 reducing droplet size (Table 2.1) after heating and cooling. No significant difference in $d_{3,2}$ for emulsions prepared with NaCas and different concentrations of Tween 20 (Table 2.1) agreed with similar Abs_{600} of emulsions (Figure 2.1B).

In a previous study for emulsions prepared with 10% w/w lemon oil and 9% w/w Tween 80 using the PIT method, about 1% w/v lemon oil was emulsified by 1% w/v Tween 80, with the droplet diameter greater than 200 nm.[10] However, 1.5% w/v lemon oil was emulsified by 2% w/v NaCas and 1% w/v Tween 20 with a smaller droplet diameter of 102.9 nm (Table 2.1), which means only 1% w/v Tween was need to emulsify 1.5% w/v lemon oil into nanoemulsions. Therefore, the combination of NaCas and Tween 20 significantly decreases the amount of synthesized SMS required to prepare nanoemulsions with relatively small droplets.

2.3.1.2. Effects of heating conditions on emulsion formation

To further study emulsion formation conditions, Abs_{600} of samples prepared with 2% w/v NaCas, 1% w/v Tween 20, and 1.5% w/v lemon oil was determined after heating at 50-98 °C for 1 h and cooling down in ice/water bath. As presented in Figure 2.2A, Abs_{600} decreased sharply at 70 °C and continued to decrease until 90 °C, followed by no further significant decrease at 98 °C. When the temperature is raised to a point higher than the PIT, the coarse emulsion transfers to a W/O emulsion that can change to a W/O/W emulsion and finally to an O/W emulsion during cooling.[38] The PIT can be determined as the lowest temperature that can make the emulsion with smaller particle size and better clarity. The data in Figure 2.2B indicated the PIT of the mixture being 90 °C or lower.

When samples were heated at 90 °C for different durations (Figure 2.2B), Abs_{600} decreased significantly with an increase of heating time until 1 h, followed by no significant changes. Therefore, heating for 1 h at 90 °C was selected for further studies.

2.3.1.3. Emulsifying capacity of NaCas and Tween 20 mixture

The emulsifying capacity of 2% w/v NaCas and 1% w/v Tween 20 after heating at 90 °C for 1 h was measured for Abs_{600} (Figure 2.3). There was no significant increase of Abs_{600} at a lemon oil content between 1.0 and 2.5% w/v. At 3.0 and 3.5% w/v lemon oil, Abs_{600} of emulsions became significantly higher. Free oil can be observed on the top for emulsions with 2%

w/v or more lemon oil. Therefore, 1.5% w/v lemon oil was treated to be the emulsifying capacity of with 2% w/v NaCas and 1% w/v Tween 20.

2.3.1.4. Influence of ionic strength on phase inversion

Because ionic strength can facilitate nanoemulsion formation in the PIT method,[39] the mixture with 2% w/v NaCas, 1% w/v Tween 20 , and 1.5% w/v lemon oil was studied at 0-0.8 M NaCl. The Abs_{600} of emulsions after heating at 90 °C for 1 h and quenching is shown in Figure 2.4. Abs_{600} became higher when NaCl increased from 0 to 0.4 M, followed by dramatic decrease at 0.6 M, and there was no significant difference between the treatment without NaCl and those prepared with 0.6 and 0.8 M NaCl.

The Abs_{600} of emulsions can be affected by NaCl in two different ways. An increase in ionic strength increases the hydrophile-lipophile deviation of nonionic surfactants, which lowers the PIT and favors phase inversion.[38] However, emulsions had higher Abs_{600} at 0.2 and 0.4 M NaCl, which suggests NaCl also affected emulsion droplet stability. An increase of NaCl concentration suppresses the Debye length and therefore weakens inter-droplet electrostatic repulsion when droplets are charged.[39] In addition, the weakened intra-molecular repulsion by NaCl can suppress the train conformation of NaCas on emulsion droplets and therefore weaken the steric repulsion.[39] The overall weakened inter-particle repulsion can induce droplet flocculation, which can increase sample turbidity and even result in creaming.[40] At sufficiently high NaCl concentrations, effects of ionic strength on NaCas charge may become insignificant, [41] and the effects of ionic strength on the phase inversion become dominant, resulting in formation of finer droplets and lower turbidity of emulsions.[42]

2.3.2. Phase inversion temperature (PIT) of NaCas Tween 20 lemon oil mixture

Turbidity and viscosity are commonly used to determine the PIT of emulsion systems.[13,38,43] In this set of experiments, Abs_{600} and viscosity of mixtures prepared with 1% w/v Tween 20 and 2% w/v lemon oil, with 0 or 0.8 M NaCl, and 0 or 2% w/v NaCas were measured (Figure 2.5). For mixtures without NaCas and NaCl, Abs_{600} was similar at 25-90 °C and viscosity decreased continuously (Figure 2.5A), which indicates no PIT. At 0.8 M NaCl, the Tween 20/lemon oil mixture showed a dramatic reduction of Abs_{600} and viscosity at 87 °C and 85 °C, respectively (Figure 2.5C). For mixtures with NaCas, the dramatic reduction of Abs_{600} was

observed at 85 °C, but viscosity decreased continuously at 0 M NaCl (Figure 2.5B). At 0.8 M, a dramatic decrease of Abs_{600} of the mixture with NaCas was observed at 80 °C and a sudden drop was observed for viscosity at around 83 °C (Figure 2.5D).

During heating of an O/W emulsion, two transitions, to bicontinuous structures and a W/O emulsion occur, because of the increased hydrophobicity of SMSs, resulting from the dehydration of the head groups of SMSs.[43,44] If the water-to-oil volume ratio is very high, there is not enough oil to form a W/O emulsion, and phase separation can occur during heating to a temperature called cloud point.[43] The PIT can be estimated the sudden change of Abs_{600} and viscosity.

In the present study, phase inversion was not observed for the mixture prepared with 1% w/v Tween 20 and 2% w/v (Figure 2.5A), which may be due to an insufficient amount of surfactants. The similar phenomenon was reported for the turbidity of emulsion prepared with 7% Tween 80 and 10% lemon oil in PG and water (mass ratio 1:2) solutions, during heating from 20 to 90 °C.[10] When 0.8 M NaCl was added into the mixture of 1% w/v Tween 20 and 2% w/v lemon oil, the PIT was observed based on (Figure 2.5C), which is due to impacts on the hydrophilic lipophilic deviation, as discussed previously.[38] For the system with 2% w/v lemon oil and 1% w/v Tween 20, the addition of 2% w/v NaCas enabled the phase inversion based on Abs_{600}, which indicates the adsorption of both NaCas and Tween 20 on the interface. At 0.8 M NaCl, the PIT became more apparent and was evident in both Abs_{600} and viscosity data (Figure 2.5D).

2.3.3. Dimension and structure of emulsion droplets

The AFM structures of NaCas solution and emulsions prepared with 2% w/v lemon oil, 1.2% w/v Tween 20 only or 2% w/v NaCas and 0.2% w/v Tween 20 mixture are presented in Figure 2.6. The sample prepared with 1.2% w/v Tween 20 had bigger particles (98.3 nm) while no significant difference was observed for the emulsion prepared with NaCas-Tween 20 mixture (86.7 nm) and NaCas solution (87.2 nm).

To study the adsorption of NaCas on oil droplets as affected by $R_{s\text{-}p}$, the reduction of free protein concentration (proteins % at interface) and the amount of protein on unit area of droplet surface (surface load, Γ_s) were quantified (Figure 2.7). Using respective molecular weights of 1227 and 23,000 Da for Tween 20 and NaCas, [23] the $R_{s\text{-}p}$ in treatments with 0.2-1.2 w/v Tween

20 and 2% w/v NaCas is about 2-12. As shown in Figure 2.7, both protein adsorbed on the interface and Γ_s decreased significantly from $R_{s\text{-}p}$ of 0-6, while no significant difference was observed at $R_{s\text{-}p}$ of 6-12. For soybean oil emulsions prepared with Tween 60 (HLB=14.9) and NaCas using high pressure homogenization, Γ_s varied from 2.7 to 1.7 mg/m^2 at $R_{s\text{-}p}$ of 2-10,[20] while is within the range of 1.6-6.9 mg/m^2 in the present study. The data in Figure 2.7 at $R_{s\text{-}p}$ of 2 was higher than proteins on It was also reported that the saturation protein coverage of soybean oil droplets is about 3 mg/m^2.[45] The difference may due to the different type and amount of oil used in two studies. At increased $R_{s\text{-}p}$, the more surface-active Tween 20 can compete with interfaces better, resulting in the decreased amount of interfacial NaCas. The consistent presence of NaCas on the interface further verifieds the co-adsorption of Tween 20 and NaCas on surfaces after heating and cooling. This agrees with the presence of NaCas at $R_{s\text{-}p}$ as high as 100.[20]

2.3.4. Stability of nanoemulsions

To study the stability of nanoemulsions prepared with both NaCas and Tween 20, $d_{3,2}$ of samples with 0.2-1.2% w/v Tween 20 was measured before and after 15-day storage at ambient temperature (Table 2.1). There were no significant difference ($P > 0.05$) for $d_{3,2}$ of samples with 0.4-1.2% w/v Tween 20 before or after storage, while the sample with 0.2% w/v Tween 20 became unmeasurable after 15-day storage. The droplet size distributions of emulsions with 0.6 and 1% w/v of Tween 20 before and after 15-day storage are compared in Figure 2.8. Despite some growth of droplet size distribution (Figure 2.8), $d_{3,2}$ and polydispersity index (Table 2.2) did not change significantly. These results indicated the short-term storage stability of these emulsions prepared with 0.4-1.2% w/v Tween 20 and 2% NaCas. However, creaming appeared for all the samples after one-month storage.

2.4. Conclusions

In the present work, lemon oil nanoemulsions were formed by the Tween 20 and NaCas combination using the PIT method. The emulsion turbidity and droplet size reduced significantly using the combination when compared with those prepared with each emulsifier individually. An increase in Tween 20 concentration resulted in a smaller amount of NaCas on the interface, but NaCas was present on the interface in all treatments. The addition of NaCas enabled the PIT between 80 °C and 90 °C. The presence of NaCas on droplet surface resulted in negative phase

inversion at 0.2 and 0.4 M NaCl but the impact was positive at 0.6 and 0.8 M NaCl. Emulsions prepared with 2% w/v NaCas and 0.4-1.2 % w/v Tween 20 provided a short-term stablility at ambient temperature for about 15 days. This work provided an option to use amphiphilic biopolymers in the PIT method to prepare nanoemulsions using a decreased dosage of synthesized SMSs.

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Appendix

Table 2.1. Volume-area mean diameter (nm) of samples prepared with 1.5% w/v lemon oil, 2% w/v NaCas and 0.2-1.2% w/v Tween 20 before and after storage at 21 °C for 15 days. *

Tween 20 (% w/v)	0.2	0.4	0.6	0.8	1.0	1.2
Day 1	66.78±19.50^{bc}	57.38±5.97^{c}	99.26±18.30^{abc}	105.66±45.51^{abc}	98.37±18.46^{abc}	120.33±4.93^{ab}
Day 15	N/A	104.39±3.26^{abc}	96.35±14.40^{abc}	113.44±7.67^{abc}	124.34±2.29^{a}	104.17±0.98^{abc}

* Numbers are mean±standard deviation (n=3). Different superscript letters indicate difference in mean ($P< 0.05$). "N/A" indicates this sample cannot be tested by the instrument.

Table 2.2. Polydispersity index of samples prepared with 1.5% w/v lemon oil, 2% w/v NaCas and 0.2-1.2% w/v Tween 20 before and after storage at 21 °C for 15 days. *

Tween 20 (% w/v)	0.2	0.4	0.6	0.8	1.0	1.2
Day 1	0.299 ± 0.011^{abc}	0.310 ± 0.073^{abc}	0.255 ± 0.047^{abc}	0.201 ± 0.008^{c}	0.224 ± 0.007^{bc}	0.307 ± 0.036^{abc}
Day 15	N/A	0.334 ± 0.019^{a}	0.322 ± 0.010^{ab}	0.311 ± 0.012^{ab}	0.284 ± 0.013^{abc}	0.298 ± 0.017^{abc}

* Numbers are mean±standard deviation (n=3). Different superscript letters indicate differences in mean ($P< 0.05$). "N/A" indicates this sample cannot be tested by the instrument.

Figure 2.1. Appearance of emulsions with 2% w/v lemon oil prepared with 2% w/v NaCas, 0.2% w/v Tween 20 or 80, or both. (B) Absorbance at 600 nm (Abs_{600}) of samples prepared with 1.5% w/v lemon oil, 0-1.2% w/v Tween 20, with or without 2% w/v NaCas. All samples were heated at 90°C for 1 h. Error bars are standard deviations (n = 3). Different letters above symbols indicate significant differences of mean ($P < 0.05$).

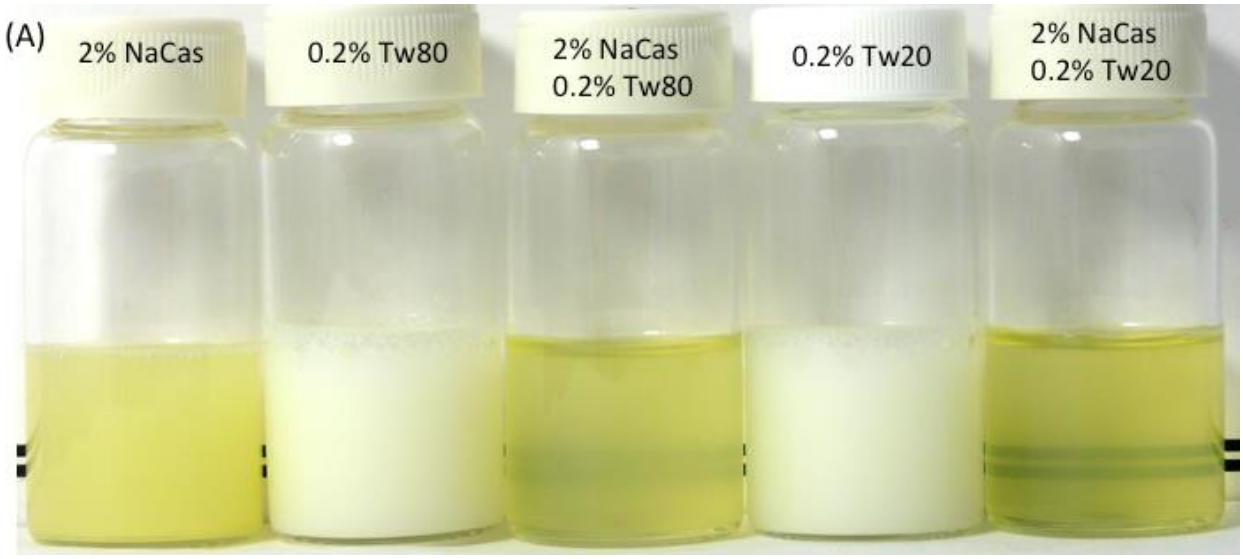


Figure 2.1. continued.

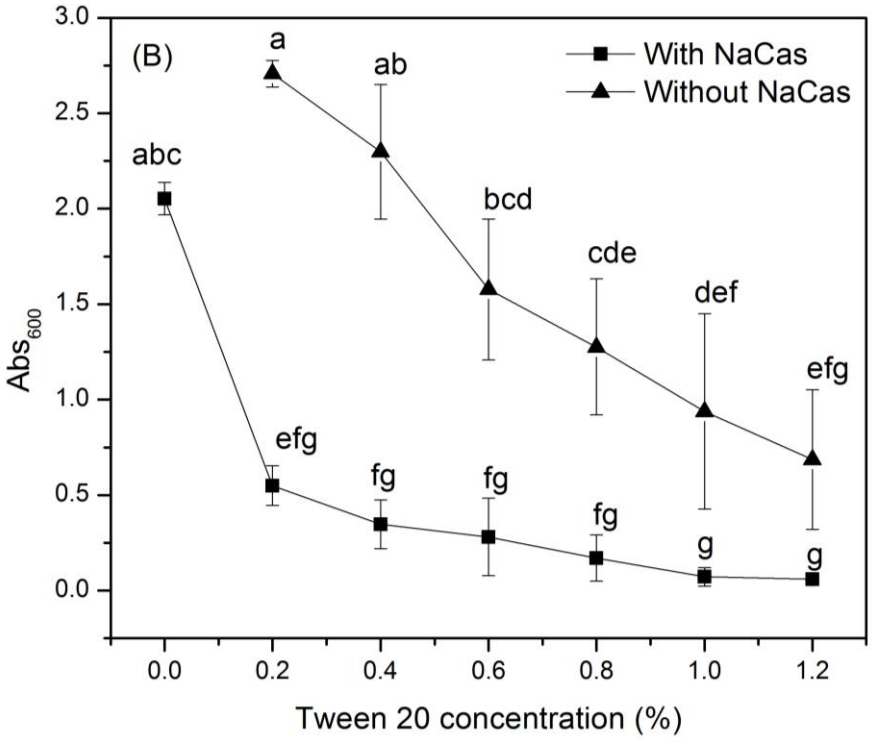
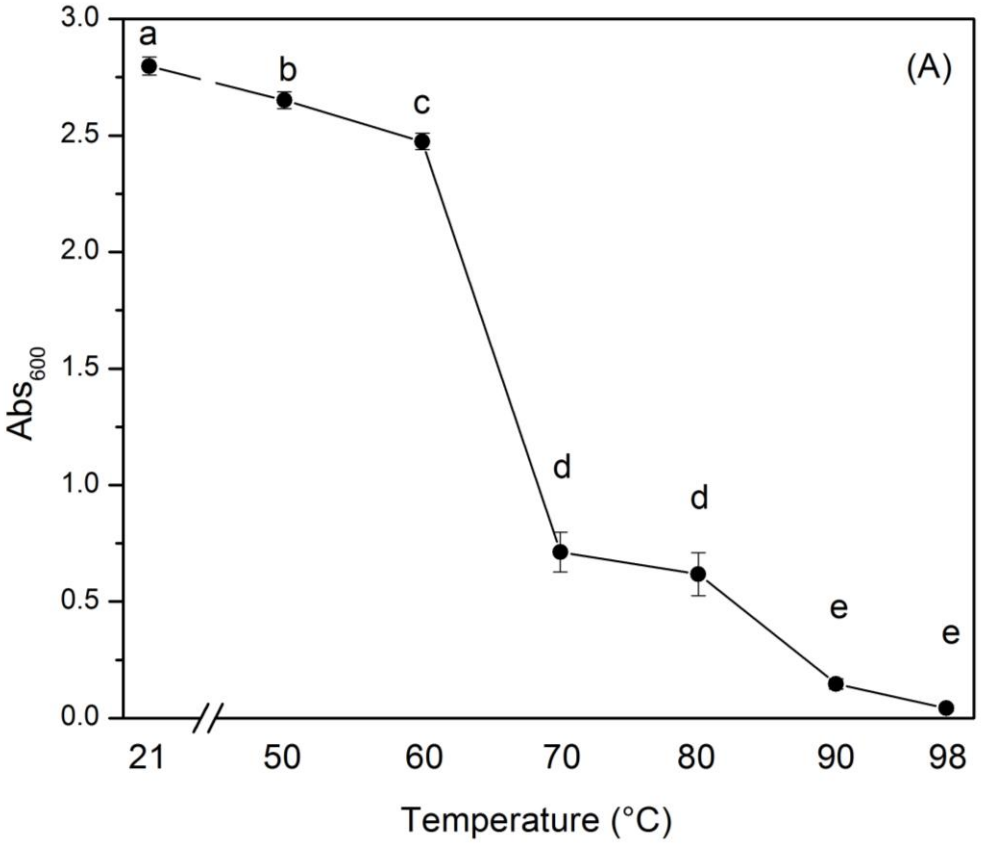


Figure 2.1. continued.

Figure 2.2. Absorbance at 600 nm (Abs_{600}) of emulsions prepared with 2% w/v NaCas, 1% w/v Tween 20 and 1.5% w/v lemon oil without NaCl after heating at (A) 50-98 °C for 1 h or (B) 90 °C up to 3 h followed by quenching in an ice/water bath. Error bars are standard deviations (n = 3). Different letters above symbols indicate significant differences of mean ($P < 0.05$).



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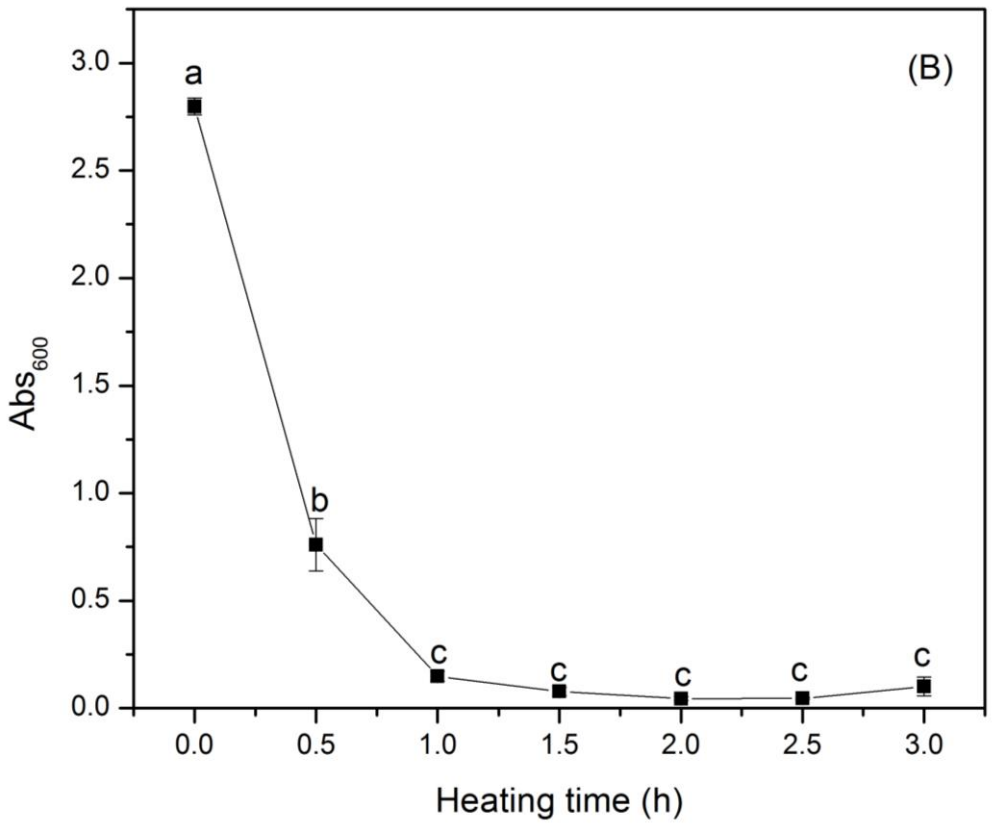

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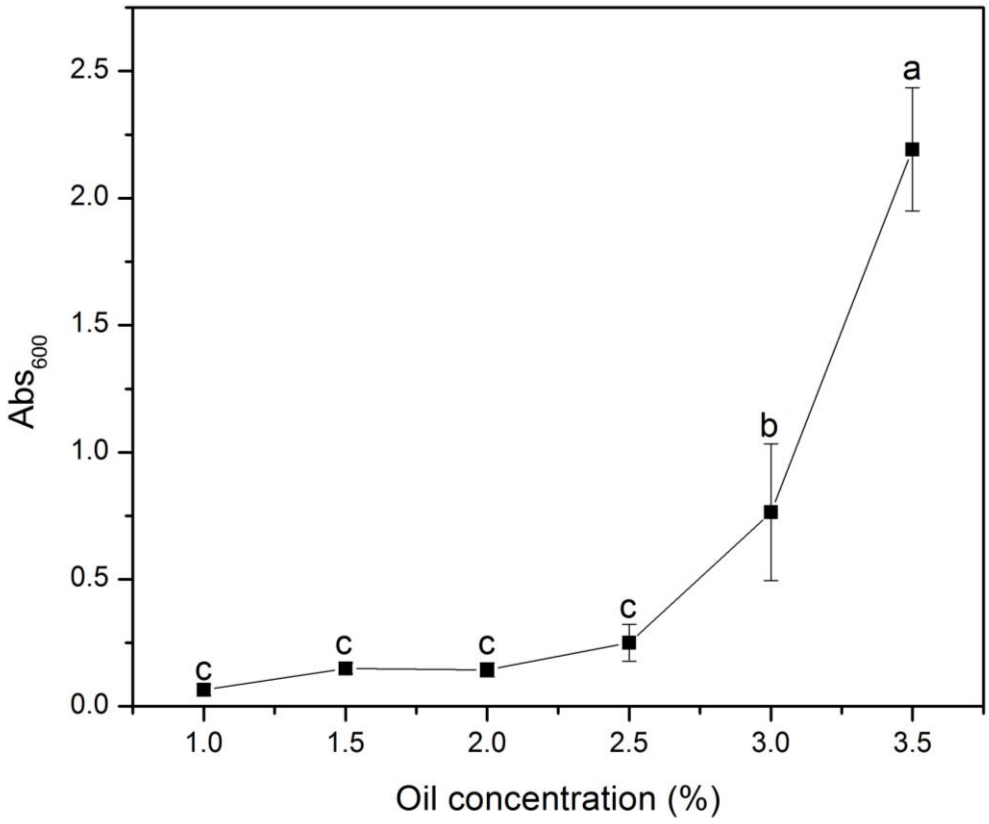


Figure 2.3. Absorbance at 600 nm (Abs_{600}) of samples prepared with 2% w/v NaCas, 1% w/v Tween 20, and 1.0-3.5% w/v lemon oil after heating at 90 °C for 1 h and quenching in an ice/water bath. Error bars are standard deviations (n = 3). Different letters above symbols indicate significant differences of mean ($P < 0.05$).

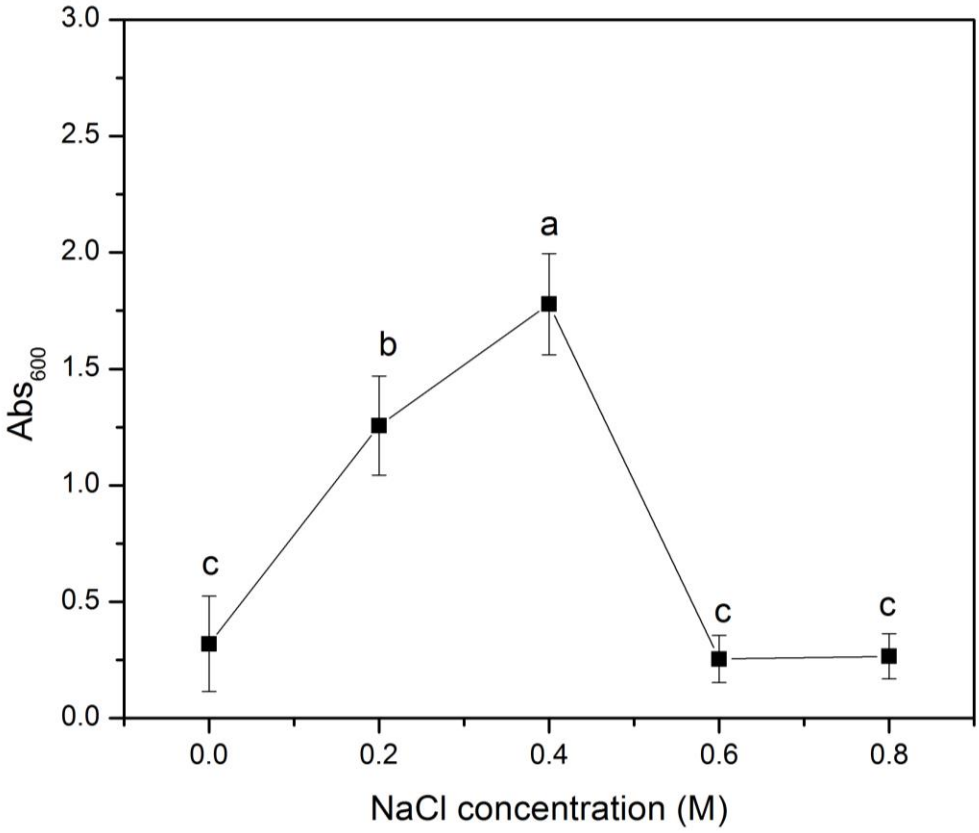
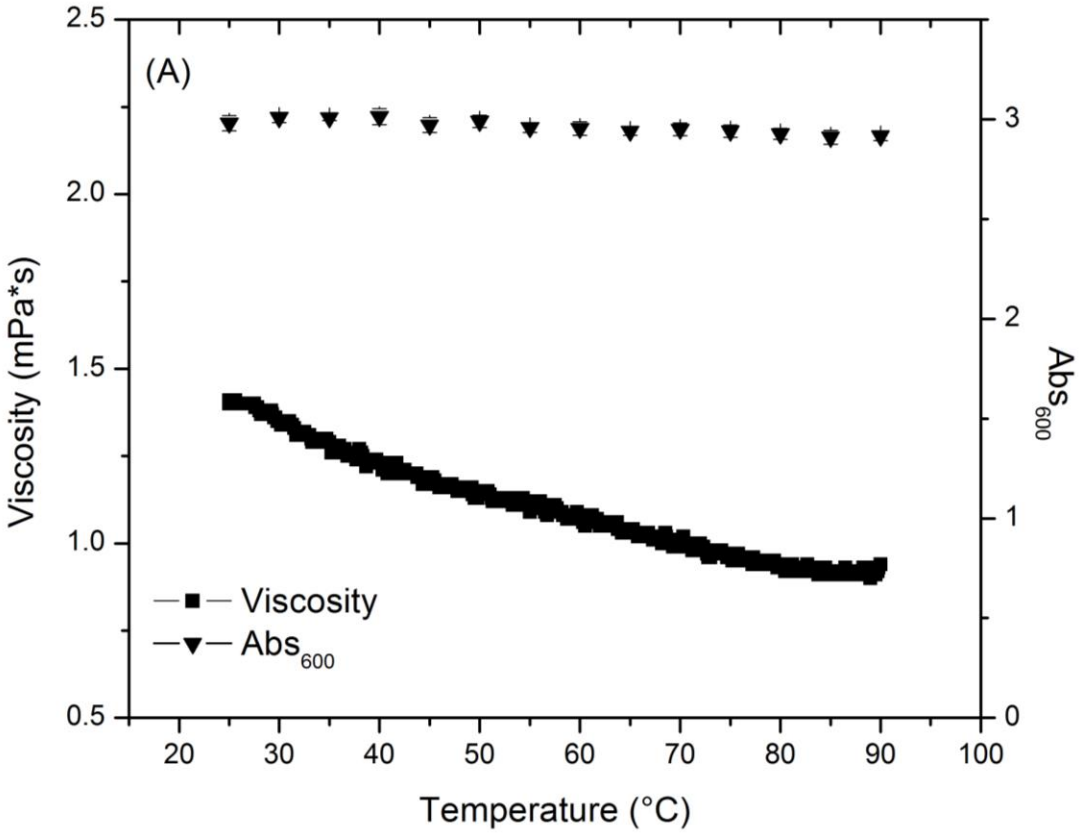


Figure 2.4. Absorbance at 600 nm (Abs_{600}) of emulsions prepared with 2% w/v NaCas, 1% w/v Tween 20, 1.5% w/v lemon oil, and 0-0.8 M NaCl by heating at 90 °C for 1 h, followed by quenching in an ice/water bath. Error bars are standard deviations (n = 3). Different letters above bars/symbols indicate significant differences of mean ($P < 0.05$).

Figure 2.5. Absorbance at 600 nm (Abs_{600}) at 25-90 °C and viscosity during heating from 25 to 90 °C at 1 °C/min of coarse emulsions prepared with (A) 2% w/v lemon oil, 1% Tween 20, and 0 M NaCl; (B) 2% w/v NaCas, 1% Tween 20, and 0 M NaCl; (C) 1% Tween 20, and 0.8 M NaCl; or (D) 2% w/v NaCas, 1% Tween 20, and 0.8 M NaCl. Error bars are standard deviations (n = 3).



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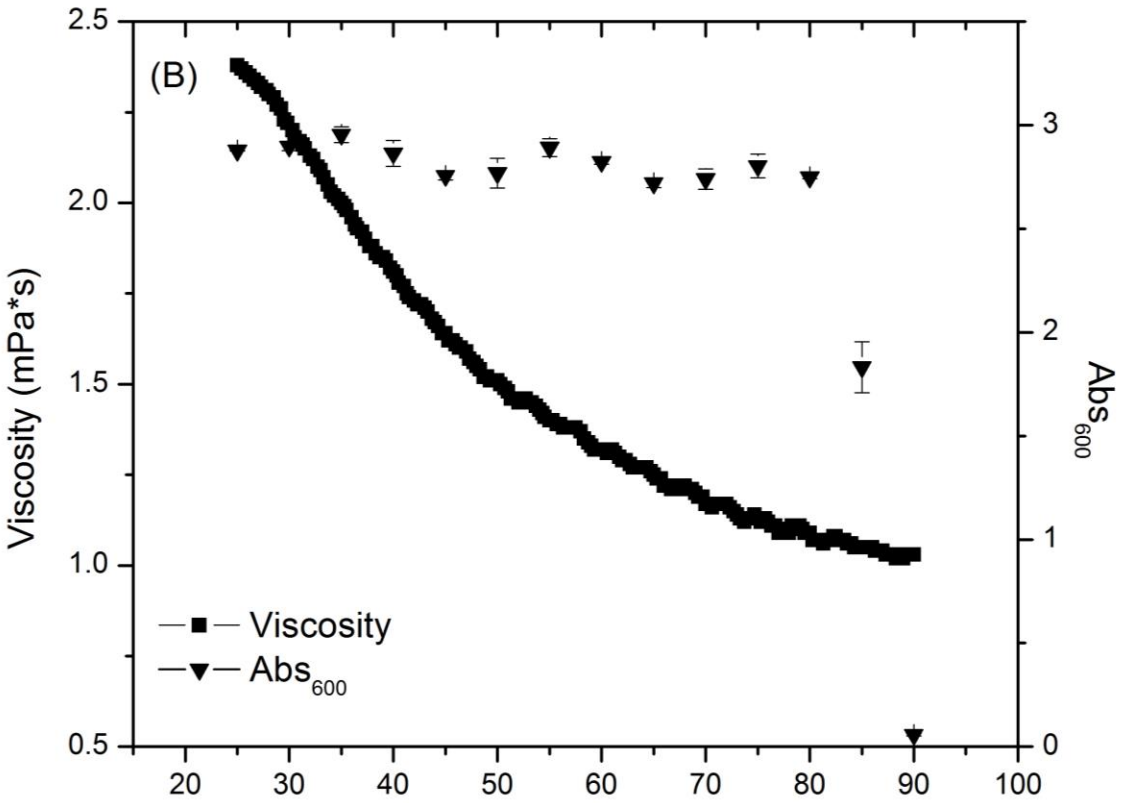

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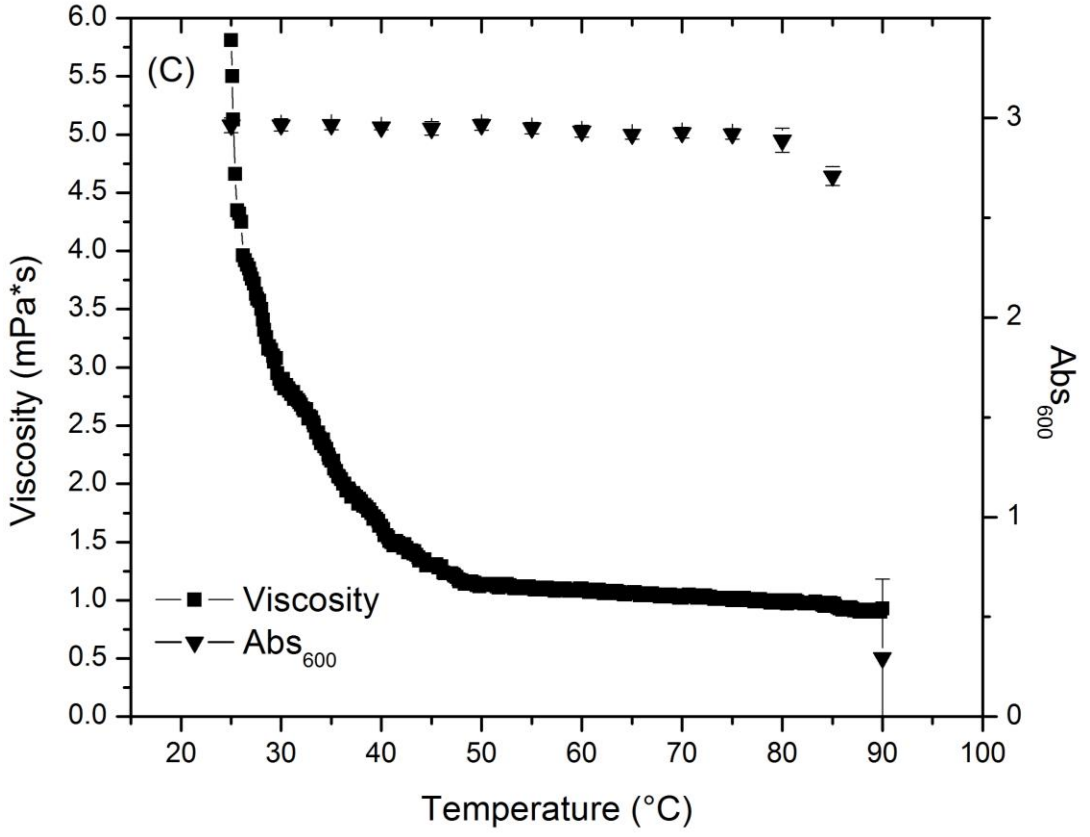

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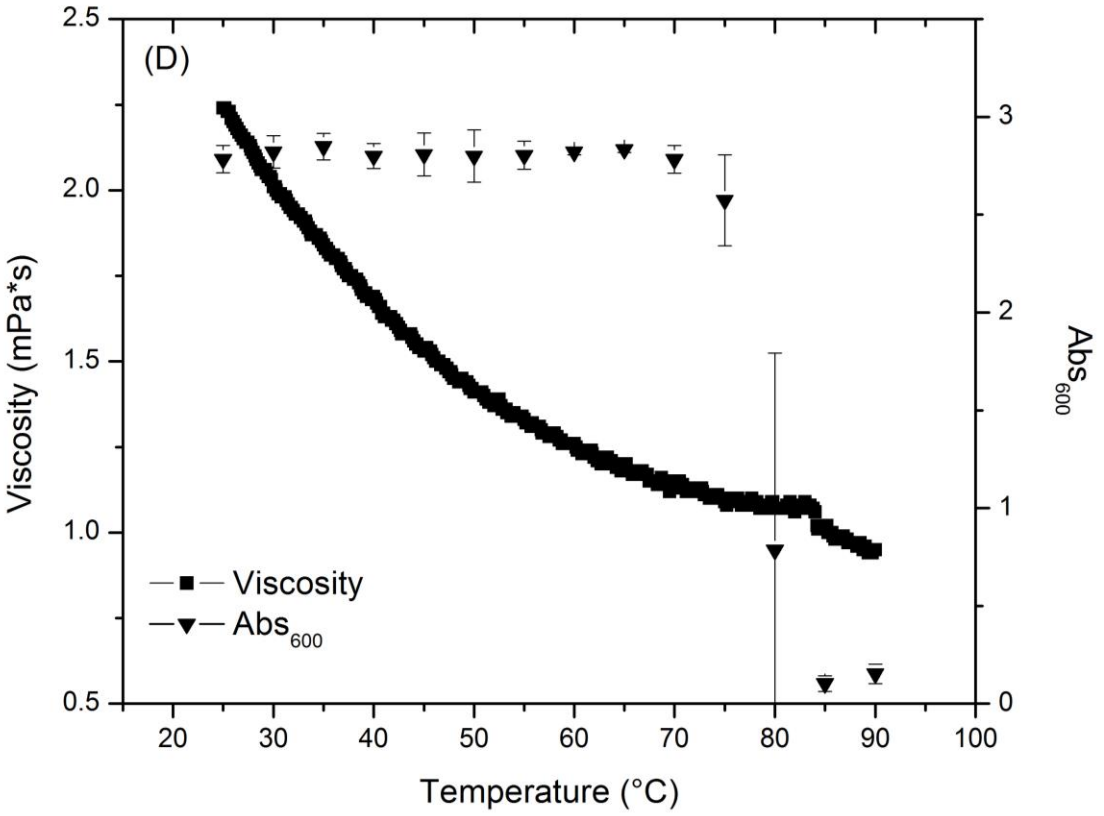

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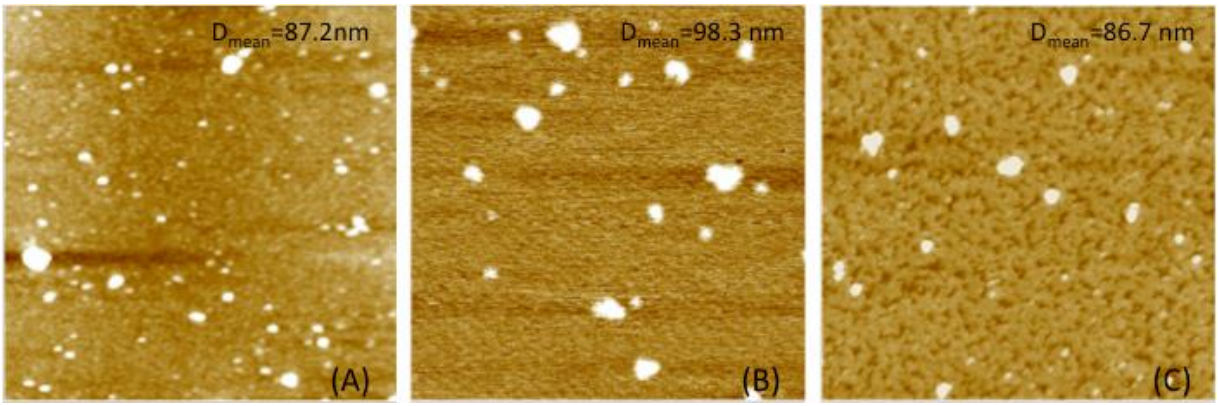

Figure 2.6. AFM images of samples with (A) 2% w/v NaCas, (B) 2% w/v lemon oil emulsion prepared with 1.2% w/v Tween 20, and (C) 2% w/v lemon oil emulsion prepared with 0.2% Tween 20 and 2% NaCas after heating at 90 °C for 1 h and quenching in ice/water bath. Image dimension is 2 μm × 2 μm.

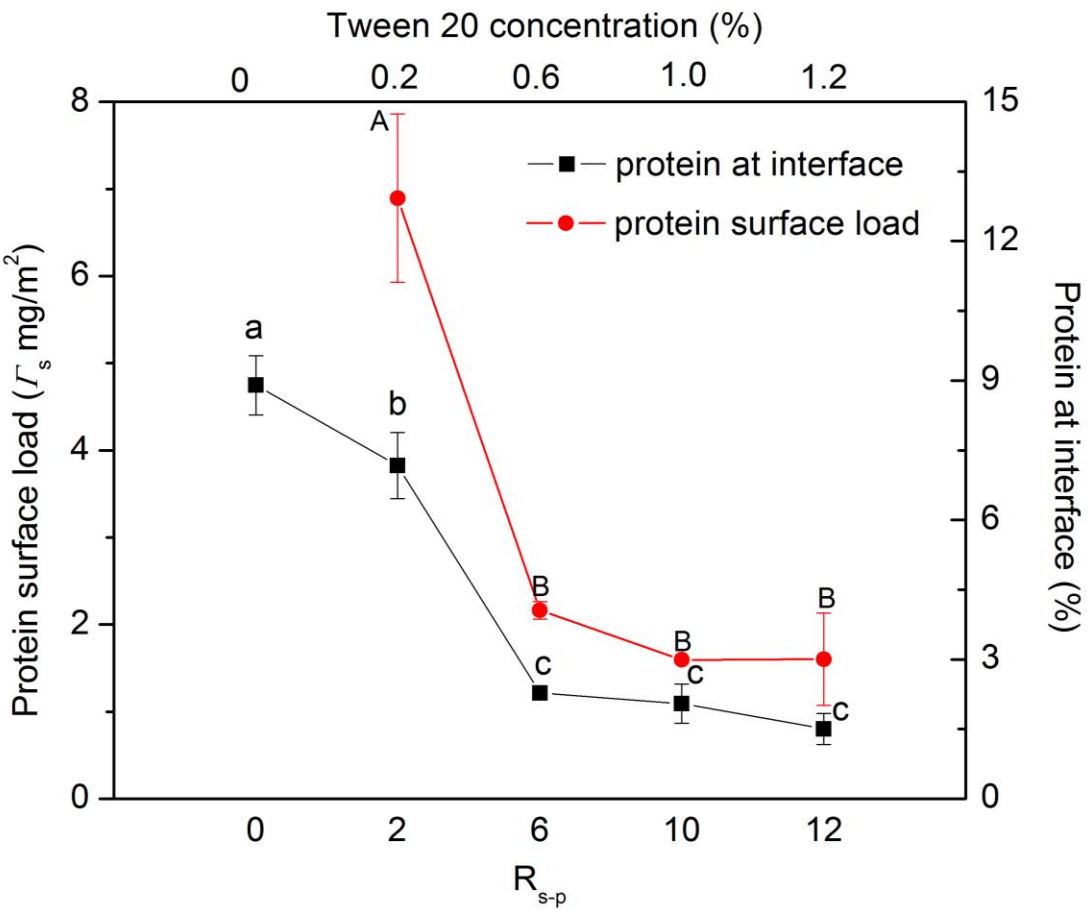

Figure 2.7. Surface load of NaCas and proteins % at interface in emulsion prepared with 1.5% w/v lemon oil and 2% w/v NaCas alone without heating and emulsions prepared with 1.5% w/v lemon oil, 2% w/v NaCas, and 0.2-1.2% w/v Tween 20 by heating at 90 °C for 1 h. Error bars are standard deviations (n = 3). Different letters above symbols indicate significant difference of mean ($P < 0.05$).

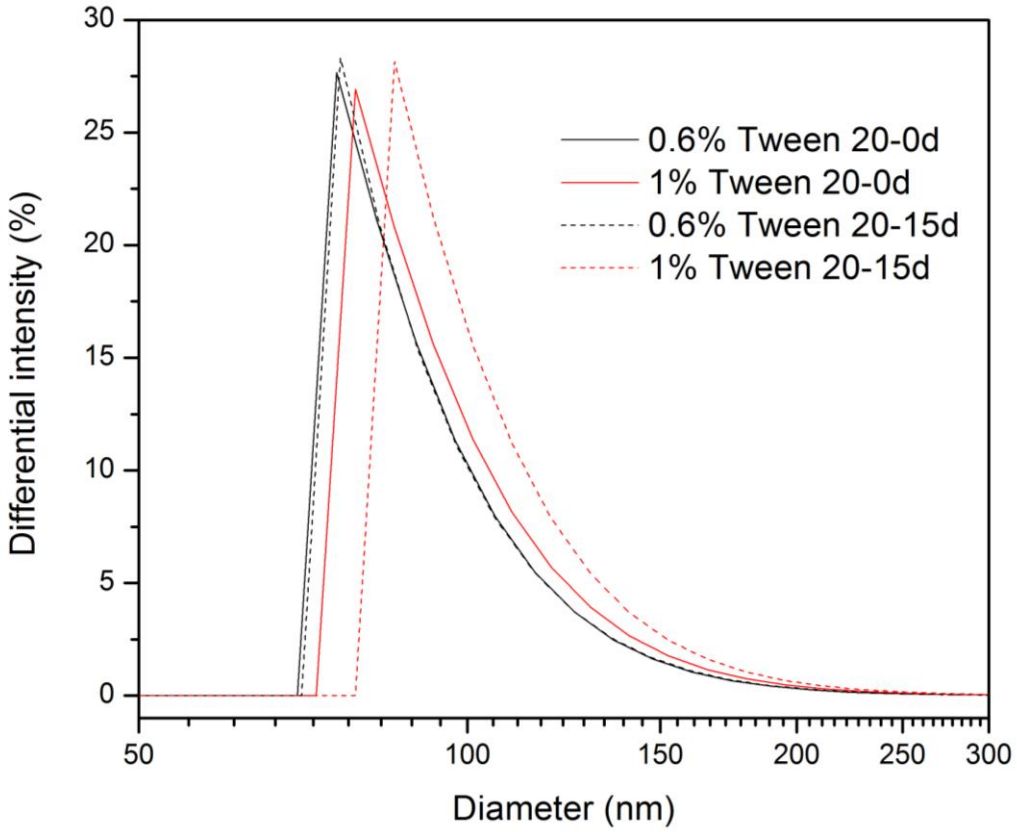

Figure 2.8. Droplet size distributions of emulsions prepared with 1.5% w/v lemon oil, 2% w/v NaCas, and 0.6 or 1% w/v Tween 20 by heating at 90 °C for 1 h, before and after 15-day storage at ambient conditions (21 °C).

CHAPTER 3 FORMATION OF THYMOL NANOEMULSIONS WITH COMBINATIONS OF CASEIN HYDROLYSATES AND SUCROSE STEARATE

Abstract

To improve the stability of emulsions prepared with caseins, casein hydrolysates (CH) were used because of its high diffusion efficiency and improved flexibility. Sucrose stearate (SS) also can principally increase system stability and oil loading of emulsions prepared with caseins. The objective of the present work was to fabricate thymol nanoemulsions using the combinations of pancreatic CH and SS. Casein hydrolyzed for 10 min (10mCH) was used at 2% w/v and mixed with SS to prepare emulsions by homogenization at 10,000 rpm for 2 min and heating at 90 °C for 20 min. Emulsification capacity increased from 1 to 3% thymol with the addition of SS from 0 to 0.25% w/v. Addition of SS from 0.5 to 1.5% w/v improved the emulsion clarity than using 10mCH alone. Turbidity and particle dimension (from ~ 50 nm to ~150 nm) of emulsions significantly increased, when SS content increased from 1.5 to 2% w/v. Heat treatment further improved the emulsion stability because of the improved solubility of SS and the strengthened interactions between 10mCH and SS during heating. With increased thymol concentration, emulsion stability improved. Emulsions prepared with 1.5-3% w/v thymol and 0.25% w/v SS, and emulsions prepared with 3% w/v thymol and 1% w/v SS were stable for over 60-day storage. The strengthened interactions between SS and 10mCH during heating can also enable the stable thymol emulsions at pH5.0.

Keywords: casein hydrolysates, sucrose stearate, nanoemulsions, thymol, stability

3.1. Introduction

Oil-in-water emulsions are commonly used to deliver lipophilic bioactive components such as beta-cartene, [1] curcumin, [2] and essential oils [3,4] in aqueous products. Nanoemulsions have advantages when compared to conventional emulsions. Droplets in nanoemulsions are relatively small (< 200 nm in diameter) and scatter light weakly. The light scattering properties provide opportunities of nanoemulsions for use in transparent products. The small dimension of droplets improves the stability against particle aggregation and gravitational separation.[5]

Nanoemulsions have been studied extensively using non-ionic synthetic surfactants and surface-active biopolymers.[6,7] For food applications, generally-recognized-as-safe (GRAS) emulsifiers such as polysaccharides, proteins, and select sugar-fatty acid esters are preferred to meet regulatory requirements.[8] Whey protein isolate,[9] soy protein isolate,[10] and sodium caseinate (NaCas)[11] are commonly used as emulsifiers in preparation of emulsions.[8] NaCas has excellent emulsification ability due to its copolymer-like flexible structure.[12] However, with the isoelectric point (pI) at pH 4.6, NaCas reaches a net zero charge near pH 5.0 and precipitates due to weakended electrostatic repulsion and thus the stability of prepared emulsions is impaired.[13]

To improve functionalities of proteins, hydrolysis of proteins to smaller molecules is a common approach. [14] The reduced mass of proteins improves the flexibility and possibly the efficiency of emulsifying oils. [15] Hydrolysis of caseins using proteases is the most commonly studied method. [16] Casein hydrolysates (CHs) have been shown to have the improved functional properties than caseins such as inhibition of angiotensin I-converting enzyme (ACE) and antihypertensive properties, [17] hypersensitiveness, [18] antioxidant activity, [19] and antimicrobial activity. [20] Therefore, it is of great interest to prepare nanoemulsions using CH as not only an emulsifier but also a functional ingredient.

However, there are a few limitations of using CHs to prepare nanoemulsions. CHs do not have stearic repulsion provided by NaCas, and the stability of the prepared emulsions is poor, especially nearby pH 4.6 when the net charge of CH becomes zero. [16] To overcome this limitation, nonionic emulsifiers may be used to co-emulsify oil with CH, because the nonionic head groups provide steric hindrance to stabilize emulsions and are insensitive to pH and ionic strength. [21]

The objective of the present work was to fabricate thymol nanoemulsions using CHs and sucrose stearate (SS). SS is produced by esterification of sucrose with edible stearic acid[22] and is GRAS.[23] Because there are eight hydroxyl groups available on one sucrose molecule, different amounts of stearic acid molecules can be grafted on sucrose to form mono-, di- or poly-esters to obtain a wide range of hydrophile-lipophile-balance (HLB) values. [24] Besides, as the confirmation of stearate is temperature-sensitive, [22] the flexibility and emulsification properties of SS can be controlled using temperature as a fabrication factor. Thymol, the major component in the essential oil extracted from *Carum copticum* or *Thymus vulgaris,* [25] was chosen as a model lipophilic compound because it is classified as GRAS by the U.S. Food and Drug Administration.[26] The excellent antioxidation [27] and antimicrobial activities[28,29] of thymol have been widely reported. The solubility of thymol in water is 0.75 mg/mL at 21 °C, and nanoemulsification of thymol by NaCas improves its antimicrobial activity in complex food matrices such as milk.[5]

3.2. Materials and methods

3.2.1. Materials

NaCas, thymol (> 99% purity) and pancreatin (catalogue number P1750) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Propylene glycol (PG) was purchased from Fisher Scientific (Pittsburgh, PA, USA). The commercial SS product (S1170) donated by Mitsubishi Chemical Co. (Tokyo, Japan) contained 55% monoester and 45% di-/poly-esters.

3.2.2. Preparation of casein hydrolysates

NaCas was dissolved at 5% w/v in 200 mL of 0.01 M sodium phosphate-buffered saline (PBS), and pancreatin was dissolved at an enzyme: substrate mass ratio of 1:1000.[14] The mixture pH was adjusted to 8.0 before incubation in a shaking water bath (C 76 classic series, New Brunswick Scientific, NJ, USA) operating at 120 rpm and 37 °C. Fifty milliliters of samples were withdrawn after hydrolysis for 10 min, 1 h, 4 h, and 20 h without pH adjustment. The withdrawn samples were heated at 95 °C for 10 min to inactivate the enzyme. After cooling in an ice/water bath, the samples were centrifuged (SORVALL RC5B Plus centrifuge, DuPont, Wilmington, DE, USA) at 5000 *g* for 20 min to remove insoluble contents. The supernatants

were freeze-dried (VirTis AdVantage Plus EL-85 benchtop freeze dryer, SP Scientific Inc., Gardiner, NY, USA), and the powder obtained was stored at -20 °C for further use.

3.2.3. Preparation of emulsions

Thymol was dissolved in PG at different concentrations. SS was mixed with CH solutions, followed by stirring overnight to ensure compete hydration. The thymol/PG solution was added into the emulsifier dispersion. The original acidity was tested to be about pH 6.8. The mixtures were then homogenized at 10,000 rpm for 2 min using a Cyclone I.Q. microprocessor homogenizer (VirTis Co., Gardiner, NY) to prepare coarse emulsions. The coarse emulsions were heated at 90 °C for 20 min in a water bath, followed by quenching in an ice/water bath under static conditions.

3.2.4. Turbidity measurements

Turbidity of samples was measured for absorbance at 600 nm (Abs_{600}) as an indicator using a UV-Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA, USA). Deionized water was used as a blank.

3.2.5. Particle size determination

The particle size of samples was measured using a Delas Nano particle analyzer (Beckman Coulter, Fullerton, CA). All samples were diluted to an appropriate concentration in deionized water prior to analysis.

3.2.6. Atomic force microscopy (AFM)

The morphology and dimension of particles were characterized using a Multimode VIII microscope (Bruker Corporation, Billerica, MA, USA). Nanoemulsions were diluted to 50 ppm CH using deionized water. Five microlitters of the diluted sample was dropped on a freshly cleaved mica disk and dried under ambient temperature (21 °C) in a chemical fume hood overnight. Images were collected at the tapping mode and analyzed using the instrument software.

3.2.7. Fluorescence spectroscopy

The interactions between SS and CH were studied by fluorescence spectroscopy using a spectrofluorometer (model RF-1501, Shimadzu Corp., Kyoto, Japan). SS was dissolved at 0-4

mg/mL in 0.5 mg/mL aqueous CH (pH 6.8) solutions and incubated at room temperature (21 °C) or 90 °C for 2 h (and cooled down to room temperature in an ice/water bath) before measurement. Fluorescent spectroscopy was carried out with an excitation wavelength of 280 or 295 nm. The slit width for excitation and emission was set at 10 nm.

3.2.8 Stability of dispersions at different pHs

The nanoemulsions prepared with 2% w/v CH, 1% w/v SS and 2% w/v thymol by heating at 90 °C for 20 min were acidified through two approaches. The first group of emulsions were heated to and adjusted at 75 °C to pH 3-6 with 1.0 M and 0.5 M HCl. Another group of nanoemulsions was acidified at room temperature (21 °C) with 1.0 M and 0.5 M HCl. All the acidified samples were diluted to a thymol concentration of 0.1% w/v using deionized water for taking photos.

3.2.9. Zeta (ζ)-Potential measurement

ζ-potential was measured using Malvern Zetasizer Nano ZS (ZEN3600) instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Emulsions were prepared with 2% w/v thymol using 2% w/v CH with 0 or 1% w/v SS and heating at 90 °C for 20 min. Emulsions were acidified at 75 °C to pH 3.0-6.8 with 1.0 M and 0.5 M HCl and then diluted 20 times with deionized water before ζ-potential measurement. Because of the low solubility of SS in water at ambient temperature, emulsions with thymol emulsified by SS only were prepared by dissolving 10% w/v SS and 20% w/v thymol in PG and heating at 75 °C for complete dissolution, followed by mixing 0.375 mL of the PG solution with SS and thymol into 14.625 mL water with stirring at room temperature. After pH adjustment to pH 3.0-6.8, the dispersions were diluted 5 times for ζ-potential measurement.

3.2.10. Statistical analysis

Statistical analyses were performed using the SAS software (version 9.4, SAS Institute, Cary, NC). One-way analysis of variance was carried out. Differences between pairs of means were compared using a Tukey's test. The significance level was set at 0.05.

3.3. Results and discussions

3.3.1. Effects of hydrolysis duration on emulsification ability of hydrolysates

Figure 3.1 shows the Abs_{600} of emulsions prepared using 2% w/v thymol, 0.5% SS w/v and 2% w/v NaCas or CH. Before heating, only the samples prepared with CH hydrolyzed for 10 min (10mCH) resulted in a clear emulsion, which was stable for more than 1 week. Emulsions prepared with NaCas had Abs_{600} that was significantly higher than the 10mCH treatment but was significantly lower than those with CH hydrolyzed for 1-20 h. Emulsions prepared with NaCas or 1-20 h CH all precipitated after one-day storage. After heating at 90 °C for 20 min, Abs_{600} of the emulsion prepared with NaCas significantly reduced, while no influence on other emulsions ($p > 0.05$) was observed. Heated emulsions prepared with NaCas and 10mCH were optically stable for more than 60 days, while other heated emulsions precipitated after one-day storage.

When examined using SDS-PAGE (Figure 3.2), a partial reduction of NaCas molecular weight was observed after 10-min hydrolysis, while other CHs had a substantial reduction of molecular weight, with the 20-h treatment showing no visible bands. Results in Figure 3.1 and Figure 3.2 show that a sufficient molecular weight is needed to maintain good emulsifying properties of CHs. [30] This agrees with a previous study reporting the reduced emulsifying capacity of CHs produced by tryptic hydrolysis at pH 5.5-8.0 for a longer duration.[30] It was also suggested that a minimum length of 20 amino acid residues and distinct zones of hydrophobic and hydrophilic residues are required for peptides with good emulsifying and interface properties.[31] Based on the results in Figure 3.1, the 10mCH was selected for further studies.

3.3.2. Influence of sucrose stearate concentration on nanoemulsion formation

To further study the nanoemulsion formation, the first group of emulsions was prepared with 2% w/v 10mCH, 0-3% w/v thymol, and 0, 0.25, or 1% w/v SS, with and without heat treatment. For emulsions without SS, there was no big difference of Abs_{600} between samples with and without heat treatments when thymol was used at 1% w/v or higher (Figure 3.3A). Without thymol, the heated 10mCH solution had significantly higher Abs_{600} than the unheated sample, which may be caused by the aggregation of hydrophobic hydrolysates during heating. The Abs_{600} of both heated and unheated emulsions was higher at a higher content of thymol, and there was visible oil on the top when thymol concentration was higher than 1% w/v, which indicated the emulsifying capacity of 10mCH is about 1% at the studied conditions. The stability

of heated and unheated emulsions with 1% w/v thymol was quite different. The unheated samples turned turbid and precipitated after one month, while the heated samples with $d_{3,2}$ around 40 nm was stable for two months (Table 3.1; Figure 3.4). This set of results showed that emulsifying thymol as colloidal particles stabilized 10mCH from aggregation, and the stability improved after heat treatment. This improved stability should be attribute to the hydrophobic interactions between 10mCH and thymol. It is reported that complexes of NaCas and thymol can be formed due to strong hydrophobic interactions,[5] which can also be the case for 10mCH due to the preservation of some NaCas structures (Figure 3.2). The 10mCH can be more hydrophobic than NaCas, [16] and the strengthened interactions between the hydrophobic amino acid residuals of CH and thymol can cause the restructuring of complexes to improve the stability of the heated emulsion.[5]

When 0.25% w/v SS was added into the 10mCH-thymol mixture to prepare emulsions, samples had the improved clarity after heat treatment, except the one with 3% w/v thymol showing no significant difference (Figure 3.3B). Because the water solubility of SS is poor at ambient temperature, the reduced turbidity after heating can be attributed to both reduced oil droplet size and reduced amount and size of insoluble SS particles. Heating improves water-solubility of SS and therefore interfacial activity to restructure oil droplets. SS dissolved during heating also can aggregate as smaller particles after cooling to ambient conditions. These two aspects can reduce emulsion turbidity after heating. The improved interfacial activity of SS after heating was evident for the ability to emulsify a higher content of thymol as stable nanoemulsions (Table 3.1). For the heated samples, turbidity increased with the increase of thymol concentration. Because there is no significant difference in particle size of the samples with thymol (Table 3.1), the higher turbidity at a higher thymol concentration resulted from the increased population of oil droplets. At a thymol concentration of 3% w/v, the contribution of turbidity may be similar for oil droplets and SS particles, resulting in no difference in sample turbidity before and after heating. Although there is no difference in the turbidity of emulsions with 3% thymol, the heated sample with volume-area mean diameter ($d_{3,2}$) of 59.02 nm was stable for two months (Table 3.1; Figure 3.4), while the unheated sample precipitated after only one day. The results indicate SS particles are not stable, while the co-adsorption of 10mCH and SS on oil droplets stabilized the emulsions.

When more SS (1%) was added, the Abs_{600} of emulsions was overall lowered after heating, except at 1% w/v thymol concentration (Figure 3.3C) that can be attributed to similar effects of SS and heating at this thymol level. The lowest Abs_{600} was observed 1.5% and 2% w/v thymol after heating. The data further supports the improved emulsifying ability of SS after heating. For samples with 1.5-3% w/v thymol, unheated samples precipitated after one day, while heated samples with $d_{3,2}$ around 45 nm (Table 3.1), were stable at least for one month.

To further investigate how SS concentration influenced the emulsion clarity, another set of emulsions was prepared with 2% w/v 10mCH, 2% w/v thymol and 0-2% w/v SS with and without heating at 90 °C for 20 min. Without heating, the Abs_{600} of emulsions increased with an increase of SS concentration (Figure 3.5A), which likely is due to the increased concentration of insoluble SS particles. In contrast, the heated emulsions had the lowest Abs_{600} at 1% SS (Figure 3.5A) due to the enhanced interfacial activity of SS at an elevated temperature, and the highest Abs_{600} of the 2% w/v SS treatment can be contributed by excess SS. For unheated samples, the storage stability, measured by the appearance of visible precipitation, decreased from less than 30-day to 1-day at 0-2% w/v SS. For heated samples, emulsions prepared with 0-2% w/v SS were all stable for at least one month, and the stability also decreased with an increase of SS concentration.

The $d_{3,2}$ and polydispersity index (PDI) of heated emulsions prepared with 2% w/v 10mCH, 2% w/v thymol and 0-2% w/v SS are shown in Figure 3.5B. The $d_{3,2}$ was around 50 nm and not significantly different among samples prepared with up to 1.5% w/v SS. The $d_{3,2}$ of the emulsion with 2% SS (>150 nm) was significantly larger than other emulsions with less SS, which is due to excess SS on the interface or SS aggregates in the aqueous phase. Free oil was observed on the top of emulsions for those prepared with 2% w/v 10mCH and 0-0.125% w/v SS, which indicates the limited emulsifying capacity of systems with a low SS concentration. When more than 0.25% w/v was used, PDI increased with an increase of SS concentration. This result indicated that various forms of SS may exist at higher SS concentration, likely due to the heterogeneity of SS aggregates.

3.3.3. Effects of thymol concentration on nanoemulsion formation

$d_{3,2}$ of emulsions prepared with 2% w/v 10mCH, 0, 0.25 or 1% w/v SS and 0-3% w/v thymol are shown in (Table 3.1). For freshly made samples, $d_{3,2}$ decreased with the presence of

thymol in the system, and was no significantly different at different thymol concentrations. This is because thymol may enhance the packing of both 10mCH and SS molecules due to hydrophobic attraction, which otherwise would result in bigger particles with a higher thymol concentration.[5] This was further evidenced for the precipitation of treatments with low concentrations of thymol (0 and 1% for 0.25% SS; 0-2% for 1% SS).

3.3.4. Emulsion structures studied by AFM

Emulsions containing 2% w/v thymol prepared with 2% w/v 10mCH and 0, 0.25, and 1% w/v SS were imaged using AFM (Figure 3.6). Particles were discrete and mostly spherical. There was no big difference between average particle dimensions estimated in AFM in different samples, which agreed with $d_{3,2}$ obtained using dynamic light scattering (Figure 3.5B).

3.3.5. Interactions between casein hydrolysate and sucrose stearate

Binding between proteins and small molecules in aqueous solutions can be conveniently investigated using intrinsic fluorescence properties of proteins. For example, fluorescence data showed that thymol formed complexes with caseins by binding with tyrosine (Tyr).[5] Due to composition similarities between caseins and 10mCH, similar interactions are expected between thymol and 10mCH. In the present study, fluorescence spectroscopy was used to further investigate interactions between 0-4 mg/mL SS and 0.5 mg/mL 10mCH before and after heat treatment. As shown in Figure 3.7, fluorescence intensity at an excitation wavelength of both 295 nm and 280 nm significantly increased with an increase of SS concentration, especially after heating, and the increases were greater at 280 nm. At an excitation wavelength of 280 nm, both tryptophan (Trp) and Tyr residues have fluorescence emission, while at 295 nm, it only shows fluorescence emission of Trp residue.[32] The excitation of both Trp and Tyr resides (Figure 3.7) indicates a global change of protein structure.[33] It has been shown that hydrophobic tails of SS molecules prefer to interact with hydrophobic regions of caseins to change the conformation of caseins and therefore the exposure of Trp and Tyr residues,[34] which in turn result in the increase of fluorescence intensity with increasing concentrations of SS. The more significant increase in fluorescence intensity at both excitation wavelengths after heating indicates the strengthened interactions between SS and 10mCH after heating, partially due to the enhanced solubility and therefore mobility and flexibility of SS. In thymol emulsion system, the increased exposure of hydrophobic residues of 10mCH and the flexible conformation of SS during thermal treatments

can provide more binding sites for thymol,[35] which enhances the emulsification properties of 10mCH with and without SS, as presented previously.

3.3.6. Storage stability of emulsions

Destabilization of nanoemulsions may occur through multiple physicochemical phenomena, such as creaming, flocculation, coalescence and Ostwald ripening.[36] A presented previously, 2% w/v 10mCH was able to stabilize only 1% thymol, and an insufficient amount of thymol resulted in precipitation (of likely SS particles) at each combination of 10mCH and SS (Table 3.1). Particle size and PDI are two quantitative parameters comparing physical stability of emulsion systems, and a smaller growth of particle size and PDI after storage indicates a better physical stability.[37] The $d_{3,2}$ and PDI of thymol nanoemulsions before and after storage at room temperature for 60 days are listed in Table 3.1 and Table 3.2, respectively. For samples remaining dispersed after storage, no significant differences of $d_{3,2}$ and PDI were observed (Table 3.1 and Table 3.2). These samples also shown similar appearance and particle size distribution before and after storage (Figure 3.4).

3.3.7. Stability at different pHs

Nanoemulsions prepared with 2% w/v 10mCH and 2% w/v thymol, and with or without 1% w/v SS by heating at 90 °C for 20 min were studied for pH stability tests. Figure 3.8 shows the appearance of the 20-fold diluted emulsions acidified to pH 3.0-6.0 at 21°C and 75°C. When pH was adjusted at the system temperature of 21°C, no precipitation was observed for samples with or without SS at pH 3.0 and pH 6.0, while samples at pH 4.0-5.0 all precipitated immediately. When pH was adjusted at the system temperature of 75 °C, emulsions also precipitated at pH 4.0 and pH 4.6, but samples with SS did not precipitate at pH 3.0, pH 6.0 and pH 5.0, with the pH 3.0 sample showing some turbidity. The results indicate that thymol nanoemulsions prepared with both 10mCH and SS can be stabilized at pH 5.0 by acidification at 75 °C, which is able to dissolve SS based on our observation.

To understand the stabilization mechanism of emulsions acidified at 75 °C, lowest ζ-potential of thymol emulsions prepared with 10mCH, SS or both was measured (Figure 3.9). At pH 4.0 and 4.6, emulsions prepared with 10mCH with and without SS had a low ζ- potential magnitude, corresponding to precipitation (Figure 3.8). The high magnitude of ζ- potential at pH 3.0, 6.0 and 6.8 agreed with the stability of these samples (Figure 3.8). The much higher ζ-

potential of the emulsion prepared with both 10mCH and SS than that of emulsion prepared with only 10mCH at pH 5.0 supported the stability of the former and the instability of the latter (Figure 3.8). Therefore, electrostatic repulsion provided stability of these emulsions against aggregation. Despite steric repulsion can be provided by sucrose head group of SS, it is not evident for emulsions at pH 4.0-5.0 when the ζ- potential magnitude is low. For emulsions prepared with SS only, the ζ- potential was all negative at pH 3.0-6.8, and the smaller magnitude at pH 3.0 and 4.0 is likely due to the protonation of some free stearate molecules.

3.4. Conclusions

In conclusion, several advantages have been shown in the present work for stable and transparent thymol nanoemulsions prepared using combinations of food grade emulsifiers 10mCH and SS. Interactions between SS and 10mCH caused the enhanced exposure of both Trp and Tyr residues to favor the binding with thymol during heating and therefore the improved capability of emulsifying a greater amount of thymol and the enhanced activity in reducing droplet dimension. The reorientation of SS molecules after heating and cooling improved the stability of nanoemulsions at neutral pH and also pH 5.0 that would otherwise be impossible with 10mCH alone. The transparent nanoemulsions had $d_{3,2}$ of around 50 nm and were stable during 60-day storage at room temperature. These transparent stable nanoemulsions prepared with food grade emulsifiers have great potential to incorporate lipophilic bioactive compounds like thymol in functional beverages.

Acknowledgements

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Appendix

Table 3.1. Volume-area mean diameter (nm) of samples prepared with 0-3% w/v thymol, 2% w/v 10-min casein hydrolysate, and 0, 0.25, or 1% w/v sucrose stearate (SS) before and after storage at 21 °C for up to 60 days. *

Thymol (% w/v)	0% SS		0.25% SS		1% SS	
	Day 0	Day 60	Day 0	Day 60	Day 0	Day 60
0	117.81±5.01 ^{bc}	precipitated	75.13±23.19 ^{bcd}	precipitated	132.63±65.17 ^{b}	precipitated
1	40.93±5.72 ^{d}	40.14±0.93 ^{d}	42.28±4.97 ^{d}	precipitated	193.38±29.83 ^{a}	precipitated
2	51.67±6.91 ^{d}	N/A	58.08±3.50 ^{d}	38.67±0.93 ^{d}	41.09±.54 ^{d}	precipitated
3	50.87±5.50 ^{d}	N/A	59.02±5.31 ^{d}	61.57±4.36 ^{cd}	45.94±1.37 ^{d}	43.33±1.56 ^{d}

* Numbers are mean±standard deviation (n=3). Different superscript letters indicate differences in mean ($P< 0.05$). "N/A" indicates samples that have free oil on the top, and "precipitated" indicates samples showing precipitation.

Table 3.2. Polydispersity index of samples prepared with 0-3% w/v thymol, 2% w/v 10-min casein hydrolysate, and 0, 0.25, or 1% w/v sucrose stearate (SS) before and after storage at 21 °C for up to 60 days. *

Thymol (% w/v)	0% SS		0.25% SS		1% SS	
	Day 0	Day 60	Day 0	Day 60	Day 0	Day 60
0	0.240±0.018 ^{c}	precipitated	0.248±0.060 ^{bc}	precipitated	0.290±0.026 ^{abc}	precipitated
1	0.344±0.124 ^{a}	0.246±0.020 ^{bc}	0.333±0.014 ^{a}	precipitated	0.329±0.024 ^{ab}	precipitated
2	0.265±0.023 ^{abc}	N/A	0.242±0.011 ^{c}	0.288±0.050 ^{abc}	0.283±0.018 ^{abc}	precipitated
3	0.291±0.025 ^{abc}	N/A	0.233±0.022 ^{c}	0.243±0.028 ^{c}	0.259±0.014 ^{abc}	0.268±0.034 ^{abc}

* Numbers are mean±standard deviation (n=3). Different superscript letters indicate differences in mean ($P< 0.05$). "N/A" indicates samples that have free oil on the top, and "precipitated" indicates samples showing precipitation.

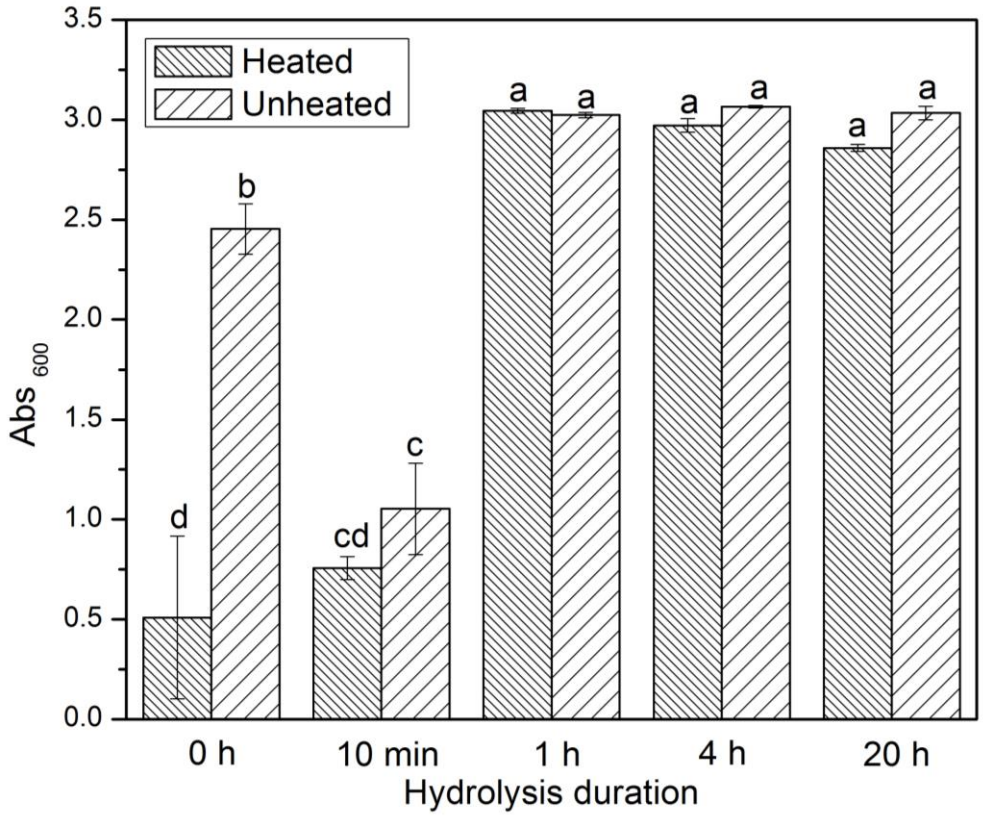


Figure 3.1. Absorbance at 600 nm (Abs_{600}) of emulsions prepared with 2% w/v casein hydrolysates produced by pancreatin hydrolysis for 0-20 h, 0.5% w/v sucrose stearate, and 2% w/v thymol with and without heating at 90 °C for 20 min.

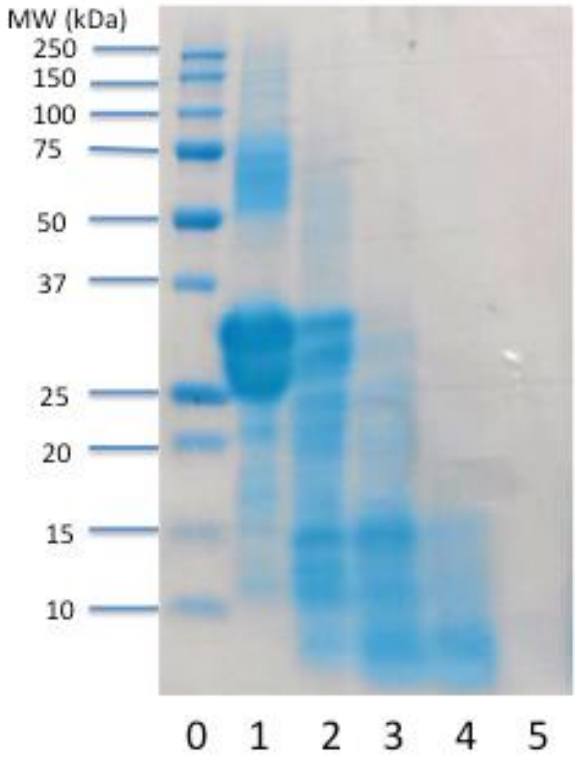


Figure 3.2. SDS-PAGE analysis of protein markers (lane 0), sodium caseinate (lane 1), and its hydrolysates produced by pancreatin hydrolysis for 10 min (lane 2), 1 h (lane 3), 4 h (lane 4), and 20 h (lane 5).

Figure 3.3. Absorbance at 600 nm (Abs_{600}) of emulsions prepared with 0-3% w/v thymol, 2% w/v 10-min casein hydrolysate and (A) 0, (B) 0.25%, or (C) 1% w/v sucrose stearate with and without heating at 90 °C for 20 min.

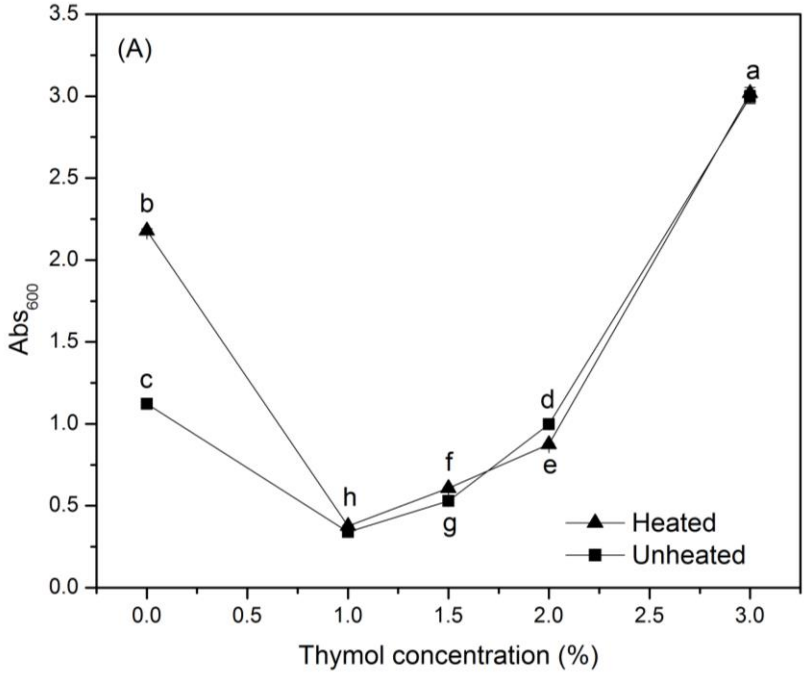
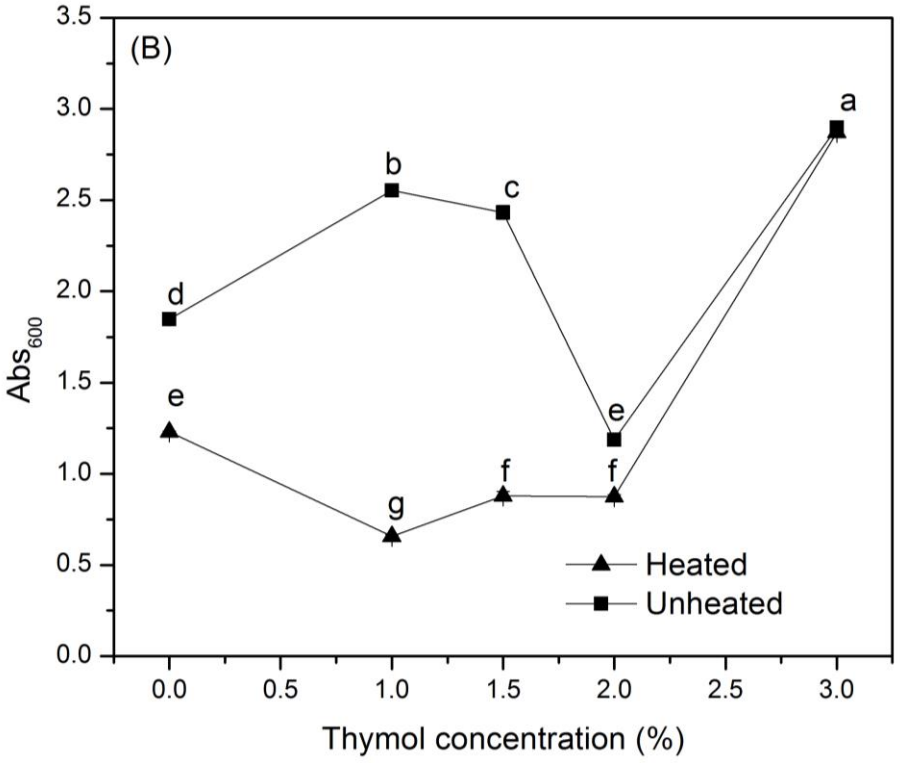


Figure 3.3. continued



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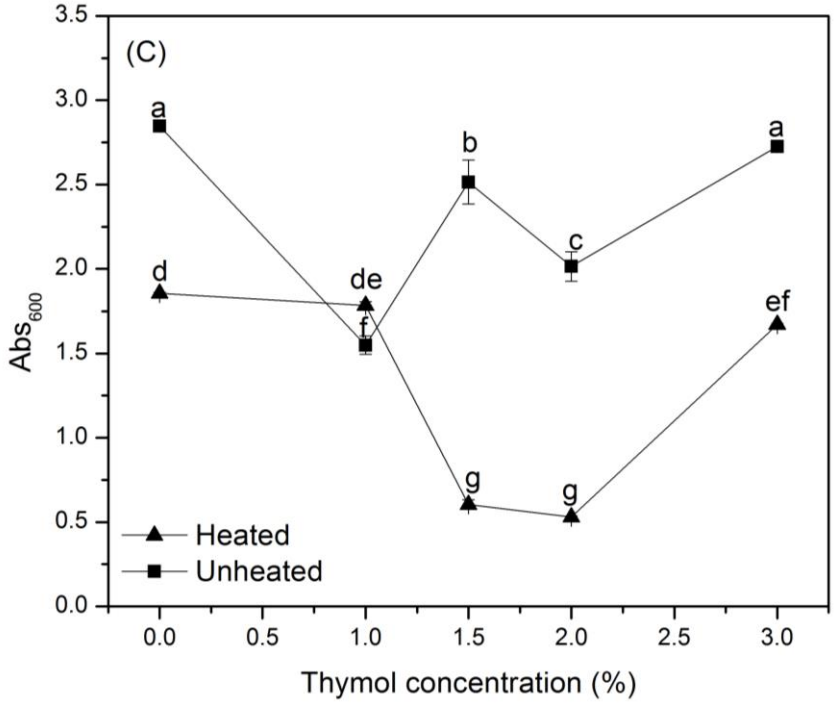
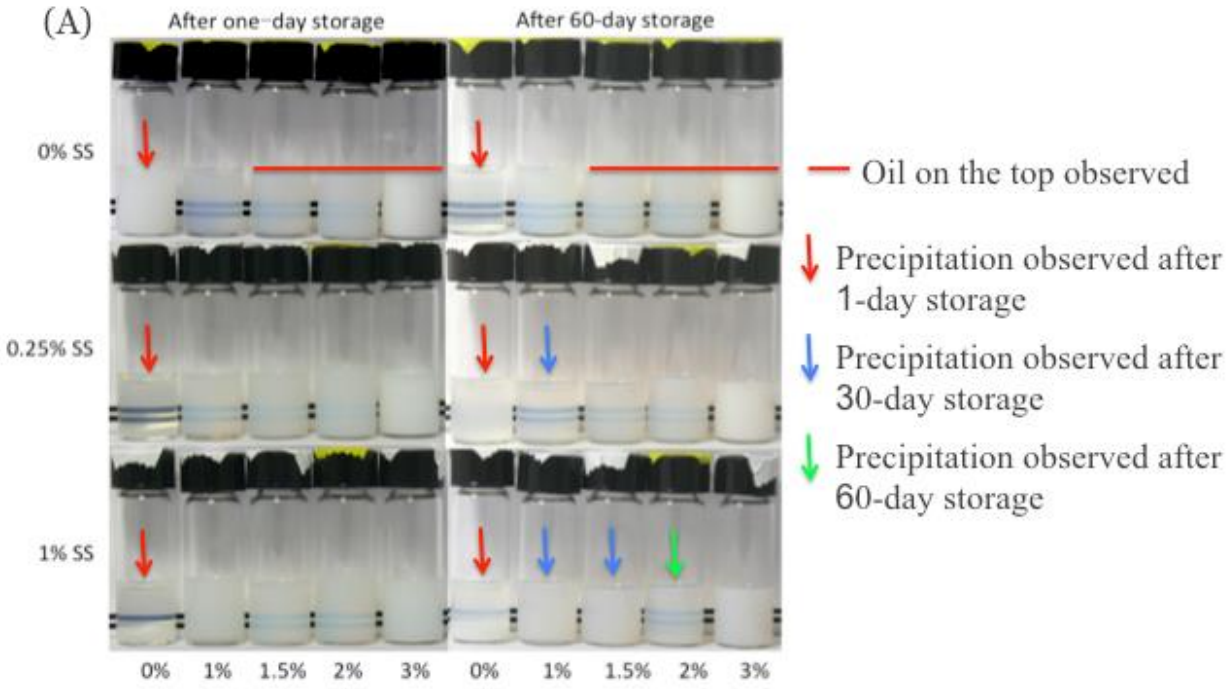


Figure 3.3. continued.

Figure 3.4. (A) Appearance of samples prepared with 0-3% w/v thymol, 0-1% w/v sucrose stearate (SS), and 2% w/v 10-min casein hydrolysate (10mCH) after 1-day and 60-day storage; (B) particle size distributions of samples prepared with 2% w/v 10mCH only and 1% w/v thymol, or with 2% w/v 10mCH, 0.25 or 1% w/v SS, and 3% w/v thymol, before and after 60-day storage.



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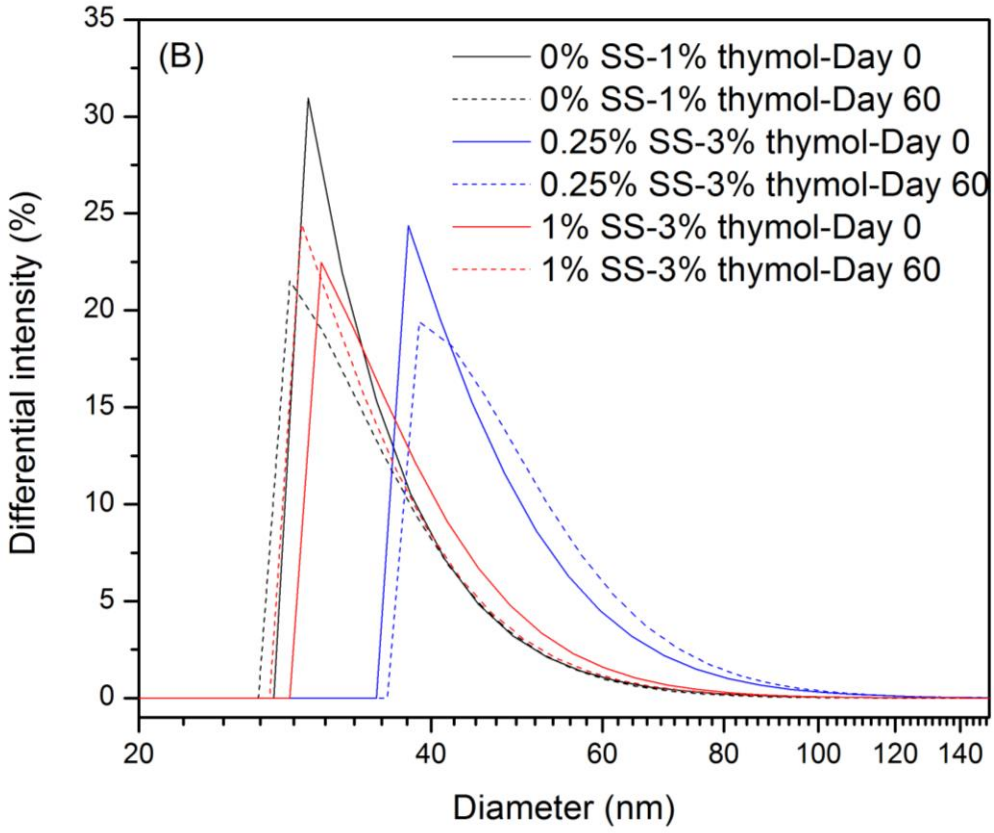
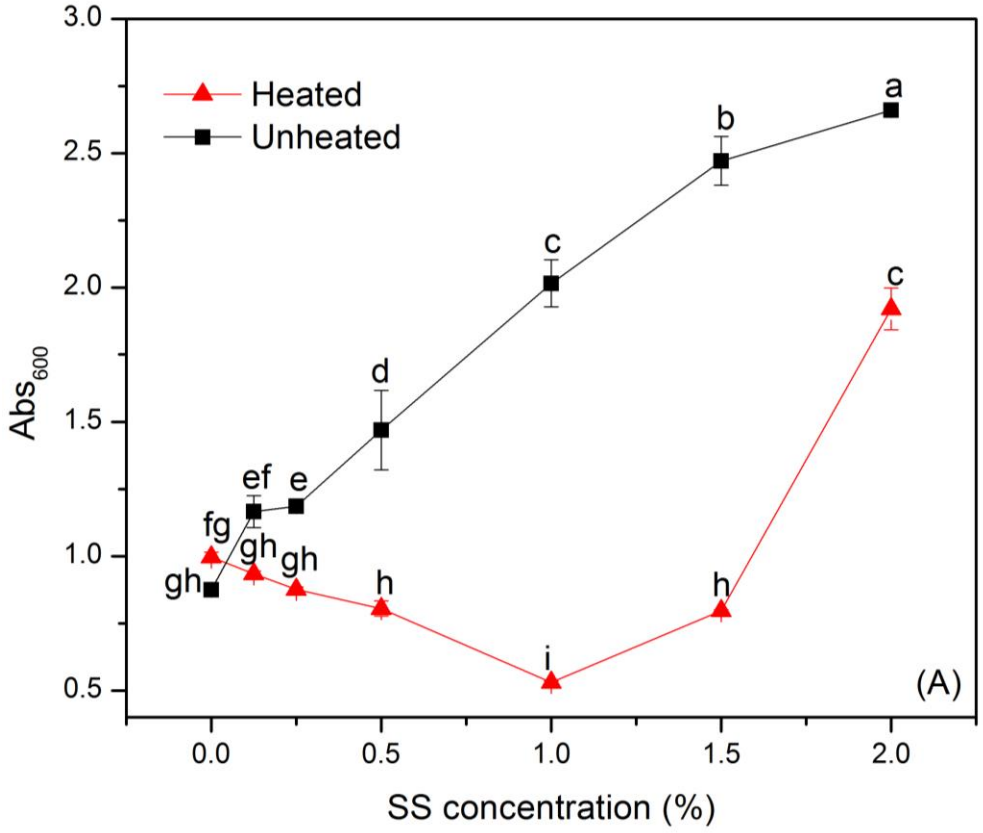


Figure 3.4. continued.

Figure 3.5. (A) Absorbance at 600 nm (Abs_{600}) of freshly-made emulsions prepared with 2% w/v 10-min casein hydrolysate, 2% w/v thymol, and 0-2% w/v sucrose stearate (SS) with and without heating at 90 °C for 20 min. Volume-area mean diameter ($d_{3,2}$) and polydispersity index of heated samples are shown in Figure (B).



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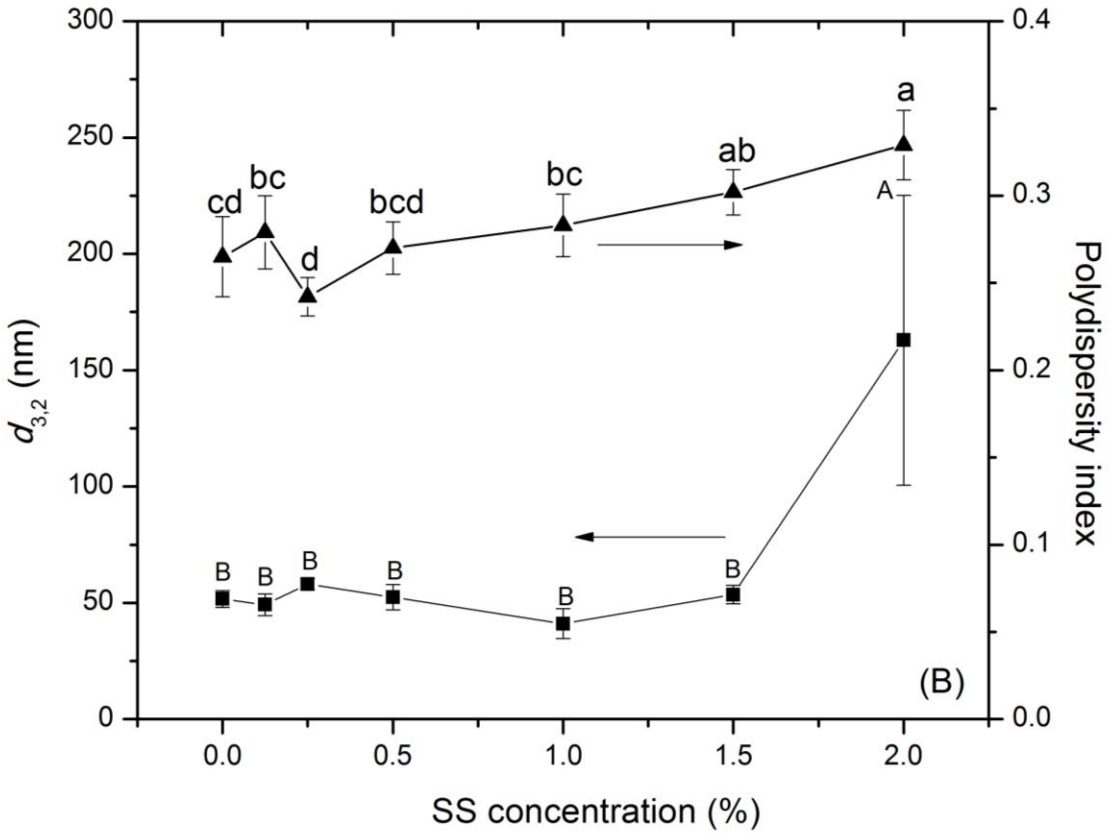

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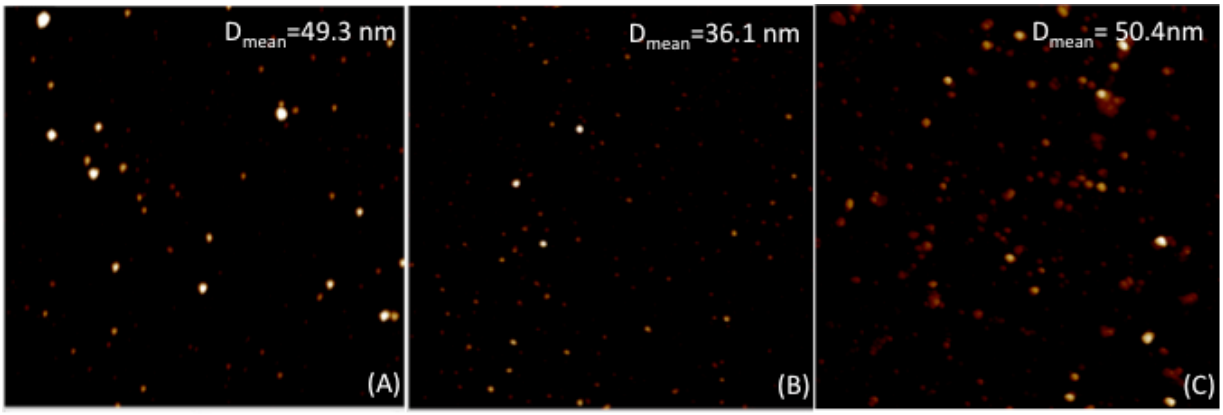
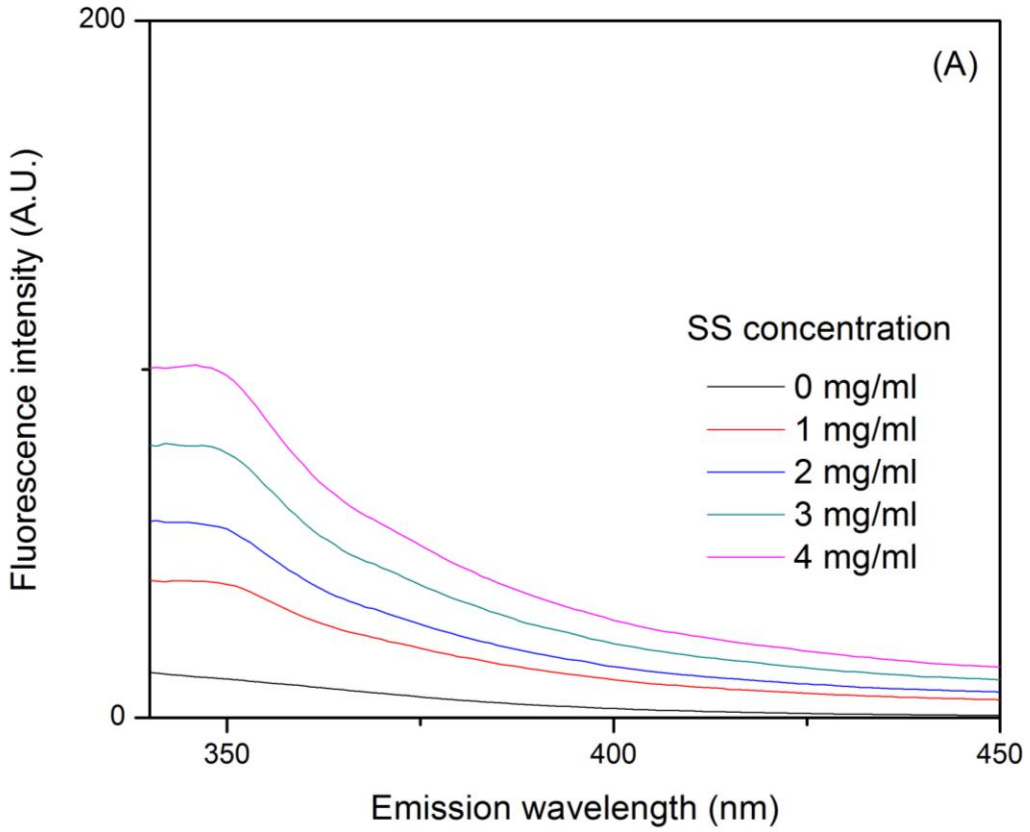


Figure 3.6. AFM topography images of nanoemulsions prepared with 2% w/v 10-min casein hydrolysate, 2% w/v thymol, and (A) 0 or (B) 0.25% w/v (C) 1% w/v sucrose stearate. Image dimension is 2 μm × 2 μm.

Figure 3.7. Fluorescence emission spectra of 0.5 mg/mL 10-min casein hydrolysate aqueous solution at an excitation wavelength of 295 nm for (A) unheated and (B) heated samples, and at excitation wavelength of 280 nm for (C) unheated and (D) heated samples after interacting with 0-4 mg/mL sucrose stearate (SS) at pH 6.8 for 2 h. Unheated samples were incubated at ambient temperature and heated samples were incubated at 90 °C and cooled down to room temperature in an ice/water bath.



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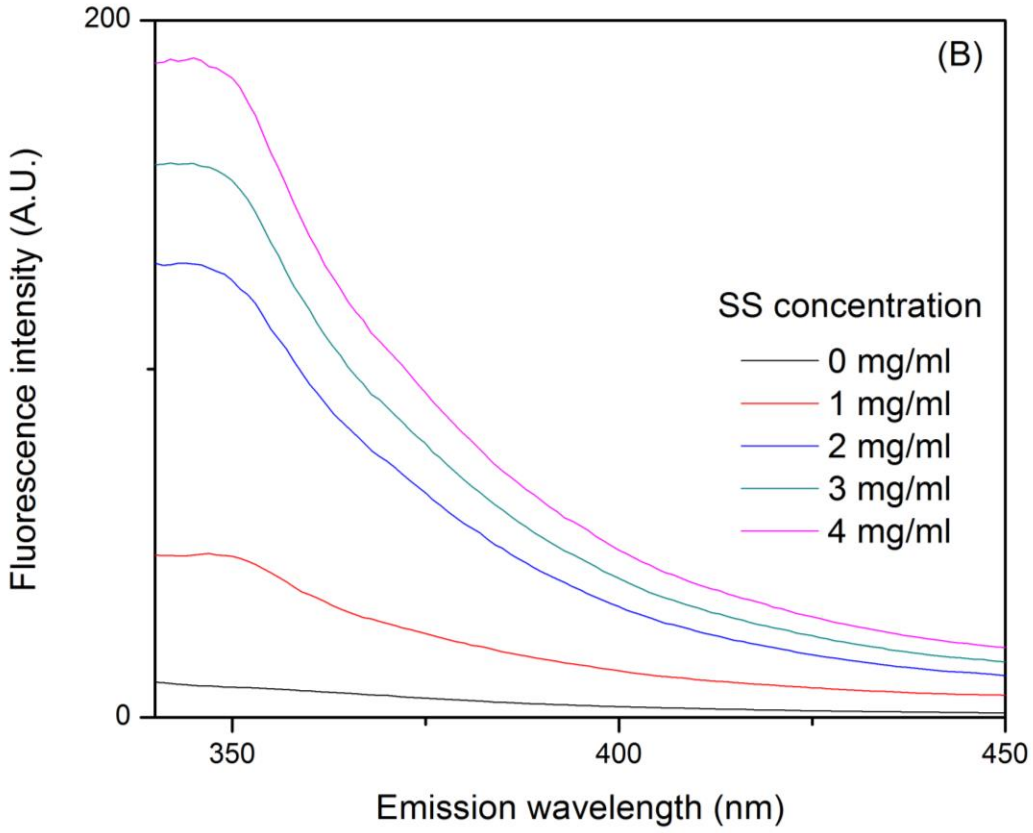

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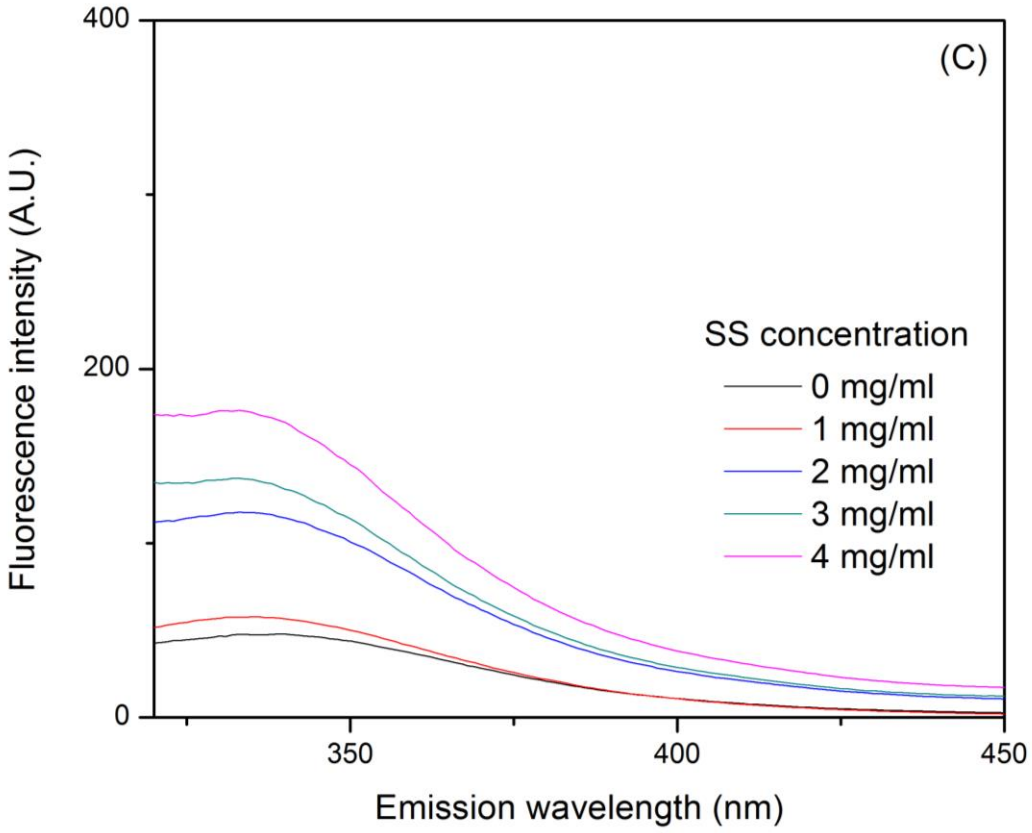

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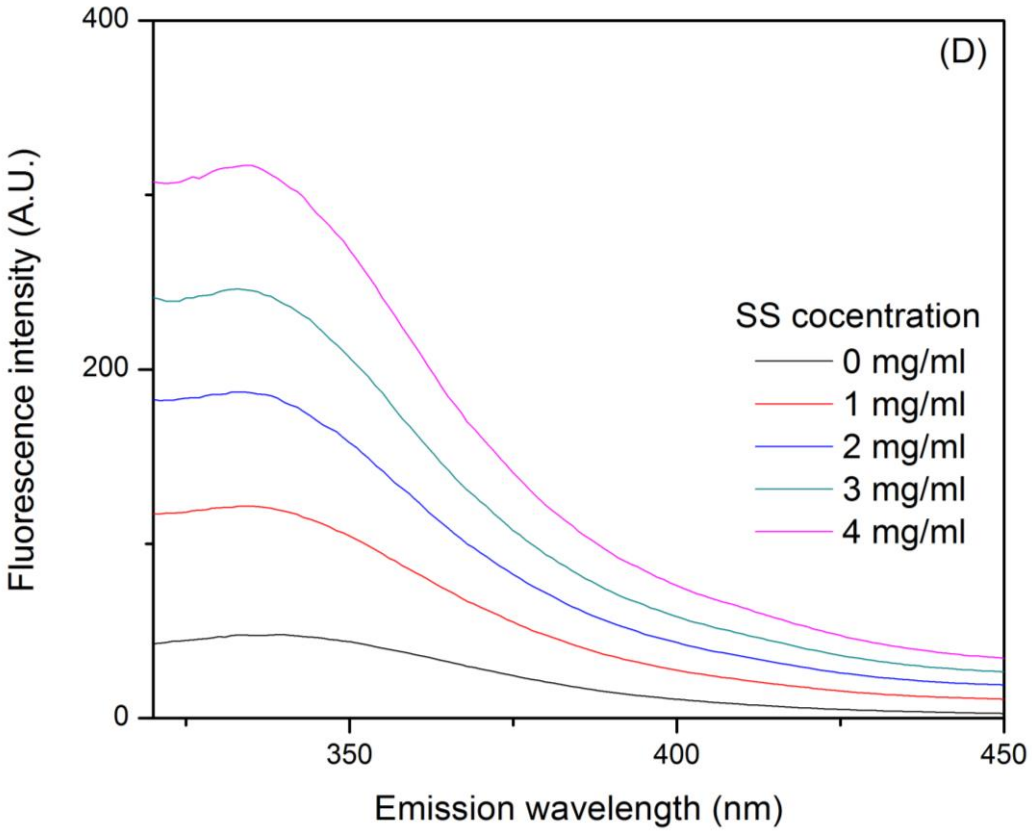

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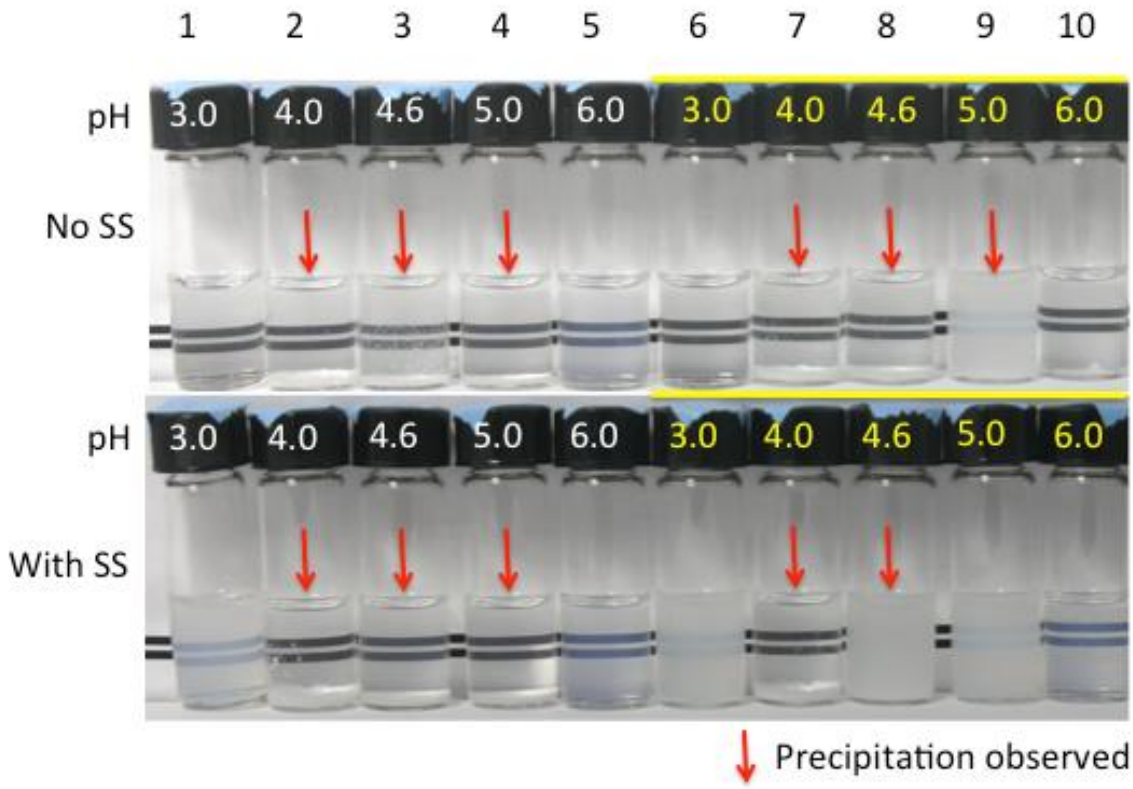

Figure 3.8. Appearance of 20-fold diluted dispersions of nanoemulsion acidified to pH 3.0-6.0 at 21 (vials 1-5) or 75 °C (vials 6-10). Before acidification, nanoemulsions were prepared with 2% w/v thymol, 2% w/v 10mCH and 0 (rop panel) or 1% w/v (bottom panel) sucrose stearate (SS) at pH 6.8 by heating at 90 °C for 20 min and cooling down in an ice/water bath.

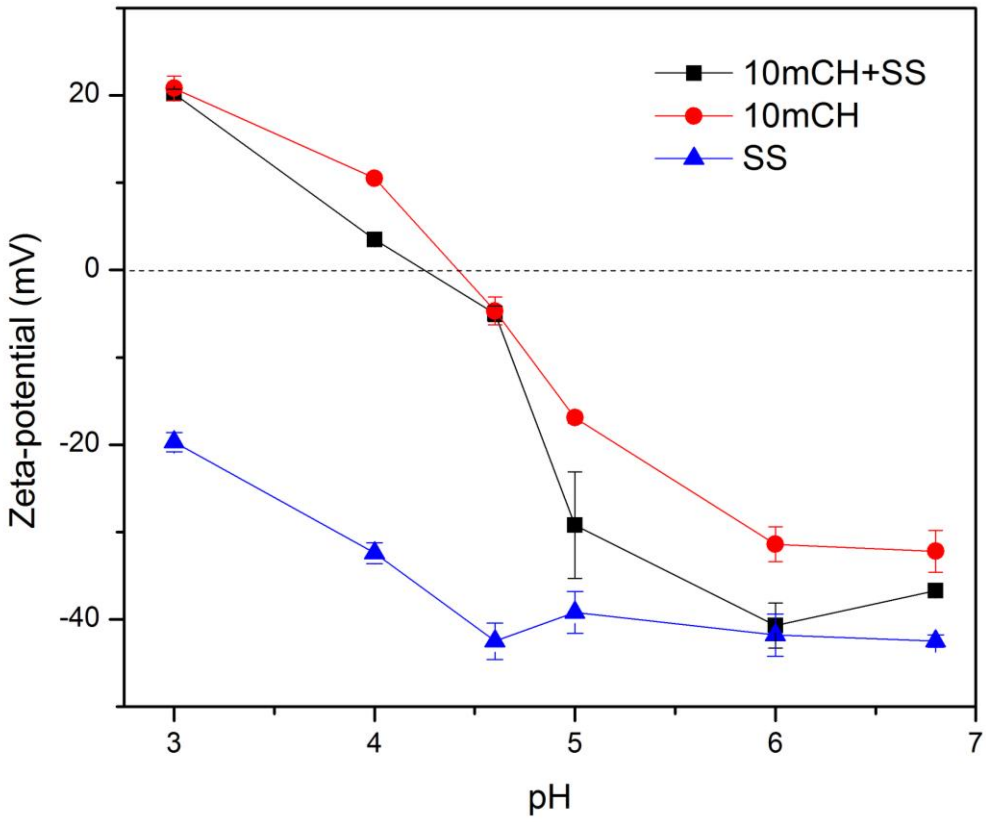

Figure 3.9. Zeta-potential of emulsions with 2% w/v thymol emulsified by 2% w/v 10-min casein hydrolysate (10mCH) alone, 1% w/v sucrose stearate (SS) alone, or both. Emulsions were prepared by heating at 90 °C for 20 min and cooling down in an ice/water bath before acidification to pH 3.0-6.8 at 75 °C. The acidified emulsions were cooled to room temperature and diluted before zeta-potential measurements.

CHAPTER 4 CONCLUSIONS AND FUTURE DIRECTIONS

In this study, two nanoemulsion systems were studied for essential oils. The Tween 20 and sodium caseinate (NaCas) combination was used to form lemon oil nanoemulsions using the phase inversion temperature method. Trubidity and droplet size of the emulsions were significantly reduced using the combinations than using the emulsifiers individually, which significantly decreased the concentration of Tween 20 required for making nanoemulsions. Turbidity and viscosity results showed a PIT between 80 °C and 90 °C. The addition of 0.2-0.4 M NaCl negatively affected the formation of nanoemulsion while 0.6-0.8 M NaCl was in favor of the formation of fine emulsions. Competitive adsorption existed in emulsion systems with both Tween 20 and NaCas. With a higher Tween 20 to NaCas molar ratio (R_{s-p}) from 2 to 6, surface load of NaCas decreased. At R_{s-p} of 6-12, there were no significant changes in the surface load of NaCas. Therefore NaCas co-adsorbed with Tween 20 at all studied conditions. Nanoemulsion with 2% NaCas and 0.4-1.2% Tween 20 were visible stable during short-term storage (15 days), but creaming appeared after longer time storage. This work provided an option to use surface-active biopolymers in the PIT method and decrease the dosage of synthetic surfactants required to make emulsions with small droplets.

To improve the nanoemulsion stability, thymol emulsions were fabricated with casein hydrolysates (CH) and sucrose stearate (SS). These transparent nanoemulsions have mean diameters smaller than 100 nm and some emulsions were stable during two-month storage at ambient temperature. Interactions between SS and CH exposed Trp and Tyr residues, during heating to favor the binding with thymol, resulting in the improved emulsification properties. Reorientation of SS molecules at a high temperature and interactions between SS and CH molecules during heating provided the excellent nanoemulsion stability, even at pH 5. These transparent stable nanoemulsions prepared with food grade emulsifiers have great potential to incorporate lipophilic bioactive compounds like thymol in functional beverages. The overall conclusion of this thesis research is that synergistic properties of caseins and small molecule surfactants can be used to fabricate essential oil nanoemulsions with desired properties.

In the present work, a transparent and stable thymol nanoemulsions were prepared using CH and SS. However, some further studies are needed before the applying these nanoemulsions in the food industry. Firstly, antioxidative activity and antimicrobial activity of CH can be studies, which have potential properties to protect the encapsulated bioactive compounds from oxidation and to increase chemical and microbial shelf-life stability. Secondly, antimicrobial

activity of the nanoemulsion system is to be characterized. Although both CH and SS may have some antimicrobial activity, the antimicrobial activity of thymol in nanoemulsions can also be affected by the interactions between the three ingredients, which in some cases can be reduced when compared to free antimicrobials. Thirdly, as both SS and essential oils can be dissolved in PG at 75 °C as a stock solution, nanoemulsion can be studied using the anti-solvent precipitation method by dispersing the stock solution in water.

VITA

Dan Su was born in Cangzhou, Hebei, China on December 30, 1990. After graduation from Cangxian High School in Cangzhou in 2009, she continued her education at Nanjing Agricultural University and earned a B.E. degree majoring in Food Quality and Safety. In May 2015, she will earn an M.S. degree in Food Science and Technology with a concentration in Food Chemistry from the University of Tennessee, Knoxville.