Emulsifying Properties of Bile Salt and Its Competitive Adsorption with Other Emulsifiers

A thesis presented in partial fulfillment of the requirements for the degree of

Master of Technology
in
Food Technology

at Massey University, Albany,
New Zealand

He Jing

2021

Abstract

Emulsion is a common form of food products that additionally offers food functionalization, such as encapsulation and delivery of vitamins, etc. Emulsion formation and stabilization as well as the rate of digestion has been extensively studied. During the digestion, the presence of emulsifiers in the emulsion may compete for the oil-aqueous interface with bile salts (BS), a bio-surfactant synthesized in a body. Since bile salts play an important role in fat digestion, the aim of this study is to explore the interaction of bile salts with commercially available emulsifiers at the oil-water interface. In order to examine the capability of bile salts to adsorb at the interface, the droplet size, zeta-potential and creaming index of emulsions stabilized by bile salts were investigated and compared with those of WPI and Tween stabilized emulsions. Subsequently, the competitive adsorption of bile salts and its ability to displace WPI and Tween on the oil-water interface was studied in the similar manner, i.e., through investigation of the emulsion properties as well as interfacial tension. The results suggested that BS had better emulsifying ability than WPI and Tween, indicating that it had a better ability to adsorb onto oil-water interfaces. The results for competitive adsorption suggested that BS was able to displace almost entirely the Tween 80 from the oil-water interface but it might not displace the WPI completely. These results complement available information on the fat digestion mechanism and processes.

Acknowledgement

First of all, I would like to express my gratitude to my supervisor, Dr. Nowak Emilia, for her careful guidance and enthusiastic help given to me, during the whole process of finishing my thesis. I am really grateful for the time and effort she spent on reading my thesis and giving me constructive feedback.

Secondly, I would like to thank all the lab technicians, who gave me a lot of help during my lab work. I have learned professional knowledge and mastered solid skills from them, which made my thesis better.

Finally, I would like to thank my family for their love and encouragement during this period. It is due to their support and care that I was able to study at ease and complete this work.

Table of Contents

List of Table

List of Figures

Chapter 1. Introduction

In recent years, the production of low-fat or fat-free foods has become a major innovative task for the food industry, since consumers have an increasing appetite for healthier foods. However, many foods with a low fat content are often perceived by consumers as having lower organoleptic qualities, making the task of producing such foods even more difficult (Maldonado-Valderrama et al., 2011). In order to improve the nutritional value of products while maintaining their sensory qualities, food scientists are working to find alternative strategies for producing healthier products other than reducing fat content. One idea is to control the digestion of fat molecules as they pass through the intestine, thus reducing the fat intake by body (Tiss et al., 2001).

Fat can be consumed by body in various forms, of which oil-in-water emulsion is a very common form (e.g., milk and salad dressings) where lipids are consumed as droplets contained in continuous water-phase (Dapčević Hadnađev et al., 2013). In oil-in-water emulsions, the surface of fat droplets is coated by surface-active molecules. These amphiphilic molecules are known as emulsifiers; their adsorption on the droplet surface is not only an important step in the formation of emulsions, but also has a significant effect on the rate of fat digestion (McClements & Gumus, 2016). Therefore, it is necessary to focus on the surface-active molecules adsorbed on the droplet surface and to select suitable surface-active substance as emulsifiers to control fat digestion.

Bile salt (BS) is a bio-surfactant that plays a vital role in fat digestion process, during which lipase adsorbs to the lipid surface to facilitate the hydrolysis of triglycerides and the release of monoglycerides and fatty acids (Sarkar et al., 2016). In this process, BS adsorbs on the lipid surface and removes other surface active molecules (especially proteins), allowing lipolysis to occur as lipase adsorbs on the lipid surface (Holm et al., 2013). This means that the ability of BS to displace surface-active substances adsorbed on lipid surface has an important effect on the digestion process (Maldonado-Valderrama et al., 2011). Therefore, investigating the ability of BS to displace surfactant from surface of fat droplets provides important information for the control of lipid digestion. Figure 1 shows the interfacial action of bile salt during digestion and lipolysis.

Figure 1. Bile salt in digestion and transport of lipids (Macierzanka et al.,2019)

In order to regulate fat digestion by manipulating interfacial structure, the aim of this study is to investigate the interaction of bile salts with commonly used emulsifiers in emulsions. Considering that emulsifying ability of bile salt and the performance of emulsion it formed can indicate its ability to adsorb onto the oil-water interface (Bai et al., 2017), this study characterized the emulsifying properties (e.g., droplet size and distribution) of bile salt in comparison to two common emulsifiers (Whey protein and Tween). Moreover, the competitive adsorption of these three surface-active substances was investigated through mixing the emulsions with bile salts and through the interfacial tension measurements, in order to understand their affinity to the oil-water interface.

Chapter 2. Literature Review

2.1 Bile Salt

2.1.1 Importance of BS in Digestion

Bile salt is a kind of steroidal detergent bio-synthesized from cholesterol in the liver and stored in the gall bladder (Maldonado-Valderrama et al., 2011). They are essential for the fat digestion in the gastrointestinal tract (GIT), largely due to their ability to adsorb onto the lipid surface (Macierzanka et al.,2019). As the fat in food is digested, BS competes with other surfactant molecules to displace them from the oil-water interface in preparation for the hydrolysis of triglycerides catalysed by lipase (Sarkar et al., 2016). Eventually the fatty acids released by lipolysis are transported as part of the bile salt micelles to the intestinal wall for absorption (Maldonado-Valderrama et al., 2014).

2.1.2 Molecular Structure of BS and Its Synthesis

The function of BS in the human body originates from their complex and specific structure. As a special surfactant it differs from both the classical head-and-tail surfactants whose molecular structures have been extensively studied and from other amphiphilic molecules in the GIT (Euston et al., 2011). Unlike classical amphiphilic molecules, BS exhibit planar polarity, but they do not have a well-defined hydrophilic head and hydrophobic tail (Maldonado-Valderrama et al., 2008). BS molecule is made up of a rigid sterol core and a flexible fatty tail, where the steroid backbone contains a five-membered ring and three six-membered rings (O'connor & Wallace, 1984). As shown in Figure 2.1, the hydrophilic hydroxyl groups are located on the concave side of the sterol skeleton, while the convex side of the steroid ring system is hydrophobic. This planar polarity allows the BS to have a high affinity to the hydrophobic phase and the ability to self-assemble into micelles in solution (Madenci & Egelhaaf, 2010).

There are different BS within the gastrointestinal tract, due to the different pathways by which they are synthesized. Chenodeoxycholic acid and cholic acid are two primary bile acids that are formed in the liver by the enzymatic oxidation of cholesterol (Euston et al., 2013). Some of these two bile acids are dehydroxylated in the intestine by intestinal bacteria to form two secondary bile acids: lithophanic and deoxycholic acids. These four bile acids may be taken back to the liver via the bloodstream. In the liver, these molecules may be bound to taurine or glycine via an amino group located on the concave side of the steroid backbone (as shown in Figure 2.1). Ultimately, eight bile acids may be present in the human gastrointestinal tract, which are usually found in the form of sodium salts and are collectively referred to as bile salts (Euston et al., 2013).

The molecular structures of bile salt have important impacts on their surface activity and biological function. The position of the methyl substituent, the number and

position of hydroxyl groups on the sterol backbone, and the type of amino acid, all have an influence on the physicochemical properties of the bile salts (di Gregorio et al., 2018). The molecular structures of four different bile salt: sodium cholate (NaC), sodium deoxycholate (NaDC), sodium taurcholate (NaTC), and sodium glycodeoxycholate (NaGDC) are given to illustrate the BS structure (Figure 2.2) (Euston et al., 2013). It can be seen that NaC and NaDC have three and two ring hydroxyls respectively, and that neither of them contains conjugated amino acid residues. On the other hand, NaTC and NaGDC have three and two ring hydroxyl groups respectively, and contain conjugated taurine and glycine residues, respectively.

Figure 2.1 The structure of bile salts (Maldonado-Valderrama et al., 2011)

Figure 2.2 Molecular structure of the four bile salts: (a) NaC, (b) NaDC, (c) NaTC, and (d) NaGDC (Euston et al., 2013)

2.1.3 Competitive Adsorption of BS with Other Surface-active Substance at Oil-water Interface

Due to the unique surface property of BS and its important role in digestion, food scientists have investigated its surface properties and the competitive adsorption with other surface active molecules. Euston et al. (2013) investigated the competitive adsorption of four kinds of bile salts with casein and whey protein in oil-in-water emulsion. They measured and compared the coverage of proteins and bile salts on the droplet surface by colorimetric methods. The results showed that all bile salts (NaTC, NaC, NaDC, and NaGDC) displaced proteins from the surface of the emulsion droplets, but to different extents, and to a lesser extent for sodium caseinate than for whey protein. The study highlighted that both the type of protein and the structure of bile salt could influence the displacement of protein by BS from the oil-water interface. Sarkar et al. (2010) researched the impact of added bile salt on milk protein emulsions by monitoring confocal microstructure, zeta potential and droplet size under simulated intestinal conditions. They concluded that after adding BS, protein was pushed into the continuous phase from the droplet surface, while BS were gradually adsorbed onto due to their high surface activity. This study also mentioned that the interaction of bile salt with the interface stabilized by protein was significantly influenced by the type and charge of protein and the concentration of bile salt added into emulsion. Maldonado-Valderrama et al. (2011) investigated the mechanism of BS addition influencing the β-lactoglobulin interfacial membrane. They observed the changes on the interface upon addition of BS using Atomic Force Microscopy. They suggested that the bile salt penetrates, weakens and disrupts the β-lactoglobulin interfacial network by an orogenic mechanism (involving the growth of bile salt structural domains), and finally displaces protein. In addition, the effect of bile salt addition on non-protein stabilized-interfaces has also been reported by Bellesi et al. (2014).

2.2 Emulsion

Emulsions are made up of at least two immiscible phases (usually oil and water), with one liquid dispersed in the form of small droplets in another one (Lam & Nickerson, 2013). In the food industry many products are produced and digested in the form of emulsion, which are often described as oil-in-water type (e.g., salad dressings, creams, milk, etc.) or water-in-oil type (e.g., butter and margarine). In addition to these, there are other multiple emulsions such as water-in-oil-in-water (W/O/W) type and oil-in-water-in-oil (O/W/O) type, which are often applied in functional foods and targeted pharmaceuticals (McClements & Gumus, 2016). Figure 2.3 shows different type of emulsions. Food products are produced in the form of emulsion to improve their sensory properties such as colour and texture, and also to enhance their nutritional value (Lam & Nickerson, 2013). For example, oil droplets can act as delivery systems in emulsions to encapsulate and release fat-soluble nutrients (McClements & Gumus, 2016). Emulsions are thermodynamically unstable systems and may encounter many environmental stresses during processing. The use of

emulsifiers to promote emulsion formation and stabilization is therefore essential in order to improve the functional properties of products and extend their shelf life.

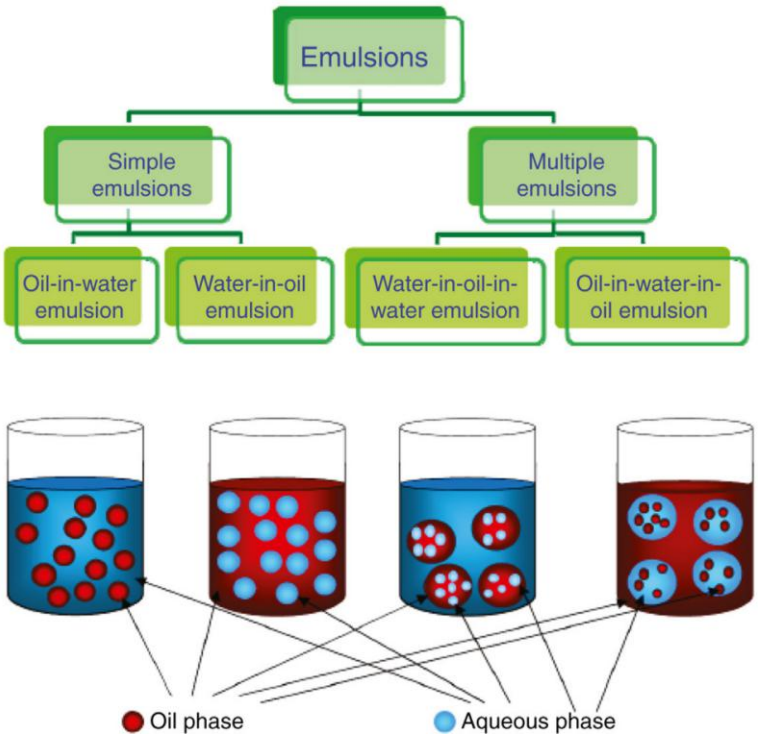


Figure 2.3 Classification of emulsions.

2.3 Emulsifier

The aim of this study is to understand the interaction of BS with commonly used emulsifiers at the oil-water surface, therefore it is necessary to understand the molecular characteristic of emulsifiers, their surface activity and the mechanisms by which they stabilize emulsion. Also, BS is also a kind of surface active molecule, hence, the study of emulsifiers such as surfactants can help to understand the behaviour of BS at the oil-water interface.

Emulsifier is a surface-active substance that can rapidly adsorb to the oil-water interface during homogenizing process, reduce surface tension and form an interfacial layer on the droplet surface to prevent droplet aggregation during manufacturing, storage, transportation and use of emulsion (Ozturk & MacClements, 2016). Emulsifiers available in food industry include phospholipids, small-molecule surfactants, and amphiphilic biopolymers. Due to the difference in the molecular and physicochemical property of different emulsifiers, their ability to form and stabilize emulsion varies considerably (Ozturk & MacClements, 2016).

2.3.1 Surfactants

Surfactants are relatively small surface-active molecules consisting of hydrophilic head groups and hydrophobic tail groups. Food grade surfactants are chemically

produced using various raw materials (e.g., glycerin, fats, organic acids, and polyols) (Hasenhuettl & Hartel, 2008). In fact, commonly used surfactants are a mixture of many different chemical substances. Thus, when studying surfactants, it is necessary to identify the type and concentration of chemicals in them. Surfactant plays a vital role in the formation and stabilization of emulsion. Additionally, they can modify emulsion properties in many ways, such as by altering the structure of fat crystals, interacting with biopolymers, and forming surfactant micelles (Hasenhuettl & Hartel, 2008).

- **Molecular Characteristics**

The properties of surfactants rely on the nature of their head and tail groups. For the head groups, they may be cationic, anionic, amphoteric (both positively and negatively charged) or non-ionic (McClements, 2015). On the other hand, the tailing groups of surfactants usually comprise one or more hydrocarbon chains, which may be aliphatic or aromatic, linear or branched, saturated or unsaturated (McClements, 2015). Commonly used surfactants in food industry are amphoteric (e.g., lecithin), anionic (e.g. fatty acid salts, stearoyl lactates, DATEM and CITREM), or non-ionic (e.g. monoglycerides, Tweens, Spans, ACETEM and LACTEM).

- **Molecular Organization of Surfactants in Solution**

Surfactants are present in solutions as monomers at sufficiently low concentrations; and when added above a certain level (at which point the concentration is referred to as critical micelle concentration i.e. CMC), they can spontaneously form various thermodynamically stable structures as associated micelles (Meyer et al., 2006), driven by hydrophobic effect (Figure 2.4). Above CMC, when adding more surfactants into solution, the number of micelle instead of the shape or size of single micelle increases; and the monomer concentration remains constant (McClements, 2015). Surfactants will form multiple liquid crystalline structures at higher concentrations (Kralova & Sjöblom, 2009).

Surfactant molecules present in micelles have different properties to ones present as monomers. For instance, as an amphiphilic molecule, surfactants have high surface activity, in contrast to micelles which have low surface activity due to their surfaces being covered by hydrophilic head groups (Kralova & Sjöblom, 2009). Accordingly, the surface tension of the solution decreases as the surfactant concentration increases, but it remains constant when the surfactant concentration reaches CMC. In addition, the physicochemical properties of surfactant in solution such as turbidity, osmotic pressure and electrical conductivity will change abruptly when their concentration is above the CMC (McClements, 2015).

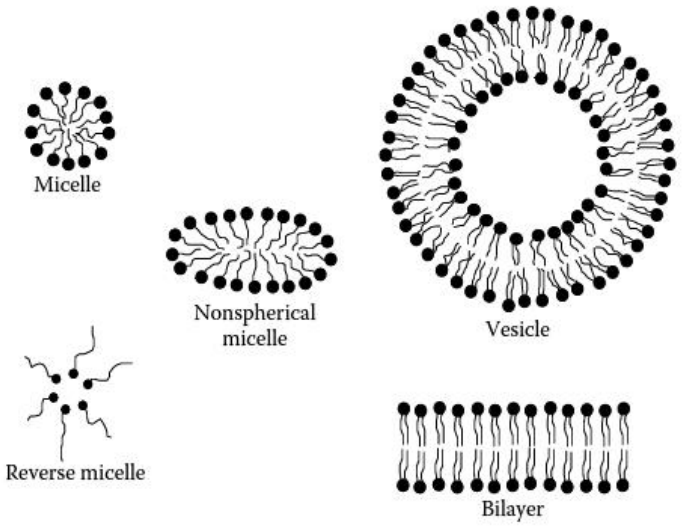


Figure 2.4 Some typical structures (micelles, bilayers, vesicles, and revers micelles) (McClements, 2015)

- **Interfacial Activity and Emulsion Stability**

The process of surfactants facilitating the formation and stabilization of emulsion is as follow:
(i) The surfactant molecules are rapidly adsorbed on the oil-water interface by adopting an orientation where the hydrophilic part is situated in water-phase and the hydrophobic tail group is located in oil-phase.
(ii) The interfacial tension is greatly reduced when the surfactant molecules adsorb to the oil-water interface, which is very important in homogenization process because this promotes the destruction of large droplets in the emulsion.
(iii) When surfactants adsorb to the droplet surface, they are able to provide sufficient repulsive force to prevent aggregation between droplets. For ionic surfactants, they provide stability mainly through electrostatic repulsion by making all the droplets have the same charge, whereas non-ionic surfactants maintain stability mainly through the generation of short-range repulsive forces (i.e., steric repulsion).

It is worth mentioning that, when the concentration of surfactant in the continuous phase is above the CMC, micelles formed by self-assembly of surfactant molecules have a negative impact on emulsion stability. For example, micelles may facilitate oil molecules to transport between droplets, thus inducing depletion flocculation (Hasenhuettl & Hartel, 2008).

2.3.2 Amphiphilic Biopolymers

The amphiphilic biopolymers commonly used as natural emulsifiers in food industry are polysaccharides and proteins, which are polymers of monosaccharides and amino acids respectively (Lad & Murthy, 2016). In general, the properties of the molecules that make up a biopolymer, such as molecular weight, polarity, hydrophobicity and

conformation (Figure 2.5), determine functional properties of the biopolymer as an emulsifier (e.g., thickening and surface activity); and the properties of these molecules are determined by the number, type and order of the monomers that make up the polymer chain (McClements, 2015). These monomers have different sizes, polarities and chemically reactive groups.

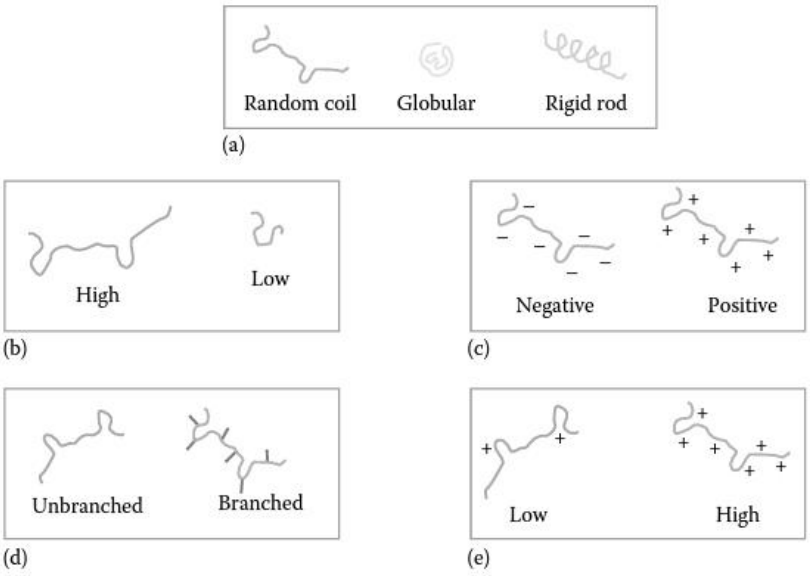


Figure 2.5 Biopolymers with different molecular characteristics. (a) Configurations, (b) molecular weight, (c) charge, (d) branching, and (e) charge density (McClements, 2015)

- **Molecular Characteristics**

The most common configurations adopted by biopolymer chains in solution are three types: rod-like, random coil, and globular (Figure 2.5). Random-coil biopolymer has a highly flexible and dynamic structure; rod-like biopolymers usually are rigid extended; the structure of globular biopolymers are rigid dense (Hasenhuettl & Hartel, 2008). Practically, biopolymers often adopt rather well-defined configurations in order to minimize free energy of the system under environmental conditions (McClements, 2015). However, they do not only have one exclusive configuration, but rather containing regions that adopt different conformations. In addition to configuration, the degree of branching of biopolymer chains also significantly affects their functional properties. Polysaccharides have branched (e.g., amylopectin) or linear (e.g., amylose) chains, while most proteins chains are linear (McClements & Gumus, 2016). It should be noted that the configurations and aggregation state of the biopolymer may change when the environmental condition such as temperature, ionic strength, solvent composition and pH changes, which can influence its functional properties (Lam & Nickerson, 2013).

- **Interfacial Activity and Emulsion Stabilization**

Many biopolymers have lipophilic and hydrophilic regions attached to their skeletons, so they are highly surface-active. For instance, some polysaccharides have nonpolar side chains distributed along with a polar backbone (Lam & Nickerson, 2013), and most proteins have a large number of exposed nonpolar amino acid side chain (Small et al., 2009). When biopolymers are dispersed in water-phase, they adsorb onto oil-water interface through hydrophobic effects and adopt the conformation of hydrophilic groups in contact with water (located in the aqueous phase) and nonpolar groups away from water (located in the oil phase) (Figure 2.6), thereby reducing interfacial tension (Ozturk & MacClements, 2016).

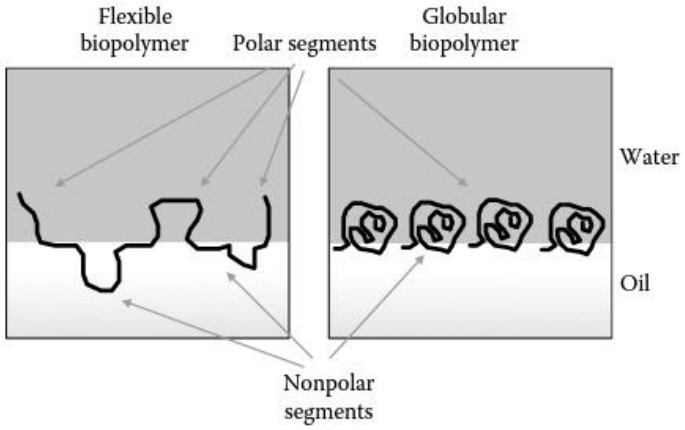


Figure 2.6 Two different configuration adsorbed on the oil-water interface (McClements, 2015).

The molecular structure and interactions of the bio polymers determine their conformation at the interface. Random-coil bio polymers with flexible structure adopt a conformation with neutral region lying flatly at the interface, major polar region protruding into water-phase, and major non polar segments protruding into oil-phase (Figure 2.6). This type of molecules is more likely to form relatively thick and open interface with low viscoelasticity (Ozturk & MacClements, 2016). By contrast, the interface formed by globular bio polymers tend to be relatively dense and thin, and to have high degree of viscoelasticity (Small et al., 2009). This is due to that globular bio polymers (usually proteins) adopt a different conformation to adsorb to the interface, in which their predominantly polar regions face water-phase while their predominantly non polar regions face oil-phase, resulting in a specific orientation at interface (Figure 2.6) (McClements, 2015). In addition, unfolded globular proteins will expose amino acids initially located within the hydrophobic interior of molecules. This may enhance the interaction with adjacent protein molecules through the formation of disulfide bonds or the hydrophobic attraction (Lad & Murthy, 2016). Once bio polymers adsorb to the interface, they tend to undergo structural rearrangements that allow their non-polar groups can be contacted with oil-phase as much as possible. Globular bio polymers tend to rearrange more slowly than random-coil bio polymers do, since the molecules of globular bio polymers are more

rigid whilst random-coil bio polymers have more flexible molecular structures (Lad & Murthy, 2016).

In addition to being able to rapidly adsorb to oil-water interface, as an effective emulsifier, bio polymers also need to form interfacial coatings between droplets, preventing droplets from aggregating with each other. Interfacial coatings formed by bio polymers can prevent droplets in emulsion from aggregation through different mechanisms such as hydration, electrostatic and steric repulsion.

It is worth mentioning that droplet aggregation could be induced by the change of solvent composition, ionic strength, pH and temperature during homogenization or after emulsion formation, because the biopolymer ingredient may be influenced by these environmental conditions (Lad & Murthy, 2016). For instance, it is difficult for protein to stabilize emulsion under high salt concentrations or at pH values close to its isoelectric point, where there is no sufficient electrostatic repulsion between droplets to prevent droplet aggregation.

2.3.3 Selected Emulsifiers Used in This Project

As a base for comparison, the commonly used emulsifier (whey protein) used for the bile salts displacement in this study will be introduced in this section.

Whey Protein

Protein is used as a common natural emulsifier in many emulsion-based foods, such as ice cream, frozen desserts, infant formula, etc. The proteins commonly used as emulsifiers are isolated from milk and can be divided into two main groups: casein (~ 80 wt%) and whey protein (~ 20 wt%) (Pilosof, 2017). The whey and casein fractions of milk are usually separated by precipitating the casein from the solution. Casein and whey components contain many different types of proteins that can be separated by fractionation (e.g., β-casein or β-lactoglobulin) (Pilosof, 2017). However, fractionated purified fractions are so expensive that they are usually used for research rather than as emulsifier in the food manufacturing.

Since protein-formed interfacial coating is usually electrically charged and relatively thin, the main mechanism for protein to prevent droplet in emulsion from flocculation is electrostatic repulsion rather than steric repulsion (Ozturk & MacClements, 2016). Accordingly, protein-stabilized emulsions are sensitive to ionic strength and pH values; and flocculation will occur in these emulsions at pH values close to the isoelectric point of protein or under high salt concentrations. In addition, some globular protein-stabilized emulsions are sensitive to heat treatment (Lam & Nickerson, 2013). This is because when the temperature is above a certain value, some globular proteins will be unfolded, and the sulfhydryl groups and other nonpolar reactive groups within them are exposed to the emulsion, resulting in increased mutual attraction between droplets (Lam & Nickerson, 2013).

The protein emulsifier used in this study was whey protein, which is a mixture of different proteins including β-lactoglobulin (~ 55%), α-lactalbumin (24%), serum albumin (~ 5%) and immunoglobulin (15%) (McClements, 2015). Among these proteins, β-lactoglobulin dominates the functional properties of whey protein due to its relatively high concentration and specific physicochemical properties. Whey protein-stabilized emulsions will destabilize and flocculate when heating temperature is above the heat denaturation temperature of whey protein, at high salt concentrations, and when the pH is close to the isoelectric point of whey protein.

2.4 Emulsion Stability and Testing Method

Emulsions are usually stabilized through the electrostatic or steric repulsion brought about by emulsifier, suggesting that the nature of the emulsifier is one of the most important factors in determining the stability of the emulsion, in another word, emulsifying property of emulsifier can be characterized by emulsion stability. In fact, there are some parameters used to describe the effectiveness of emulsifiers, such as emulsifying capacity (EC) and emulsion stability index (ESI) (Mcclements 2007). However, a single parameter cannot be used to quantitatively compare the effectiveness of emulsifiers in stabilizing emulsions in different emulsification systems. Hence, the emulsifying properties of the emulsifier needs to be characterized by emulsion stability. One of the objectives of this study is to understand the surface activity of BS, i.e., one of the emulsification properties, which can be characterized by emulsion stability. It is therefore important to understand the emulsion stability and its testing methods. The mechanism by which emulsifiers stabilize emulsions (electrostatic and steric repulsion) has been described in the previous section for introducing emulsifiers (section 2.3), so this section reviewed the instability mechanism of emulsions, and methods for testing emulsion stability.

2.4.1 Instability Mechanisms

The term "emulsion stability" refers to the ability of an emulsion to resist changes on its characteristics (McClements, 2015). Understanding the major physical or chemical mechanisms that lead to emulsion instability is critical to improving emulsion stability. The main destabilization phenomena in emulsion include gravitational separation (sedimentation/creaming), flocculation, coalescence, partial coalescence, Ostwald ripening, and phase inversion (Figure 2.7) (Mcclements 2007). These types of breakdown phenomena are determined by different emulsion characteristics. For example, the two main factors affecting gravitational separation are: (1) the difference in density between the droplets and the continuous phase, (2) the distribution of droplets with different sizes in the emulsion. The occurrence of flocculation is related to the magnitude of the attractive and repulsive forces between droplets. Ostwald ripening is caused by the different solubility of droplets with different sizes in the continuous phase. Coalescence is influenced by the stability of the liquid film between the droplets.

It is worth mentioning that these instability mechanisms are often interrelated. For instance, droplet size may increase as a result of Ostwald ripening occurring in the emulsion, which may further lead to gravitational separation. Similarly, flocculation and coalescence may also lead to an increase in droplet size, which may result in gravitational separation (Chanamai & McClements, 2000). On the other hand, after flocculation or gravitational separation occurred, some droplets would contact closely over a long period of time and thus inducing coalescence. The factors influencing emulsion instability are therefore complex, and emulsion instability is not only caused by the observed mechanisms. For example, gravity separation is an easily observed phenomenon, however it may be caused by flocculation, coalescence or Ostwald ripening. Accordingly, emulsions should be analyzed in a more detailed way before strategies are developed to reduce destabilization in emulsions.

Figure 2.7 Common instability mechanisms in food emulsions (Mcclements 2007)

- **Gravitational Separation (Creaming and Sedimentation)**

Gravitational separation is the process by which droplets in an emulsion move upwards or downwards under the influence of an external force (usually gravity), due to the difference in density between the droplet and the surrounding continuous phase (Vélez et al., 2003). Usually, this process does not cause changes in droplet size (Mcclements 2007). When droplets are less dense than the surrounding liquid, they will move upwards, which is known as creaming, while the process by which droplets move downwards because their density is greater than that of the continuous phase is known as sedimentation (Figure 2.7). Due to the fact that edible oils are less dense than water, creaming generally occurs in O/W emulsions, whereas sedimentation often occurs in W/O emulsions. The following is explained by example of oil-in-water emulsion.

In a monodisperse emulsion, all droplets have the same size, so they move upwards at

the same velocity and eventually accumulate on the top of the emulsion (Newling et al., 1997). The tightness of the accumulation of droplets in the upper part of the emulsion depends on the forces of attraction and repulsion between them (Mcclements 2007). When creaming occurs, a "cream layer" rich in droplets forms at the top of the emulsion, while a "serum layer" containing a small number of droplets is located at the bottom of the emulsion, and the "emulsion layer" between them contains the similar number of droplets as the initial emulsion (Newling et al., 1997). The serum layer is usually transparent and the cream layer is optically opaque, with the middle emulsion layer having a similar appearance to the original emulsion (Mcclements 2007). Eventually, the emulsion layer disappears as all the droplets accumulate at the top of the emulsion.

In practice, most food emulsions are polydisperse systems in which the droplets move upwards at different velocity (Newling et al., 1997). The smaller droplets move more slowly while the larger ones move more quickly. Therefore, for polydisperse emulsions, sometimes it can be difficult to accurately determine the extent of creaming occurring in the emulsion, since the smaller droplets may be dispersed in the "serum layer" located at the bottom of the emulsion (Newling et al., 1997).

Figure 2.8 Creaming occurring in an monodiperse O/W emuslion (Mcclements 2007)

Figure 2.9 Creaming in a polydisperse oil-in-water emulsion (Mcclements 2007)

In practice, gravitational separation is one of the most common instability mechanisms in food emulsions. Determining the extent to which gravity separation occurs in the product over a given period of time and assessing the susceptibility of the product to gravitational separation is essential to improve the stability of the product.

- **Flocculation**

Flocculation is the process by which two or more droplets become associated with each other but maintain their individual integrity (Chanamai & McClements, 2000). In this process the droplets are close to each other but are not tightly bound (coalesce) (Mcclements 2007). Flocculation is mainly resulted from the van der Waals attraction, which is primarily due to the London dispersion forces generated by the charge fluctuations of atoms or molecules in the dispersed droplets (Chanamai & McClements, 2000). The degree of flocculation varies depending on the magnitude of the attraction energy. The nature and extent of flocculation significantly influences the macroscopic properties of emulsion foods, including appearance, sensory properties, and rheology (McClements, 2015). Generally, flocculation is critical to the quality of the emulsion food product. For instance, for some relatively dilute emulsions (e.g., nutritional drinks, soft drinks, infant formula), flocculation leads to an increase in particle size, which accelerates gravitational separation, thus resulting in a shorter shelf life of products (Chantrapornchai et al., 2001). As another example, flocculation may lead to an increase in viscosity and even the formation of gels in some concentrated emulsions, which is disadvantage for some products that need to have a lower viscosity. In some cases, flocculation can be used to obtain desirable textural properties (gels) (McClements, 2007), which can be beneficial to the quality of the product. Therefore, understanding the causes of flocculation is essential to improve the quality of emulsion food products.

- **Coalescence**

Coalescence is the process by which the liquid film between droplets thins and breaks down, resulting in the merging of two or more droplets to form larger droplets. Coalescence tends to occur in creaming and sedimentation layers, flocs, or concentrated emulsions (McClements, 2007). This process results in a large change in droplet size distribution, which in turn accelerates the gravitational separation (creaming and sedimentation) of the emulsion. At this point, the appearance of the emulsion is likely to exhibit less turbidity and more intense color, as larger droplets can scatter light more efficiently than smaller droplets (McClements, 2007). In extreme cases, the emulsion is separated completely into two distinct liquid phases: a layer of oil at the top of the material (for oil-in-water emulsions) or a layer of water at the bottom (for water-in-oil emulsions), a phenomenon known as oiling off. Characterization of the coalescence of food emulsions in such cases must be approached with caution because the sample chosen for analysis may not be

representative of the entire emulsion.

- **Phase Inversion**

Phase inversion is the exchange of a dispersed phase with a continuous phase. For example, a water-in-oil emulsion may change to an oil-in-water emulsion as conditions change or as time passes (Binks & Lumsdon, 2000). Very often the process of phase inversion is accompanied by the creation of multiple emulsions. For example, for water-in-oil emulsions, the continuous phase of oil may be emulsified in aqueous droplets to form an O/W/O emulsion until the entire oil phase is emulsified into the aqueous phase, producing an oil-in-water emulsion.

- **Ostwald Ripening (Disproportionation)**

There is limited but non-negligible solubility between immiscible liquids. For polydisperse emulsions, smaller droplets have a higher solubility than larger droplets (Kabalnov, 2001). As a result, over time the smaller droplets will dissolve and diffuse in the continuous phase and their molecules will be deposited on the larger droplets (Kabalnov, 2001), causing the droplet size distribution to shift towards larger values.

2.4.2 Experimental Determination of Physical Stability of Emulsion

- **Droplet Characteristics**

Droplets in food emulsions significantly influence the physicochemical properties of the emulsion. Therefore, measuring the characteristic of droplets (including size, charge, etc.) can provide important information about the stability of emulsions and the emulsifying properties of emulsifiers.

Droplet Size

The size of droplets in emulsions has a significant impact on their stability (e.g., gravitational separation, flocculation and coalescence), optical properties (e.g., colour), rheology (e.g. viscosity) and their sensory properties (McClements, 2015). Therefore, it is particularly important to reliably determine the droplet size of an emulsion. When all droplets in an emulsion have the same size, the emulsion is “monodisperse”, whereas in practice, most food emulsions contains droplet with different size and the emulsion is described as “polydisperse” (McClements, 2007) (Figures 2.10 and 2.11). For monodisperse emulsions, the droplet size can be characterized directly by the radius or diameter of the droplet. For polydisperse emulsions, they can be characterized by their “particle size distribution” (Figure 2.11), which defines the concentration of droplets in different particle size classes (McClements, 2007).

Figure 2.10 Photomicrograph of a polydisperse oil-in-water emulsion (McClements, 2007)

Figure 2.11 Particle size distribution of a polydisperse emulsions (McClements, 2007)

When interpreting the particle size distribution (PSD) of polydisperse emulsions, the particle concentration is usually expressed as the number percentage (number %) or the volume percentage (volume %) of droplets within a given size class, and the droplet size is usually expressed as the mid-point particle diameter or radius of the particle size class (McClements, 2007). Figure 2.12 shows the same data for a flocculated emulsion expressed as a number and volume percentage. It can be seen from the graph that although large particles make up a considerable proportion of all droplets (>25% by volume), when particle concentration is expressed as the number percentage, large particles in the emulsion cannot be observed in the particle size distribution. It is therefore important to choose the appropriate particle concentration parameter for the y-axis of the particle size distribution graph. One peak, two peaks and multiple peaks shown in the particle size distribution of polydispersed emulsions can be described as “monomodal”, “bimodal” and “multimodal”, respectively (McClements, 2007). In food science research it is important to know the full particle size distribution of an emulsion, because it provides information on the size of all the

droplets in the emulsion and it is very helpful for figuring out the sources of instability in the system.

Figure 2.12 Particle size distribution represented as either Volume% or Number% versus Diameter (McClements, 2007)

In addition, many different mean particle sizes can be derived from a full particle size distribution, which have different magnitude and physical significance. The three most commonly used mean particle size values are the volume-weighted mean diameter, the surface-weighted mean diameter and the number-weighted mean diameter (McClements, 2007). In general, the volume-weighted mean diameter is more sensitive to the presence of large particles than the number-weighted mean diameter (McClements, 2015). Therefore, it is important to take care when reporting or interpreting particle size data to determine which average size value is being used.

Droplet Charge

Droplets in many food emulsions have electrical charges because their surfaces are adsorbed by ionized or ionizable molecules (McClements, 2015), such as proteins and ionic surfactants. These charges cause electrostatic repulsion between droplets, thus keeping the emulsion stable. In addition to this, these charges also influence the interaction of droplets with other charged substances in the colloidal system. The charge on the droplet is therefore very important. Furthermore, the type and concentration of ionized charges on the droplet surface, as well as the ionic composition and nature of the surrounding liquid, determine the electrical properties of the droplet surface. In this section, the zeta potential, which can characterize the charge density on the droplet interface, is highlighted.

Zeta Potential

The liquid layer surrounding the droplet consists of an external (diffusion) zone and an internal zone (Stern region). In the inner zone the ions are tightly bound while in the outer zone the ions are not strongly linked. Within the diffusion layer there is a

boundary, within which the particle and the surrounding ions can be considered as a stable entity (McClements, 2015). The electric potential at this boundary is the zeta potential.

When droplets have a large positive or negative electric potential, they will repel each other and are unlikely to clump together. Conversely, when they have a low electric potential, they will not repel each other by electricstatic repulsion, and flocculation or coalescence can occur. The magnitude of the zeta potential is therefore an indication of the stability of a colloidal system. The usual dividing line between stable and unstable particles is ± 30 mV, i.e., droplets with zeta potentials more than 30 mV in absolute value are usually considered stable (McClements, 2015). Measuring zeta potentials to assess the stability of a colloidal system is a widely used method.

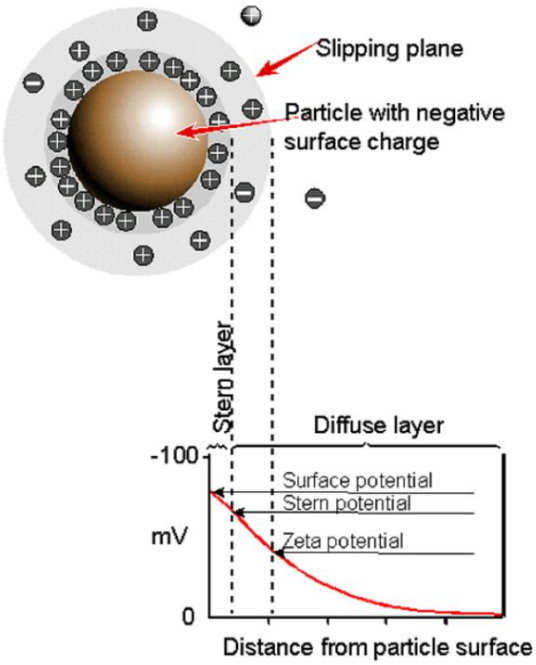


Figure 2.13 Schematic representation of zeta potential (adapted from Technical Notes, Malvern Instruments Ltd)

- **Experimental determination of gravitational separation**

The most common and convenient way to determine gravity separation in emulsions is visual observation. For example, after creaming has occurred, two or three layers can be observed in an oil-in-water emulsion. Ideally, the cream layer should be opaque while the serum layer is optically transparent; the emulsion layer is similar in appearance to the original emulsion (Darling, 1982). The extent of creaming can then be characterized by a creaming index (CI):

$$CI = 100 \times \frac{H_S}{H_E} \qquad (1)$$

where, H_E is the total height of the emulsion and H_S is the height of the serum layer.

The determination of gravitational separation by visual observation has two main disadvantages: (i) In many cases it is difficult to clearly determine the location of the boundary because the cream layer is opaque or because the boundaries of the layers are blurred; (ii) This method does not allow the distribution of droplet concentrations in the vertical direction to be determined (McClements, 2007).

- **Experimental characterization of droplet flocculation**

The most common and simplest method of determining flocculation in emulsions is microscopic observation (Darling, 1982). The optical microscope, an instrument available in almost all laboratories, has long been used extensively to determine the flocculation of emulsions. When the droplets of the observed emulsion are evenly distributed on the image and can be clearly observed to be separated, it can be determined that no flocculation is occurring in the emulsion (Chantrapornchai et al., 2001). Conversely, if droplets are observed to be closely packed in the emulsion and aggregated, it can be assumed that flocculation is occurring in this emulsion. Furthermore, by observing the microstructure of the flocs it is possible to understand the degree of attraction between the droplets and to distinguish between depletion flocculation and bridging flocculation (Chantrapornchai et al., 2001). This is because when the attractive force is strong, the droplets tend to form irregularly shaped structures of flocs (bridging flocculation), whereas when the attraction is weak, the flocs are more compact and regular in structure (depletion flocculation) (McClements, 2007). In practice, microscopic observation is usually combined with droplet size analysis to obtain information about particle aggregation faster.

2.5 Interfacial Properties and The Characterization

2.5.1 Introduction

Interface is the narrow region that separates two phases (e.g., gas and liquid, liquid and solid, liquid and liquid, etc.) (Fisher et al., 1985). Although an interface separating the two phases occupies only a small part of the volume of the emulsion, its physicochemical properties have an important influence on the formation and stabilization of the emulsion. Also, interfacial properties significantly affect the physicochemical properties, sensory characteristics and rheological properties of food emulsions (Berry et al., 2015). Therefore, studying the influence of interfacial properties (including its composition, structure, energy, electrical properties and rheology) on emulsions, as well as the influence of surface active substance on interfacial properties, can provide important information for developing strategies to improve the quality of food emulsions.

The aim of this study is to understand the interaction of BS with commonly used emulsifier molecules at the oil-water interface. BS, as a surface active molecule, has a significant effect on interfacial properties, especially on the interfacial tension. This

section therefore provides a review on the interfacial tension, and also describes the effect of surface active molecules on it and the method of measuring surface tension.

2.5.2 Interfacial Tension and Its Measurement

- **Interfacial Tension**

At the molecular level, the various molecules (in this study mainly oil and water molecules) mix and interact with each other in the interfacial region. For instance, water molecules form strong hydrogen bonds in the water phase, while oil molecules interact with each other in the oil phase through weaker van der Waals bonds (McClements, 2015). At the oil-water interface, oil molecules can only form van der Waals bonds with water molecules as they have no polar groups capable of forming hydrogen bonds (McClements, 2015). The unfavourable interactions between oil and water molecules make the interfacial region thin and inflexible, so that free energy needs to be supplied to the system to increase the contact between oil and water molecules (O'Connor et al., 1983). Conceptually, interfacial tension is a measure of the free energy required to increase the interfacial area by a unit amount, usually in units of energy per unit of interfacial area (Jm^{-2}) or force per unit of interfacial length (Nm^{-1}) (McClements, 2015). In general, the magnitude of imbalance of interaction between molecules at an interface determines the amount of interfacial tension: the greater the imbalance of interaction, the greater the interfacial tension.

Interfacial tension has an important influence on the generation and stabilization of food emulsions, for example, it affects the size of the droplets generated after homogenization and it also influences the stability of emulsions against coalescence and Ostwald ripening. In addition, the measurement of interfacial tension provides valuable information for studying emulsifiers, such as their adsorption rate, surface activity and critical micelle concentration.

- **Effect of Surface Active Substances on Interfacial Tension**

In the food industry there are many surface active molecules (as described earlier) which can adsorb and accumulate at interfaces and change interfacial properties. Surface active molecules affect interfacial tension by the following processes：
(i) When the free energy of the adsorbed state is lower than that of the non-adsorbed state, amphiphilic solutes tend to accumulate at the interface and separate the oil and water phases.
(ii) At this point, the polar segment of the solute molecule is in contact with the water molecule and the non-polar segment is in contact with the oil molecule, which means that there is less direct contact between the water and oil molecules.
(iii) Due to the presence of surface active substances at the interface, the thermodynamically unfavorable contact between the oil and water phases is reduced and the free energy of the system changes, which is expressed as a change in interfacial tension. The reduced interfacial tension is referred to as surface pressure (McClements, 2015).

Different types and concentrations of surface active molecules have different effects on interfacial tension. The higher the concentration of the surface active substance at the interface, the greater the reduction in interfacial tension. Figure 2.14 illustrates the variation in interfacial tension with increasing concentrations of the different solutes added (McClements, 2015). For solutes with negative surface activity (e.g., NaCl), the interfacial tension increases as their concentration increases. For solutes with no or little surface activity, such as sucrose, the surface tension barely changes with increasing concentration. For solutes with moderate surface activity, such as methanol, the surface tension decreases slowly with increasing solute concentration. For solutes with high surface activity, such as SDS, the surface tension decreases rapidly with increasing solute concentration and then reaches a relatively constant value when the surface is saturated (McClements, 2015).

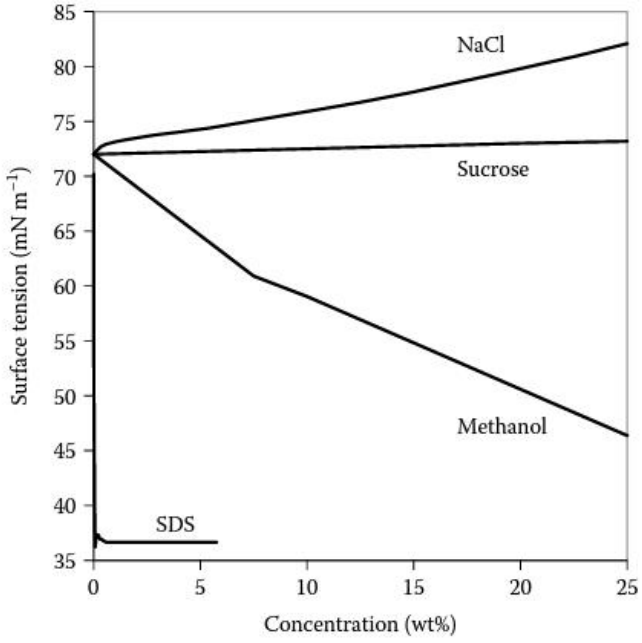


Figure 2.14 The change of interfacial tension with increasing concentration of different surface active solutes (McClements, 2015)

- **The Measurement of Interfacial Tension**

A number of methods have been used to measure surface (or interfacial) tension in research (Figure 2.15). These methods are different in mechanical design, operation principle, and the measurement is dynamic or static. Among these methods, pendant drop tensiometry is one of the simplest (Berry et al., 2015).

Figure 2.15 Schematics of experimental techniques used to determine interfacial tension (Berry et al., 2015)

Pendant Drop Tensiometry

The instruments required for the pendant tension method are very simple: light sources, needles, and cameras (as shown in Figure 2.16a). By fitting the droplet image recorded by the camera to the Young-Laplace equation (Eq. 2), the computer can obtain the interfacial tension of the droplet.

$$\Delta P = \sigma \left(\frac{1}{R_1} + \frac{1}{R_2}\right) \quad (2)$$

The surface tension σ can be calculated, if R_1 and R_2, the two principal radii of curvature of the surface, and ΔP, the pressure difference across the interface, are known. Surface pressure π is calculated by $\pi = \sigma_0 - \sigma$ with σ_0 as the surface tension of the empty surface (water; σ_0=72.8 mN/m).

A typical image that is suited to fitting is shown in Figure 2.16b.

Figure 2.16 (a) A basic experimental setup for pendant drop tensiometry; (b) a typical drop image (Berry et al., 2015)

Chapter 3. Materials and Methods

3.1 Materials

Whey protein isolate (WPI) was obtained from the Isopure Company. Porcine bile extract B8631 was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Commercial canola oil was obtained from The Warehouse, Albany, New Zealand. Prior to experiments, solutions were freshly prepared using Milli-Q water (purified by a Milli-Q apparatus; Millipore Corp., Bedford, MA, USA) as the solvent.

3.2 Preparation of Emulsions

3.2.1 Preparation of WPI Emulsion

Aqueous solutions of WPI were prepared by dispersing 2 g WPI in 78 g Milli-Q water and mixing them with overhead stirrer at 300 rev/min for 5 minutes to ensure complete dissolution (Sarkar et al., 2010). Then, 20g canola oil was slowly poured into water phase and mixed with WPI solution using the same overhead stirrer at 300 rev/min for 5 minutes for pre-homogenizeation to produce a coarse emulsion (final concentration of WPI in the emulsion is 2.0% wt). Immediately, the beaker was transferred underneath the high shear mixer (Silverson L5M-A Laboratory Mixer) to homogenize at 7000 rev/min for 3 minutes to get fine emulsion. The L5M-A Laboratory Mixer is suitable for the widest range of applications including mixing, emulsifying, homogenizing, disintegrating and dissolving, with an efficiency and flexibility unmatched by other machines. However, the commercial oil which contains varying levels of polar lipids was not be cleaned, and this could impact on the results.

3.2.2 Preparation of BS Emulsion

Similarly, aqueous solutions of bile salt (BS) were prepared by dispersing 2 g BS in 78 g Milli-Q water and stirring with overhead stirrer at 300 rev/min for 2 h at 45℃ until all the BS had dissolved (Jódar-Reyes et al., 2010). Pre-emulsion was prepared by mixing 20.0 wt% canola oil (20 g) with 80.0 wt% BS solution (80 g) using the same overhead stirrer at 300 rpm for 5 min. The coarse emulsions were homogenized by a high shear mixer at 7000 rev/min for 3 minutes.

3.2.3 Preparation of Tween Emulsion

2 g Tween 80 was dispersed in 78 g Milli-Q water by mixing them with using a overhead stirrer at 300 rev/min for 10 min at room temperature (Ariyaprakai et al., 2013). Then, 20 g canola oil was slowly added to the aqueous phase for pre-homogenizing using the same overhead stirrer at 300 rpm for 5 min. Pre-emulsion was continued homogenized by a high shear mixer at 7000 rpm for 3 minutes. The WPI, BS and Twen emulsions were prepared at least in duplicate.

3.3 Droplet Size Determination

The mean droplet size distribution was determined using Mastersizer 2000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The refractive index of canola oil was taken 1.5 and the refractive index of aqueous phase was 1.33, i.e., the relative refractive index of the emulsion was 1.128.

The measurement of droplet size was carried immediately after the emulsion was prepared in order to avoid the influence from coalescence of droplets and phase separation. The droplet size distribution was also recorded after making emulsion 1 hour, 2 hours, 4 hours, 24 hours and 48 hours, in order to compare the stability of emulsion stablized by WPI, BS and Tween. The droplet size was measured twice.

3.4 Zeta-Potential Measurements

The zeta-potential of emulsions was estimated using a Malvern Zetasizer Nano ZS (ZEN 3600) instrument (Malvern Instruments Ltd). The emulsion samples were diluted 100 times and placed in a disposable capillary cell with electrodes (Model DTS1070, Malvern Instruments Ltd). An individual zeta-potential measurement was calculated from at least three readings for an individual sample. The zeta-potential of the sample was measured at different pH value in order to study the stability of the emulsion stabilized by WPI, BS and Tween, because pH value has significant effect on the zeta-potential of emulsion.

3.5 The Creaming Stability of Emulsion

The creaming stability of the emulsion stabilized by the three emulsifiers was compared by the simplest direct observation method. The emulsions to be analyzed were placed in a test tube and left for a certain period, and then the height of any boundaries between different layers is measured. The creaming index was recorded at 0h, 1h, 2h, 4h, 24h and 48h after the emulsion was prepared. For better observation, the emulsion was stained with methyl blue and Sudan III red after preparation.

Instability of emulsions was characterized by the creaming index (CI) (Arancibia et al., 2017):

$$CI = 100 \times \frac{H_S}{H_E} \qquad (1)$$

where, H_E is the total height of the emulsion (mm) and H_S is the height of cream layer (mm). All measurements were performed in triplicate for each emulsion.

3.6 The Displacement of WPI and Tween by BS

3.6.1 The Displacement of WPI and Tween by BS

The WPI and Tween emulsion was prepared as the procedure described in 3.2.1. Bile salt solutions of concentrations of 2.5% (wt) were made up in Mili-Q water (2g BS dissolved in 78g water). Twenty-five milliliters of these solutions were mixed with 25mL of two kinds of emulsions respectively and left to stand for at least 2h at room temperature for competitive adsorption between the bile salt and protein and between BS and Tween to take place. Although the purpose of the project is to investigate the role of bile during the digestive process, in this experiment, the competitive adsorption was occurring under room temperature, because it has been able to provide the temperature environment required for competitive adsorption at room temperature. In previous literature, it had been shown that the displacement of protein is complete within 2h (Euston et al., 2013). Twenty-five milliliters of two kinds of emulsions were mixed with Mili-Q water respectively in order to compare with those emulsions mixed with BS solution.

3.6.2 Droplet Size Determination

The determinations of droplet size distribution for WPI (or Tween) emulsion mixed with BS solution and for WPI (or Tween) emulsion mixed with Mili-Q water were carried in the meantime according to the process in 3.3. This means that the emulsions were either mixed with the BS solution or diluted with water. The first measurement was made immediately after the emulsion was prepared, and the subsequent determinations were carried after 1h, 2h, 4h and 24 hours.

3.6.3 Zeta-potential Measurement

Similarly, the zeta-potential of WPI (or Tween) emulsion mixed with BS and of WPI (or Tween) emulsion with Milli-Q water was measured in the same way described in 3.4. In the same way, this means that the emulsions were either mixed with the BS solution or diluted with water. The measurements were carried after 2h of emulsion preparation.

3.7 Surface Activity of Three Surface-Active Substances at Oil-water Interface

The oil-water interfacial tension after adding three surface-active substances was measured by using a Theta Flex Tensiometer (Biolin Scientific, Gothenburg, Sweden) at room temperature. The solution samples of WPI, BS and Tween were prepared according to the process in 3.2. The measurement was carried every 10s, and the total measurement duration was 60s. The analysis mode is based on Young-Laplace. Water with surface tension equal to 72.5 mNm^{-1} was selected as testing liquid.

Chapter 4. Results and Discussions

4.1 Emulsifying Properties of Three Emulsifiers

4.1.1 Droplet Size Determination

The droplet size in emulsion has important effects on its stability (including against coalescence, flocculation and gravity separation), sensory properties, rheology and optical properties (McClements, 2015), and it can be used to characterize the emulsifying ability of emulsifier. Therefore, the droplet size in emulsions made with the BS, WPI and Tween was measured, in order to analyse the emulsion stability and then to compare the emulsification properties of these three emulsifiers. The droplet size in emulsions was determined using a Mastersizer. Figure 4.1 shows the particle size and distribution in the canola oil emulsions prepared with 2% (wt) BS, WPI and Tween.

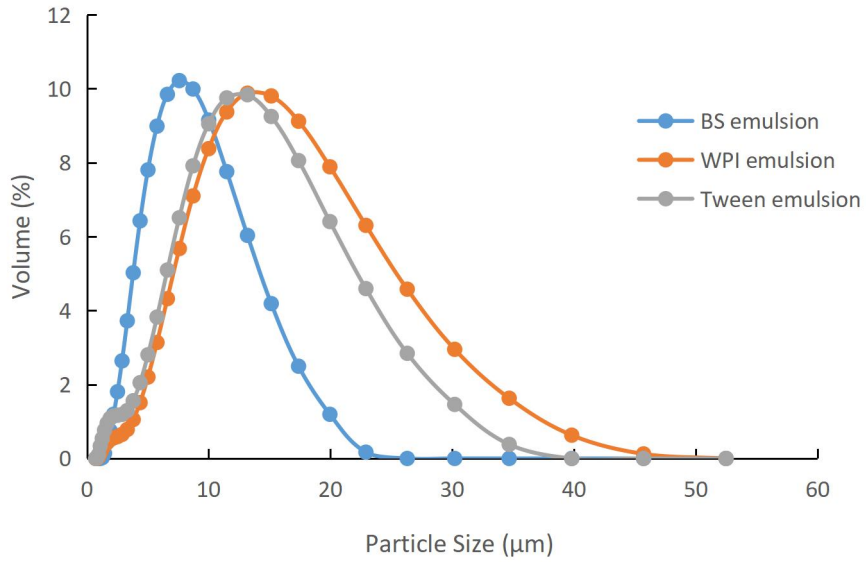
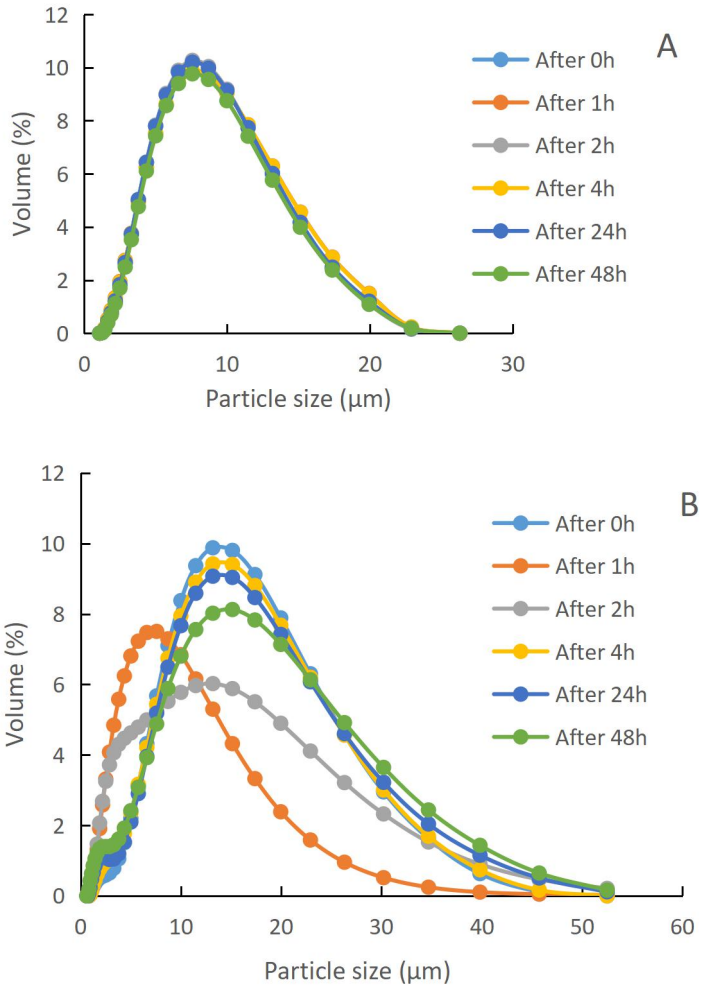


Figure 4.1 Comparison of the particle size distribution of BS, WPI and Tween fresh emulsion.

The particle size distribution in all three emulsions were monomodal, ranging from 0.9μm to 45μm. As can be seen from the graph, the emulsions prepared with WPI and Tween had similar droplet size distributions and had wide peaks in a similar range, demonstrating their similar emulsifying ability. It is worth noting that BS has the same or even better emulsifying ability compared to Tween and WPI, as evidenced by the smaller droplet size and narrower peak widths in the distribution of the emulsion prepared from BS, although the biological role of BS is not actually to stabilize emulsions. In another word, BS can act as an efficient emulsifier. This result is consistent with previous works done by (Sarkar et al., 2016), which highlighted that BS is a well-performing emulsifier and is able to adsorb rapidly onto the interface and form stable emulsions. It should be noted that this experiment is to evaluate the

emulsifying ability of the three emulsifiers by evaluating the results of the emulsification procedures, and one of the common methods of studying the results of the emulsification procedures is to compare the droplet size of the emulsion prepared by different emulsifiers. Determining droplet size distribution as a function of process and product variables is by far the best method to study emulsification.

To further compare the emulsifying ability of the three emulsifiers, the change of droplet size in the emulsions prepared using these three emulsifiers was also measured over 48 hours of storage and used to compare the stability of the three emulsions; the results of which are shown in Figure 4.2. Since the emulsion prepared by Tween after 24 hours had obviously different particle size distribution with the emulsion prepared by WPI and BS, the droplet size of the Tween emulsion after 48 hours was not recorded.



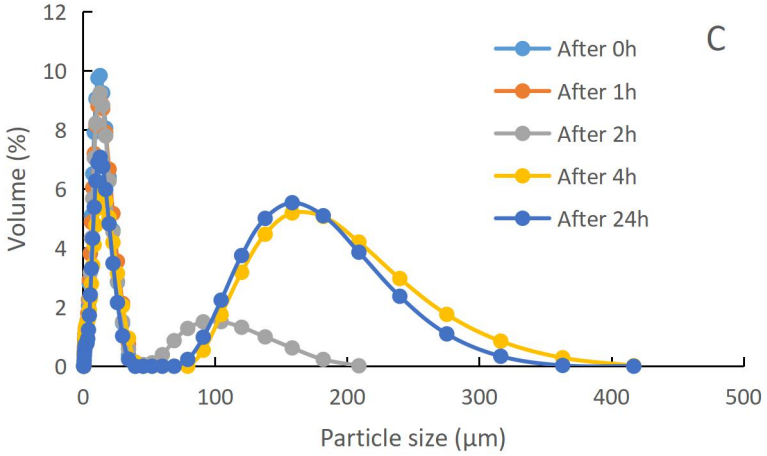


Figure 4.2 Volume droplet size distribution of emulsion containing BS (A), WPI (B) and Tween (C), freshly prepared and after storage at 25°C for 1h, 2h, 4h, 24h and 48h.

Figure 4.2 shows the volume droplet size distribution curves of canola oil emulsion containing BS (A), WPI (B) and Tween (C), obtained after preparation (0h) and after storage at 25°C for 1h, 2h, 4h, 24h and 48h. It is clear from the Figure 4.2 that the droplet size distribution for the BS-emulsified emulsion remained almost unchanged over 48 hours and the droplet size remained a monomodal distribution with peak widths ranging from 1 µm to 25 µm. For the WPI containing emulsion, the droplet size also remained a monomodal distribution over 48 hours and the range of droplet size distribution also remained constant (from 1 µm to 50 µm). However, as time progressed, the peaks of the distribution decreased, indicating a decrease of small droplets and an increase of larger droplets in the emulsion. It is worth noting that after 1h and 2h, the droplet size reduced. This may be due to the fact that the larger droplets had creamed to the top of the sample and were not collected with pipette. For the emulsions prepared with Tween, the droplet size showed a bimodal distribution after 2 hours of storage, and another peak with a higher size (100µm) was observed. Over time, the first peak gradually decreased while the second peak increased significantly and shifted to right, and the maximum droplet size had exceeded 400 µm after 24 hours, indicating that the droplet size of the emulsion containing Tween increased significantly over 24 hours. By comparing the droplet size distribution of emulsions containing the three emulsifiers over time, it is possible to compare their ability to stabilize emulsion. Considering that increased droplets size indicates the emulsifier's decreased capability to stabilize an interfacial area (Arancibia et al., 2017), it seems that BS has better ability to adsorb and stabilize interface than WPI and Tween does.

The particle size distribution of an emulsion provides information on the size of all particles in the emulsion, and is very helpful for determining the sources of instability in the system. However, in order to further understand the detailed information on droplet size in an emulsion, it is essential to analyze the central tendency of the size distribution, which is usually measured by the mean, median or modal particle size (Mcclements 2007). The three most commonly used average particle size values are

the surface-weighted average diameter (d_{32}), the number-weighted average diameter (d_{10}), and the volume-weighted average diameter (d_{43}). Each mean particle size has different magnitude and physical significance. Literature has pointed that the volume-weighted average diameter (d_{43}) is more sensitive to the presence of large particles than the number-weighted average diameter (d_{10}) (Mcclements 2007). In addition, the differences between the mean diameters are important for analyzing the particle size distribution. Significant differences between d_{10}, d_{32} and d_{43}, for example, usually indicate that the particle size distribution is broad or multimodal (McClements, 2015). Therefore, this experiment also compares the d_{32} and d_{43} values of droplets in emulsions containing the three emulsifiers, as shown in Table 4.1. Additionally, the variations of d_{43} values over time over a 48 h period are plotted (Figure 4.3). The diameter $D_{3,2}$ (surface volume mean diameter) (Eq. (3)) and $D_{4,3}$ (volume weighted mean diameter) (Eq. (4)) are used to represent the droplet diameter of the sample.

$$D_{3,2} = \sum n_i d_i^3 \Big/ \sum n_i d_i^2 \quad (3)$$

$$D_{4,3} = \sum n_i d_i^4 \Big/ \sum n_i d^3 \quad (4)$$

In the equations, n_i is the number of particles with diameter d_i. D_{43} is related to droplet size change during instability like droplet aggregation. D_{32} represents the average diameter of most droplets.

Table 4.1 The d_{32} and d_{43} diameters of droplets from emulsions containing the three emulsifiers

Emulsifier / Mean diameter	BS	WPI	Tween
$D_{3,2}$	5.654	8.867	7.047
$D_{4,3}$	7.389	13.161	11.194

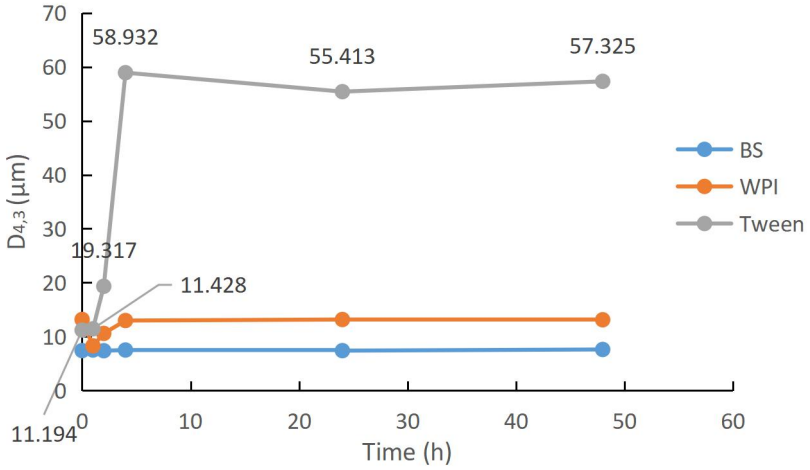


Figure 4.3 $D_{4,3}$ diameter of droplets in the emulsions containing BS, WPI and Tween over time.

It can be seen from the Figure 4.3 that the d_{43} diameter for BS and WPI stabilized emulsions remained almost unchanged within two days. On the contrary, the d_{43} values of Tween emulsions increased within one hour after emulsion preparation and was always higher than that of BS and WPI emulsions (see data tagged on the graph). As mentioned above, the d_{43} value is rather sensitive to the presence of large particles. Hence, the increased d_{43} values for Tween emulsion showed that there were larger droplets existing in the Tween emulsion within 1 hour, which was not shown in the full droplet size distribution (Figure 4.2). Still, these results showed that the droplet size for the stored Tween emulsions significantly increased during 48 hours, which is in line with the results obtained from the droplet size distribution. This suggested that Tween had worse ability to stabilize droplet in emulsion than WPI and BS. In addition, it should be noted that there were higher differences between d_{32} and d_{43} values for WPI and Tween emulsions than BS emulsion (Table 4.1). Previous research has pointed that higher differences between d_{32} and d_{43} could indicate a higher tendency for destabilization (McClements, 2015). This means that Tween and WPI emulsions are more likely to destabilize than BS emulsions. The changes in stability over time may due due to the fact that the emulsions were in a creamed state, thus the Tween emulsions were destabilizing more than other two emulsions.

4.1.2 Zeta-potential Measurements

Droplets in many food emulsions have electrical charges because their surfaces are adsorbed by ionized or ionizable molecules (McClements, 2015), such as proteins and

ionic surfactants. These charges cause electrostatic repulsion between droplets, thus keeping the emulsion stable. In addition to this, these charges also influence the interaction of droplets with other charged substances in the colloidal system. The charge on the droplet is therefore very important. The electric potential on the droplet interface can be characterized by measuring the zeta-potential (as introduced in 2.4.2). Therefore, the zeta potential for emulsion with three emulsifiers and its change with pH was measured to compare the emulsion stability.

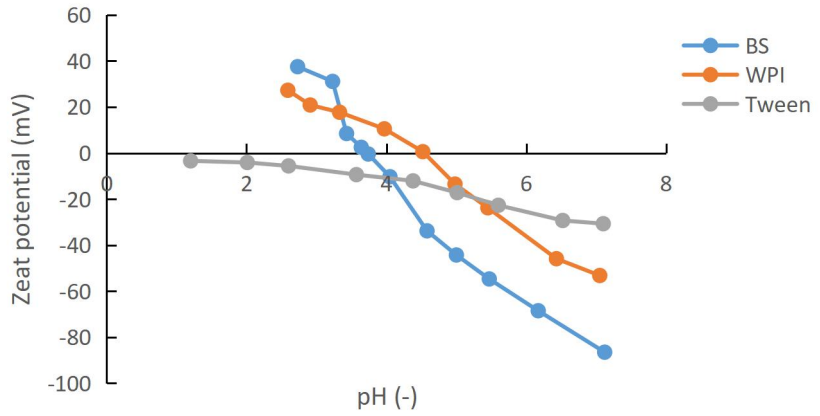


Figure 4.4 The zeta potential of BS, WPI and Tween emulsion changed with pH

Figure 4.4 shows the z-potential value of the emulsion containing three emulsifiers as pH changes. In general, it was observed that at the initial pH (around 6.5), all emulsions exhibited a negative electrical charge, being samples with WPI and BS significantly more electronegative than ones with Tween 80. The low amount of charge of Tween is related to its mechanism for emulsion stabilization. As a non-ionic emulsifier, Tween stabilize emulsion by sterical repulsion (large size) rather than electrostatic repulsion. Therefore, the z-potential value of Tween emulsion cannot be compared with that of other two emulsion. It is worth noting that, in this experiment, Tween-stabled emulsions unexpectedly exhibit rather significant electronegativity, possibly due to the presence of anionic impurities in the emulsion.

As mentioned before, the usual dividing line between stable and unstable particles is ± 30 mV, i.e., droplets with zeta potentials more than 30 mV in absolute value are usually considered stable. In this case, WPI and BS emulsions showed z-potential absolute values over 30 mV. Therefore, these emulsions could be considered to have a satisfying stability against coalescence. It should be noted that, there are some other factors influencing the emulsion stability such as environmental conditions, which means these results cannot guarantee emulsion stability. In addition, at the initial pH (around 6.5), absolute values of z-potential for BS emulsions were higher than that of WPI emulsions, indicating the better ability of BS to reduce the tendency to destabilization.

In addition to measuring the z-potential values at the original pH, the change in

z-potential values with pH was also measured. As can be seen in Figure 4.4, the z-potential values for BS and WPI emulsions increased significantly with decreasing pH (from 7 to 3) and reached zero at pH 3.7 and 4.5 respectively. These differences between emulsifiers can be due to electrical charge differences between these emulsifiers. It is well known that at the isoelectric point, the protein molecules exist as amphoteric ions with zero net molecular charge (i.e., equal positive and negative charges), at which point there is no electrostatic repulsion between protein molecules. Therefore, protein-stabilized emulsions are particularly sensitive to pH and will tend to flocculate at pH values close to the isoelectric point of the adsorbed proteins. In this study, the z-potential of WPI-stabilized emulsions reached 0 at pH 4.7, which means they are unstable at pH 4.7. This agreed with previous literature which shows that whey protein-stabilized emulsions tend to flocculate at pH values (~ 4–5.5) close to their isoelectric point (pI ~ 5) (Small, 2009).

4.1.3 Creaming Stability of The Emulsions

Gravitational separation is one of the most common instability mechanisms in the food industry. It is important to assess the degree of creaming or sedimentation occurred in a product and its susceptibility to this kind of instability. The most common and convenient way to determine gravity separation in emulsions is visual observation. In this case, the creaming stability of the emulsion stabilized by the three emulsifiers was compared by the simplest direct observation method. The creaming index was recorded at 0h (Figure 4.6A), 1h (Figure 4.6B), 2h (Figure 4.6C), 4h (Figure 4.6D), 24h (Figure 4.7) and 48h (Figure 4.8) after the emulsion was prepared. For better observation, the emulsion was stained with methyl blue and Sudan III red after preparation (Figure 4.5). In Figure 4.5, Figure 4.6, Figure 4.7 and Figure 4.8, the three samples from left to right are prepared by WPI,BS and Tween respectively.

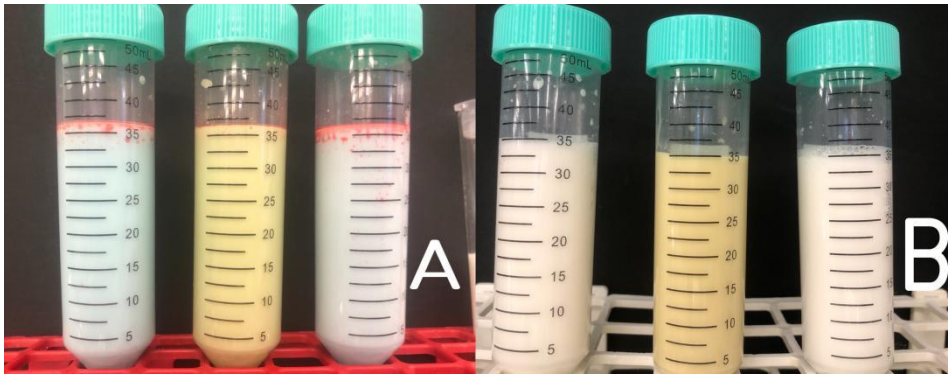


Figure 4.5 The WPI, BS, and Tween emulsions after (A) and before (B) dyeing

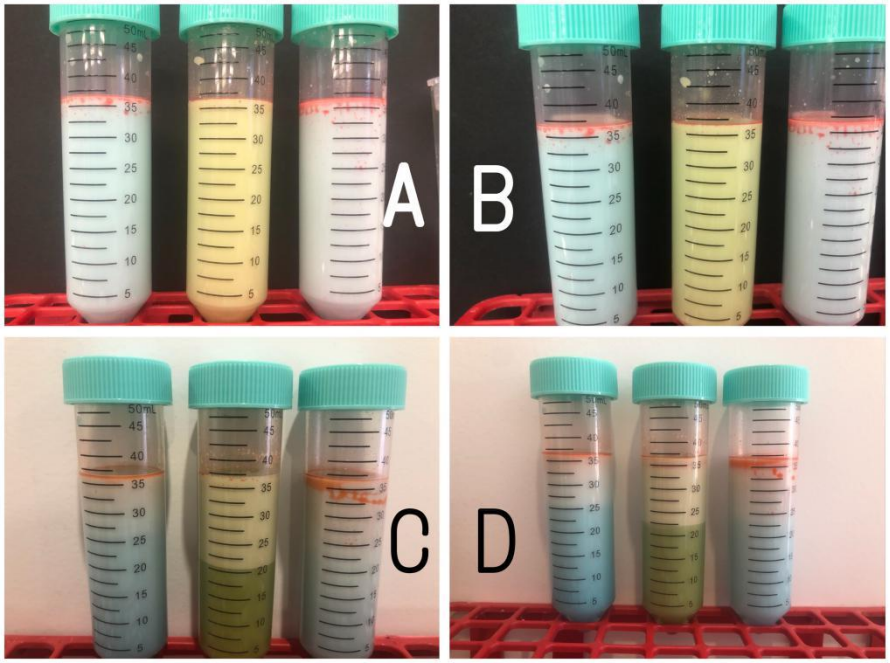


Figure 4.6 The cream layers in the WPI, BS, and Tween emulsion formed after 0h (A), 1h (B), 2h (C) and 4h (D)

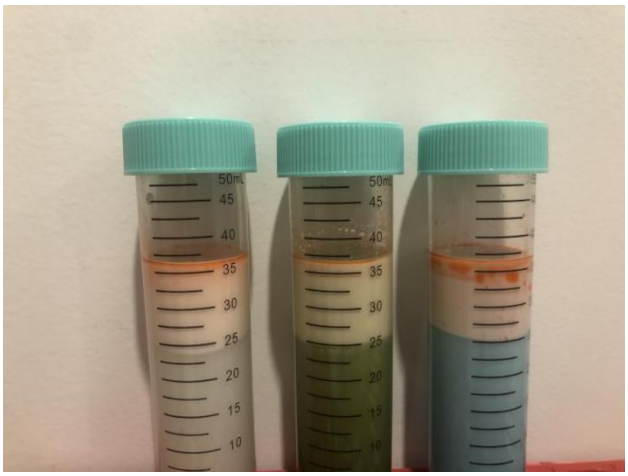


Figure 4.7 The cream layers in the WPI, BS, and Tween emulsion formed after 24h

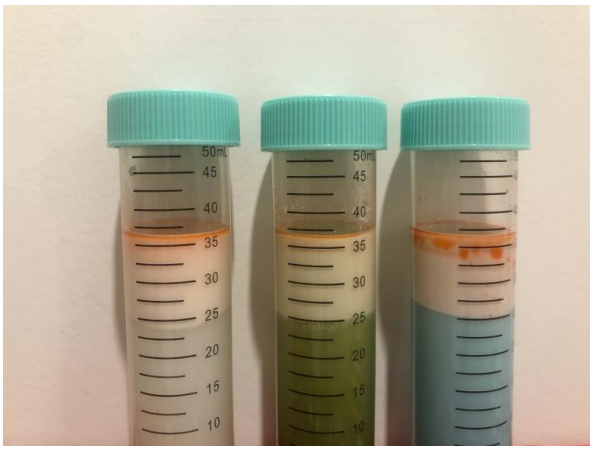


Figure 4.8 The cream layers in the WPI, BS, and Tween emulsion formed after 48h

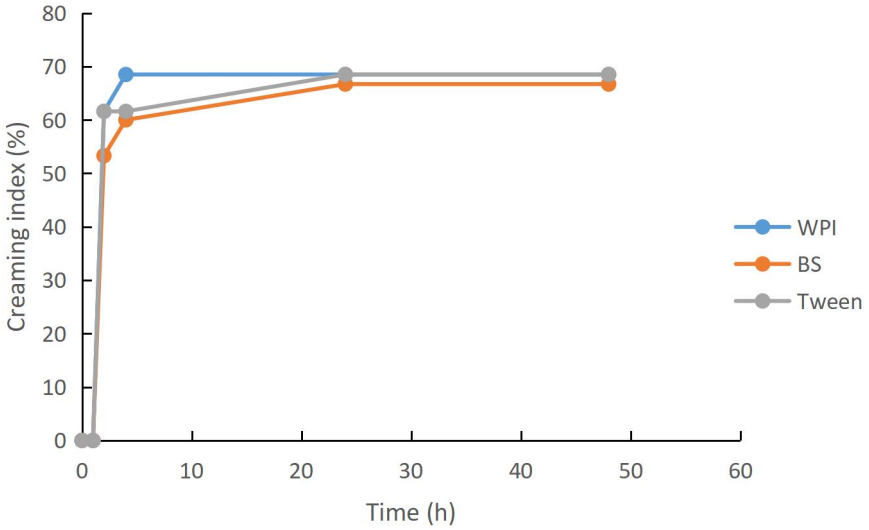


Figure 4.9 The creaming index of emulsions containing WPI, BS and Tween during storage.

Initially, the WPI and Tween emulsions had a homogeneous whitish appearance, and the BS emulsion showed uniform pale yellow (Figure 4.5). All emulsions remained stable for 1h storage (Figure 4.6B). After 2h storage, a visible cream layer formed on top of all the emulsions (Figure 4.6C). In order to compare the creaming stability of emulsions stabilized by different emulsifiers more intuitively, the height of the separated serum layer was recorded and was expressed as the creaming index (CI), and they were plotted as a function of time (Figure 4.9). This value is known to be related to the extent of the droplet aggregation in an emulsion (McClements, 2007). It can be observed that all the emulsions creamed after 2h of their preparation. WPI and Tween emulsions showed higher values of CI than BS emulsions. Finally, a similar behavior was observed for all systems, with creaming index reaching a constant value after 24h of storage.

As indicated in the literature, viscosity and thickness of bulk phase has important influence on emulsion stability (Vélez et al., 2003). Accordingly, the better stability of BS-stabilized emulsion might be due to the way BS molecules adsorbed on to the droplet surface, in which they could occupy the large area of interface, possibly increasing the viscosity and reducing the movement of droplet (Malik, 2016). It is worth mentioning that, gravitational creaming would be the dominant instability mechanism for emulsion stabilized by BS, instead of flocculation or coalescence, because the droplet size distribution in BS emulsion did not change during the examined time period.

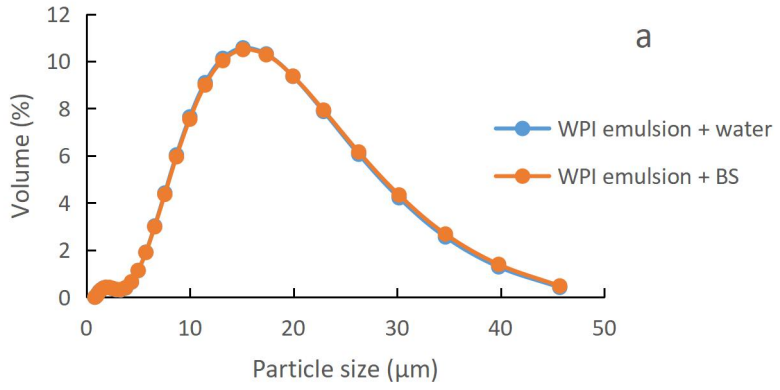
For the other two emulsifiers, it is well known that they can rapidly adsorb to the droplet surface and reduce the interfacial tension during the homogenization process,

resulting in the formation of emulsion with small droplet (Mcclements & Jafari, 2017). However, in this case, they do not seem to exhibit a stronger emulsifying property than BS, especially the ability to stabilize emulsions (including resistance to gravity separation). In addition to the different surface activity related to their different molecular structures, thus resulting in different ability to stabilize droplets, creaming observed in the BS, WPI and Tween-stabilized emulsions could be governed by droplet size in these emulsions.

4.2 The Competition for The Interface and Displacement of WPI and Tween by BS

4.2.1 The Droplet Behaviour of The WPI Emulsions in The Presence of Added BS

These results aim to investigate the droplet behaviour of the WPI emulsion in the presence of added BS and figure out whether WPI adsorbed onto the droplet surface can be displaced by BS at room temperature and pressure. Equal concentration amount of BS solution was added immediately after the emulsion was prepared using WPI as the emulsifier as described in materials and methods. The control sample has been also used as a base for comparison. The change in droplet size and in z-potential was observed to determine whether potential interfacial replacement of WPI with bile salts has occurred (Figure 4.10, 4.11). The change in droplet size was measured in time and recorded after adding BS in the time intervals: 0h, 1h, 2h and 4h. The change in zeta potential was measured after 2h from initial BS addition. This time span has been chosen to make sure the displacement can occur because previous work has shown that the displacement of protein is complete within 2h (Euston et al., 2013).



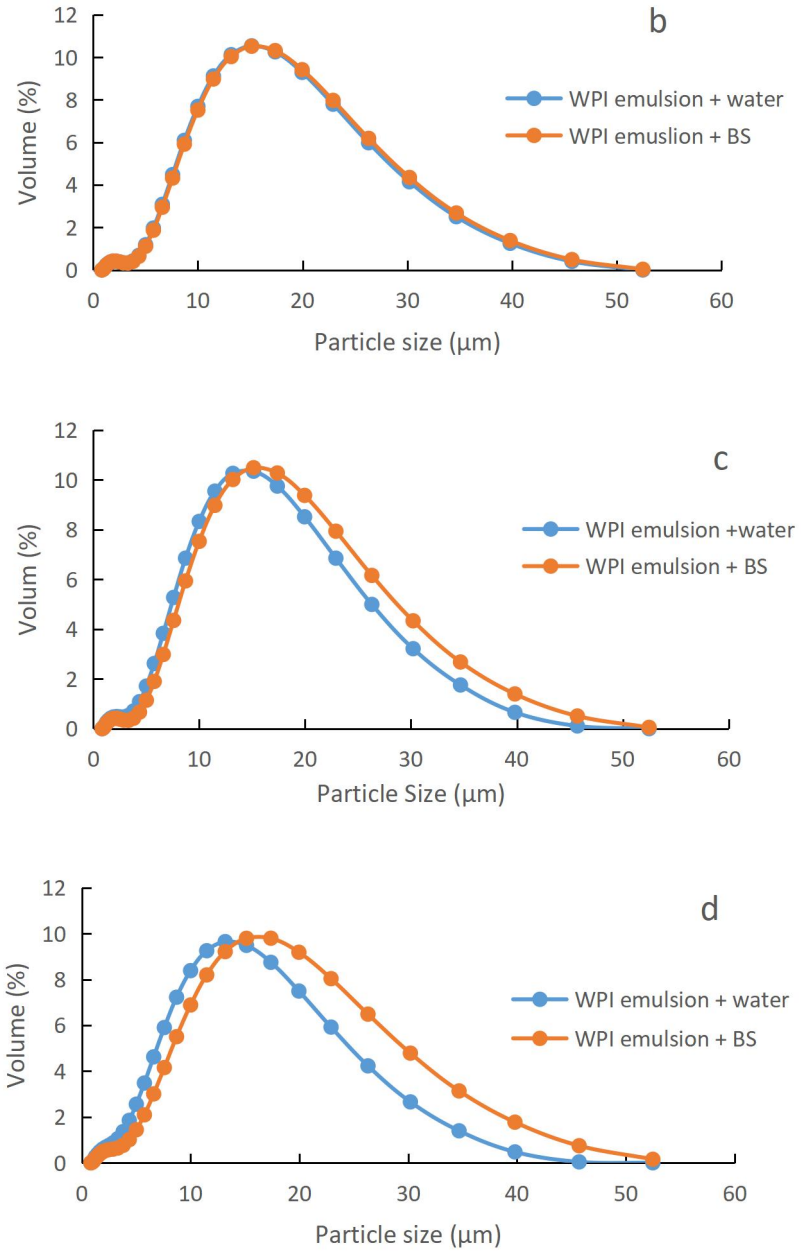


Figure 4.10 Particle size distribution of WPI emulsion after adding BS for 0h (a), 1h (b), 2h (c) and 4h (d)

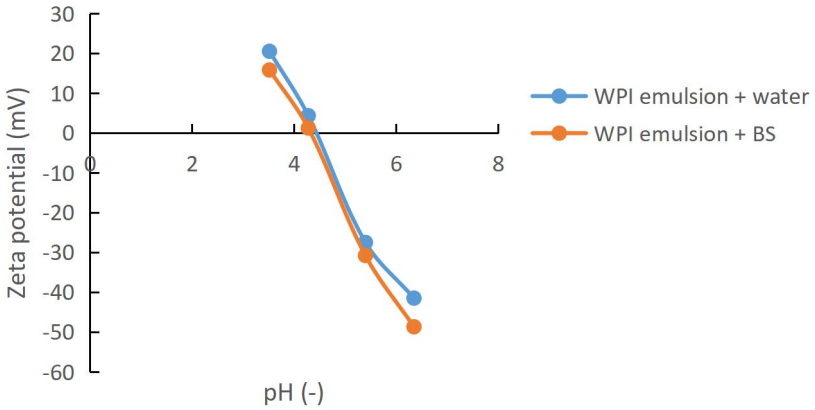


Figure 4.11 Z-potential of WPI Emulsion after adding BS for 2h.

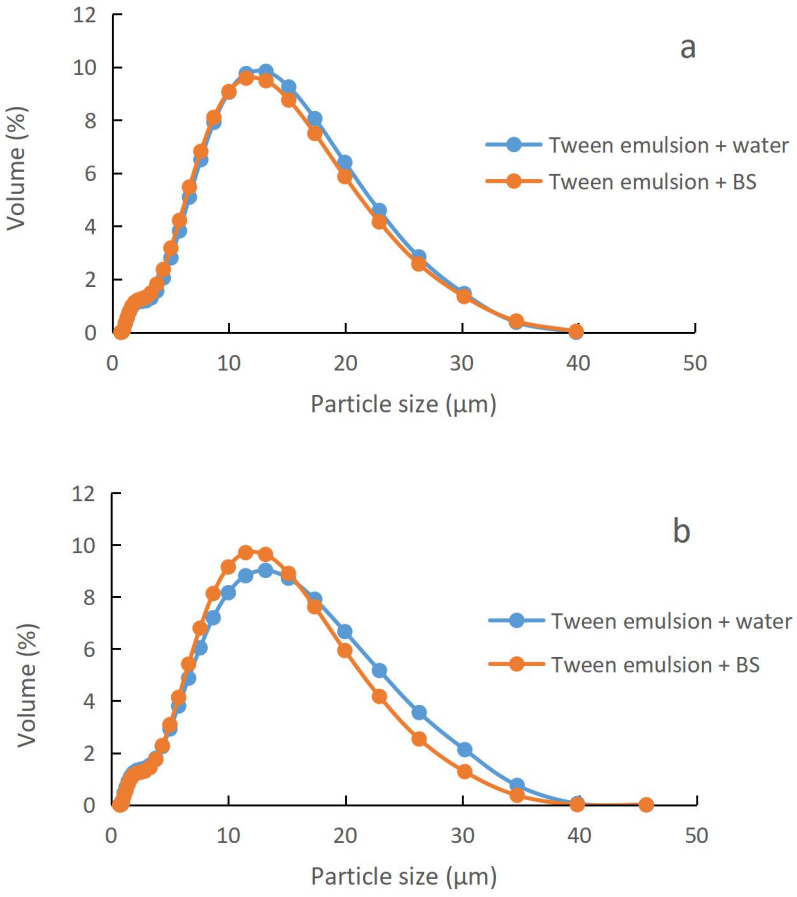
The droplet size distributions and the z-potentials of WPI emulsions with the addition of BS solution are shown in Figure 4.10 and Figure 4.11. As shown in Figure 4.10, there was no significant change on the particle size distribution of the WPI emulsion after adding BS, suggesting there was no any coalescence or droplet aggregation induced by BS. This was in agreement with the results obtained by Sarkar et al., (2016). They discussed the droplet behaviour of emulsions stabilized by Nacas (sodium caseinate) in the presence of BS under simulated intestinal conditions. They found that the droplet diameter of the Nacas emulsion did not change significantly after adding 0.2-5.0 mg/mL bile-extra solution. From the distribution of particle size, it is difficult to determine whether BS competition behavior with protein occurs on the oil-water interface. Therefore, in order to conclude whether the protein on the droplet surface was displaced by BS in WPI emulsion, the change of z-potential needs to be analyzed.

As shown in Figure 4.11, after adding BS solution, the z-potential curve maintains the same trend with the original one, but with a slightly overall downward shift. This result is also consistent with the results of work by Sarkar et al., (2016), in which they found that the z-potential increased slightly from -26 to -30mV after adding aqueous bile-extract (BE). They thought that the increase in negative charge may be due to some anionic component of the BE displacing the original Nacas from the surface of the droplet. However, they found that, the zeta-potential of the Nacas emulsion could not reach the value of that of the BE-stabilized emulsions, even after adding 5 mg/mL of BE solution. This finding also was consistent with our results. The results indicated that, although there were some WPI molecules displaced by BS, some remnants of adsorbed WPI still existed on the interface, resulting in a WPI and BS mixed interface coating on the droplet (Sarkar et al., 2016).

4.2.2 The Droplet Behaviour of The Tween Emulsions in The Presence of Added BS

In order to investigate the competitive behaviour of BS molecules on the surface of

Tween-stabilized emulsion droplets, the same approach to WPI emulsion has been used. Here again, the emulsions were mixed either with water (control sample) or with bile salt solution and the droplet size distribution and z-potential of Tween emulsions before and after the addition of BS has been recorded. Figures 4.13 and 4.14 show the particle size distribution with time and the z-potential curves with pH, respectively. It can be seen from both sets of plots that the droplets in the Tween emulsions changed considerably before and after the addition of BS.



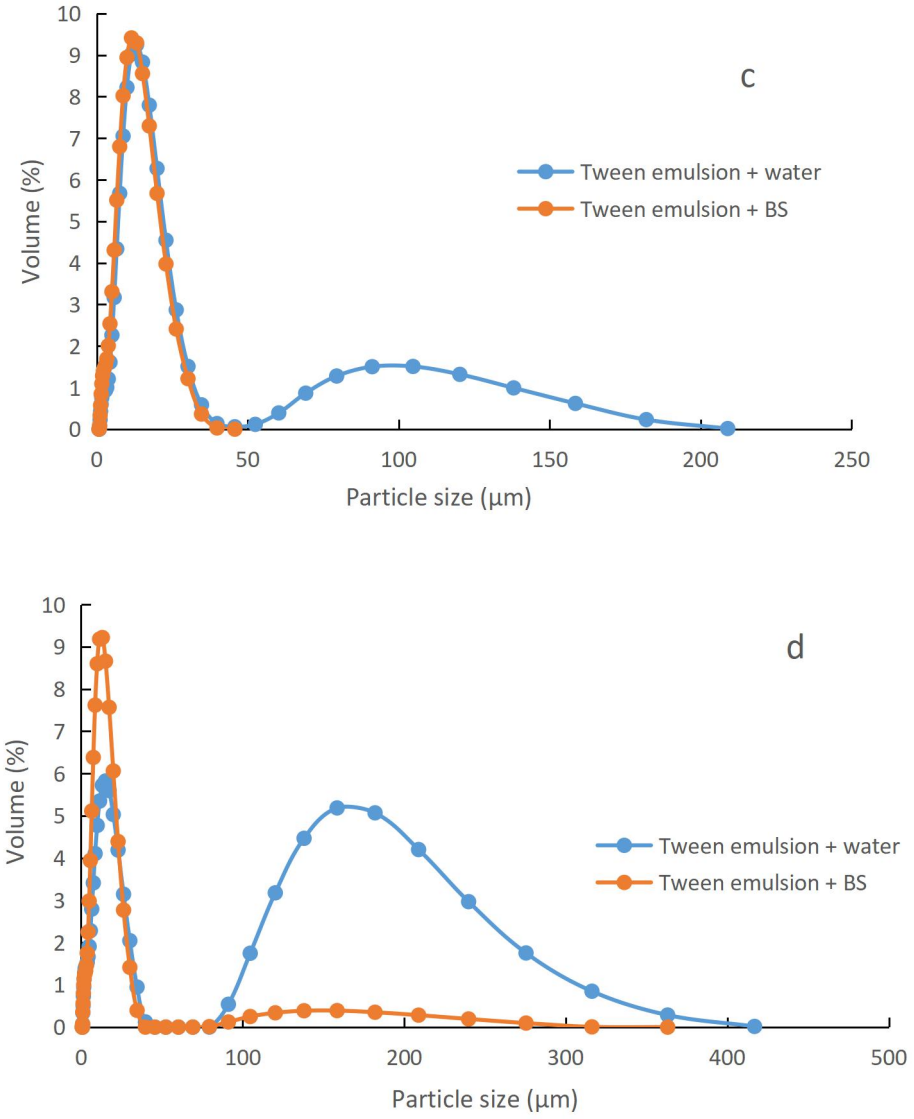


Figure 4.12 Particle size distribution of Tween emulsion after adding BS for 0h (a), 1h (b), 2h (c) and 4h (d)

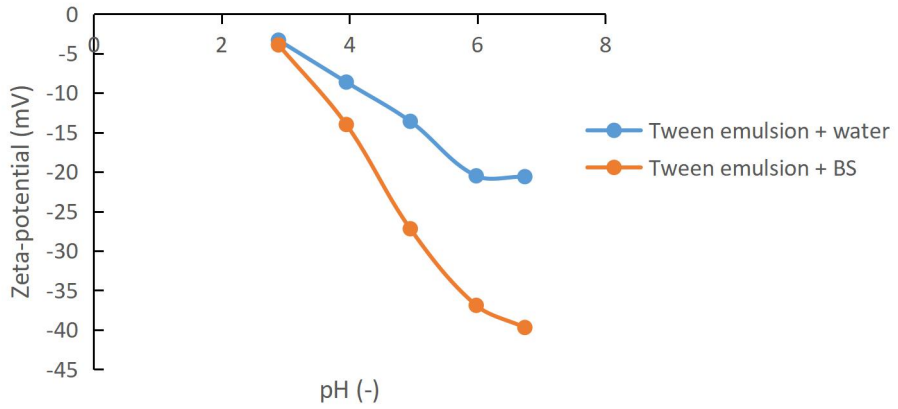


Figure 4.13 Z-potential of WPI Emulsion after adding BS for 2h.

As can be seen in Figure 4.12a and 4.12b, when BS was added (0h and 1h), the overall particle size distribution plot shifted slightly to the right and the peak remained more or less constant, indicating that the smaller droplets in the Tween emulsion were reduced or the droplet size became larger after the addition of BS. This result may be due to the flocculation or aggregation of droplets in the emulsion caused by BS. After 2 hours (shown as Figure 4.13c), the droplet size distribution changed significantly. The curves for the Tween emulsion without BS addition produced bimodal droplet size distribution, which is consistent with the results in chapter 4.1.1, while the curves for emulsion added BS still remained monomodal. After 4 hours, the particle size distribution of the BS-added emulsion also showed a second peak and expanded to 400 μm. These significant changes in droplet size suggested that the BS molecules had a significant effect on the Tween-stabilized emulsion droplets, most likely due to the displacement of Tween on the droplet surface by BS. To further investigate whether the competitive behaviour and displacement occurred, the z-potential of the Tween emulsions was measured after adding BS for 2 hours.

From Figure 4.13 it can be seen that the z-potential of the Tween emulsion with added BS decreased significantly with increasing pH, whereas the change of z-potential of the Tween emulsion without BS were relatively insignificant. This further evidenced that BS adsorption on the surface existed in this Tween emulsion, i.e., displacement of BS for Tween occurred, but the extent of displacement is difficult to derive from the changes in droplet behaviour.

4.2.3 Surface Activity of Three surface-active Substances

In the food industry, emulsions contain a wide variety of surfactants which can adsorb and accumulate at the interface, thus changing the interfacial properties. Although the interface separating the two phases represents only a small fraction of the total volume of a conventional emulsion, it has an important influence on the formation and stability of food emulsions, as well as other physicochemical and sensory properties such as rheology and flavor. Therefore, food scientists are committed to improving the quality of food emulsions by studying the effects of interfacial properties. From a practical point of view, the study of interfacial tension is important to improve the quality of emulsion-based foods. Interfacial tension has an important influence on the size of the droplets produced by homogenization and also determines the stability of the emulsion against Ostwald ripening and coalescence (McClements, 2015). In addition, the measurement of interfacial tension provides valuable information on emulsifiers such as adsorption rate (dynamic), surface activity and critical micelle concentration (McClements, 2015). Therefore, in this study, the interfacial tension of oil-water interface was measured after adding three different emulsifiers in order to compare their ability to reduce the interfacial tension indicating the ability to form emulsion through adsorption at the interface. In addition, from the change of interfacial tension with time after adding emulsifiers, their long time-scale kinetics of adsorption can be compared in order to provide information on the possible rearrangements on the interface when WPI and Tween or BS are present. The

interfacial tension measured for each emulsifier at the oil-water interface is shown in Figure 4.14.

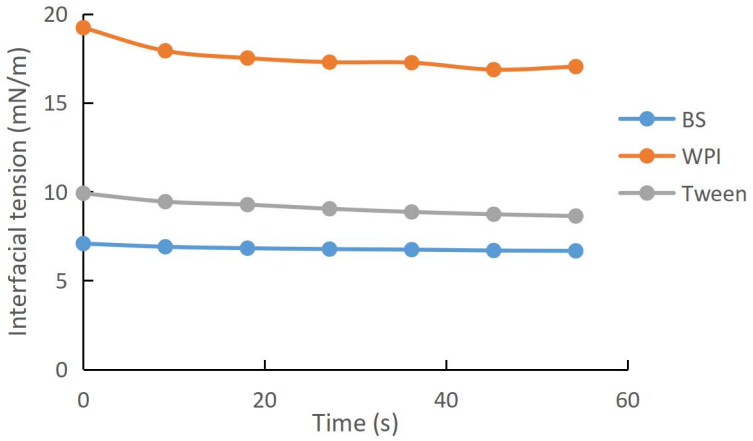


Figure 4.14 The variation of interfacial tension of O/W interface containing BS, WPI and Tween.

Based on previous literature and measurements in this study (Fisher et al., 1985), the oil-water interfacial tension (ITF) was 23.45 mNm^{-1} before the addition of any surface active substance while the ITF was 7.08, 19.23 and 9.91 mNm^{-1} after the addition of BS, WPI and Tween respectively. From the data and Figure 4.14, it can be seen that BS can reduce interfacial tension more effectively than WPI and Tween (i.e., BS provides larger interfacial tension reduction), possibly attribute to their amphiphilic character and different orientation at interface. This result was in line with research realized by Bellesi et al. (2014) which found that BS can adsorb onto the interface more rapidly and effectively than protein and Tween.

Protein adsorption at the oil-water interface decreased the interfacial tension in a function of time. This adsorption can be divided into three steps: protein molecules diffusing to the oil-water interface; protein molecules unfolding and adsorbing onto the surface; adsorbed proteins rearranging (Small et al., 2009). Nevertheless, compared with the adsorption kinetics of BS, the adsorption of proteins at the interface is relatively slow and requires denaturation and rearrangements. This result indicates that there is a possibility that surfactant (BS) with the lower interfacial tension value is going to be present at the interface and possibly displace the surfactant (WPI) with higher value. Although the energy of desorption ought to be compared in this case. In order to confirm this assumption, Bellesi et al. (2014) also measured the dynamics of coadsorption of the mixed systems and the sequential adsorption of BS at protein covered films. They found that cooperation between these components occurred, suggesting that proteins may not be completely displaced by the BS molecules from the oil-water interface, although BS can occupy the interface rapidly. This result is consistent with the result from the change of droplet size and

zeta-potential. However, different results were obtained by Maldonado-Valderrama and co-workers (2008) who investigated the competitive displacement of β-lactoglobulin by bile salts from oil−water interfaces in vitro under model duodenal digestion conditions. They found that the complete displacement of β-lactoglobulin by BS occurred under duodenal conditions. They also explained the mechanism of the displacement of intact protein network by BS: the dilatational modulus of the adsorbed β-lactoglobulin film at olive oil−water interfaces was severely decreased by the addition of BS. They believe that bile salts can penetrate into, weaken, and break up the interfacial β-lactoglobulin networks. In addition, their AFM images suggested that displacement occurs via an orogenic mechanism. The different results obtained in the present study from Maldonado-Valderrama's findings (2008) may be due to the fact that the present experiment was carried out at ambient temperature rather than under conditions simulating duodenal digestion. In addition, the observed differences might also be resulted from different proteins or surfactants used in previous study.

Comparing the kinetics of BS and Tween adsorption (Figure 4.14), it is obvious that BS can adsorb onto the O/W interface and reduce the interfacial tension more effectively than Tween does. This is in agreement with the result obtained by Bellesi et al. (2014). In order to observe the displacement of Tween by BS, they also measured the interfacial behavior of mixed Tween-BS films in simultaneous and sequential adsorption. They found that Tween 20 can neither resist the displacement by BS nor compete with the BS molecules at the oil-water interface. BS molecules would penetrate in the interface and displace almost totally the Tween 20 molecules previously adsorbed (Bellesi et al., 2014).

In order to explain the better ability of BS absorbing onto the O/W interface, it is important to note their unique molecular structure. Tiss (2001) and co-workers, revealed that BS adopt a planar conformation at the interface by calculating the area of BS molecules, which is due to their facial amphiphilic molecular structure. As BS adopts a planar conformation at the interface, it is possible for just one molecule to occupy a relatively large area at the interface, facilitating the rapid adsorption of BS molecules at the interface, creating large desorption energy.

Chapter 5. Conclusion

It is well known that bile salts play an important role in human body during fat digestion. This study aims to investigate the interaction of bile salt with commonly used emulsifiers in formulated emulsions to deepen the understanding of the interfacial phenomena and mechanisms. This may be helpful to manipulate the interfacial structure and design a stable layer in food emulsions so that they are resistant to the activity of lipase to regulate the digestion of fats.

This study examined the performance of BS as an emulsifier and evaluated the effect of BS on previously covered surfaces. By comparing the droplet size, z-potential and creaming index of emulsions stabilized by BS, WPI and Tween, it can be concluded that BS would seem to have better emulsifying properties than WPI and Tween, indicating its better ability to adsorb onto the oil-water interface. In order to investigate the effect of BS on the droplet interface covered by two common emulsifiers, WPI and Tween, the droplet size and z-potential of WPI and Tween stabilized emulsions were measured after adding BS solution, and the oil-water surface tension in the presence of the three substances was determined. The results indicated that although the molecules of BS tend to occupy the oil-water interface very quickly, they are not able to completely displace the proteins from the oil-water interface, whereas BS molecules would displace almost totally the Tween 80 molecules previously adsorbed. In conclusion, the research demonstrated that BS had a better ability to adsorb onto the oil-water interface than two commercially available emulsifiers (WPI and Tween), and it was able to displace them from the oil-water interface. However, the extent of displacement and the influencing factors could not be quantified exactly in the present study.

As discussed previously in the literature, the structure and nature of BS and the type of surface-active substance has an important influence on displacement and competitive adsorption (Torcello-Gómez et al., 2012). Accordingly, subsequent studies need to focus on the effect of different types of BS on different surface active substances in order to understand digestion of emulsion better. It is recommended to use dynamic interfacial tension measurements for the future examination of the displacements of the emulsifiers in the oil-water systems.

Chapter 6. References

Ariyaprakai, S., Limpachoti, T., & Pradipasena, P. (2013). Interfacial and emulsifying properties of sucrose ester in coconut milk emulsions in comparison with tween. *Food Hydrocolloids, 30*(2013), 358-367. http://dx.doi.org/10.1016/j.foodhyd.2012.06.003

Arancibia, C., Riquelme, N., Zúñiga, R., & Matiacevich, S. (2017). Comparing the effectiveness of natural and synthetic emulsifiers on oxidative and physical stability of avocado oil-based nanoemulsions. *Innovative Food Science and Emerging Technologies, 44*(2017), 159-166. http://dx.doi.org/10.1016/j.ifset.2017.06.009

Binks, B. P., & Lumsdon, S. O. (2000). Transitional phase inversion of solid-stabilized emulsions using particle mixtures. *Langmuir, 16*(8), 3748-3756. https://doi.org/10.1021/la991427q

Bai, L., Huan, S., Li, Z., & Mcclements, D. J. (2017). Comparison of emulsifying properties of food-grade polysaccharides in oil-in-water emulsions: gum arabic, beet pectin, and corn fiber gum. *Food Hydrocolloids, 66*(2017), 144-153. http://dx.doi.org/10.1016/j.foodhyd.2016.12.019

Berry, J. D., Neeson, M. J., Dagastine, R. R., Chan, D. Y., & Tabor, R. F. (2015). Measurement of surface and interfacial tension using pendant drop tensiometry. *Journal of Colloid and Interface Science, 454*(15), 226-237. https://doi.org/10.1016/j.jcis.2015.05.012

Bellesi, F. A., Ruiz-Henestrosa, V. M. P., & Pilosof, A. M. (2014). Behavior of protein interfacial films upon bile salts addition. *Food Hydrocolloids, 36*(2014), 115-122. https://doi.org/10.1016/j.foodhyd.2013.09.010

Chantrapornchai, W., Clydesdale, F. M., & McClements, D. J. (2001). Influence of flocculation on optical properties of emulsions. *Journal of Food Science, 66*(3), 464-469. https://doi.org/10.1111/j.1365-2621.2001.tb16130.x

Chanamai, R., & McClements, D. J. (2000). Creaming stability of flocculated monodisperse oil-in-water emulsions. *Journal of Colloid and Interface Science, 225*(1), 214-218. https://doi.org/10.1006/jcis.2000.6766

Darling, D. F. (1982). Recent advances in the destabilization of dairy emulsions. *Journal of Dairy Research, 49*(4), 695-712. https://doi.org/10.1017/S0022029900022834

Dapčević Hadnađev, T., Dokić, P., Krstonošić, V., & Hadnađev, M. (2013). Influence of oil phase concentration on droplet size distribution and stability of oil-in-water emulsions. *European Journal of Lipid Science and Technology, 115*(3), 313-321. https://doi.org/10.1002/ejlt.201100321

di Gregorio, M. C., Travaglini, L., Del Giudice, A., Cautela, J., Pavel, N. V., & Galantini, L. (2018). Bile salts: natural surfactants and precursors of a broad family of complex amphiphiles. *Langmuir, 35*(21), 6803-6821. https://doi.org/10.1021/acs.langmuir.8b02657

Euston, S. R., Bellstedt, U., Schillbach, K., & Hughes, P. S. (2011). The adsorption and competitive adsorption of bile salts and whey protein at the oil–water interface. *Soft Matter, 7*(19), 8942-8951. https://doi.org/10.1039/C1SM05840A

Euston, S. R., Baird, W. G., Campbell, L., & Kuhns, M. (2013). Competitive adsorption of dihydroxy and trihydroxy bile salts with whey protein and casein in oil-in-water emulsions. *Biomacromolecules, 14*(6), 1850-1858. https://doi.org/10.1021/bm4002443

Fisher, L. R., Mitchell, E. E., & Parker, N. S. (1985). Interfacial tensions of commercial vegetable oils with water. *Journal of Food Science, 50*(4), 1201-1202. https://doi.org/10.1111/j.1365-2621.1985.tb13052.x

Hasenhuettl, G. L., & Hartel, R. W. (2008). *Food emulsifiers and their applications.* New York: Springer Science. https://doi.org/10.1007/978-3-030-29187-7

Holm, R., Müllertz, A., & Mu, H. (2013). Bile salts and their importance for drug absorption. *International Journal of Pharmaceutics, 453*(1), 44-55. https://doi.org/10.1016/j.ijpharm.2013.04.003

Jódar-Reyes, A. B., Torcello-Gómez, A., Wulff-Pérez, M., Gálvez-Ruiz, M. J., & Martín-Rodríguez, A. (2010). Different stability regimes of oil-in-water emulsions in the presence of bile salts. *Food Research International, 43*(6), 1634-1641. https://doi.org/10.1016/j.foodres.2010.05.005

Kabalnov, A. (2001). Ostwald ripening and related phenomena. *Journal of Dispersion Science and Technology, 22*(1), 1-12. https://doi.org/10.1081/DIS-100102675

Kralova, I., & Sjöblom, J. (2009). Surfactants used in food industry: a review. *Journal of Dispersion Science and Technology, 30*(9), 1363-1383. https://doi.org/10.1080/01932690902735561

Lad, V. N., & Murthy, Z. V. P. (2016). Natural materials as additives in food emulsions. In A. M.Grumezescu, (Eds.), *Novel Approaches of Nanotechnology in Food* (pp. 97-132). https://doi.org/10.1016/B978-0-12-804308-0.00004-2

Lam, R. S. H., & Nickerson, M. T. (2013). Food proteins: a review on their emulsifying properties using a structure–function approach. *Food Chemistry, 141*(2013), 975–984. http://dx.doi.org/10.1016/j.foodchem.2013.04.038

Mcclements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition, 47*(7), 611-649. https://doi.org/10.1080/10408390701289292

McClements, D. J. (2015). *Food emulsions: principles, practices, and techniques* (3rd ed.). CRC Press. https://doi.org/10.1201/b18868

Malik, N. A. (2016). Solubilization and interaction studies of bile salts with surfactants and drugs: a review. *Applied Biochemistry and Biotechnology, 179*(2016), 179-201. https://doi.org/10.1007/s12010-016-1987-x

Madenci, D., & Egelhaaf, S. U. (2010). Self-assembly in aqueous bile salt solutions. *Current Opinion in Colloid & Interface Science, 15*(1-2), 109-115. https://doi.org/10.1016/j.cocis.2009.11.010

Mcclements, D. J., & Gumus, C. E. (2016). Natural emulsifiers — biosurfactants, phospholipids, biopolymers, and colloidal particles: molecular and physicochemical basis of functional performance. *Advances in Colloid & Interface Science, 234*(2016), 3-26. http://dx.doi.org/10.1016/j.cis.2016.03.002

Mcclements, D. J., & Jafari, S. M. (2017). Improving emulsion formation, stability and performance using mixed emulsifiers: a review. *Advances in Colloid and Interface Science, 251*(2018), 55–79. https://doi.org/10.1016/j.cis.2017.12.001

Maldonado-Valderrama, J., Muros-Cobos, J. L., Holgado-Terriza, J. A., & Cabrerizo-Vílchez, M. A. (2014). Bile salts at the air–water interface: adsorption and desorption. *Colloids and Surfaces B: Biointerfaces, 120*(2014), 176-183. https://doi.org/10.1016/j.colsurfb.2014.05.014

Maldonado-Valderrama, J., Woodward, N. C., Gunning, A. P., Ridout, M. J., Husband, F. A., Mackie, A. R., ... & Wilde, P. J. (2008). Interfacial characterization of β-lactoglobulin networks: Displacement by bile salts. *Langmuir, 24*(13), 6759-6767. https://doi.org/10.1021/la800551u

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., & Mackie, A. (2011). The role of bile salts in digestion. *Advances in Colloid and Interface Science, 165*(1),

36-46. https://doi.org/10.1016/j.cis.2010.12.002

Macierzanka, A., Torcello-Gómez, A., Jungnickel, C., & Maldonado-Valderrama, J. (2019). Bile salts in digestion and transport of lipids. *Advances in Colloid and Interface Science, 274*(2019), 102045. https://doi.org/10.1016/j.cis.2019.102045

Meyer, E. E., Rosenberg, K. J., & Israelachvili, J. (2006). Recent progress in understanding hydrophobic interactions. *Proceedings of the National Academy of Sciences, 103*(43), 15739-15746. https://doi.org/10.1073/pnas.0606422103

Newling, B., Glover, P. M., Keddie, J. L., Lane, D. M., & McDonald, P. J. (1997). Concentration profiles in creaming oil-in-water emulsion layers determined with stray field magnetic resonance imaging. *Langmuir, 13*(14), 3621-3626. https://doi.org/10.1021/la9610056

O'Connor, C. J., Ch'ng, B. T., & Wallace, R. G. (1983). Studies in bile salt solutions: 1. Surface tension evidence for a stepwise aggregation model. *Journal of Colloid and Interface Science, 95*(2), 410-419. https://doi.org/10.1016/0021-9797(83)90200-X

Ozturk, B., & McClements, D. J. (2016) Progress in natural emulsifiers for utilization in food emulsions. *Current Opinion in Food Science, 7*(2016), 1–6. http://dx.doi.org/10.1016/j.cofs.2015.07.008

O'connor, C. J., & Wallace, R. G. (1984). Studies in bile salt solutions: The effect of pH on the cholate and taurocholate stimulation of human milk lipase catalyzed hydrolysis of *p*-nitrophenylacetate. *European journal of biochemistry, 141*(2), 379-383. https://doi.org/10.1111/j.1432-1033.1984.tb08202.x

Pilosof, A. M. (2017). Potential impact of interfacial composition of proteins and polysaccharides stabilized emulsions on the modulation of lipolysis. The role of bile salts. *Food Hydrocolloids, 68*(2017), 178-185. https://doi.org/10.1016/j.foodhyd.2016.08.030

Sarkar, A., Horne, D. S., & Singh, H. (2010). Interactions of milk protein-stabilized oil-in-water emulsions with bile salts in a simulated upper intestinal model. *Food Hydrocolloids, 24*(2-3), 142-151. https://doi.org/10.1016/j.foodhyd.2009.08.012

Small, D. M., Wang, L., & Mitsche, M. A. (2009). The adsorption of biological peptides and proteins at the oil/water interface. A potentially important but largely unexplored field. *Journal of Lipid Research, 50*(2009), S329-S334. https://doi.org/10.1194/jlr.R800083-JLR200

Sarkar, A., Ye, A., & Singh, H. (2016). On the role of bile salts in the digestion of emulsified lipids. *Food Hydrocolloids, 60*(2016), 77-84. https://doi.org/10.1016/j.foodhyd.2016.03.018

Tiss, A., Ransac, S., Lengsfeld, H., Hadvàry, P., Cagna, A., & Verger, R. (2001). Surface behaviour of bile salts and tetrahydrolipstatin at air/water and oil/water interfaces. *Chemistry and Physics of Lipids, 111*(1), 73-85. https://doi.org/10.1016/S0009-3084(01)00149-9

Torcello-Gómez, A., Jódar-Reyes, A. B., Maldonado-Valderrama, J., & Martín-Rodríguez, A. (2012). Effect of emulsifier type against the action of bile salts at oil–water interfaces. *Food Research International, 48*(1), 140-147. https://doi.org/10.1016/j.foodres.2012.03.007

Vélez, G., Fernández, M. A., Muñoz, J., Williams, P. A., & English, R. J. (2003). Role of hydrocolloids in the creaming of oil in water emulsions. *Journal of Agricultural and Food Chemistry, 51*(1), 265-269. https://doi.org/10.1021/jf020664n

Appendix: Experiment Raw Data

Table A1 The particle size of WPI emulsion after 0h, 1h, 2h, 4h, 24h and 48h

Particle size (μm)	Volume (%)					
	0h	1h	2h	4h	24h	48h
0.630957	0	0	0	0	0	0
0.724436	0	0	0	0	0	0.053222
0.831764	0	0	0	0	0.078777	0.188701
0.954993	0.043844	0.035797	0.047506	0.035951	0.23293	0.430113
1.096478	0.140133	0.160396	0.196056	0.13005	0.471128	0.633181
1.258925	0.263024	0.475147	0.542207	0.297291	0.661156	0.855113
1.44544	0.354695	0.836899	0.942793	0.446745	0.855251	1.057836
1.659587	0.443365	1.323632	1.468473	0.612076	1.003646	1.225273
1.905461	0.510319	1.910477	2.066685	0.762362	1.092673	1.339134
2.187762	0.5559	2.581928	2.683053	0.883877	1.110591	1.391896
2.511886	0.591786	3.314465	3.251867	0.97362	1.074389	1.398566
2.884031	0.651077	4.078396	3.718557	1.054268	1.031695	1.399191
3.311311	0.784784	4.84339	4.062723	1.17374	1.049607	1.450884
3.801894	1.052948	5.579029	4.300704	1.393401	1.196903	1.611972
4.365158	1.509911	6.244124	4.472117	1.771293	1.530774	1.924917
5.011872	2.201902	6.80841	4.624703	2.357282	2.095738	2.416259
5.754399	3.136728	7.22834	4.790646	3.166975	2.902264	3.082846
6.606934	4.322071	7.474481	4.994831	4.215457	3.958143	3.92352
7.585776	5.676514	7.506046	5.241116	5.437845	5.187361	4.878487
8.709636	7.101192	7.295777	5.518695	6.748467	6.492629	5.884221
10	8.379495	6.844626	5.776298	7.947712	7.669425	6.803896
11.481536	9.371671	6.156406	5.969032	8.90405	8.589153	7.55878
13.182567	9.879465	5.298829	6.024489	9.423641	9.072942	8.021831
15.135612	9.804896	4.319814	5.882116	9.40431	9.038107	8.126776
17.378008	9.12058	3.328339	5.509015	8.807353	8.466577	7.829667
19.952623	7.888286	2.386677	4.898775	7.680816	7.420384	7.136819
22.908677	6.304151	1.585483	4.110422	6.202305	6.075203	6.132542
26.30268	4.578016	0.955304	3.214994	4.563287	4.605965	4.91631
30.199517	2.951398	0.516744	2.325245	3.005625	3.22207	3.646076
34.673685	1.628465	0.245977	1.528523	1.693655	2.039957	2.437725
39.810717	0.629848	0.10617	0.900503	0.740911	1.152167	1.434211
45.708819	0.123537	0.047914	0.465928	0.165635	0.507772	0.648397
52.480746	0	0.027015	0.211425	0	0.114622	0.161639

Table A2 The particle size of BS emulsion after 0h, 1h, 2h, 4h, 24h and 48h

Particle size (μm)	Volume (%)					
	0h	1h	2h	4h	24h	48h
1.096478	0	0	0	0	0	0
1.258925	0.023321	0.031444	0.021505	0.03022	0.022534	0.018495
1.44544	0.139396	0.182106	0.132016	0.175362	0.138006	0.116782
1.659587	0.430133	0.52619	0.425239	0.508989	0.442283	0.393012
1.905461	0.751242	0.874756	0.746755	0.850727	0.771532	0.697528
2.187762	1.195013	1.335127	1.190222	1.307324	1.220456	1.118391
2.511886	1.804858	1.941684	1.799512	1.914525	1.83137	1.69672
2.884031	2.639739	2.745567	2.635025	2.723832	2.6636	2.48955
3.311311	3.722451	3.762053	3.721133	3.750661	3.740312	3.520389
3.801894	5.022736	4.961237	5.028461	4.963883	5.0324	4.76205
4.365158	6.427316	6.244417	6.443378	6.262275	6.428222	6.107326
5.011872	7.804816	7.503032	7.833046	7.534212	7.797825	7.430325
5.754399	8.987195	8.59677	9.026814	8.636213	8.974244	8.56889
6.606934	9.847288	9.420458	9.895202	9.461476	9.830637	9.400146
7.585776	10.219754	9.827481	10.270369	9.863202	10.201636	9.764452
8.709636	9.992452	9.712376	10.037762	9.737418	9.974115	9.553013
10	9.153631	9.044131	9.184395	9.056251	9.13527	8.752944
11.481536	7.758111	7.847313	7.765463	7.846722	7.739681	7.416449
13.182567	6.032956	6.294626	6.011996	6.284434	6.014923	5.762022
15.135612	4.188654	4.557537	4.138817	4.541578	4.173982	3.989065
17.378008	2.494762	2.863424	2.429336	2.847229	2.486259	2.378739
19.952623	1.193719	1.49681	1.113083	1.477116	1.205068	1.087954
22.908677	0.170457	0.23146	0.150472	0.226348	0.175645	0.190319
26.30268	0	0	0	0	0	0

Table A3 The particle size of Tween emulsion after 0h, 1h, 2h, 4h, and 24h

Particle size (μm)	Volume (%)				
	0h	1h	2h	4h	24h
0.724436	0	0	0	0	0
0.831764	0.017887	0.036869	0	0	0
0.954993	0.105966	0.166851	0.071954	0.073211	0.061221
1.096478	0.336733	0.451864	0.228171	0.338781	0.192839
1.258925	0.541168	0.667003	0.430123	0.508582	0.359029
1.44544	0.764013	0.899769	0.598073	0.733302	0.495976
1.659587	0.951298	1.094025	0.758533	0.944479	0.625566
1.905461	1.084507	1.240647	0.87021	1.136719	0.713633
2.187762	1.150167	1.326874	0.923754	1.284893	0.75219
2.511886	1.168113	1.368463	0.931001	1.380065	0.750409
2.884031	1.191687	1.411953	0.935758	1.430607	0.743546
3.311311	1.296352	1.527432	1.004045	1.465818	0.784509
3.801894	1.561373	1.787847	1.208276	1.528907	0.930532
4.365158	2.049396	2.243589	1.61087	1.664701	1.230616
5.011872	2.804573	2.920978	2.261213	1.910448	1.722884
5.754399	3.823434	3.802979	3.168628	2.282297	2.414186
6.606934	5.097017	4.874079	4.337608	2.792019	3.306913
7.585776	6.50997	6.040753	5.674509	3.412293	4.328375
8.709636	7.915095	7.199055	7.052022	4.105457	5.380738
10	9.05084	8.16107	8.222639	4.772657	6.275441
11.481536	9.751669	8.815888	9.021688	5.348637	6.888659
13.182567	9.837215	9.021901	9.251395	5.722237	7.071194
15.135612	9.249273	8.717963	8.830946	5.822409	6.761048
17.378008	8.056816	7.912641	7.795165	5.595613	5.980662
19.952623	6.406151	6.666781	6.272816	5.03137	4.820282
22.908677	4.594265	5.167646	4.545562	4.190132	3.481302
26.30268	2.843827	3.551607	2.869984	3.144312	2.153672
30.199517	1.460714	2.123677	1.509849	2.045398	1.025564
34.673685	0.38048	0.748701	0.585633	0.947624	0.239632
39.810717	0	0.051095	0.134951	0.124653	0
45.708819		0	0.056687	0	0
52.480746			0.115242	0	0
60.255959			0.39046	0	0.000622
69.183097			0.866216	0	0
79.432823			1.278329	0.000575	0.23006
91.201084			1.503061	0.54059	0.983213
104.712855			1.508983	1.747484	2.236049
120.226443			1.318336	3.177194	3.745856

138.038426			0.992855	4.466944	5.003278
158.489319			0.619718	5.185805	5.533548
181.970086			0.229848	5.069512	5.09323
208.929613			0.014889	4.201389	3.859173
239.883292				2.968104	2.366912
275.42287				1.75408	1.093721
316.227766				0.849061	0.337698
363.078055				0.285349	0.026646
416.869383				0.016292	0

Table A4 The particle size of BS, WPI and Tween emulsion

Particle size (μm)	Volume (%)		
	BS	WPI	Tween
0.724436	0	0	0
0.831764	0	0	0.017887
0.954993	0	0.043844	0.105966
1.096478	0	0.140133	0.336733
1.258925	0.023321	0.263024	0.541168
1.44544	0.139396	0.354695	0.764013
1.659587	0.430133	0.443365	0.951298
1.905461	0.751242	0.510319	1.084507
2.187762	1.195013	0.5559	1.150167
2.511886	1.804858	0.591786	1.168113
2.884031	2.639739	0.651077	1.191687
3.311311	3.722451	0.784784	1.296352
3.801894	5.022736	1.052948	1.561373
4.365158	6.427316	1.509911	2.049396
5.011872	7.804816	2.201902	2.804573
5.754399	8.987195	3.136728	3.823434
6.606934	9.847288	4.322071	5.097017
7.585776	10.219754	5.676514	6.50997
8.709636	9.992452	7.101192	7.915095
10	9.153631	8.379495	9.05084
11.481536	7.758111	9.371671	9.751669
13.182567	6.032956	9.879465	9.837215
15.135612	4.188654	9.804896	9.249273
17.378008	2.494762	9.12058	8.056816
19.952623	1.193719	7.888286	6.406151
22.908677	0.170457	6.304151	4.594265
26.30268	0	4.578016	2.843827
30.199517	0	2.951398	1.460714

34.673685	0	1.628465	0.38048
39.810717	0	0.629848	0
45.708819	0	0.123537	0
52.480746	0	0	0

Table A5 The change of zeta-potential of WPI emulsion with pH

pH	Zeta-potential
2.59	27.25
2.91	20.85
3.33	17.7
3.97	10.5
4.52	0.588
4.67	-1.54
4.98	-13.5
5.45	-23.8
6.43	-45.9

Table A6 The change of zeta-potential of BS emulsion with pH

pH	Zeta-potential
3.43	8.46
3.57	4.74
3.64	2.47
3.74	-0.435
4.05	-10.3
5.00	-44.3
5.47	-54.7
6.17	-68.5
7.12	-86.5

Table A7 The change of zeta-potential of Tween emulsion with pH

pH	Zeta-potential
1.2	-3.42
2.01	-4.12
2.6	-5.6
3.57	-9.39
4.38	-12.1
5.01	-17.2
5.60	-22.7
6.52	-29.3
7.10	-30.7

Table A8 The droplet size of WPI emulsion after adding BS (0h)

Particle size (μm)	Volume (%)	
	WPI emulsion + water	WPI emulsion + BS
0.831764	0	0
0.954993	0.043903	0.036134
1.096478	0.136649	0.117918
1.258925	0.244348	0.228324
1.44544	0.320173	0.308354
1.659587	0.385648	0.379813
1.905461	0.418057	0.418665
2.187762	0.410267	0.416958
2.511886	0.366531	0.378253
2.884031	0.315384	0.330327
3.311311	0.304308	0.320248
3.801894	0.390595	0.405148
4.365158	0.641435	0.651213
5.011872	1.130804	1.130965
5.754399	1.907832	1.892981
6.606934	3.017384	2.98227
7.585776	4.416484	4.358253
8.709636	6.033224	5.952482
10	7.645261	7.548206
11.481536	9.098158	8.995053
13.182567	10.12605	10.03087
15.135612	10.570386	10.49856
17.378008	10.313446	10.278196
19.952623	9.362545	9.373065
22.908677	7.874645	7.931871
26.30268	6.059672	6.156412
30.199517	4.213832	4.335294
34.673685	2.553772	2.677082
39.810717	1.284228	1.391036
45.708819	0.414976	0.47605

Table A9 The droplet size of WPI emulsion after adding BS (1h)

Particle size	WPI emulsion + water	WPI emulsion + BS
0.831764	0	0
0.954993	0.036238	0.033158
1.096478	0.118412	0.110234
1.258925	0.229607	0.219771
1.44544	0.309829	0.300376
1.659587	0.381283	0.373676
1.905461	0.420004	0.415405
2.187762	0.418539	0.416856
2.511886	0.381184	0.380658
2.884031	0.336487	0.333672
3.311311	0.332229	0.322457
3.801894	0.426657	0.403898
4.365158	0.687023	0.644051
5.011872	1.186207	1.115713
5.754399	1.971475	1.868706
6.606934	3.086014	2.949651
7.585776	4.484845	4.320342
8.709636	6.094274	5.914552
10	7.691377	7.516928
11.481536	9.12181	8.977283
13.182567	10.122632	10.031231
15.135612	10.538383	10.518481
17.378008	10.256471	10.314026
19.952623	9.287718	9.41724
22.908677	7.792172	7.974079
26.30268	5.980246	6.187728
30.199517	4.146427	4.349712
34.673685	2.504521	2.680715
39.810717	1.254394	1.384598
45.708819	0.403543	0.490198
52.480746	0	0.034607

Table A10 The droplet size of WPI emulsion after adding BS (2h)

Particle size (μm)	Volume (%)	
	WPI emulsion + water	WPI emulsion +BS
0.831764	0	0
0.954993	0.010697	0.033506
1.096478	0.067828	0.110912
1.258925	0.244904	0.219429
1.44544	0.434596	0.298402
1.659587	0.681198	0.369673
1.905461	0.966251	0.409463
2.187762	1.280172	0.409544
2.511886	1.614394	0.372934
2.884031	1.969477	0.326726
3.311311	2.358447	0.317423
3.801894	2.801998	0.401806
4.365158	3.315058	0.645961
5.011872	3.913266	1.122569
5.754399	4.586515	1.880775
6.606934	5.331719	2.966427
7.585776	6.096523	4.340277
8.709636	6.828398	5.935281
10	7.417542	7.535598
11.481536	7.793752	8.991005
13.182567	7.865735	10.037886
15.135612	7.586464	10.517006
17.378008	6.957761	10.305089
19.952623	6.015905	9.40285
22.908677	4.881458	7.957428
26.30268	3.668309	6.172233
30.199517	2.527676	4.33812
34.673685	1.552702	2.674264
39.810717	0.82543	1.382712
45.708819	0.332162	0.49043
52.480746	0.073665	0.034272

Table A11 The droplet size of WPI emulsion after adding BS (4h)

Particle size (μm)	Volume (%)	
	WPI emulsion + water	WPI emulsion + BS
0.831764	0	0
0.954993	0.041508	0.032899
1.096478	0.134185	0.109736
1.258925	0.255615	0.21953
1.44544	0.342741	0.299622
1.659587	0.421857	0.372554
1.905461	0.470387	0.414437
2.187762	0.484764	0.416868
2.511886	0.4738	0.382544
2.884031	0.47058	0.338367
3.311311	0.527909	0.330978
3.801894	0.70999	0.417435
4.365158	1.083143	0.663616
5.011872	1.71014	1.141375
5.754399	2.61767	1.898865
6.606934	3.828943	2.981232
7.585776	5.272497	4.349085
8.709636	6.850734	5.935655
10	8.326988	7.526382
11.481536	9.542575	8.972102
13.182567	10.257959	10.011005
15.135612	10.345136	10.484998
17.378008	9.74565	10.27187
19.952623	8.51231	9.372532
22.908677	6.849439	7.933523
26.30268	4.991203	6.157179
30.199517	3.213408	4.332853
34.673685	1.754969	2.678426
39.810717	0.655521	1.394932
45.708819	0.108378	0.507902
52.480746	0	0.051498

Table A12 The zeta-potential of WPI emulsion after adding BS

pH	Zeta-potential	
	WPI emulsion + water	WPI emulsion + BS
3.52	20.5	15.8
4.28	4.33	1.22
5.4	-27.5	-30.8
6.35	-41.5	-48.7

Table A13 The droplet size of Tween emulsion after adding BS (0h)

Particle size (μm)	Volume (%)	
	WPI emulsion + water	WPI emulsion + BS
0.724436	0	0
0.831764	0.017887	0.013164
0.954993	0.105966	0.099489
1.096478	0.336733	0.34065
1.258925	0.541168	0.554892
1.44544	0.764013	0.788353
1.659587	0.951298	0.986301
1.905461	1.084507	1.132509
2.187762	1.150167	1.216769
2.511886	1.168113	1.262953
2.884031	1.191687	1.32732
3.311311	1.296352	1.485929
3.801894	1.561373	1.815089
4.365158	2.049396	2.368177
5.011872	2.804573	3.176238
5.754399	3.823434	4.220078
6.606934	5.097017	5.477215
7.585776	6.50997	6.822145
8.709636	7.915095	8.103401
10	9.05084	9.073301
11.481536	9.751669	9.581826
13.182567	9.837215	9.485286
15.135612	9.249273	8.756248
17.378008	8.056816	7.497221
19.952623	6.406151	5.869112
22.908677	4.594265	4.160893
26.30268	2.843827	2.575163
30.199517	1.460714	1.349133
34.673685	0.38048	0.425014
39.810717	0	0.036131

Table A14 The droplet size of Tween emulsion after adding BS (1h)

Particle size (μm)	Volume (%)	
	WPI emulsion + water	WPI emulsion + BS
0.724436	0	0
0.831764	0.036869	0
0.954993	0.166851	0.067147
1.096478	0.451864	0.33843
1.258925	0.667003	0.549202
1.44544	0.899769	0.79289
1.659587	1.094025	0.995998
1.905461	1.240647	1.143636
2.187762	1.326874	1.222313
2.511886	1.368463	1.255483
2.884031	1.411953	1.300894
3.311311	1.527432	1.437535
3.801894	1.787847	1.745302
4.365158	2.243589	2.282099
5.011872	2.920978	3.083449
5.754399	3.802979	4.134142
6.606934	4.874079	5.414359
7.585776	6.040753	6.797898
8.709636	7.199055	8.129427
10	8.16107	9.150984
11.481536	8.815888	9.704196
13.182567	9.021901	9.633637
15.135612	8.717963	8.904913
17.378008	7.912641	7.618421
19.952623	6.666781	5.939976
22.908677	5.167646	4.171849
26.30268	3.551607	2.534206
30.199517	2.123677	1.278061
34.673685	0.748701	0.3669
39.810717	0.051095	0.006654

Table A15 The droplet size of Tween emulsion after adding BS (2h)

Particle size (μm)	Volume (%)	
	WPI emulsion + water	WPI emulsion + BS
0.724436	0	0
0.831764	0	0.013622
0.954993	0.071954	0.096123
1.096478	0.228171	0.334546
1.258925	0.430123	0.572737
1.44544	0.598073	0.845656
1.659587	0.758533	1.091627
1.905461	0.87021	1.283783
2.187762	0.923754	1.403388
2.511886	0.931001	1.4705
2.884031	0.935758	1.541218
3.311311	1.004045	1.693769
3.801894	1.208276	2.006872
4.365158	1.61087	2.534688
5.011872	2.261213	3.307517
5.754399	3.168628	4.306649
6.606934	4.337608	5.510248
7.585776	5.674509	6.797565
8.709636	7.052022	8.021645
10	8.222639	8.942608
11.481536	9.021688	9.413767
13.182567	9.251395	9.294656
15.135612	8.830946	8.55563
17.378008	7.795165	7.296021
19.952623	6.272816	5.67341
22.908677	4.545562	3.974935
26.30268	2.869984	2.410256
30.199517	1.509849	1.214879
34.673685	0.585633	0.362909
39.810717	0.134951	0.028775
45.708819	0.056687	0
52.480746	0.115242	0
60.255959	0.39046	0
69.183097	0.866216	
79.432823	1.278329	
91.201084	1.503061	
104.712855	1.508983	
120.226443	1.318336	
138.038426	0.992855	
158.489319	0.619718	

181.970086	0.229848	
208.929613	0.014889	

Table A16 The droplet size of Tween emulsion after adding BS (4h)

Particle size (μm)	Volume (%)	
	WPI emulsion + water	WPI emulsion + BS
0.724436	0	0
0.831764	0	0
0.954993	0.073211	0.074264
1.096478	0.338781	0.361732
1.258925	0.508582	0.565214
1.44544	0.733302	0.803693
1.659587	0.944479	1.002955
1.905461	1.136719	1.153124
2.187762	1.284893	1.240037
2.511886	1.380065	1.282935
2.884031	1.430607	1.333368
3.311311	1.465818	1.464696
3.801894	1.528907	1.751596
4.365158	1.664701	2.247129
5.011872	1.910448	2.982542
5.754399	2.282297	3.943085
6.606934	2.792019	5.112367
7.585776	3.412293	6.381085
8.709636	4.105457	7.618053
10	4.772657	8.597405
11.481536	5.348637	9.180494
13.182567	5.722237	9.217053
15.135612	5.822409	8.658971
17.378008	5.595613	7.565899
19.952623	5.03137	6.060167
22.908677	4.190132	4.39168
26.30268	3.144312	2.769815
30.199517	2.045398	1.414612
34.673685	0.947624	0.3964
39.810717	0.124653	0
45.708819	0	0
52.480746	0	0
60.255959	0	0
69.183097	0	0
79.432823	0.000575	0.011871
91.201084	0.54059	0.121404
104.712855	1.747484	0.250799

120.226443	3.177194	0.338479
138.038426	4.466944	0.387245
158.489319	5.185805	0.39055
181.970086	5.069512	0.351924
208.929613	4.201389	0.283121
239.883292	2.968104	0.192762
275.42287	1.75408	0.095446
316.227766	0.849061	0.006026
363.078055	0.285349	0
416.869383	0.016292	

Table A17 The zeta-potential of Tween emulsion after adding BS

pH	Zeta-potential	
	WPI emulsion + water	WPI emulsion + BS
2.89	-3.32	-3.92
3.95	-8.63	-14
4.95	-13.6	-27.2
5.98	-20.5	-36.9

Table A18 Interfacial tension of O/W interface containing BS, WPI and Tween

Time (s)	Surface tension		
	BS	WPI	Tween
0	7.08	19.23	9.91
9.04	6.9	17.93	9.44
18.09	6.82	17.52	9.27
27.13	6.77	17.29	9.04
36.18	6.74	17.26	8.86
45.22	6.69	16.87	8.73
54.26	6.67	17.04	8.63