The competitive adsorption of surfactants in model dairy emulsions

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Abstract

Emulsion structure is an important factor effecting the properties and stability of dairy products. The formation of new surface area and the adsorption of emulsifiers in the homogenizer influences the final particle size distribution. In addition, this provides insight on what surfactants may be present on the oil droplet surface. This in turn effect what interactions might occur between droplets, the surfactants and other components in the product. Often there are multiple potential surfactants in the system and the ability to predict which will adsorb onto newly created oil droplet surface area is valuable.

The objective of this study was to investigate how mixtures of dairy proteins and chemical surfactants stabilize oil droplet surfaces during emulsion formation. Characterizing the surface loading and emulsion surface areas were done in a simple oil-in-water emulsions with WPI and Tween.

Initial investigations were carried out to characterize the emulsion structures formed with Tween surfactants.

There were several factors limiting the minimal particle size and amount of new surface area created through high pressure homogenization stabilized by Tween. Two different types of Tweens were used to at various surfactant concentrations at 200 bar for 3 passes to determine the particle size distribution and specific surface area. There were no differences between Tween 60 and 80 emulsions. At concentrations above 3% Tween (wt%/oil) minimal changes in particle size and surface area were observed. Similarly, no significant difference was observed when oil concentration was adjusted from 15% to 40% at the same surface area to volume ratio. Further, the effect of temperature from 40°C to 70°C showed minimal differences in specific surface area and particle size. Under these conditions where there is excess surfactant present, the specific surface area created is limited by the capacity of the homogenizer to further break up particles. At high surfactant concentration 5% Tween 60 (wt%/oil) the effect of increasing homogenization pressure was linear and if higher pressure is used, additional surface area may be created.

At low surfactant concentration (less than 2% Tween 60 wt%/oil), the limits of new surface area are attained at 100-200 bar (3 passes) due to the availability of surfactant where increasing pressure or number of homogenizer passes showed minimal change in specific surface area and particle size. The surface coverage of emulsion was in approximate agreement with a theoretical CMC model that measures the critical micellar concentration (CMC) which is a specific concentration for surfactants in solution above which forms micelles. The model assumes a theoretical area covered by each molecule of Tween.

When Tween emulsions were created in the presence of excess whey proteins, more complex absorption behaviour occurred. At high Tween concentrations, the specific surface area created was similar to what occurred in the absence of whey proteins. At low Tween concentrations there was evidence of coalescence as bimodal particle size distributions were found, and the specific surface area was lower than what could be achieved in the same conditions in the absence of Tween.

These observations were extended by measuring the protein loading, adsorbed onto the oil droplet surface. The mass of protein adsorbed per area of oil droplet decreased as Tween concentration increased, until at about 2% Tween, no whey protein adsorption was measured.

In the formation of emulsions at different whey and Tween concentrations, Tween outcompetes whey onto the surface even at very low Tween concentration (0.3% (wt%/oil)). The relative size of whey is large compared to monomers of Tween however the rate of adsorption of whey, suggesting it should adsorb faster. However, Tween forms micelles, even at very low aqueous phase concentrations, and the average size of these micelles are similar or slightly larger than the whey proteins. Conformational changes are required for whey unfold and orientate in order to adsorb onto the oil surface. A Tween micelle can quickly spread on impact which exposes the hydrophobic tails and allows spreading on the surface. For these reasons Tween adsorption dominates the surface of the emulsion even at low concentrations. Only when the aqueous phase of the emulsion is depleted of Tween, does the higher concentration whey protein have a chance to adsorb and contribute to stabilizing surface area created in the homogenizer. As the concentration of Tween increases at 2.2% (wt%/oil), whey is outcompeted completely by Tween onto the surface because there at these concentrations there is enough Tween available to stabilize the surface area that can be created by the homogenizer. The findings of the work

suggest how the competitive adsorption between surfactants can be explored to identify how to design emulsions with specific components stabilizing the surface of the oil phase.

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Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

1.1 Background

Most dairy products are emulsions, which form when two immiscible fluids create a stable mixture. For example, butter is a water-in-oil (w/o) emulsion while creams tend to be oil-in-water (o/w) emulsions. Casein and whey have amphiphilic properties which means they can be used as a surfactant to use in emulsions. Whilst whey and casein are known emulsifiers, chemical surfactants are often used to alter the characteristics of the emulsion or to increase stability to changes during formulation, processing or storage.

Chemical surfactants are typically classified depending on their solubility in either oil or water using the Hydrophilic-lipophilic balance (HLB). Emulsifier selection also depends on the continuous phase of emulsions where the surfactant should be most soluble in (Bancroft's rule). One particular class of emulsifier used in o/w emulsions is known as polysorbates or Tween. Tweens are produced by chemical esterification of sorbitol with fatty acids at high temperature before adding ethylene oxide to form polyoxyalkylene groups. There are variations in Tweens depending on the chain length of their fatty acids. In addition, Tween is non-ionic and has lower interaction compared with ionic surfactants in dairy emulsions.

Homogenizers are used widely due to their effectiveness of oil droplet break-up. This method is employed on commercial scale in dairy food processing but has also been scaled down for use under laboratory conditions. During emulsification of dairy o/w systems, both proteins and chemical surfactants may adsorb on the surface of oil droplets. Because the functionality of the emulsion is largely dependent on which surface active agents are on the fat surface after homogenization, it is important to understand how the competition between the different potential surfactants onto newly created surface can be controlled.

The adsorption of proteins and chemical surfactants has been researched extensively (Courthaudon et al., 1991; Demetriades & McClements, 2000; Euston et al., 1995, 1996; Fuller et al., 2018; Mackie et al., 1993; Zhang et al., 2021), and it is known that chemical surfactants such as Tween are more surface active than dairy proteins. It has been shown that over time, the surface of protein stabilized emulsions can be replaced by Tween molecules, pushing off whey or casein molecules. As the concentration of surfactants increases, the surface loading of protein decreases (Chen & Dickinson, 1993; Courthaudon et al., 1991). Aged protein emulsions can form a robust biopolymer film at the interface (perhaps due to protein aggregation reactions), which once established are difficult to displace by Tween. In addition, the adsorption of dairy proteins can be influenced by changes in pH, temperature and ionic interaction in the presence of Tween (Demetriades & McClements, 1998; Dickinson et al., 2003; Euston et al., 2001; Gomes et al., 2021). In many emulsion applications, this exchange of surfactants is important as they can occur during storage of the product. In other applications such as spray drying, there is little time between homogenization and drying, so exchange is less important, and the oil droplet surface is stabilized during drying by the molecules that first adsorb when the emulsion is created.

Less well studied are the differences in adsorption rates during homogenization of emulsions where multiple surfactants are present. Some reports state that the smaller molecular weight surfactants can diffuse more rapidly onto the surface as newly created surface area is created as opposed to the large protein polymers (Gomes et al., 2018). Under high shear conditions in a homogenizer however, the frequency of collisions between surfactants and newly created surface area is dependent on the concentration and size of the molecules, larger molecules being more likely to collide (Håkansson & Nilsson, 2023). Further complicating this process is that if enough Tween is present, the molecules will exist as micelles which increases their size and therefore the chances of collision (Szymczyk et al., 2018).

Another factor is the amount of surfactant needed to cover the fat surface to stabilize it. Adsorption of a single larger molecule can cover more surface than a single smaller molecule. As a result, the composition of an emulsion immediately after homogenization

is dependent on the relative concentrations, surface activity, surface coverage and rates of mass transfer between the available surfactants in the system.

Given the various surfactants typically found in dairy emulsions, it is important to understand the limiting factors and the minimum requirement to stabilize oil droplet surfaces during emulsion formation. This study aims to identify the limiting factors in the competitive adsorption of dairy proteins and non-ionic surfactants under various homogenization conditions and formulation.

1.2 Objectives

The objective of this study is to characterize the surfactant loadings and possible emulsion surface areas that can be created with Tween. Characterize the surfactant loadings and possible surface areas that can be created with combinations of dairy proteins and Tween.

Chapter 2: Literature review

Surfactants are widely used in food applications as a means of reducing the interfacial tension to create or stabilise emulsion systems. They adsorb onto the surface of oil droplets and form an interfacial layer between the aqueous and dispersed emulsion phases. In terms of adsorption during emulsification, low molecular weight surfactants are mobile and tend to coat newly created interface efficiently during emulsification. Classes of surfactants include monoglycerides, lecithins, glycolipids, fatty acids and fatty alcohols (Kralova & Sjöblom, 2009).

Higher molecular weight surfactants in the food industry primarily include plant or dairy proteins such as casein and whey protein. The rate of adsorption is less efficient for proteins during emulsification when used as a co-surfactant with low molecular weight surfactant in oil-in-water emulsions (Zhang et al., 2021).

Surfactants have amphiphilic properties which is due to their molecular structure made up of hydrophobic and hydrophilic regions. Provided the concentrations are high enough, emulsions are stabilized through steric interactions. In some cases, the hydrophilic portion for different surfactants varies in surface charge which needs to be considered when selecting a suitable emulsifier. Surfactants with net surface charge tend to stabilize emulsions through electrostatic repulsion. Additional interactions with minerals and additives with surface charge can destabilize an emulsion (Dickinson et al., 1992).

In this work it is important to review the types and actions of surfactants, both added and those already present in dairy products together with their interactions. When both low molecular weight surfactants and dairy proteins are present, which are thermodynamically favoured, which adsorb first during homogenization and how do these processes impact the emulsion structure that can be formed?

2.1 Characterization of surfactants

2.1.1 HLB Value

The solubility of surfactants can be identified through the hydrophilic-lipophilic balance (HLB) which is a standardized numbering system that measures the balance of the size and ionic strength of hydrophilic and lipophilic component of surfactants (Griffin, 1954). This system utilizes Bancroft's rule which classifies the relative solubility of surfactants in either the oil or aqueous phase. In addition to the rule, for a given emulsion system, the phase in which the surfactant is most soluble often becomes the continuous phase i.e. water-soluble surfactants are most suitable for oil-in-water emulsions and vice-versa (Ruckenstein, 1996).

The threshold for surfactant solubility favouring oil-in-water emulsions is between 9-18 for water soluble emulsifiers, while oil soluble surfactants are assigned values from 1-8 are used for water-in-oil emulsions (Walstra, 1993). HLB value is particularly useful for surfactant selection depending on the intended continuous phase of an emulsion system. Low HLB surfactants may be added to the oil phase before making oil-in-water emulsions with high HLB surfactants. Over time after the emulsion forms, these molecules migrate to the oil droplet surface where they help stabilize and displace high HLB surfactants from the surface. This is observed in the processing of ice cream, where the emulsion mix is destabilized by the displacement of proteins by small molecular weight surfactants. In this instance, low HLB or oil soluble surfactants have demonstrated to displace dairy proteins at the surface when forming emulsions (Euston et al., 1995). At high enough concentrations, low molecular weight surfactants lower surface tension and pack more efficiently at the surface compared to proteins (Chen et al., 1993). This potentially illustrates the thermodynamic changes of emulsions when using surfactants of different HLB values.

Table 2.1 Summarises the HLB for some commonly used surfactants in food systems.

Surfactant Type	Surface charge	Molecular weight (g/mol)	CMC (mM)	HLB value	Primary Fatty Acid	Solubility	Reference
Tween 20	Non ionic	1228	0.05	16.7	Lauric acid	Hydrophilic	(Sanan & Mahajan, 2011)
Tween 60	Non ionic	1311	0.01	14.9	Stearic acid	Hydrophilic	(Sanan & Mahajan, 2011)
Tween 80	Non ionic	1310	0.01	15	Oleic acid	Hydrophilic	(Sanan & Mahajan, 2011)
β-casein	Amphoteric	24000 (monomer)	-	-	-	Amphiphilic	(Chen et al., 2024)
β lactoglobulin	Amphoteric	≈ 18000	-	-	-	Amphiphilic	(Sawyer & Kontopidis, 2000)
Glycerol monostearate	Non ionic	358	-	3.8	Stearic acid	Lipophilic	(He et al., 2019)

Molecular weight and HLB value for Tween from Sigma Aldrich (http://www.sigmaaldrich.com)

Surface charge and solubility for β-casein, β lactoglobulin and Glycerol monostearate (*Food Additive User's Handbook*, 1991)

2.1.2 Surfactant concentration and micelle formation

The stability of an emulsion is dependent on the surfactant concentration. During the emulsification process, if the amount of surfactant is not sufficient to saturate the newly created surface area, then droplets can coalesce. Surfactants at low concentrations adsorb onto oil droplet surfaces as monomers (Huang & Somasundaran, 1996). At high enough concentrations, excess surfactant monomers in the aqueous environment tend to self-associate and form aggregates known as micelles. They are structured through the packing of multiple surfactant molecules by orientating their hydrophobic tails to form an inner core whilst the hydrophilic ends interact with the aqueous solution. The tendency for surfactants to self-associate and form these structures is observed once a critical concentration of surfactant in solution has been reached. The minimum concentration of which micelles are formed is called the critical micelle concentration (CMC).

Below the CMC surfactants exist as monomers which reduces surface tension of fluids (Lin et al., 1999). The CMC is determined by measuring surface tension at different surfactant concentrations. The point at which the surfactant reaches equilibrium is where the rate of adsorption and desorption of surfactant molecules are equal and monomers form micelles (Lin et al., 1999). Once equilibrium is established, the effects of increasing surfactant concentration does not further change the surface tension and the formation of micelles (Szymczyk et al., 2018). In emulsions where the continuous phase surfactant concentration is above the CMC, it is assumed that the surface of oil droplets is fully saturated. In both modelling and practical application, there are difficulties in quantifying the adsorbed concentration of multiple surfactants between liquid phases of o/w emulsions which are dynamic due to surfactant interaction and changes in surface composition. The adsorption behaviour of an individual surfactant and the effect of surfactant concentration are best described by changes in surface tension.

Figure 2.1 shows the effect of surfactant concentration on surface tension and Gibbs surface excess concentration. At low concentration, surface tension is high and adsorption is low. As concentration increases, there is a transitional region where there is rapid adsorption and lowering of surface tension. Once the surface becomes saturated, the surface tension plateau establishes the CMC of T60 (32 mN/m). Higher temperatures reduce the surface tension by reducing the surface free energy.

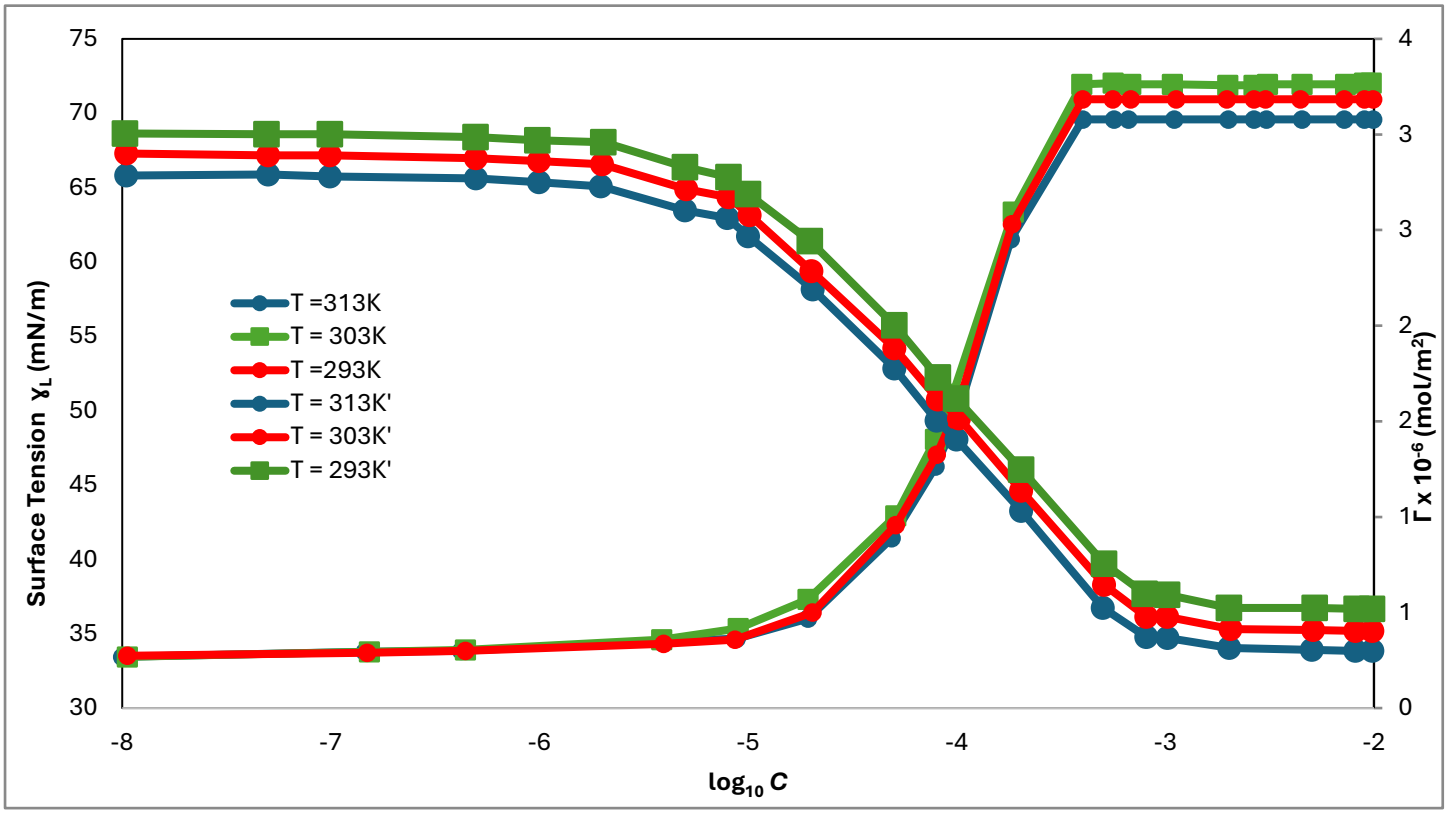

Figure 2.1 The effect of logarithmic Tween concentration on surface tension (γ/L) and Gibbs surface excess concentration (Γ) of aqueous solution of Tween 60 at various temperatures (Szymczyk et al., 2018). ‘ indicates values for Gibbs surface excess concentrations.

2.2 Classes of surfactants

Surfactants can be broadly grouped according to their charge and stabilising properties as being cationic, anionic, non-ionic and amphoteric (*Food Additive User's Handbook*, 1991). This is because one common predictor of emulsion stability is the ionic strength of surfactant. An important control parameter for surfactant selection is the net surface charge. In the competitive adsorption of anionic surfactant and whey, once the pH is near isoelectric point of protein often lead to loss in either steric or electrostatic repulsive forces (Surh et al., 2006). Anionic surfactants reduce net positive charge below isoelectric point causing loss in electrostatic repulsion (Demetriades & Mcclements, 2000). The loss in surface charge leads to increases in particle size and causes aggregation of oil droplets.

In addition, emulsions with high mineral and anionic surfactants can cause aggregation of oil droplets (Cui et al., 2008). This is related to the mineral interaction with negative surface charge which force droplets together. Similarly, adding cationic surfactant in the presence of negatively charged proteins causes ionic interaction on the oil droplet surface (Derkach et al., 2011). The ionic strength as related to the surface charge of surfactant show sensitivity to changes in emulsion properties that tend to lead to emulsion instability. Non-ionic surfactants do not have an electrical charge in solution. As such, the lack of surface charge and ionic strength increases the robustness of the emulsion against aggregation due to the addition of minerals and competing surfactants. At an extensive pH range, they can improve steric stability of emulsions when used as a co-surfactant and prevent particle aggregation (Perugini et al., 2018).

One class of non-ionic surfactants with wide use are polysorbates which consists mainly of sorbitol, ethylene oxide and a fatty acid that differs depending on the type. Variations of different polysorbates are classified based on their fatty acid component (Table 2.1). As such, their molecular structure has various carbon chain lengths and saturation resulting in different adsorption behaviour under homogenization conditions. In study by Fuller et al. (2018) o/w emulsion stabilized by Tween 60 were less shear sensitive compared to Tween 80, suggesting saturated emulsifiers with long fatty acid chain length tend to be less sensitive to shearing effects compared to short chain unsaturated surfactants. Djaković and Dokic (1978) found that viscosity of oil-in-water emulsions (an effect of decreased particle size distribution) increase with homogenization time across different polysorbates (Tween 20, 60 and 80). The effect across Tween types on viscosity is amplified with increase in surfactant concentration (see *Figure 2.2*). With respect to particle size, the type of tween could affect the creation of new surface area under controlled homogenization condition.

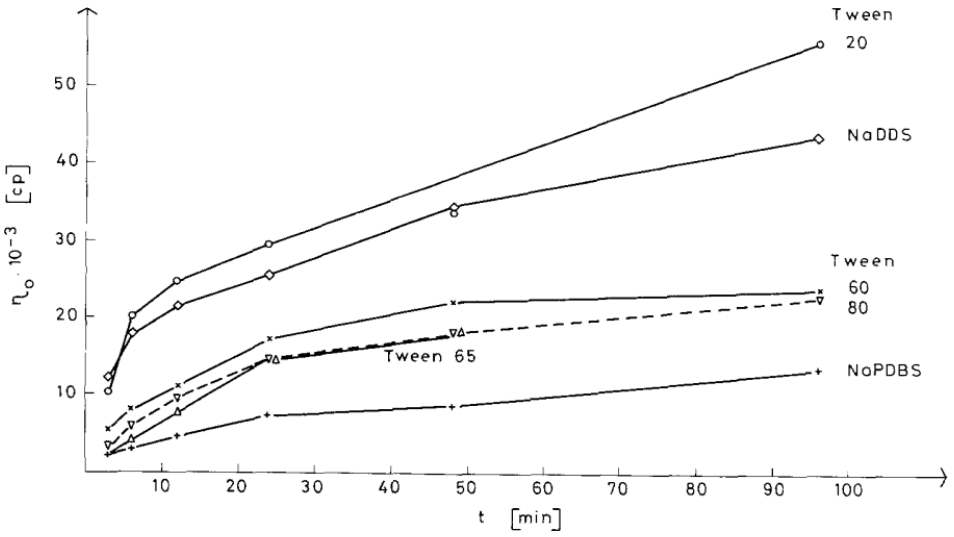

Figure 2.2 Viscosity of 70% oil in water emulsions produced using different surfactants at 3%. Taken from Djaković and Dokic (1978).

2.3 Dairy proteins as emulsifiers

Whey proteins make up around 20% of the total proteins in bovine milk. Primary protein compositions of these are beta lactoglobulin, alpha lactalbumin, bovine serum albumin and immunoglobulins (see Table 2.2). There are number of products produced in the dairy industry (for example acid whey derived as a by-product of cheese production). In whey protein concentrate (WPC) the protein concentration may vary between 30%-80%. Historically WPC has been used as animal feed but is now manufactured to a range of functional food and beverage products (Lammers et al., 1998). On the other hand, whey protein isolate (WPI) is highly refined where the protein components normally exceeds 90% (Hawks et al., 1993). Additional filtration in WPI processing removes more non protein milk solids to achieve a high protein yield.

Table 2.2 Composition and properties of major proteins in whey. (Guo & Wang, 201C)

Type of whey protein	Protein (%)	Isoelectric point	Molecular weight (kD)
Beta lactoglobulin	48-58	5.4	18
Alpha lactalbumin	13-19	4.4	14
Bovine serum albumin	6	5.1	66

The structure of whey protein is globular with repeating α-helix patterns of hydrophilic and hydrophobic amino acids (Finnegan et al., 2024). Outside of the pH range 3.5-7.5 and at temperatures above 40°C, the native structure dissociates into monomers.. Further, when the pH is less than 4.0-5.8 primary aggregates form through hydrophobic bonding. From its native form, whey protein unfolds exposing their hydrophobic components where the thiol groups of beta lactoglobulin are exposed and interact with neighbouring disulfides where denaturation is irreversible (Anema, 2020). Both temperature and pH cause changes in tertiary structure, affect protein adsorption in emulsions.

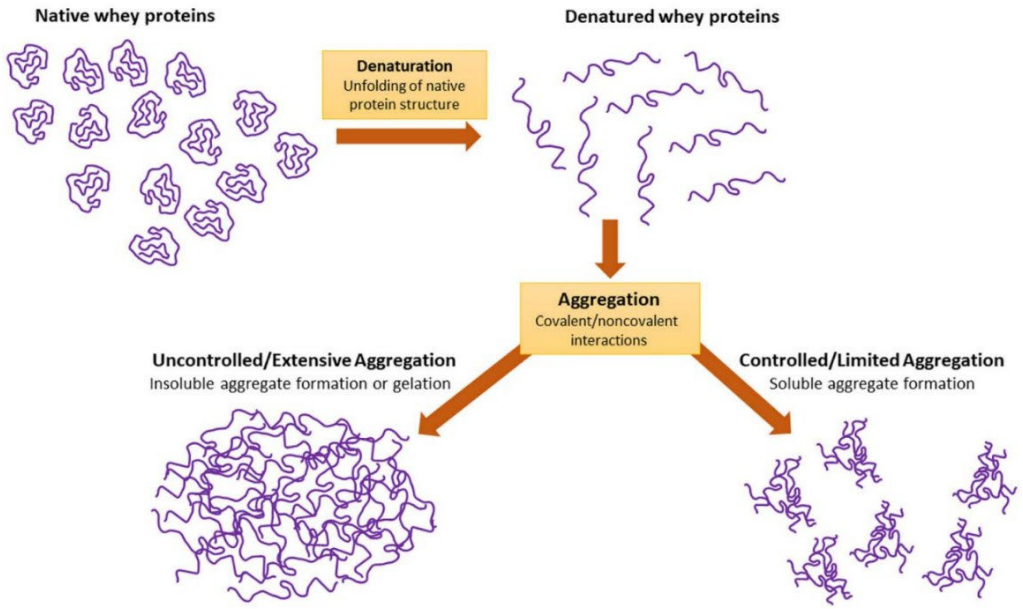

Figure 2.3 Schematic of whey protein denaturation and aggregation process. (Finnegan et al., 2024)

When the native protein structure is disrupted, it undergoes denaturation or unfolding. The changes in structure are particularly in response to change in temperature where above 70°C aggregate structures form (see *Figure 2.3*). Aggregation of whey protein is characterised according to its solubility which dictates their adsorption behaviour in emulsions. In aggregation kinetics, the surface coverage of protein in o/w emulsion increase when the aggregation rate constant increases (Euston et al., 2000). It is thought that the adsorption of whey on the oil droplet surface may form a thick interfacial layer which increases depending on the extent of aggregation. This is dictated by the time of exposure at the denaturation temperature. Below the unfolding temperature when whey proteins are in their globular state, the surface coverage is less. The surface hydrophobicity of whey is less since the hydrophobic amino acids are protected in the native structure. Changes in surface activity and hydrophobicity affect the droplet size of emulsions through protein-protein interaction βLg and α-Lac (Demetriades C McClements, 1998).

To adsorb efficently onto the fat surface, the molecule must unfold to expose the hydrophobic regions that stick onto the fat surface. This takes time, however higher temperature increases the equilibrium extent of unfolding in solution, to a point where it is all unfolded around 70°C (Yun et al., 2023). Ye (2010), showed that in whey protein emulsions, the preferential adsorption of β-lactoglobulin over α-lactalbumin increased with increasing temperature. Stabilisation of emulsions in the presence of dairy proteins is clearly complex, and, in most cases, there are several possible components that could adsorb onto newly created fat surface area. Variations in the balance between protein phases, further complicate this as a result of changing temperature and pH. In addition, these findings highlight the importance of understanding solution properties when formulating model dairy emulsions. The optimal pH conditions for a model system should be as near to the isoelectric point of the protein where their net charge is removed. Emulsions stabilised using whey (without surfactants) tend to have low surface loading (Hinderink et al., 2019). The conformation of adsorbed proteins on the surface fat droplets pre- emusification influences the composition of the interface after processing.

2.4 The influence of the oil phase

Several considerations should be made for fat/oil selection for food applications. In oil- in-water emulsions, oil must be able to disperse in an aqueous medium whilst facilitating the adsorption of surfactants. Fats are typically immiscible in water, however there are specific classes which have amphipathic properties that are useful for the formation of food emulsions. The structure of oils used in emulsion are composed of glycerol, fatty acid ester and phosphate groups (Vuorte et al., 2020). In particular, the chemical composition of fats and oils determines important physical characteristics. For instance, the frequency of double bonds and degree of saturation of fatty acids in a fat/oil determines (Diamante C Lan, 2014):

- Melting point or solids fat content (*Figure 2.4*)
- Viscosity

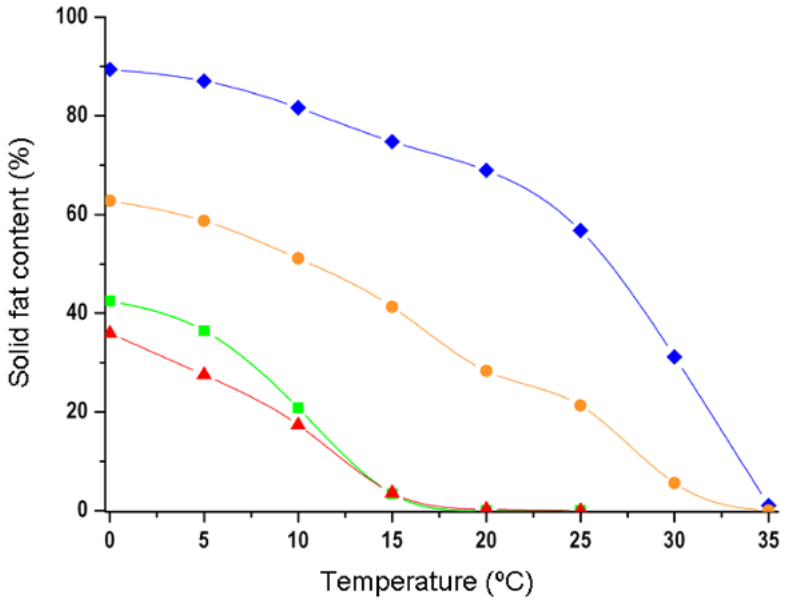

Figure 2.4 *Solid Fat Content (SFC) of some fractions, oils and butters. Yellow = high stearic sunflower stearin (dry fractionation); high stearic sunflower olein (dry fractionation); high stearic sunflower olein (dry fractionation); cocoa butter (Salas et al., 2021).*

Dairy fats can be extracted by evaporating water from cream and is known as anhydrous milk fat (AMF) which is widely used as an ingredient for food applications (Kaylegian, 1999; Szafrańska et al., 2020). The fatty acid composition of AMF is highly concentrated with triacylglycerol groups, the melting point of the different fat fractions ranges up to approximately 35°C which may affect emulsion formation at ambient conditions (i.e. there is 15 to 20% solid fat at ambient conditions). In the sample preparation for manufacturing emulsions using AMF, samples are heated above its melting point and this temperature must be maintained in both the formation of the coarse emulsion and in the final stage of homogenization (Truong et al., 2014). In addition, they found alteration of stearin and olein fraction in AMF did not change the resulting particle size of emulsions in the same way. Suggesting if completely melted, the composition of the oil itself does not affect the homogenization process significantly. Instead, the particle size was affected by the concentration of oil and the pressure used during homogenization. Moens et al. (2018) support this idea, stating that oils do not affect emulsion characteristics thus oil selection is therefore not entirely critical for the understanding of absorption of chemical surfactants and proteins.

When the fraction of AMF is higher, the average particle size of cream emulsions increases (Feng et al., 2025). As the concentration of fat and oil increases, the amount of emulsifier may not be enough to stabilize fat droplets as new surface area is created. In food systems like whipping creams where AMF is heavily used this leads to partial coalescence and aggregation of fat as indicated by bimodal distribution where large particles are formed.

In contrast to the observations from Truong et al. (2014)for medium chain triglycerides (MCT) studies of oil-in-water emulsion systems, in comparison to long chain triglycerides (LCT), smaller particle size are formed by MCT oil-in-water emulsions (Gomes et al., 2021). The characteristics of oil phase of MCT and LCT differ in both chain length and degree of unsaturation. As a result of these differences, the apparent viscosities of the oil phase affect the breakup of oil droplets during homogenization (Wooster et al., 2008). Braginsky's range describes the optimal range for which droplet breakup is most efficient. When the viscosity of oil is too high, there is greater resistance to breakup during homogenization (Floury et al., 2000). The rate of droplet breakup is considerably slow in highly viscous systems resulting in large particle size.

As shown in *Figure 2.4* oils such as AMF begin are partially crystalline at ambient temperature which requires additional precautions for sample preservation. Whilst there has been a recent preference towards MCT in o/w emulsion studies, sustaining temperature above melting point is critical throughout the process of emulsion formation. However, this may prove problematic specifically when proteins are used, and emulsion are formed near or above the denaturation temperature. To avoid potential errors from using high melting point oils, it would be practical to select a lower melting point oil to allow use at ambient temperatures.

The two main fatty acid fractions in vegetable oil which determine the melting properties are stearin and olein. Vegetable oils are refined to achieve different fractions of stearin to olein depending on the intended functional use. Stearins have a higher solid fat content than oleins and a high degree of saturation, therefore the melting point of stearin vegetable oils tend to be high compared to olein vegetable oils (De Almeida et al., 2021; Nor Aini et al., 1999; Norizzah et al., 2004). Sunflower oil is highly unsaturated with approximately 69% linoleic acid (Akkaya, 2018). Under ambient conditions the solid fat content is low compared to high saturated vegetable oil and it is fully liquid at room temperature (Teles Dos Santos et al., 2014). In o/w emulsion with competing surfactants (whey protein and Tween 80), emulsions made with sunflower oil show greater resistance to whey displacement by the Tween compared to MCT. The resistance in protein displacement is related to the degree of unsaturation of sunflower oil. Structurally, the double bonds cause bending of the molecule which makes it difficult for surface absorption of globular proteins.

2.5 Emulsification techniques

The formation of emulsions is established through the dispersion of energy by dynamic processes. High shear mixers, ultrasonication, agitators and high-pressure homogenizers are all common equipment used in the emulsification process. Despite the similarity in capability, authors have reported varying results against emulsion instability (Hall et al., 2011; Mohan & Narsimhan, 1997; Prodromidis et al., 2024). In dairy stabilized emulsions, mixers and ultrasonic device, show low creaming stability. High pressure homogenizers are widely used in food emulsions in laboratory conditions. Droplet break up in the homogenizer is determined by the pressure drop at homogenization valve where the larger the pressure drop, the smaller the particle size (Innocente et al., 2009) At a maximum most laboratory scale homogenizers are capable of up to 2000 bar.

The GEA Panda Homogenizer has been used for both food and non-food application and is effective at reducing the particle size of both emulsion and nano emulsions (Innocente et al., 2009; Perrechil & Cunha, 2010; Wu et al., 2023). The continuous flow regime allows multiple passing of an emulsion feed to ensure the minimal particle size at a range of different pressures can be observed.

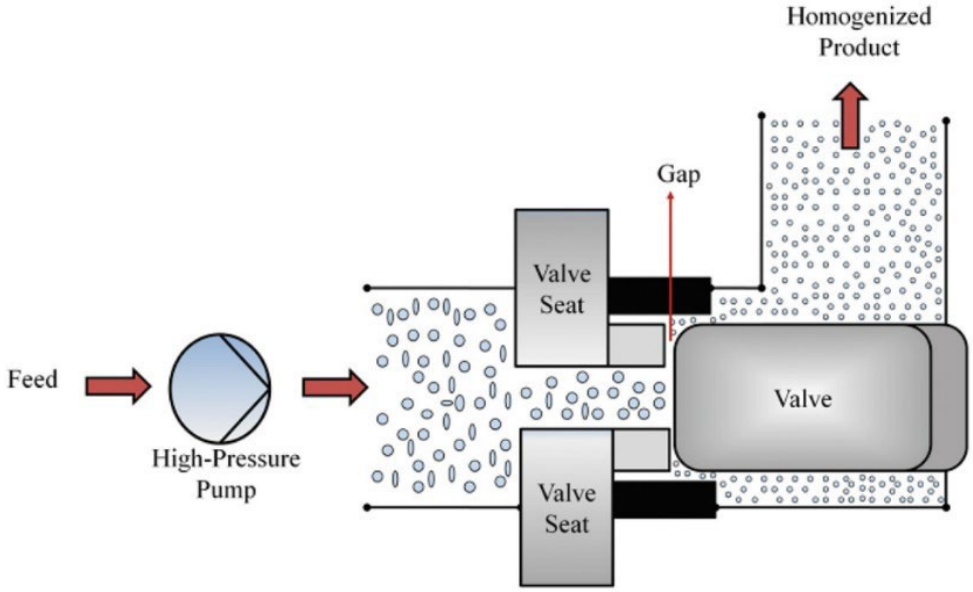

Figure 2.5 Typical design of high-pressure homogenizer. (Malik et al., 2023)

The typical design of a homogenizer as illustrated in *Figure 2.5*. consists of three major components: a high-pressure pump, valve and valve seat. The coarse emulsion is fed through the pump and flows towards the valve. Between the valve and valve seat is a narrow orifice and the fluid is forced through at high velocity which is disperses kinetic energy (Phipps, 1975). The acceleration of fluid through this gap results in elongational laminar flow causing the elongation and deformation of fluid droplets. The pressure applied to the sample depends on the distance of the valve seat to the valve which is adjusted through a dial to the correct working pressure. In a dual-stage design, a backpressure is usually applied in the second stage to regulate excess backpressure in the first stage and minimize cavitation (Håkansson, 2025).

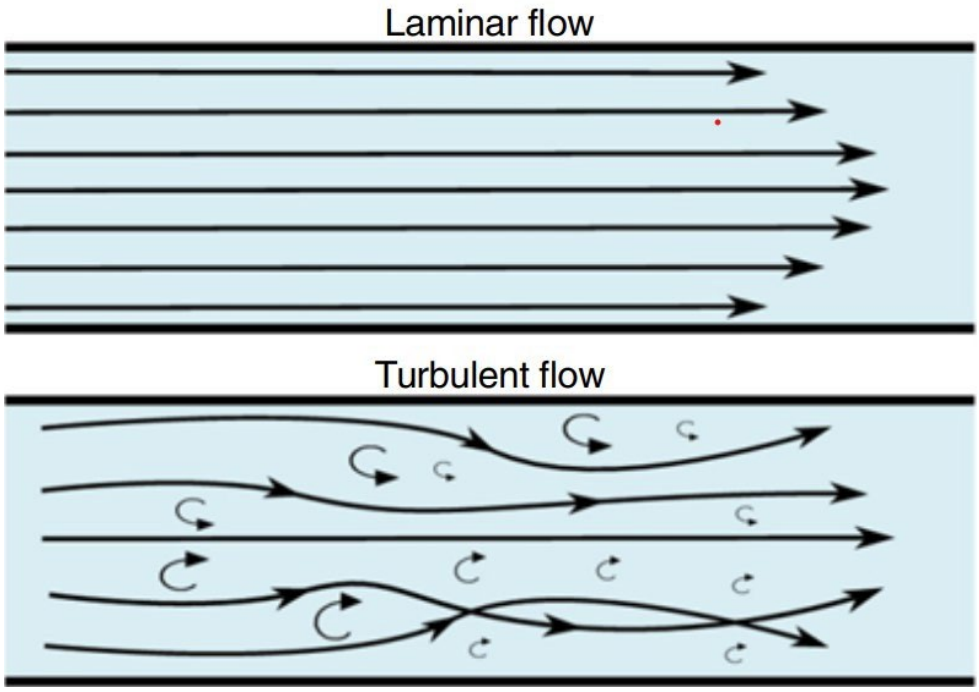

Figure 2.6 Velocity gradients of laminar and turbulent flow regime through a homogenizer. (Kotra et al., 2017)

The shear conditions during homogenization greatly vary depending on the scale of operation. In laboratory conditions, the pressure capability is limited compared to industrial homogenizers. Due to design and magnitudinal differences, the fluid dynamics governing homogenization at laboratory scale is the principal of laminar flow (see *Figure 2.6*).

Characterizing the fluid flow regime through a homogenizer using Reynolds number (Re) that depends on: density ($\boldsymbol{\rho}$), viscosity of fluid (v), and the length which corresponds to the geometric characteristics of the fluid channel (Dynamic viscosity of fluid ($\boldsymbol{\mu}$) and diameter of the pipe (d)) (Mori C Nakayama, 1967).

$$Re = \frac{\boldsymbol{\rho} v d}{\boldsymbol{\mu}}$$

Due to the difference in fluid channel diameters the Reynold number may vary for laminar flow (Avila et al., 2011; Phipps, 1975) . The general indication is that at low Reynolds number the flow is laminar (*Re* <2000) and above critical threshold (*Re* >4000) the flow is turbulent. Between these levels, the flow is said to be transitional.

One droplet break-up mechanism is known as laminar elongation flow where the velocity gradient is in the direction of the flow. As illustrated in *Figure 2.6*, when fluid is passed through the homogenizer, the outermost layer closest to the walls experiences friction which is the greatest magnitude of resistance in this system resulting in the slow velocity. Subsequent fluid layers are moving at a higher flow rate towards the centre of the pipe where the rate is greatest. If there are particles suspended in solution, the differences between flow rates of each fluid layer generates further friction causing fluid shear. Fat droplets elongate due to this shear and are fractured into smaller particles which disperse into a continuous phase (Floury et al., 2004).

In addition to the velocity gradients under laminar condition there are physical interactions the contribute to oil breakup in homogenizer. As droplets are forced through the orifice between the valve and seat there is additional friction from the walls of the homogenizer. In most designs there is an impact ring where most particles experience physical impact (*Figure 2.6*). Due to the high-pressure conditions between the valve and seat, the kinetic energy of droplets increase and at high velocity the impact with the ring contributes to the fracturing of oil droplets.

In a turbulent system, there are two mechanisms that describe droplet break up. These are turbulent inertial and turbulent viscous shear both of which are determined by their interaction with high energy regions in the flow known as eddies (Phipps, 1975). The turbulent viscous mechanism arises from the velocity gradient caused by turbulent eddies (*Figure 2.6*). As bulk fluid flows droplets are subjected to shear as they become trapped between layers of fluids moving at different rates forming turbulent eddies which shear the droplet surface. In the turbulent inertial mechanism, the interaction between the droplet and eddies differ as the shearing effects are caused by pressure fluctuations by eddies which are smaller than the oil droplets. The forces operating in the homogenizer causes the globules to stretch and elongate into irregular shapes. The shear forces which persist

rupture the elongated droplet to form smaller droplets and therefore newly created surface area (Kelemen et al., 2015).

Fluids in a high-pressure homogeniser can experience cavitation. This occurs when the static pressure reduces to the vapour pressure of fluids as they flow through the narrow gap in the valve. When the static pressure falls below the vapour pressure, vapour bubbles form in the fluid that leads to implosions and movement to high pressure (Freudig et al., 2003). In turbulent systems, the extent of cavitation in the absence of back pressure increases linearly with homogenization pressure (Håkansson, 2025). Cavitation can be controlled by applying back pressure in the second stage of homogenization but is not completely inhibited. In general, cavitation is useful for the inactivation of microbes by ultrasonication using high pressure homogenization (Coccaro et al., 2018). Cavitation during emulsification of o/w emulsions does not contribute to the breakup of fluid droplets. Instead it reduces the efficiency of fluid breakup by disruption from nucleation bubbles (Rütten et al., 2024). Though not common, cavitation may also occur under laminar conditions during laboratory scale homogenization.

2.6 Factors affecting particle size

In principle, homogenizers operate by inducing shear forces onto liquid where oil droplets of the dispersed phase are reduced. In dairy systems, the density of milk fat compared to serum is low, therefore milk fat globules float to the surface of the serum to form a cream layer. This phenomenon is governed by Stokes law where v is the rising velocity, g is gravitational acceleration, r is fat droplet radius, $\rho_p$and ρ_f are the particle (fat) and fluid densities and μ is fluid viscosity:

$$v = 2gr^2(\rho_p - \rho_f)/9\mu$$

When milk is passed through the homogenizer it is subjected to high pressure where the reduction in fat globule size can be reduced. As seen in Stokes law this will substantially reduce the rising velocity and overcome the creaming phenomena. As indicated in many studies, minimum particle size is limited by the design and pressure capacity of homogenizers at laboratory scale (Gupta et al., 2016; Lee & Norton, 2013; Tornberg & Hermansson, 1977). In addition, the concentration of oil and surfactant of an emulsion

directly impact the viscosity. The particle size of emulsions under laminar flow conditions is affected mainly by the viscosity of fluid passed through the system. The rate of droplet deformation for a high viscous system is assumably decreased under laminar conditions. High viscosity emulsions may cause changes in flow conditions where increased resistance which will require a greater amount of pressure to reduce droplet size (Walstra, 1993). In addition, heat treatment can reduce the viscosity although the extent of heat treatment depends on the heat stability of surfactants to maintain emulsion stability.

In forming an emulsion, three rate processes are occurring together. These are:

1. The rate of new surface area creation (breakup time) is typically 15 to 45 times longer than the Kolmogorov time constant ($\tau\eta = \sqrt{\nu_C/\varepsilon}$), where ν_C is kinematic viscosity ~ 10-6 m2.s-1 and ε is the dissipation rate of kinetic energy of the flow ~ 109 m2.s-3), which characterizes the time scale for turbulent eddies produced in the homogenizer to dissipate (Håkansson & Nilsson, 2023). For the emulsion to be stable, surfactant adsorption must occur before oil droplets collide and coalesce.

2. The rate of adsorption of surfactants onto the new surface being created to stabilize it (Håkansson & Nilsson, 2023).

3. The rate of collision of droplets and drainage of the film between them which may result in coalescence. Mohan and Narsimhan (1997) showed the time scale for drainage of the film present between two droplets on collision was of the order of 23 to 100 ns depending on droplet size and homogeniser step down pressure. If the contact time between droplets exceeds this drainage time, the droplets will coalesce.

As a result, the rate and extent to which surfactants can reach the newly created surface directly influences the resulting particle size distribution of the product leaving the homogeniser.

2.7 Particle size and protein loading

Droplet size distribution and protein surface loading are two important parameters in the adsorption behaviour of protein and non-ionic surfactants onto fat particles. The dispersed particles in emulsions form size distributions ranging from 0.1 to 1μm. Droplet size is an indicator of the energy input during homogenization and the effects of emulsion properties on the adsorption of surfactants. In food emulsions, droplet size affects both rheological and sensory properties.

Several techniques have been used to measure the particle size of emulsion systems. These include nanoparticle tracking analysis, coulter counter, microscopy, dynamic light scattering and laser diffraction. Techniques which determine particle size using imaging often have issues with image resolution. Further, techniques such as electron microscopy require additional sample preparation and artifacts which interfere with image analysis and is less used to measure particle distribution (Jin et al., 2017). On the other hand, techniques using the dynamic light scattering and laser diffraction have been widely used for analysing size distribution in o/w emulsions (Agboola & Dalgleish, 1996).

The adsorption of surfactants on the surface of oil droplets can be measured through the specific surface area. This is the area per unit of mass which describes the surface area available which can provide potential sites for adsorption. In context of the adsorption of surfactants in o/w emulsions, an increase in specific surface area would indicate the saturation of oil droplets suggesting more surfactants have been able to adsorb onto the surface. At low surfactant concentration, there may be not enough surfactant molecules to adsorb on the surface of oil droplet during emulsion formation which result in coalescence and a decrease in specific surface area.

In the adsorption of dairy protein, the surface loading is used as a measurement of the amount of protein per area of unit mass. It is generally measured by estimating the amount of protein lost from the aqueous phase and dividing this by the surface area of the emulsion as obtained from dynamic light scattering. In the competitive adsorption of surfactants, changes to the droplet surface composition may be monitored using the surface loading. Specifically how proteins are displaced with changes in emulsion concentration and conditions such as pH, temperature and ionic strength (Demetriades & McClements,

1998). In addition, the size range of the protein surface loading can be related to the state of protein conformation. Denatured and globular whey protein usually have a surface loading of 1-3 mg/m^2 (Euston & Hirst, 1999). As whey protein aggregates there may be multilayers of adsorbed whey which increases the protein surface loading.

The adsorption behavior of dairy protein stabilized emulsions may be influenced by the conditions both before emulsion formation and the order of processing. Li and Zhao (2020) found differences in protein loading when UHT was performed before and after homogenization. The protein surface loading was highest for emulsions prepared through double homogenization followed by UHT treatment. Single homogenization and UHT also showed high surface loading, the data illustrates that protein surface loading was higher when homogenization was conducted prior to UHT treatment. Under vigorous UHT conditions beyond the denaturation temperature of whey, the interaction undergoes a shift by forming aggregates. Under UHT conditions it can be assumed that hydrophobic interaction is increased reducing the ability of displacement by competing surfactants. In addition, the protein loading can be increased if UHT is conducted after homogenization. This provides some provision on the competitive displacement of dairy by non-ionic surfactants to help predict surface loading and achieve different protein- surfactant ratios at the emulsion interface.

The adsorption rate of milk proteins during emulsification tends to favor β-caseins over α-caseins and other dairy proteins due to their structural conformation and surface activity (Hunt & Dalgleish, 1994). This adsorption behavior depends on the conformation of proteins and favors monomer and small aggregate adsorption over micelles (Zhou et al., 2022). They found that small casein aggregates and monomers could displace whey proteins at the interface and outcompeted casein micelle adsorption. This rapid diffusion by the small aggregates and monomeric caseins compared to micellar caseins reinforces the idea that surface activity is related to molecular weight where lower molecular weight surfactants will outcompete larger surfactants. On the other hand, whey protein adsorption is dictated by temperature. At elevated temperatures above 70°C whey proteins such α-lactalbumin and β-lactoglobulin begin to denature and unfold exposing their hydrophilic regions. When these unfolded whey proteins adsorb at the oil- in-water interface they interact with κ-casein and a_{s2}-caseins, as a result the adsorbed whey proteins become increasingly robust against competitive displacement by caseins (Brun & Dalgleish,

1999).

These studies indicate the influence of temperature on dairy protein interactions oil-in-water interface. Despite the preferential adsorption of casein over whey, adjustments through heat treatment can help tailor the surface composition depending on the ratio of casein and whey protein that is required. In addition, the displacement of whey by casein must reach a limit once the concentration reaches critical point where micelle structures form similar to chemical surfactants as discussed earlier.

2.8 Competitive adsorption of whey and surfactants

There are two main mechanisms on studies of the competitive adsorption between dairy proteins and surfactants occur by either:

1. The competition of surfactants at the initial formation of the emulsion (all surfactants present at the time of homogenization).
2. The formation of emulsion with a single surfactant and the addition of the other surfactant after emulsion has formed (after homogenization).

In the competitive adsorption of glycerol monostearate with either whey and casein, the protein type had very little effect on the particle size (D_{32}) (Euston et al., 1996). Therefore, the particle size reduction is dependent on concentration of protein and is not affected by protein type. Differences in surface protein concentration is observed between whey and casein, where whey protein has lower surface loading compared to casein. The surface activity of caseins is higher than whey protein and will adsorb forming a thin layer on the surface. Caseins spread along the film and acts as a barrier for the adsorption of whey (Britten & Giroux, 1991). The efficient packing of flexible casein allows higher surface loading compared to the globular structure of whey molecules. This is also

observed in study by Ye (2008) where whey outcompetes casein up to 4% total protein content at a 1:1 weight ratio. There are conformational changes in protein structure at different relative concentration where at low concentration, globular whey spreads less and may adsorb as dimers where it outcompetes casein for the surface. At higher concentration it is likely that casein forms aggregates or micelles that pack densely at the surface. There are very little differences between oil soluble and water-soluble surfactants on the protein surface loading of emulsions using sodium caseinate (Euston et al., 1995).

In the competitive adsorption of whey and Tween, the particle size of o/w emulsions are influenced by the concentration of Tween (Chen & Dickinson, 1998; Demetriades & McClements, 1998) . Emulsions containing the highest Tween-protein ratio (*R*) showed the lowest particle size regardless of pH and temperature conditions. The adsorption of Tween is much more efficient as they pack densely on the surface as opposed to large globular whey protein. The steric stabilization of Tween also improves the heat stability of whey when heated above its denaturation temperature. In such emulsions, there is gradual protein displacement from the droplet surface. Emulsions will remain stable after the complete displacement of protein if there are enough Tween molecules to saturate the oil droplet surface (Euston et al., 2001). In addition, only partial displacement of proteins has been reported as the relative concentrations used in studies reflect the amount used in commercial applications. The limits of complete displacement of dairy proteins by non-ionic surfactant in emulsions is still an area of limited understanding.

2.9 Conclusion

From the literature it appears that nonionic surfactants will outcompete dairy proteins during the formation of emulsions. To understand the competitive adsorption of surfactants specifically the competition or displacement of protein by nonionic surfactants, it is important to understand the adsorption of each surfactant on their own. The next chapter will explore the limiting factors on the adsorption of Tween in an o/w emulsion. The experimental approach will measure changes in particle size and the adsorption of emulsifiers under different homogenization conditions and emulsion formulation. This will pave the way for the later investigation of competitive absorption between Tween and whey proteins.

Chapter 3: Characterization of Tween stabilized oil in water emulsions

3.1 Introduction

Even in a simple oil-in-water emulsion stabilized by non-ionic surfactants, predicting droplet properties such as particle size can be challenging. When adjustments are made to formulations, droplet interactions change in the high-pressure conditions through homogenization. From the literature several parameters (such as surfactant selection, or oil and surfactant concentrations) were identified which influence droplet formation, each providing levers to influence emulsion structure. In high energy emulsification methods at low surfactant concentration, the droplet size may be limited by the amount of surfactant available to stabilize newly created surface area. This has been demonstrated in studies where after several passes at high pressure (kBar) no significant differences were observed in particle size (Qian & McClements, 2011). This has been also observed in complex emulsion systems with multiple surfactants (Lee et al., 2009). In conditions with surplus surfactant availability, the droplet size distribution becomes limited by the homogeniser design and operation.

This chapter focuses on characterising and predicting the specific surface area for emulsions produced using Tween surfactants for simplified model systems.

One simple approach to predict surfactant loading (Blankart et al. (2022) uses the concept of critical micelle concentration (CMC) and assumes that a single molecule of surfactant as a sphere where half its volume occupies each phase (see *Figure 3.1*). The oil surface area that it covers is then estimated from the cross-sectional area through the centre of the sphere.

The model is a crude simplification but does account to some extent for the size of the molecule and the space it takes up on the fat surface. The expectation in using the model is that the surfactant coverage in an o/w emulsion would be useful as an initial approximation.

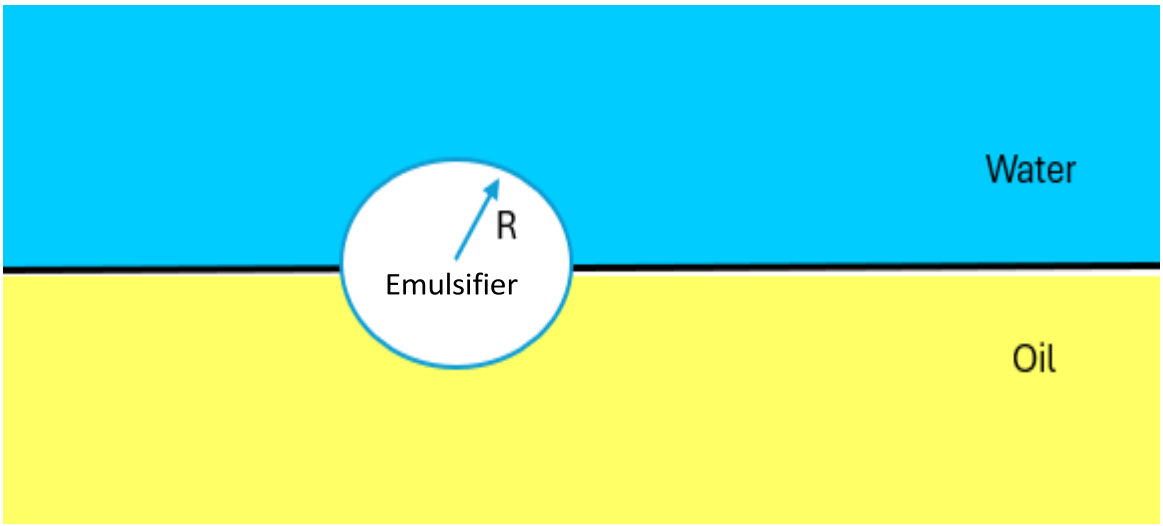

Figure 3.1 Simplified model of space utilization by a surfactant molecule Blankart et al. (2022).

The nomenclature used in the derivation provided below is defined in *Table 3.1*.

Table 3.1 Nomenclature used in surfactant estimation derivation.

Symbol	Description	Units
V_E	Volume of one molecule	m^3/molecule
M_E	Molecular weight of emulsifier	kg/mol
ρ_E	Density of emulsifier	kg/m^3
N_A	Avogadro constant	molecules/mol
R_E	Radius of the molecule	m
A_E	Area one molecule takes up	m^2/molecule
SSA	Specific surface area	m^2(oil)/kg(oil)
n_E	Number of molecules needed for a given SSA	molecules/kg(oil)
ρ_o	Density of oil	kg/m^3
K	Surface coverage of emulsifier	kg emulsifier/m^2 oil

The volume of one molecule of emulsifier:

$$V_E = \frac{N_A}{M_E \rho_E}$$

The coverage area the surfactant molecule occupies is then:

$$A_E = \pi R_E^2$$

where

$$\pi R \frac{2}{E} = \pi \left(\frac{3V_E}{4\pi}\right)^{\frac{2}{3}} = \pi \left(\frac{3V_E}{4\pi \rho_E N_A}\right)^{\frac{2}{3}}$$

For a given specific surface area: SSA (m^2 $_{oil}$/kg) the number of molecules needed to cover the surface is:

$$n_E = \frac{SSA}{A_E}$$

So:

$$C_E = \frac{N_E M_E}{N_A} = \frac{SSA}{A_E} \frac{M_E}{N_A} = \frac{SSA \times M_E}{\pi N_A} \left(\frac{3M_E}{4\pi \rho_E N_A}\right)^{-\frac{2}{3}}$$

To simplify we can define:

$$K = \frac{M_E}{\pi N_A} \left(\frac{3M_E}{4\pi \rho_E N_A}\right)^{-2/3}$$

The parameter K represents the weight (kg) of emulsifier required to cover 1 m^2 of oil and therefore the concentration of emulsifier (C_E) required to cover a droplet size distribution with a specific surface area (SSA) is given by:

$$C_E = K \times SSA$$

Table 3.2 Estimation of surfactant loading for different Tween.

Surfactant Type	M_E (g/mol)	ρ_E^* (kg/m^3)	K(mg emulsifier/m^2 oil) (Blankart et al., 2022)	K (mg emulsifier/m^2 oil) (Glenn et al., 2005)
Tween 20	1227	1095	1.11	-
Tween 60	1311	1044	1.10	1.69
Tween 80	1310	1080	1.13	2.14

Density data from Sigma Aldrich (https://www.sigmaaldrich.com)

These calculations give an initial estimate of what specific surface areas could be expected for emulsions made with different surfactant levels. They also suggest similar emulsion structures (SSA) would be achieved using Tween 20, 60 or 80. According to the K value using the model from Glenn et al. (2005), the adsorption of Tween 80 could be higher than Tween 60.

Homogenizer conditions can also influence the amount of surface area created and both temperature and pressure can be manipulated to achieve different outcomes. It is expected that in surfactant poor systems (i.e. low surfactant to oil ratio's), a maximum amount of surface area will be achieved if enough passes (or in homogenizer conditions that favour the creation of surface area) are used. In surfactant rich systems (i.e. high surfactant to oil ratio's), a point will be reached where the surface area will become limited by the ability of the homogenizer to further break up the fat particles.

This chapter provides foundational understanding of the limiting factors when creating new surface area during homogenization when one or more of these conditions are adjusted. A model system of sunflower oil in water using Tween 60 or 80 was used throughout the study. It was expected that;

- For any given formulation, a maximum specific surface area will be achieved if the emulsion is passed through the homogenizer enough times (passes).
- At low surfactant to oil ratio's the maximum SSA will be dependent on the availability of surfactant.

- At high surfactant to oil ratio's a limit to SSA will be reached where the homogenizer cannot break down the fat droplets further.
- Increasing homogenization pressure and temperature will create more surface area where there is excess surfactant.
- Tween 60 and Tween 80 will result in similar particle size distribution emulsions under the same concentrations and homogenization conditions.

3.2 Materials and methods

3.2.1 Materials

Sunflower Oil was sourced from a local grocery store. Tween 60 (T60) (Product name: SP Crillet 3 MBAL-SS-(SG), Batch no. 57245) and Tween (T80) (Product name: SP Crillet 4 MBAL LW-(SG), Batch No. 57160) were obtained from the supplier Hawkin Watts Limited, repacked from the product manufacturer Croda Singapore PTE Ltd

3.2.2 Emulsion formation

The surfactants were prepared by adding the Tween into RO water (supplied by Fonterra Research and Development Centre) and placed in a 40°C water bath. An overhead stirrer was set to 350 rpm, and the semi solid tween was mixed in with the distilled water for approximately 10 minutes to ensure completely dispersion.

The resulting aqueous phase was combined with sunflower oil and a T25 digital Ultraturrax® (IKA®, United States) was used at 17,000 rpm for 3 minutes to form a coarse emulsion.

To make the emulsions the coarse emulsion was fed into the sampling funnel of a Panda Plus 2000 Homogenizer (GEA Mechanical Instruments, Italy) at 200 bar (2 stage system where stage 1 was set to 100 bar and stage 2 set to 200 bar. The processing of the emulsion through the Panda Plus homogenizer was recycled for a total of three to four passes with sub sampling of the emulsion after each pass.

3.2.3 Characterization of emulsions

Particle size analysis was conducted using a Malvern Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK).

The Malvern Mastersizer 3000 uses the principle of laser diffraction by measuring the intensity of light scattering by directing a laser beam through a sample material. The software converts the light scattering light patterns into data which is analyzed to determine the particle size distribution of the analyte and calculation of summary parameters such as the volume average (d_{32}) and surface area average (d_{43}) mean particle diameters and the specific surface area of the emulsion (*SSA*).

Immediately after homogenization, the emulsions were fed into a wet dispersion unit and run using the Malvern Mastersizer 3000 software. The refractive index was set 1.46 approximating for sunflower oil in the emulsion samples. An oil density of 0.92 kg/m^3 was used to calculate the specific surface area (m^2/kg). The obscuration limits were adjusted to a lower limit of 5% and upper limit of 10%. At least duplicate particle size distribution measurements were made for each emulsion.

3.2.4 Surface tension of Tween 60

A 30% oil emulsion with a T60 concentration of 1% (wt%/oil) were prepared using the method described above in 3.1.2. Samples were then centrifuged at 15000 x g at 4°C for 60 minutes where the cream was removed and serum collected. The serum was again centrifuged under the same condition to ensure the removal of residual fat.

Three 10-fold serial dilutions of the aqueous phase used to create the emulsion (0.43% T60 in water) were made to obtain T60 concentrations in water of 4.3x10^{-6}, 4.3x10^{-5} , 4.3x10^{-4}, and 4.3x10^{-3} g/g water.

The surface tension of the aqueous solutions and the emulsion serum phase (undiluted and after 10-fold dilution) emulsion were measured using the Du Noüy ring method of the Sigma Force 701 tensiometer. Calibration of the force tensiometer was done by measuring the surface tension of Milli Q water (≈ 70 mN/m). Prior to each measurement

the platinum probe was cleaned by rinsing in N-Heptane and distilled water three times. Using a Bunsen burner the probe was heated to red heat. For each sample, a total of 10 surface tension measurements at 20°C were caried out.

3.2.5 Experimental plan

A series of experiments were carried out to characterize how the composition and homogenization conditions influenced the particle size distribution and specific surface area of the resulting emulsion. The experiments are summarized in Table 3.3.

Table 3.3 Summary of emulsion experiments carried out with Tween surfactants.

Trial	Tween	Oil concentration	Surfactant concentration (g/g oil)	Temperature (°C)	Pressure (bar)
1	T60	15%	1, 2, 3, 4, 5 %	40	200
2	T60	30%	1, 2, 3, 4, 5 %	40	200
3	T80	15%	1, 2, 3, 4, 5 %	40	200
4	T80	30%	1, 2, 3, 4, 5 %	40	200
5	T80	15%	0.2, 0.4, 0.6, 0.8, 1%	40	200
6	T80	5, 15, 20, 30, 40%	3%	40	200
7	T60	30%	5%	40, 50, 60, 70	200
8	T60	30%	2, 5%	40	0,100, 200, 300, 400

Experiments 1 to 4 allowed comparison of emulsions formed using two different Tweens (T60 and T80) at two different oil concentrations. It was expected that the specific surface area of each emulsion would depend on the surfactant concentration but to be similar with respect to oil concentration and Tween type (given that the surfactant concentration is a fraction of the oil concentration).

Experiment 5 extend the lower Tween concentration range below 1%. Experiment 6 was carried out to demonstrate that emulsions with different oil concentrations but the same surfactant to oil ratio would exhibit the same specific surface area. Experiment 7 was carried out to investigate the influence of temperature on emulsion formation. Experiment

8 were pressure sweeps carried out at different Tween to oil ratios to investigate surface area creation at higher pressures when surfactant levels were limiting or in excess.

3.3 Results and Discussion

Figure 3.2 shows example particle size distributions of 30% o/w emulsions, and the effect of the number of passes. This clearly demonstrates that the size distribution becomes stable after 2 to 3 passes. Similar trends were observed for emulsions created with other Tween levels, Tween type and oil concentration. Based on these results, subsequent experiments used three homogenizer passes to create emulsions and only those results are presented. Four passes were made in several experiments (prior to this decision being made) and in those cases, the data presented includes the analyses of emulsions after both 3 and 4 passes.

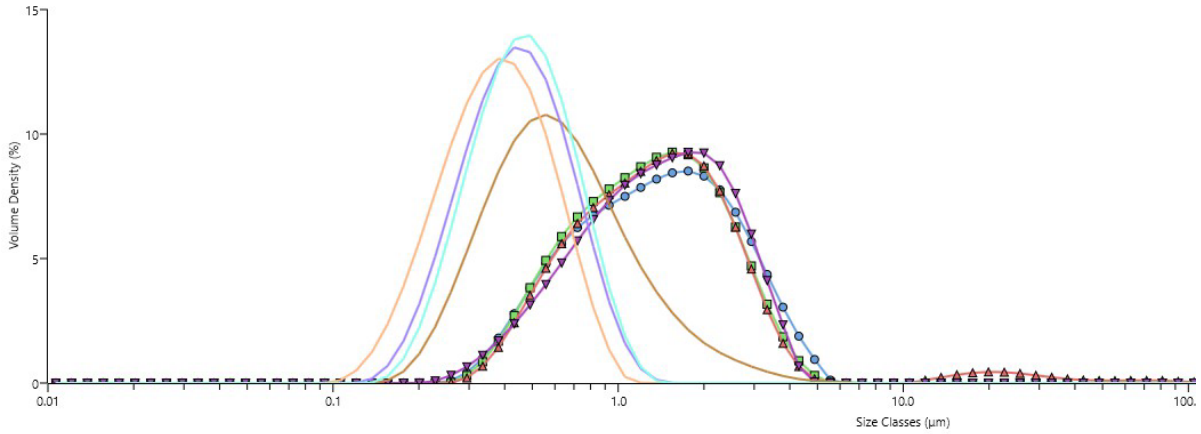

Figure 3.2 Particle size distributions of 1% (symbols) and 5% (lines) T60 emulsions (30% oil) after homogenization at 200 bar. Blue circle = 1st pass, green square = 2nd pass, red triangle = 3rd pass, purple inverted triangle = 4th pass. Brown line = 1st pass, purple line = 2nd pass, orange line = 3rd pass, turquoise line = 4th pass.

Figure 3.3 shows the particle size distributions of 30% oil emulsions made with increasing concentrations of Tween 60. The distribution shifts to the left as the Tween to oil ratio increases, suggesting that for each emulsion, the stabilisation of new surface area is limited by availability of surfactant.

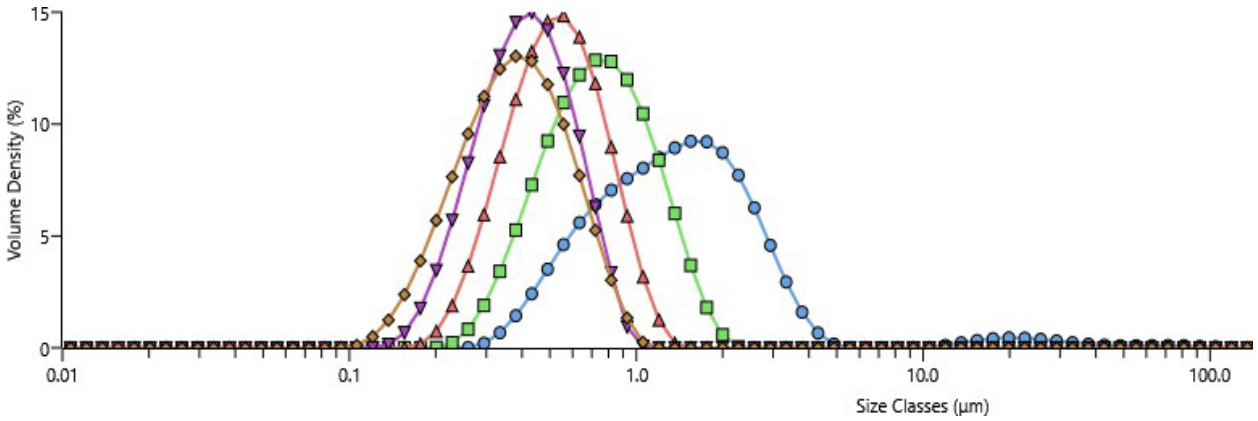

Figure 3.3 Particle size distributions of 1-5% (wt%/oil) T60 emulsions (30% oil) after 3 passes through the homogenizer at 200 bar. blue circle = 1%, green square = 2%, red triangle = 3%, purple inverted triangle = 4%, brown diamond = 5%.

Figures 3.4 to 3.6 summarizes the key aspects of the PSDs (i.e. surface average particle size (d_{32}), volume average particle size (d_{43}) and average specific surface area (*SSA*)) for the emulsions created in trials 1 to 4. The main effect of increasing surfactant concentration is a decrease in particle size.

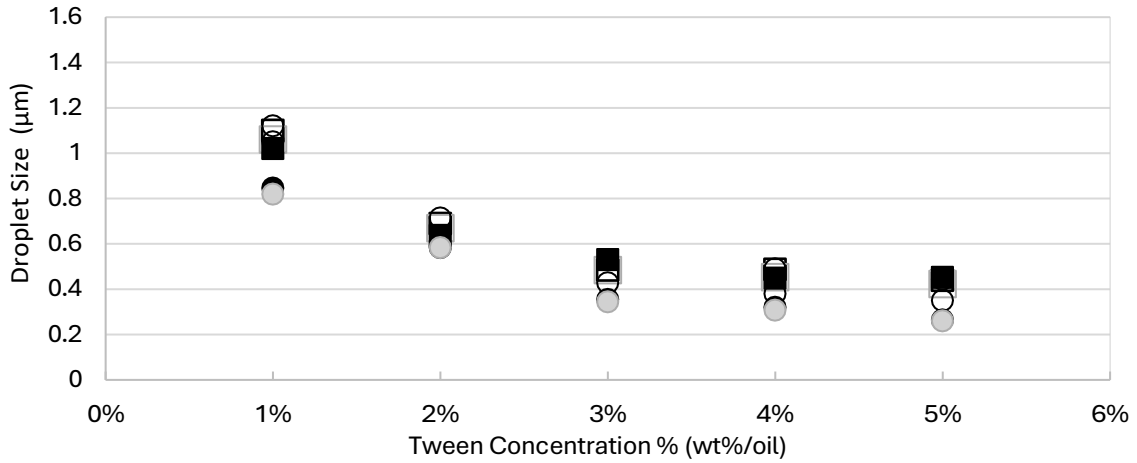

Figure 3.4 The effect of Tween concentration on d_{32}. Tween 60 = Hollow. Tween 80 = Filled. 15% oil (wt/v) = Circle. 30% oil (wt/v) = Square. 3 passes through homogenizer = Black. 4 passes through homogenizer = Grey.

Similar trends were seen for the 15 and 30% oil concentration emulsions although at high Tween concentrations the droplet size is slightly smaller lower oil samples, perhaps because in this regime the ability for the homogenizer to create new surface area is limiting and that this would be more difficult with a higher oil volume fraction. At a Tween 60 concentration above 3% (wt%/oil), the particle size does not change significantly further supporting the idea that the homogenizer is limiting the emulsion structure at high Tween to oil ratios. Reduction in droplet size to less than 1µm was achieved at 2% Tween and above, confirming that both Tween 60 and Tween 80 Both emulsions exhibited very similar trends, supporting the initial estimates provided by the simple CMC theory presented in section 3.1.

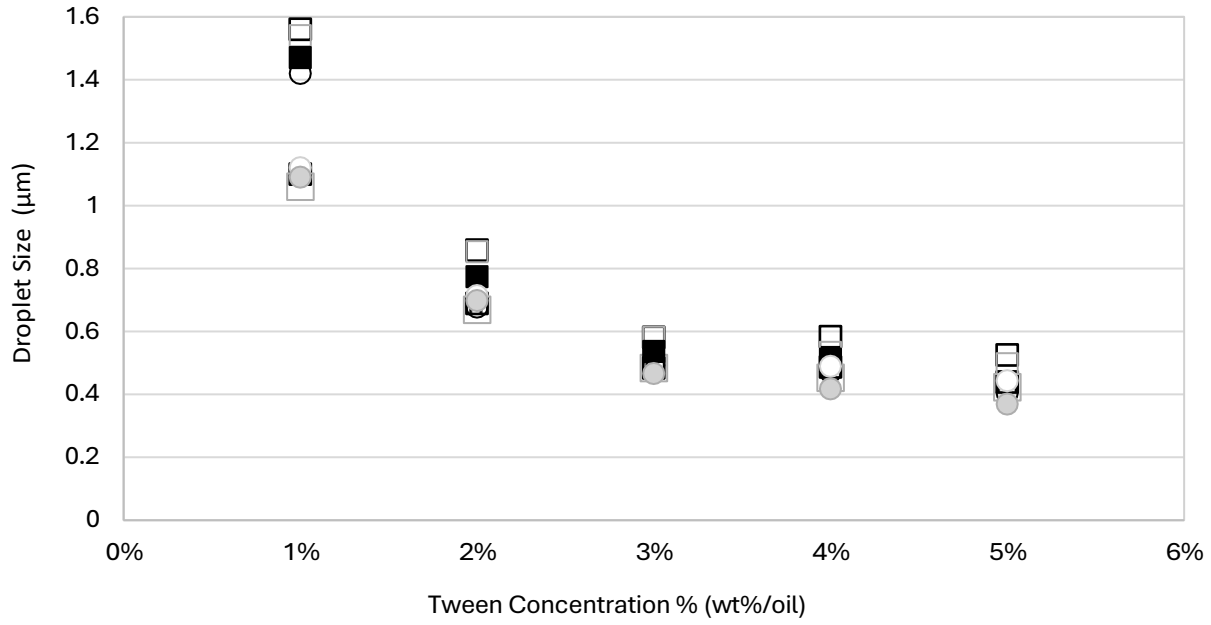

Figure 3.5 The effect of Tween concentration on d_{43}. Tween 60 = Hollow. Tween 80 = Filled. 15% oil (wt/v) = circle. 30% oil (wt/v) = square. 3 passes through homogenizer = black. 4 passes through homogenizer = grey.

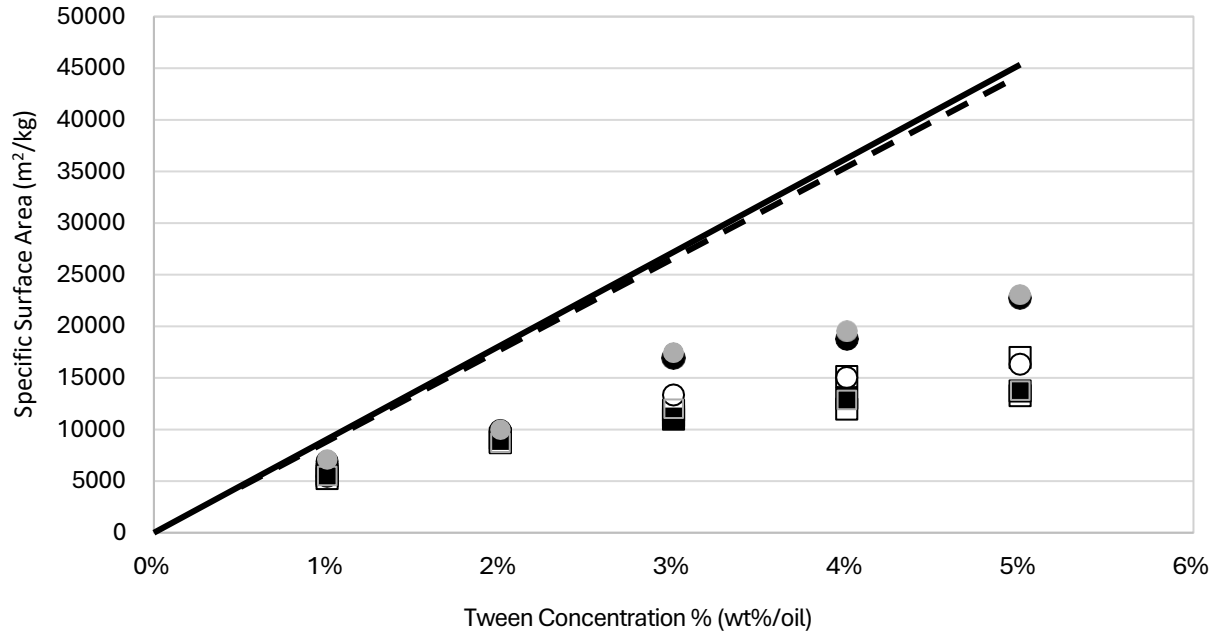

Figure 3.6 The effect of Tween concentration on average specific surface area. Tween 60 = Hollow. Tween 80 = Filled. 15% oil (wt/v) = circle. 30% oil (wt/v) = square. 3 passes through homogenizer = black. 4 passes through homogenizer = grey. Predicted TC0 = solid line, Predicted T80 = dashed line.

Figure 3.6 for the specific surface area of the emulsions shows an approximately linear trend up until 3% Tween. Also shown on the plot is the approximated specific surface area estimated using the simplified CMC model outlined in section 3.1. Although the model overestimates the specific surface area, it does predict reasonably well. Certainly, close enough to provide a good initial guide to what range of surfactant would be required to make a desired emulsion. Because the linear trend (up to 3%) has a lower slope than the estimate, it suggests that the area a surfactant molecule takes up is approximately half that assumed in the model. This make sense as the molecules are not spherical and more likely to be elongated with the hydrophobic end adsorbed in the oil phase and the hydrophilic end protruding into the water phase.

At higher concentrations (>3%) the experimental linear trend plateaus and more variability is seen. At these high relative surfactant concentrations, the mean droplet diameter remains relatively unchanged at 0.44 μm. This agrees with previous studies on Tween 80 emulsions where the mean droplet size did not change after 2% (v/v) (Roldan- Cruz et al., 2016).

It is apparent that as surfactant concentration increases there is an excess of Tween molecules but the shear conditions in the homogenizer cannot reduce the droplet diameter any further. Once an oil droplet has reached full saturation whereby surfactant molecules have coated droplet surface entirely, there is a reduction in both surface tension and intermolecular interaction. The lower oil concentration emulsions (15%) have the highest specific surface area at high Tween concentration and come closer to an extrapolated linear relationship from the surfactant limited region. With less oil present, the homogenizer needs to do less work to break down the oil droplets and be slightly more effective, although it is likely that under these conditions the creation of new surface area is still limiting.

Another important mechanism that could be observed is the formation of micelles as seen when the surfactant concentration reaches above critical concentration (Szymczyk et al., 2018). These surfactant aggregates are usually observed in the bulk of a liquid, and when this critical concentration is reached any additional surfactant molecules will integrate into the micellar structures as opposed to the oil droplet surface which presumably has reached complete saturation.

One assumption that is often made in emulsion analysis is that in a simple system stabilized by a single non-ionic surfactant, when the creation of surface area is not limiting (i.e. at low surfactant to oil ratio), all the surfactant molecules adsorb on the oil droplet surface. Alternatively, and when there is excess, surfactants could also exist in the aqueous phase of the emulsion when oil droplets have reached complete saturation. In the adsorption kinetics for low molecular weight surfactants, the release of surfactant molecules during homogenization is governed by interfacial transport of surfactant between the oil and water (Spyropoulos et al., 2020). This idea implies that an equilibrium may exist if the energy produced by the homogenizer is sufficient.

In a 30% oil-in-water emulsion stabilized by T60, the assumption is that all available surfactants must be adsorbed on to the fat droplet surface particularly at relatively low concentration. To validate this assumption, surface tension measurements were taken of the serum phase to confirm or not the presence of residual tween in aqueous solution of the emulsion. The study by Szymczyk et al. (2018), showed that measurable differences in surface tension occur over a log scale in T60 concentration.

Figure 3.7 shows the measured surface tension of a 0.0043 g/g Tween 60 dissolved in water aqueous solution and after three series 10-fold dilutions. This concentration represents the amount used in the aqueous phase to make a 1% T60 emulsion. At very low concentrations, the surface tension is that of water (70 mN/m). At high concentration the surface tension reduces and plateaus at around 35 mN/m. This is because it has reached the critical micelle concentration when the surfactant self-aggregates and cannot reduce the surface tension any further.

The surface tension of the aqueous solution used to create the emulsion was 36.6 (mN/m) in pure aqueous solution (undiluted). T60 has a surface tension of approximately 35 (mN/m) when the concentration of a solution reaches the CMC, implying T60 molecules will be monomeric for emulsions prepared near or below this concentration.

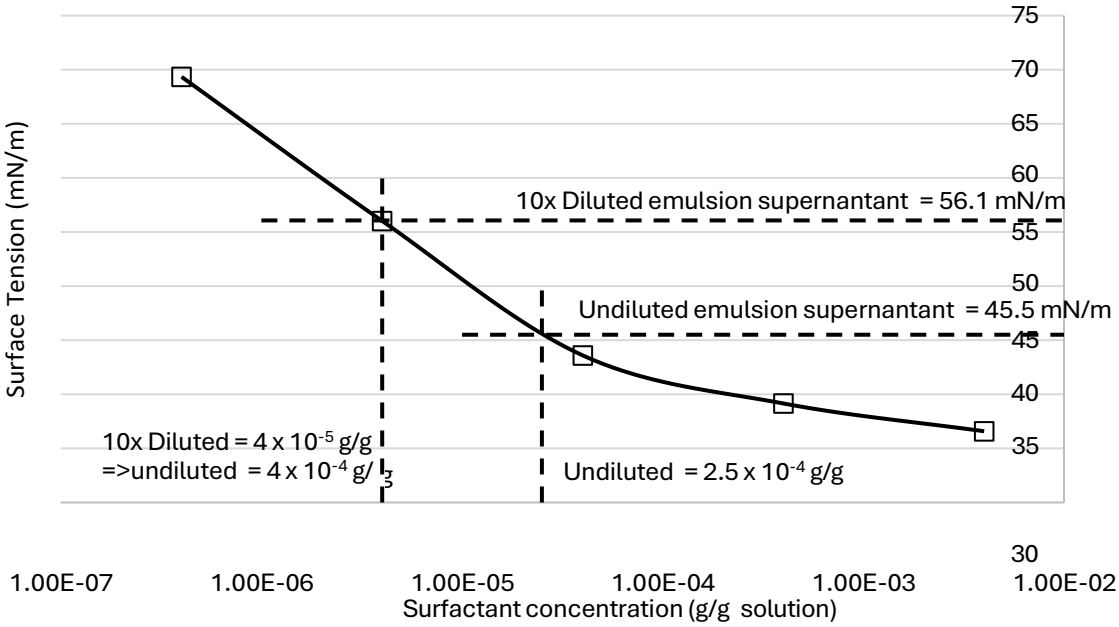

Figure 3.7 The surface tension of aqueous solutions of T60 and measured surface tension of the serum phase of a 1% Tween stabilised 30% oil emulsion (As is and after 10-fold dilution).

After forming the emulsion, the serum phase surface tension was 45.5 (mN/m). In the case of complete surfactant adsorption on the surface of oil droplets, the expectation is that the surface tension of the emulsion would be near the value in pure water. The surface tension of the serum suggests that there is residual surfactant in the aqueous solution of the serum and indicates that not all surfactant molecules adsorb at the surface. Based on the curve acquired for the diluted Tween 60 in water solution, the measured surface tension would correspond to a Tween concentration of 2.5×10^{-4} g/g (0.025%). Similar analysis of the 10x diluted serum phase and then accounting for this dilution gives an estimate of 4×10^{-4} g/g (0.04%).

This is approximately 10 to 20x lower than the amount of Tween available in the sample (c.f. 4.3×10^{-3} g/g or 0.4%) and shows the residual surfactant is approximately 10% of what is available. This indicates that the amount of tween adsorbed on the surface of oil droplet is approximately 90%. This demonstrates that complete adsorption is not

achieved but the majority of surfactant molecules will adsorb on the oil surface in a surfactant limited simple o/w emulsion system. This observation also suggests that as not all of the surfactant adsorbs, the surfactant loading factor (*K*) is closer to the estimated value made using the CMC theory than is apparent on the specific surface area vs Tween graph (*Figure 3.6*).

Experiment 5 was carried out to extend the study to very low Tween concentrations. *Figure 3.8* shows the particle size distributions for emulsions made with 0.2 to 1% Tween to oil ratios.

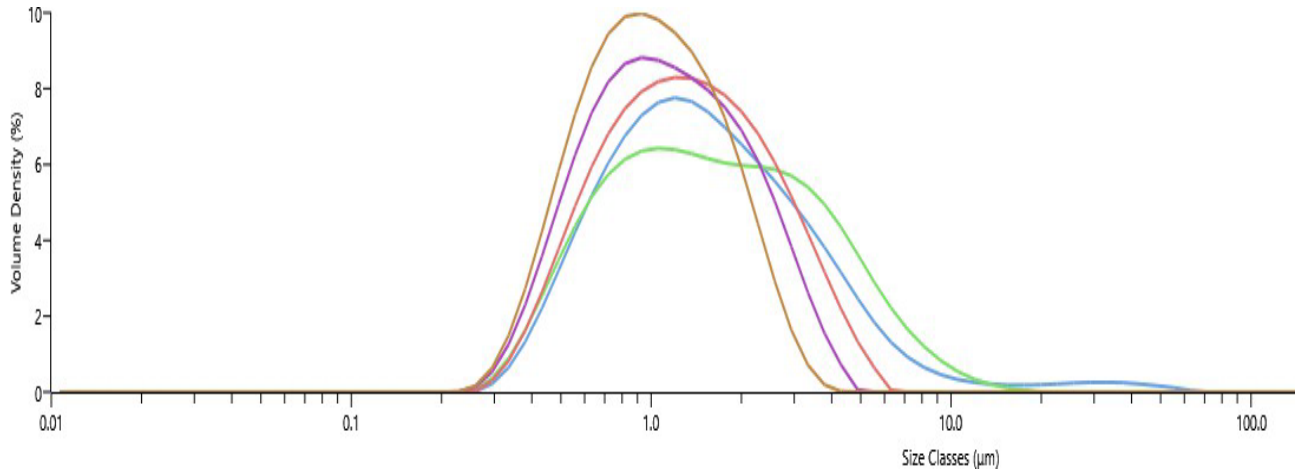

Figure 3.8 Particle size distribution of 0.2 – 1% sunffower oil-in-water emulsions (15% oil) after 3 passes at 200 bar. Blue line = 0.2%, green line = 0.4%, red line = 0.6%, purple line = 0.8%, brown line = 1.0%.

The particle size distribution shows a bimodal distribution at 0.2% and 0.4% surfactant concentration. It is likely that at these low relative concentrations there are simply not enough surfactant molecules to saturate the surface of oil droplets created by the homogenizer. Thus, if the surfactant concentration is not high enough to stabilize the oil droplets in the emulsion this may lead to instability and fat coalescence (Politova et al., 2017). The bimodal distribution at very low surfactant concentration is an indicator of this.

Figure 3.9 to 3.11 show the average particle size distributions and average specific surface area trends for the full range of Tween to oil ratios (from trials 3 to 8).

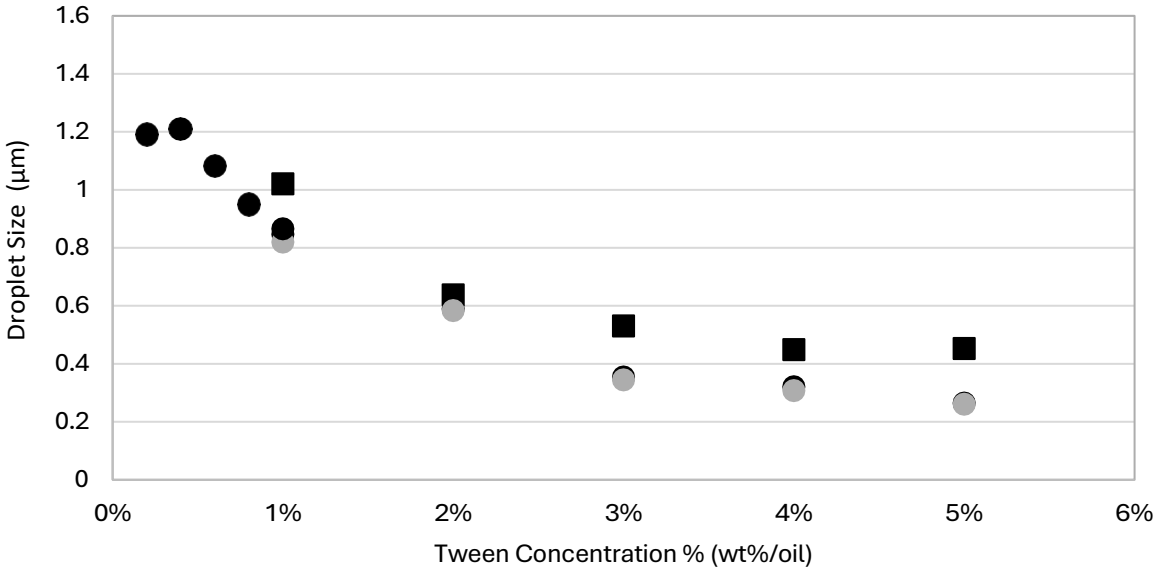

Figure 3.9 The effect of concentration on the surface average particles size (d_{32}) for Tween 80 emulsions. 15% oil (wt/v) = circle. 30% oil (wt/v) = square. 3 passes through homogenizer = black. 4 passes through homogenizer = grey.

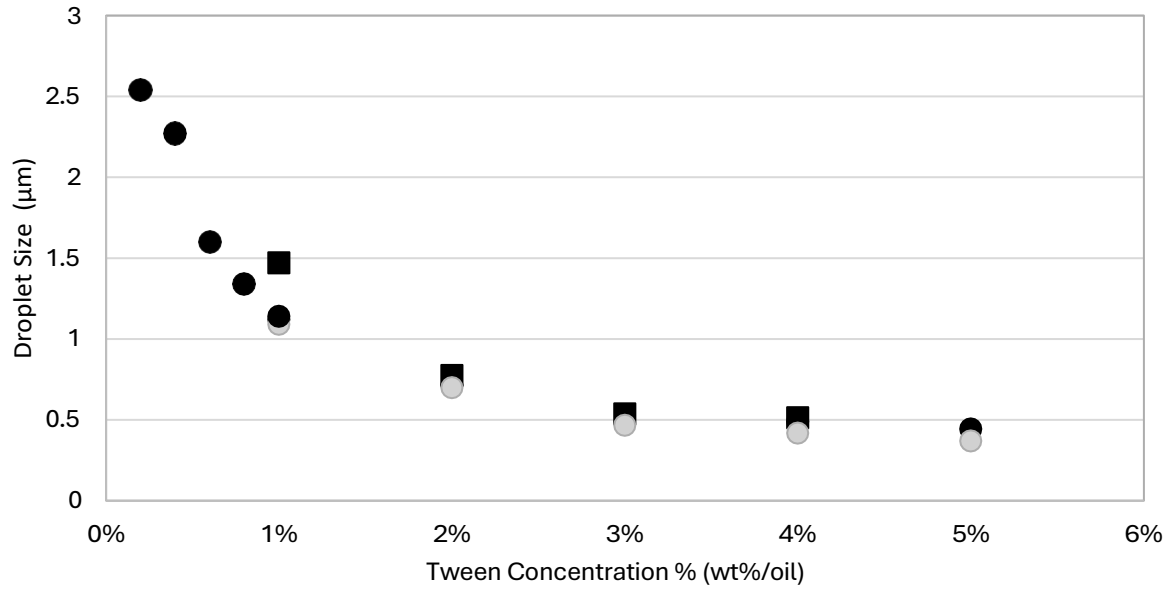

Figure 3.10 The effect of concentration on the volume average particles size (d_{43}) for Tween 80 emulsions. 15% oil (wt/v) = circle. 30% oil (wt/v) = square. 3 passes through homogenizer = black. 4 passes through homogenizer = grey.

At low relative surfactant concentrations from 0.2-1% (wt/oil) the average droplet size decreases incrementally as surfactant concentration increases. After 3 passes at 200 bar a droplet size of less than 1µm (size range of a typical microemulsion) was attained only when surfactant concentration reaches 0.8 (% wt/oil) and above. This indicates that as more surfactant molecules become available for adsorption as new surface areas are created during homogenization, smaller droplet size can be produced.

As seen at higher relative surfactant concentration in the previous trials (1 to 5% w/oil), at 200 bar the homogenizer can create smaller droplet sizes. Therefore, when the surfactant concentration is less than 1%, the minimum droplet size is limited by the amount of surfactant molecules stabilizing the oil droplets as opposed to the shear forces generated by the homogenizer.

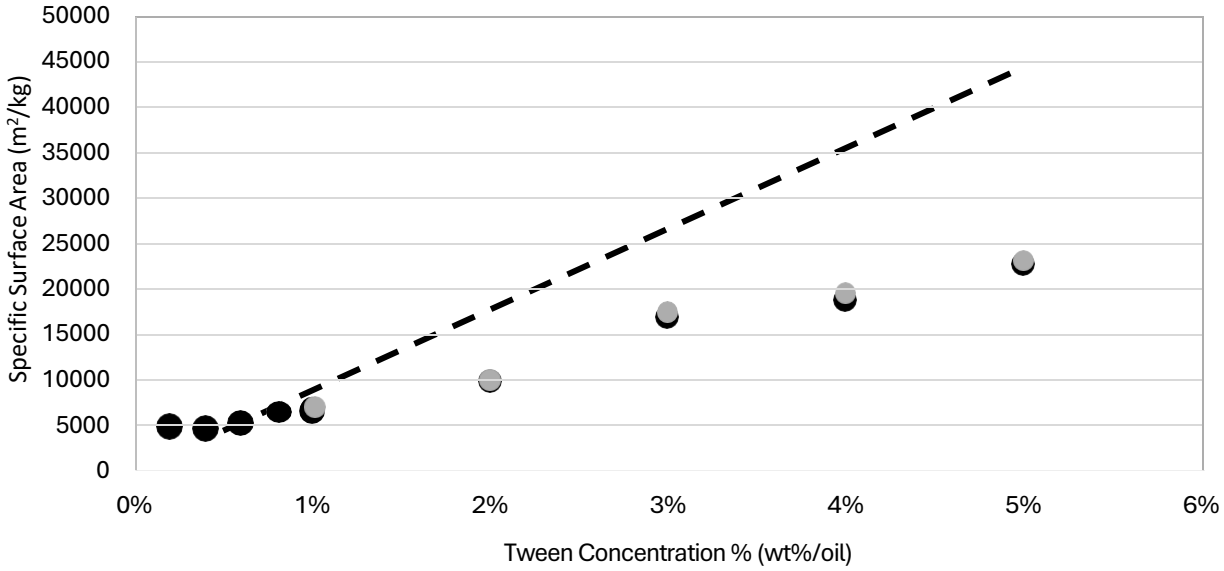

Figure 3.11 The effect of concentration on average specific surface area of 15% oil Tween 80 emulsions. 3 passes through homogenizer = black. 4 passes through homogenizer = grey. Dashed line = theoretical from the model by Blankart et al. (2022) for Tween 80.

The experiments presented from trials 1 to 4 showed that the specific surface area created for 15 and 30% oil emulsions were the same at low Tween to oil ratios. This suggests that the surface loading is independent of oil concentration. At high Tween concentrations, where surface area creation is limited by the capacity of the

homogenizer, smaller droplets were created at lower oil concentrations. The influence of oil concentration was further explored in trial 6 using a 3% Tween 80 to oil ratio.

Figure 3.12 shows the particle size distribution of emulsions made at 3% Tween 80 (wt%/oil) after 3 passes (200 bar). The effect of increasing oil concentration did not show significant change from 15% to 40%, however the lowest particle size was attained at the lowest oil concentration of 5%.

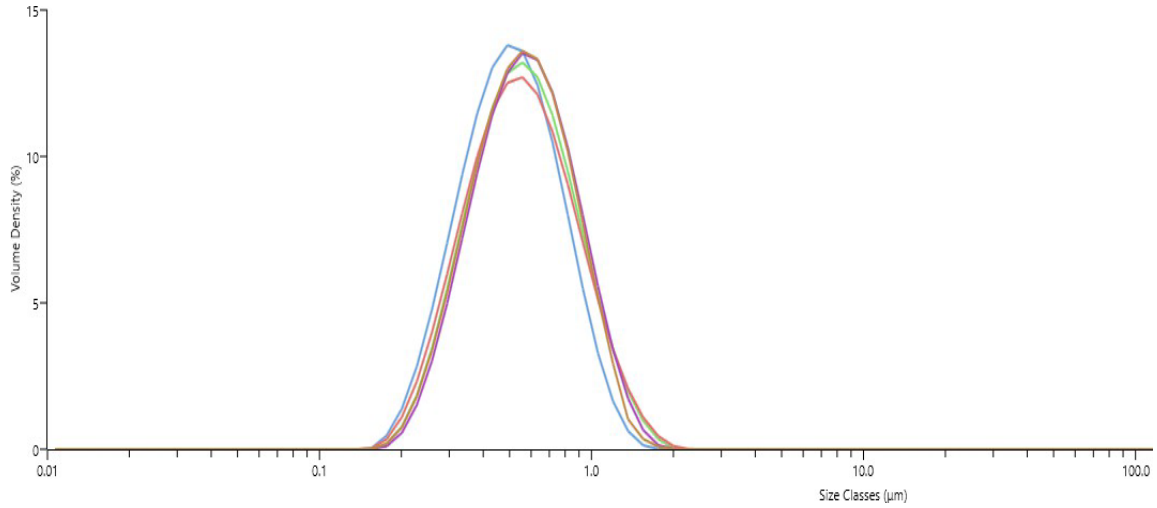

Figure 3.12 Particle size distribution of 3% Tween 80 o/w emulsions at various oil concentrations (5-40%) after 3 passes through high pressure homogenizer at 200 bar. Blue line = 5% oil. Green line = 15% oil. Red line = 20% oil. Purple line = 30% oil. Brown line = 40% oil.

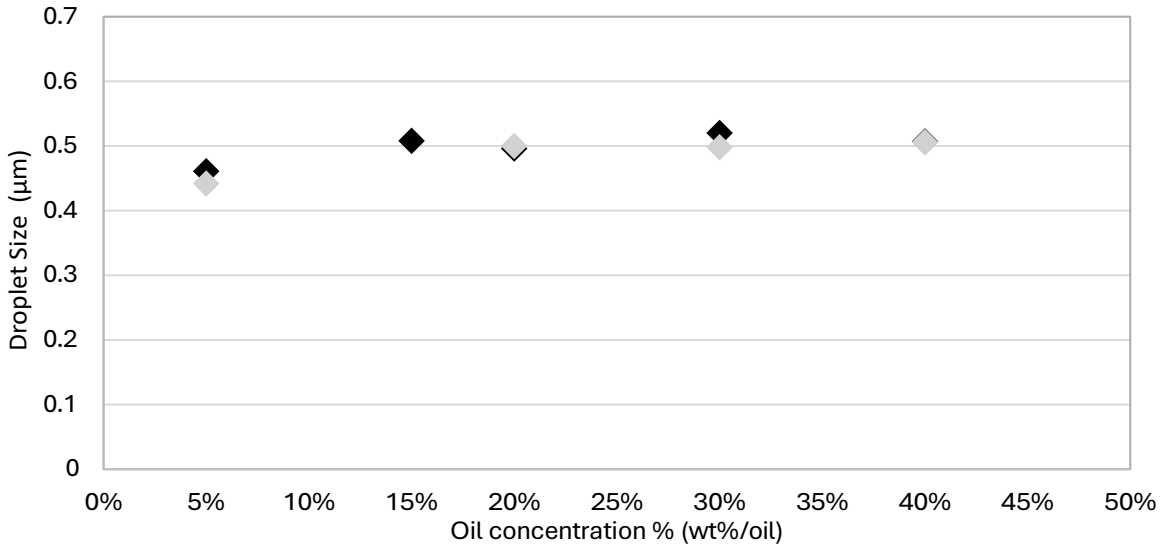

Figure 3.13 The effect of oil concentration (5 to 40 % wt/v) on surface average particle size (d_{32}) of 3% (wt%/oil) Tween 80 emulsions. 3 passes = black. 4 passes = grey.

Figures 3.13 to 3.15 summarize the key aspects of the PSD, clearly showing that there is very little difference between the emulsion structures apart from the 5% oil samples. These results are consistent with the hypothesis that the emulsion structure is limited primarily by the availability of surfactants with some impact of reduced capacity of the homogenizer to create surface area as oil concentration increases.

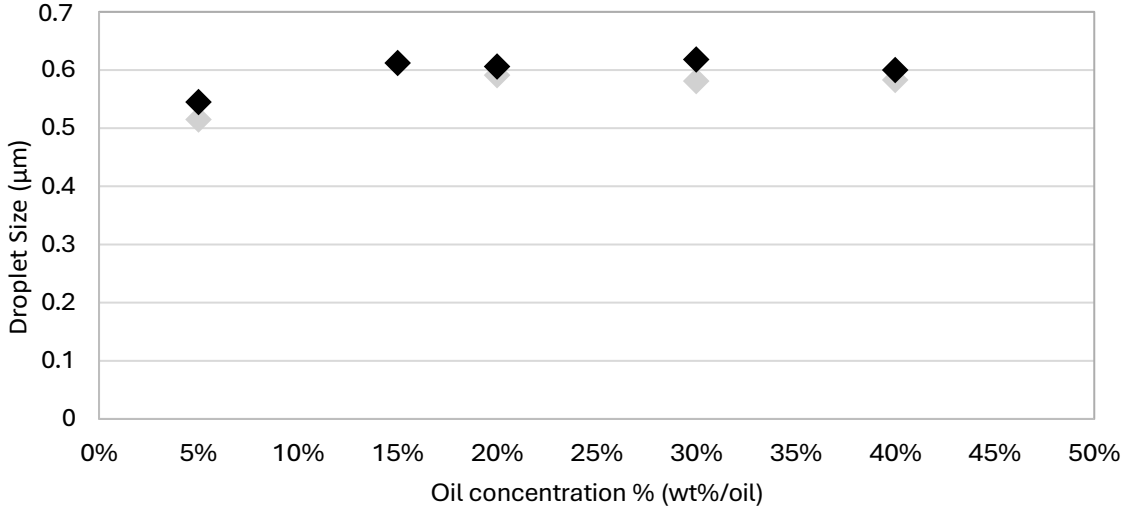

Figure 3.14 The effect of oil concentration (5 to 40 % wt/v) on volume average particle size (d_{43}) of 3% (wt%/oil) Tween 80 emulsions. 3 passes = black. 4 passes = grey.

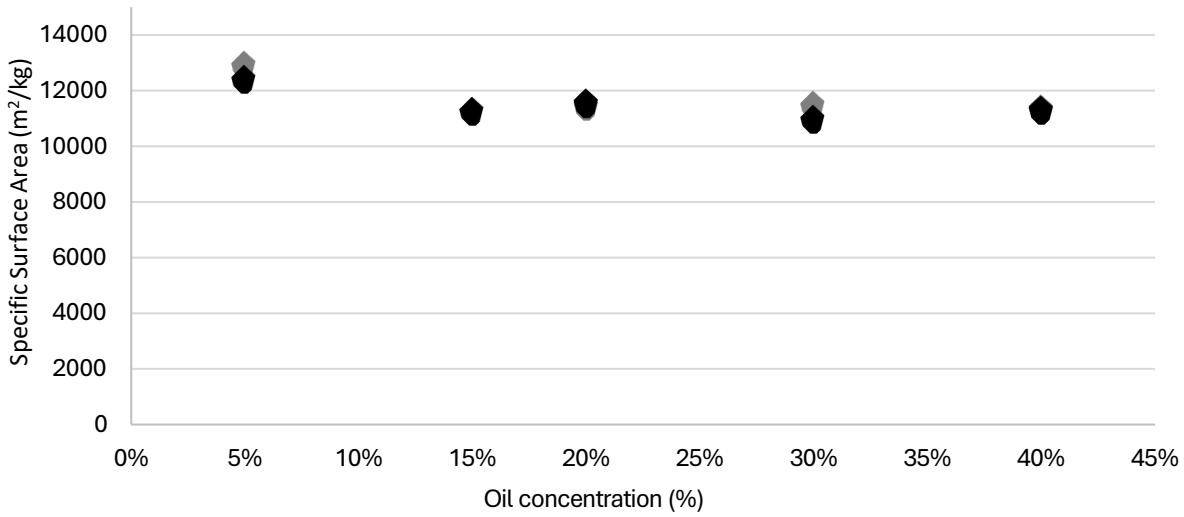

Figure 3.15 The effect of oil concentration (5 to 40 % wt/v) on average specific surface area (SSA) of 3% (wt%/oil) Tween 80 emulsions. 3 passes = black. 4 passes = grey.

The oil droplet through homogenization is affected by higher viscosity which requires greater shear force to break up oil droplets (Phipps, 1975). At higher oil fraction (wt %) the viscosity of the emulsion increases (Maindarkar et al., 2015). Interestingly, they found that at the highest surfactant concentration they used (2% w/v), the particle size does not change significantly but when reduced to low relative concentration (0.1% w/v) the particle size increases markedly with increasing oil concentration (5-50 wt%). This indicates that the changes in viscosity impact the oil droplet breakup, however the surfactant concentration and the energy produced during homogenization determine the extent of particle size reduction as illustrated in our results and finding from these studies.

Oil viscosity decreases with increasing temperature (Fasina et al., 2008) and may make it easier for the homogenizer to create new surface area. Further, when emulsions are prepared at high temperature the kinetic energy of surface-active molecules increase and tend to decrease surface tension more efficiently (Chen & Tao, 2005). The effect of temperature is likely to reduce both the viscosity and the energy requirement to disrupt oil droplets.

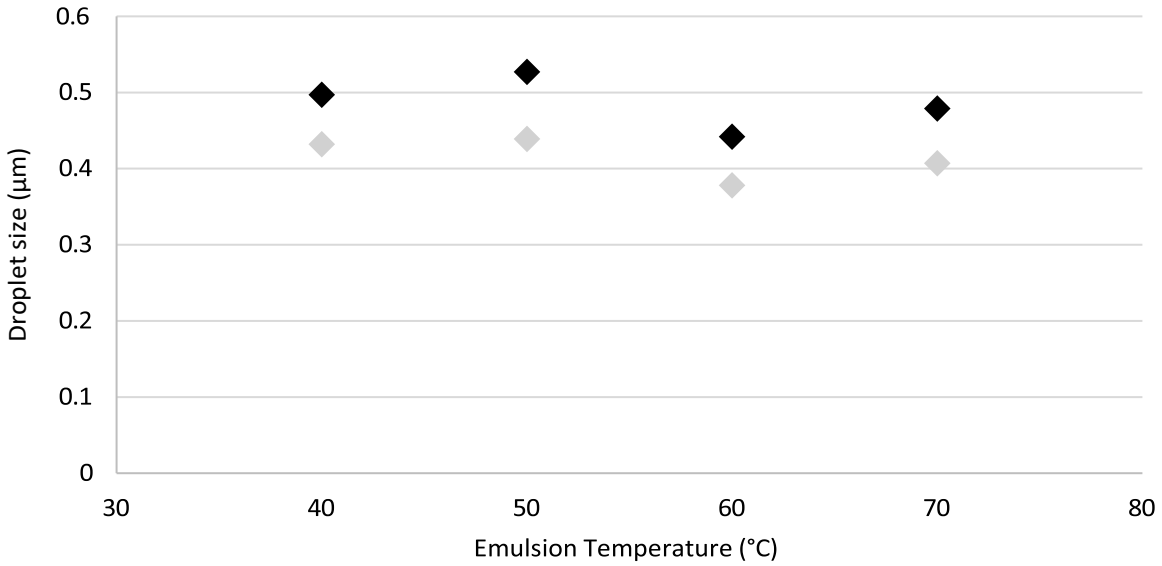

Figure 3.16 The effect of emulsion temperature (40-70°C) on average particle size for 5% (wt%/oil) Tween 60 emulsions after 3 passes at a homogenization pressure of 200 bar. Grey = d_{32}, black = d_{43}.

This was investigated in trial 7 where a 30% (w/w) oil-in-water emulsion was created using Tween 60 at 5% concentration (wt%/oil). At this high Tween to oil ratio, it has been shown in the results so far, that there is excess emulsifier and creating of surface area is limited by the homogenizer.

Figure 3.16 shows that there is no clear trend in the effect of temperature on the particle size. This is also shown in *Figure 3.17* where the highest specific surface area and lowest particle size was attained at 60°C but the opposite was observed at 50°C.

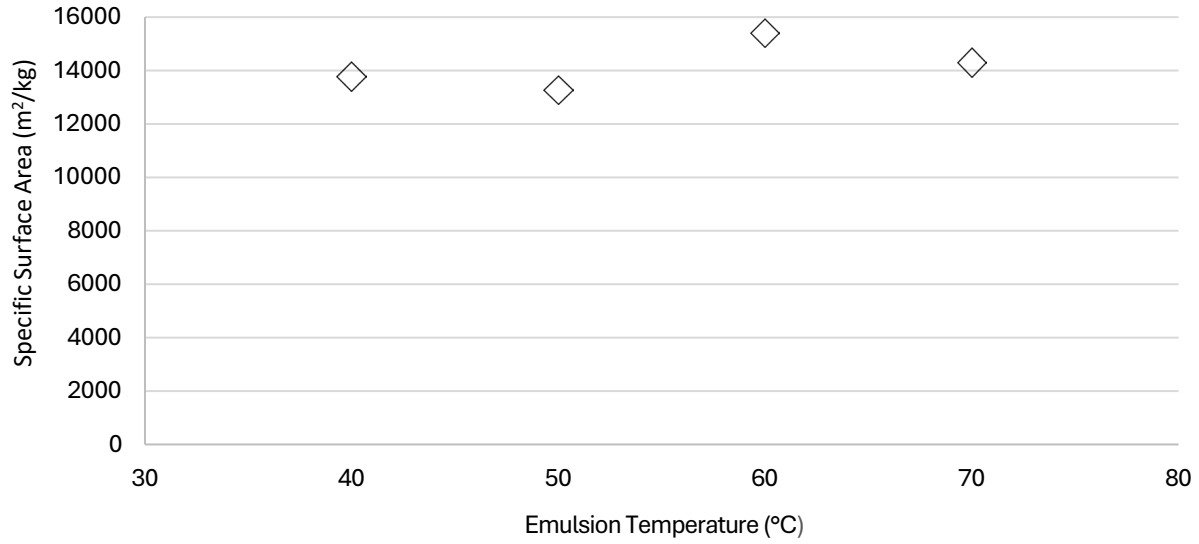

Figure 3.17 The effect of emulsion temperature (40-70°C) on average specific surface area of 5% (wt%/oil) Tween 60 emulsions after 3 passes at homogenization pressure of 200 bar.

Figures 3.18 to 3.20 shows a similar study on the effect of homogenization pressure (100-500 bar) on particle size for 30% oil-in-water emulsion stabilized by Tween 60 at 2% and 5% (wt%oil) concentrations.

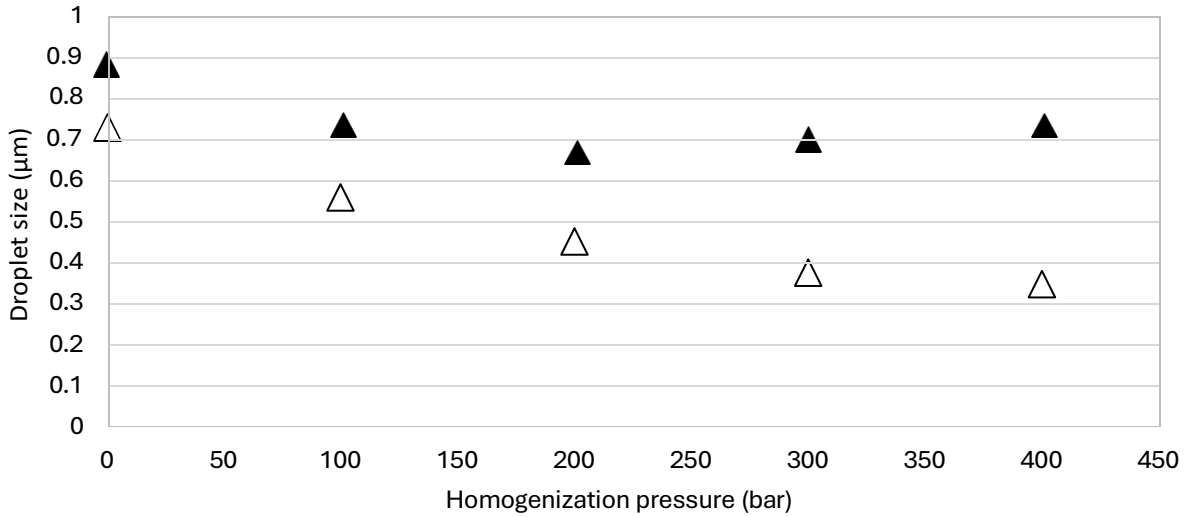

Figure 3.18 The effect of homogenization pressure (100-400 bar) on surface average particle size (d_{32}) of 30% oil Tween 60 emulsions after 3 passes. Filled = 2% T60, open = 5% T60.

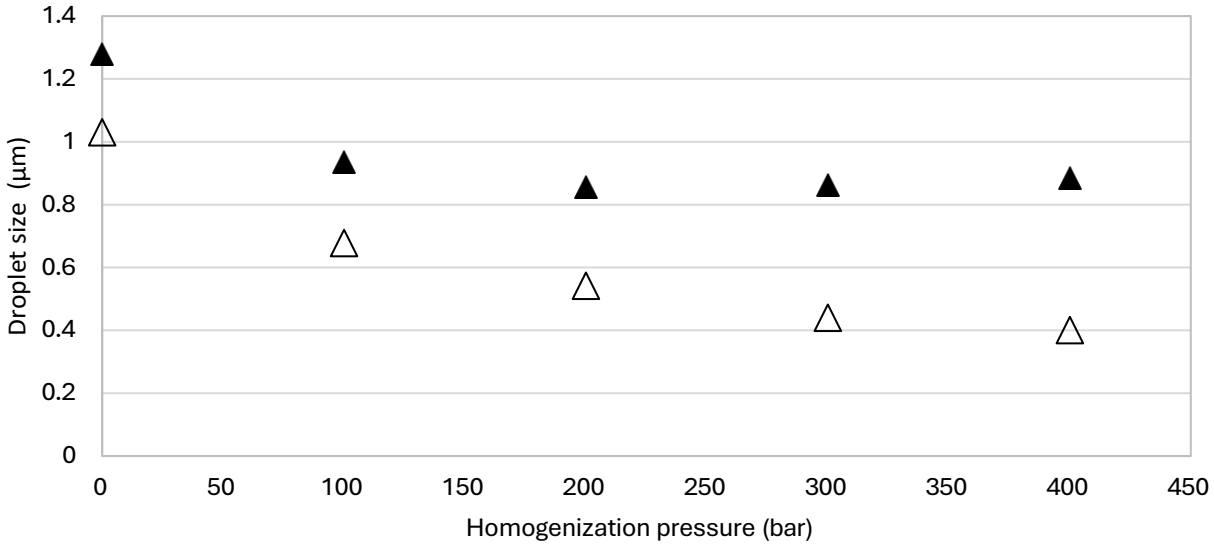

Figure 3.19 The effect of homogenization pressure (0-400 bar) on volume average particle size (d_{43}) of 30% oil Tween C0 emulsions after 3 passes. Filled = 2% T60, open= 5% T60.

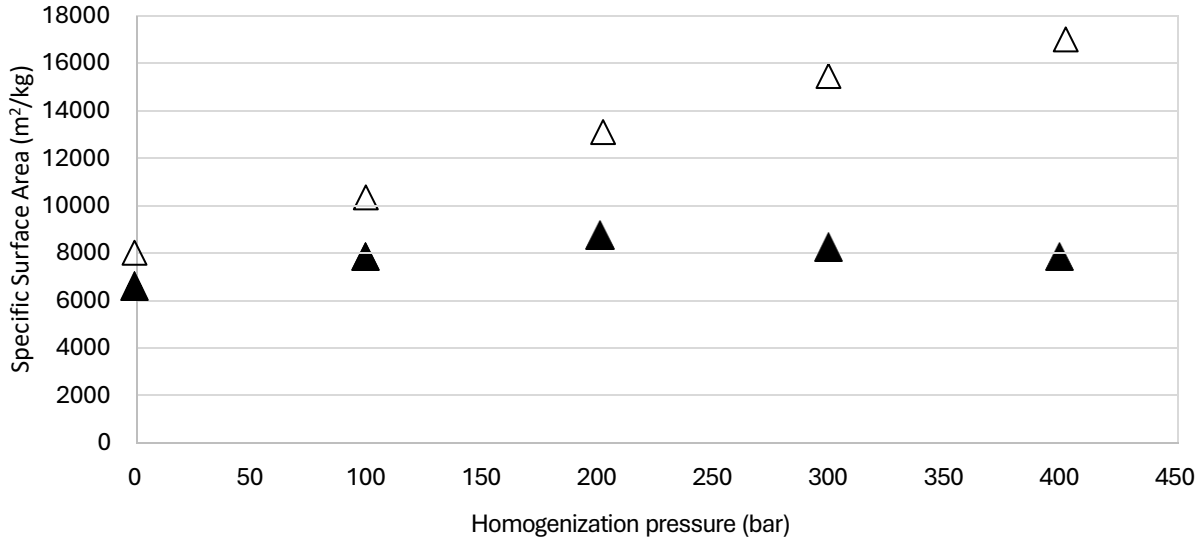

Figure 3.20 The effect of homogenization pressure (0-400 bar) on specific surface area (SSA) of 30% oil Tween 60 emulsions after 3 passes. Filled = 2% T60, open = 5% T60.

The effects of homogenization pressure on particle size show a decrease in particle size when homogenization pressure increases. The samples at 0 bar represent the particle size of the coarse emulsion prepared using the ultraturrax. At low relative pressure (below 300 bar) at both 2% and 5% T60 emulsions, the minimum particle size that can be created is limited by the shear forces created at set conditions. This is reinforced by the continual decrease in particle size when homogenization reaches higher pressures at 400 bar.

At higher relative surfactant concentration, similar decreases were observed at a pressure range from 600 – 1400 bar (Yuan et al., 2008). Interestingly, at relatively low concentration of Tween (2% T60), at low pressure (100 bar) the observed particle size is greater than compared to 5% T60 emulsions. The previous experiments showed that at low relative concentration of Tween for a 30% oil-in-water emulsion, the minimum particle size that can be created is limited by the concentration of surfactants. These effects are observed here with the 2% T60 emulsions as there is minimal difference in particle size regardless of homogenization pressure above 200 bar. It suggests in the Panda homogenizer, 100 bar is not enough to reach the surfactant limited specific surface area at 2% Tween but it is achieved when there is more surfactant available.

Figure 3.21 shows the maximum specific surface area created at different surfactant concentrations when the Tween type, oil concentration, homogenizer passes, homogenizer pressure and emulsion temperature are adjusted for. The solid line represents the theoretical surface coverage for Tween 60. By comparison, the surfactant coverage seems to be similar below 1% (wt%/oil) Tween in the experimental values. As the Tween concentration increases, the experimental values are well underestimatedby the theoretical value from the model of (Blankart et al., 2022).

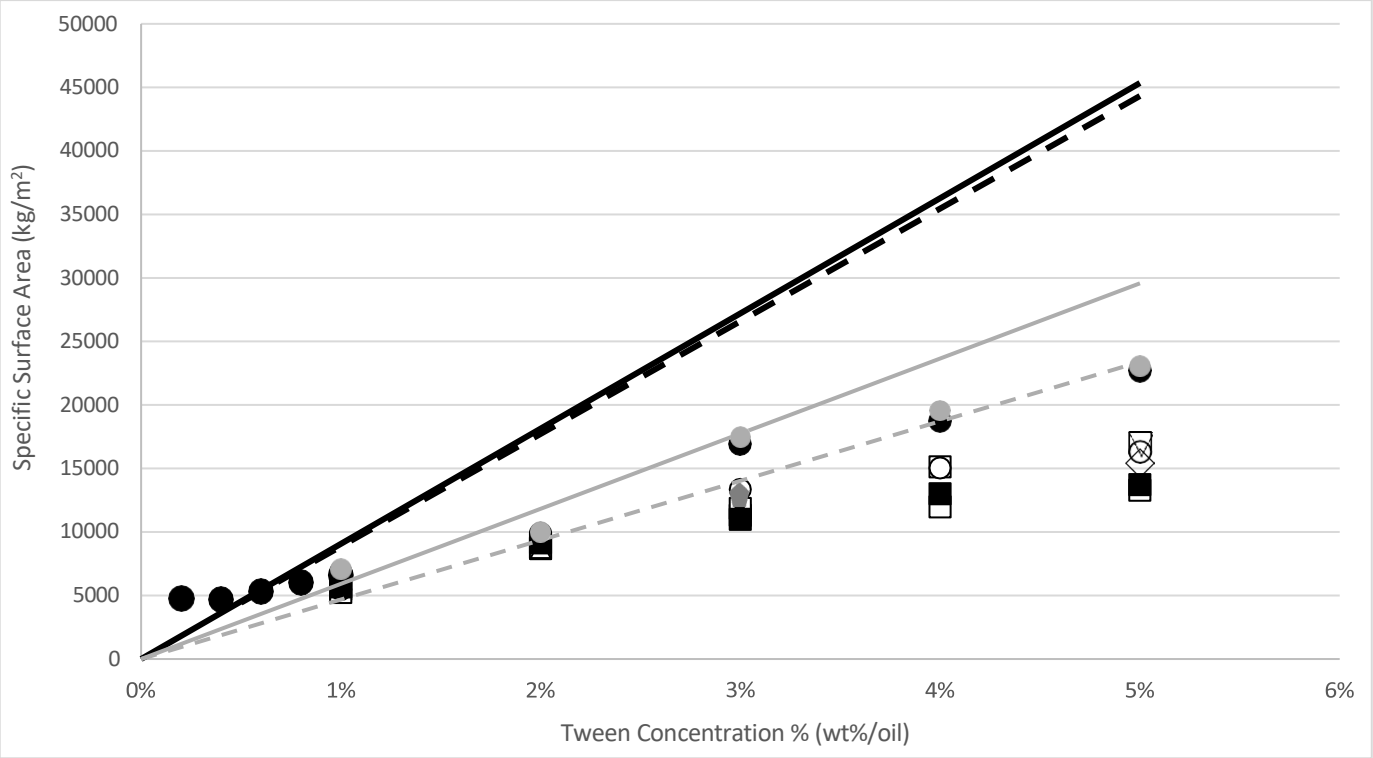

Figure 3.21 Summary of the maximum specific surface area versus surfactant concentration (wt%/oil) across all experiments conducted in Chapter 3. Tween C0 = hollow. Tween 80 = filled. 15% oil = circle. 30% oil = square. 3 passes = black. 4 passes = grey. Homogenizer pressure at 2% = triangle. Homogenizer pressure at 5% = Upside down triangle. Temperature = diamond. Oil concentration = pentagon. Theoretical value estimated from the model by Blankart et al. (2022) shown as black lines. Solid = Tween 60. Dashed = Tween 80. Theoretical value estimated from the model by (Glenn et al., 2005) shown as grey lines. Solid = Tween 60. Dashed = Tween 80.

The effect of Tween concentration above 3% (wt%/oil) does not change significantly, however when the homogenization pressure and temperature is increased at relatively high concentration the particle size decreases.

There are several limiting factors for the minimal particle size and the amount of new surface area created through high pressure homogenization for oil-in-water emulsions stabilized by non-ionic surfactants. The experimental values for emulsions against the theoretical value indicate that tween molecules occupy less area on the oil droplet surface by comparison to the theory. The surface coverage indicated by the experimental values occupies approximately 1/2 of the theoretical values. The relationship according to the model by Blankart et al. (2022) is linear between surfactant concentration and specific surface area and assumes the homogenizer never limits the creation of smaller particles. In addition, as indicated in the model by Glenn et al. (2005), the adsorption of Tween 80 can sometimes be slightly higher as indicated in our results. This may be due to the difference in fatty acid chain length on the triglyceride of Tween 60 and 80.

3.4 Conclusions

There were no differences between Tween 60 and 80 in the experimental trials carried out in this work. This is consistent with the predictions made using the Blankart et al. (2022) model although the experimental values suggest that Tween does not adsorb spherically as assumed in the model likely to the changes in conformation when above or below CMC. The effect of temperature on the specific surface area after homogenization shows minimal changes.

When a concentration of 3% surfactant concentration is reached across all experiments, there is a plateau which suggests the limits of creating new surface area at 200 bar for 3 passes has been attained. On the other hand, when oil concentration is adjusted for there were no significant differences beyond oil concentrations of 15% up to 40%.

In addition, when a concentration of 3% surfactant concentration is reached across all experiments, there is a plateau which suggests the limits of creating new surface area at 200 bar for 3 passes has been attained. Indications would suggest that at 5% surfactant concentration (wt%/oil) if higher homogenizer pressure was induced, additional surface area may be created. On the other hand, at low surfactant concentration (2% T60 (wt%/oil)) the limits of new surface area are attained within homogenizer pressures of 100-200 bar after 3 passes. There is no significant change above this pressure range due to surfactant availability.

The study carried out in this chapter provides a basis for investigating the competitive absorption of Tween and dairy proteins which is the focus of the next chapter.

Chapter 4: Competitive adsorption of whey and Tween 60

4.1 Introduction

Along with chemical surfactants, proteins can also act as an emulsifying ingredient. Dairy proteins are used extensively, providing functional as well as nutritional properties. In some cases, dairy product formulations contain native whey and/or casein proteins along with added chemical surfactants. Under these conditions during processing, there are two or more competing surfactants present particularly during emulsion formation in the homogenization step. Inevitably, both protein and chemical surfactants compete to adsorb newly created surface area as droplets form under high shear conditions. In the previous chapter it was discussed that the creation and stabilization of the specific surface area of an emulsion was determined by the availability of surfactant molecules. Specific surface area increased with emulsions with high relative surfactant concentration until surface area creation is limited by the energy created in the homogenizer.

It is widely reported that small molecular weight surfactants such as Tween may partially displace adsorbed proteins over time under appropriate condition, such as surfactant concentration and interfacial properties. (Dickinson & Tanai, 1992). Equilibrium adsorption however may not occur in the short time scales involved in stabilizing new surface area created in the homogenizer. It is suggested that the large relative size of proteins compared to the Tween molecule means that at equivalent concentration, in high shear conditions in the homogenizer whey should adsorb faster as it will cover more area for each collision. The typical particle size value for whey protein is 6.81 ± 0.75 nm (Ali, 2019). The size of Tween 80 is small but forms micelles above CMC, this means the effective size of the micelle is 9.95 nm (Bide et al., 2021). In Chapter 3 it was determined that the concentrations used to form Tween emulsions are likely to exceed CMC, therefore Tween is potentially larger and more favored to compete for new surface area compared to whey.

In mixtures of sodium caseinate and whey protein concentrate, β-casein has been shown to dominate the oil surface where β-lactoglobulin is more represented at lower total protein concentrations (Ye, 2008). To reduce complexity, this work focuses on the competition between whey proteins and Tween 60 for the oil surface area during homogenization. Whey is present in relatively high concentrations in many formulation including emulsions containing skim milk, where free serum casein levels are relatively low unless added as caseinate. As a consequence, it makes sense to investigate whey – Tween interactions in the first instance.

In the whey-Tween system, it is expected that Tween will dominate the structure of the emulsion at high surfactant to oil ratios, but that at low Tween concentrations or high protein concentrations, then the emulsions may become more dominated by proteins. Therefore, in the competitive adsorption between surfactants and protein it is important to understand:

a) The limits of adsorption of surfactants under the set homogenization conditions (200 bar for 3 passes).

b) Whether or not whey protein adsorbs on the oil droplet surface when Tween is present.

c) The critical concentration where surfactants begin to outcompete or displace protein on the oil droplet surface.

4.2 Materials and methods

The methods used in this part of the work are similar to those used in Chapter 3 with addition of whey protein. Because there were negligible differences observed between Tween 60 and 80 in the previous work, only Tween 60 was investigated.

4.2.1 Materials

Sunflower Oil was sourced from a local grocery store. Tween 60 (T60) (Product name: SP Crillet 3 MBAL-SS-(SG), Batch no. 57245) was obtained from Hawkin Watts Limited, repacked from the product manufacturer Croda Singapore PTE Ltd and Whey protein isolate (WPI895) were supplied by Fonterra Research and Development Centre. Chemical reagents for sample preparation and buffers for protein analysis were also supplied by Fonterra Research and Development Centre. The chemical reagents used in sample preparation were dithiothreitol and 6M guanidine HCl. Sample buffers that were used were 6M guanidine HCl/0.1 M Bis Tris/5.37mM sodium citrate and 4.5 M guanidine HCl. The HPLC buffers that were used were made up of 0.1% Trifluoracetic acid (TFA) (Buffer A) and 0.09% TFA with 90% Acetonitrile (Buffer B).

4.2.2 Emulsion formation

The dissolution of WPI was done with Tween 60 by adding both at the appropriate ratio into RO water (supplied by Fonterra Research and Development Centre) and placed in a 40°C water bath. An overhead stirrer was set to 350 rpm and the semi solid tween and WPI895 was mixed in with the distilled water for at least 90 minutes. The emulsion preparation including the formation of the coarse emulsion and homogenization conditions were performed as outlined in section 3.2.2.

4.2.3 Characterization of emulsions

Particle size analysis was conducted using the same methods from section 3.2.3 including the calculation for SSA (m^2/kg). Fat globule size analysis was conducted by adding 9 mL of Walstra solution to 1 mL of each emulsion to break any aggregates caused by proteins. The analysis was done as per the particle size analysis with identical settings through the Malvern Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK).

4.2.4 Preparation of emulsion serum

Homogenized emulsions were centrifuged at 15000 rpm for 45 minutes using a Kubota Centrifuge (Kubota Manufacturing Corporation, Tokyo, Japan). The fat was separated and discarded; the clear supernatant was collected. The supernatant was centrifuged under the same condition to separate and remove any residual fat and collected in a 30 mL container.

500 µL of supernatants from each T60-WPI emulsion were mixed with 1450 µL of 6M guanidine HCl and 50 µL of dithiothreitol in a 2 mL Eppendorf tube using a vortex. Samples were allowed to stand for at least 30 minutes to allow time for dithiothreitol to reduce any disulfide bonding by whey protein.

The standards contained a 2.14% WPI solution (the mass of WPI used to form a 5% WPI to oil ratio 30% emulsion) in distilled water. The preparation was as described above for the emulsion supernatants. A dilution series was performed by adding different aliquots of the 2.14% WPI solution before being made up to 500 µL using distilled water.

4.2.5 HPLC procedure

The concentration of whey proteins βlg and α-Lac in the standards and emulsion samples were determined using the Waters ACQUITY UPC2 (Waters® Corporation, United States) by RP-HPLC methods described by Elgar et al. (2000). The initial mobile phase conditions used Buffer A and Buffer B at a flow rate of 0.8 mL per minute. The sample injection volume was 50 µL and detection used was 280 nm for a run time of 25 minutes per sample.

4.2.6 Experimental plan

Figure 4.1. outlines the experimental methods used in this chapter.

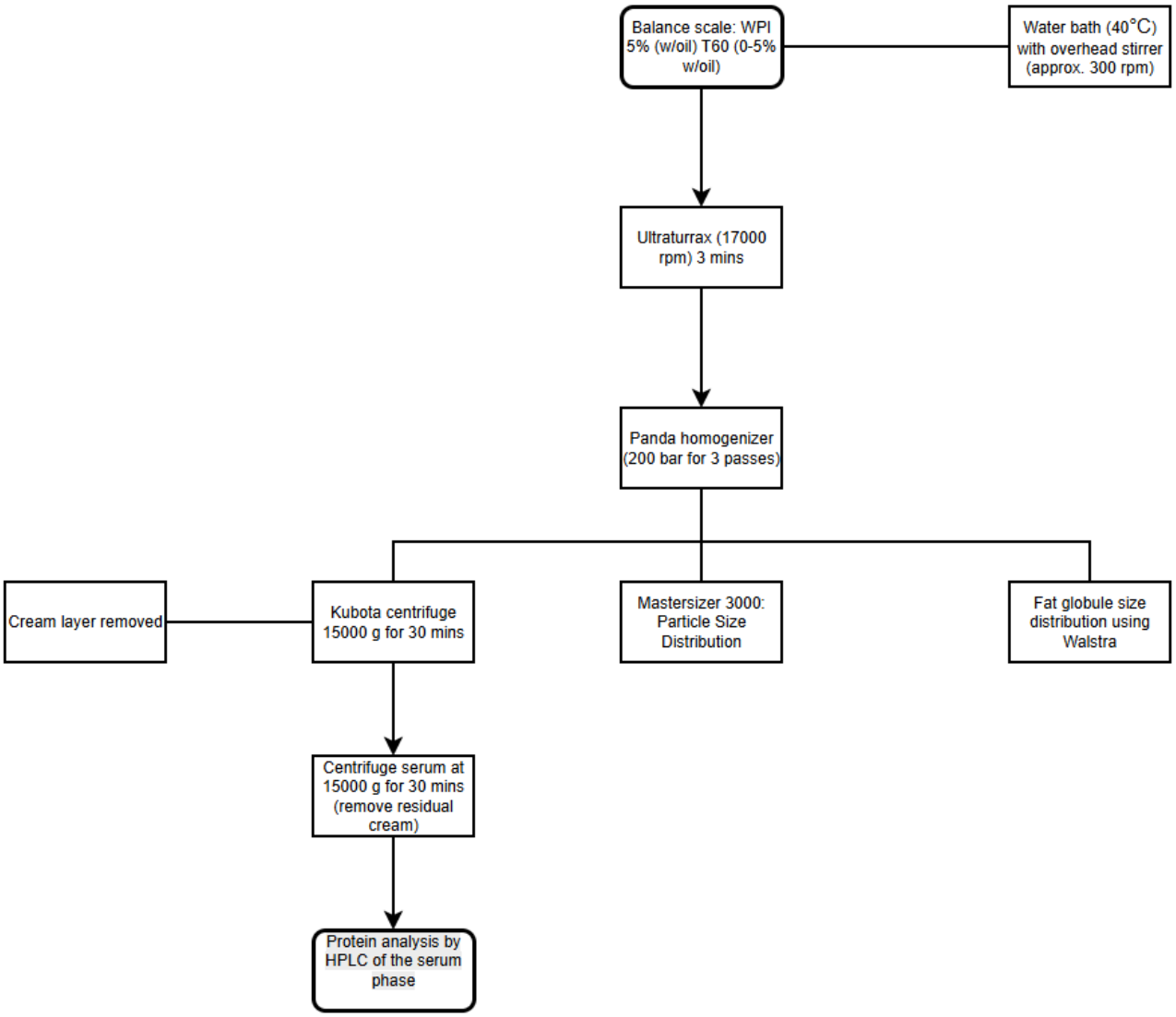

Figure 4.1 Process flow diagram of emulsion formation and protein assay of WPI-TC0 emulsions.

A series of experiments was set up to investigate the competitive adsorption of whey and Tween in oil-in-water emulsions. For most experiments the effect of Tween concentration was explored at high relative concentration of WPI (5% WPI to oil ratio). Separate experiments were carried out to assess the effect of WPI concentration at low relative Tween concentration (1% Tween to oil ratio). To characterize the surface loading of whey, HPLC was done to measure the concentrations (mg/mL) of protein in the emulsion serum. Using this concentration, a mass balance of the emulsion was done to approximate the

amount of protein adsorbed on the oil droplet surface.

4.3 Results and Discussion

Figure 4.2 shows the particle size distribution and fat globule size distributions of 30% oil emulsions made using whey protein isolate in the absence of Tween. The data shows no evidence of any oil droplet aggregation that is sometimes seen in whey protein stabilized emulsions (Ye, 2010). When there was no Tween and only WPI to stabilise the emulsion (*Figure 4.2*), the distributions are unimodal which indicates that there was enough whey to saturate the surface of oil droplets in a 30% o/w emulsion. There is a slight narrowing of the distribution shown in *Figure 4.2*, it is likely an effect of experimental error during the homogenization step where sporadic increases in homogenization pressure occur. Nevertheless, the FGSD fits within the distribution of the PSD between samples which show no evidence of aggregation or fat coalescence.

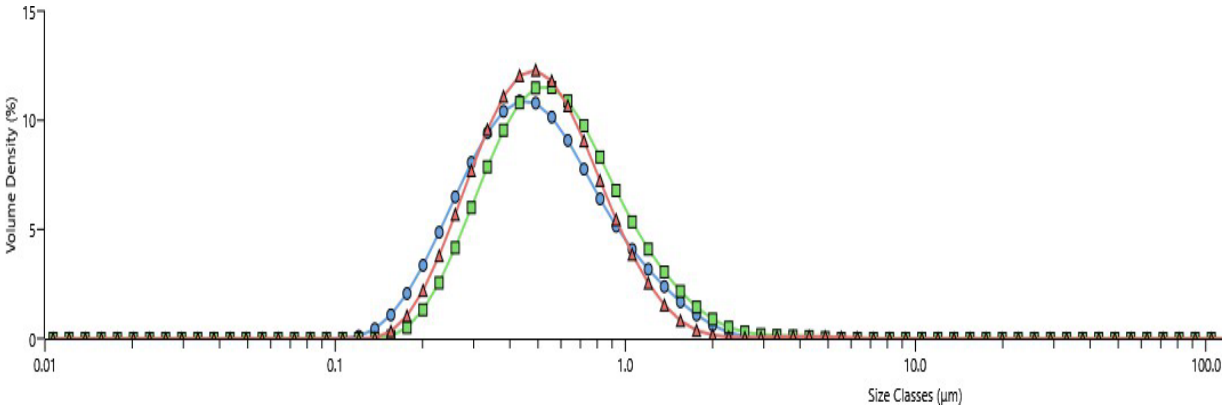

Figure 4.2 PSD and FGSD after three homogenizer passes for 30% oil emulsions stabilized with WPI (5% wt%/oil) and without T60. Square and Circle symbols = PSD. Triangles = FGSD.

The effect of T60 concentration at 5% WPI (wt%/oil) on the PSD of emulsions is shown in *Figure 4.3*.

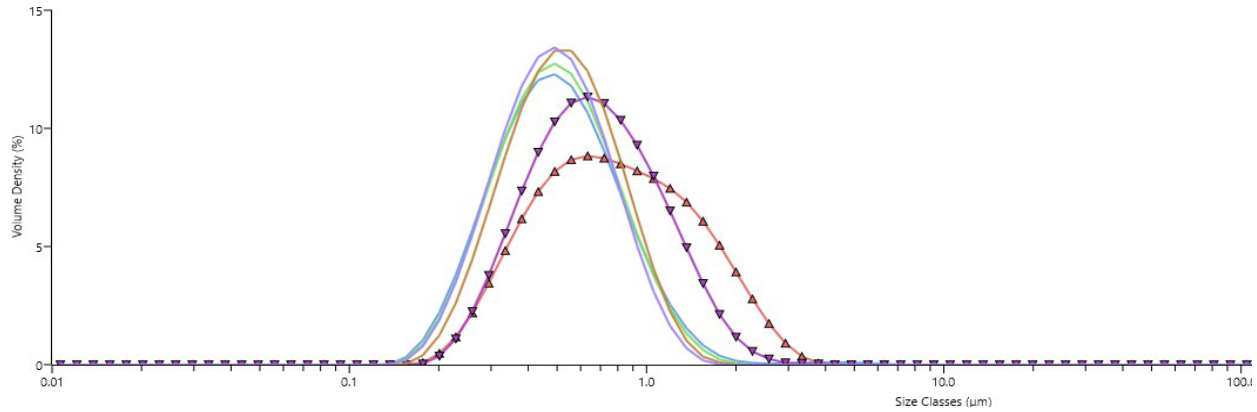

Figure 4.3 PSD after three homogenizer passes for 30% oil emulsions stabilized with WPI (5% wt%/oil) at different Tween concentration (1-5% wt%/oil). Blue line = 0% T60. Green line = 0.3% T60. Red triangles = 1.5% T60. Purple inverted triangles = 2.2% T60. Brown line = 3.6% T60. Purple line = 4.8% T60.

The PSD's show that a very similar size distribution is produced for most emulsions. This makes sense as in all the emulsions there is excess surfactant (either whey or Tween 60), and in those conditions it the PSD is expected to be limited by the capacity of the homogenizer to produce new surface area. At 1.5% and 2.2% T60 (wt%/oil) there is an increase in the distribution width and shouldering-effect which appears bimodal and FGSD is right skewed. The evidence suggests there is an initial formation of small droplets in the homogenizer but some coalescence because there is not enough surfactant present to stabilize this surface area. This is interesting as smaller particles can be stabilized in the absence of Tween 60 even though the amount of WPI was held constant which is has yet to be observed in literature to date. This suggests Tween 60 absorption is faster than whey protein absorption, droplets without sufficient Tween will coalesce before the whey proteins can adsorb and stabilize the oil droplet.

When the concentration of T60 is increased to 4.8% (wt%/oil), the distribution returns to its uniform distribution achieved in excess Tween conditions (*Figure 4.3*). This suggests that in these conditions there is enough T60 present to stabilize all the surface created in the homogenizer and that this out competes the whey proteins present in the system.

These trends are also shown in the volume and surface average diameter in the competitive adsorption of whey and T60 (30% oil 5 (wt%/oil) WPI at various T60 concentrations 0-5 (wt%/oil)) were compared in *Figures 4.4 and 4.5*.

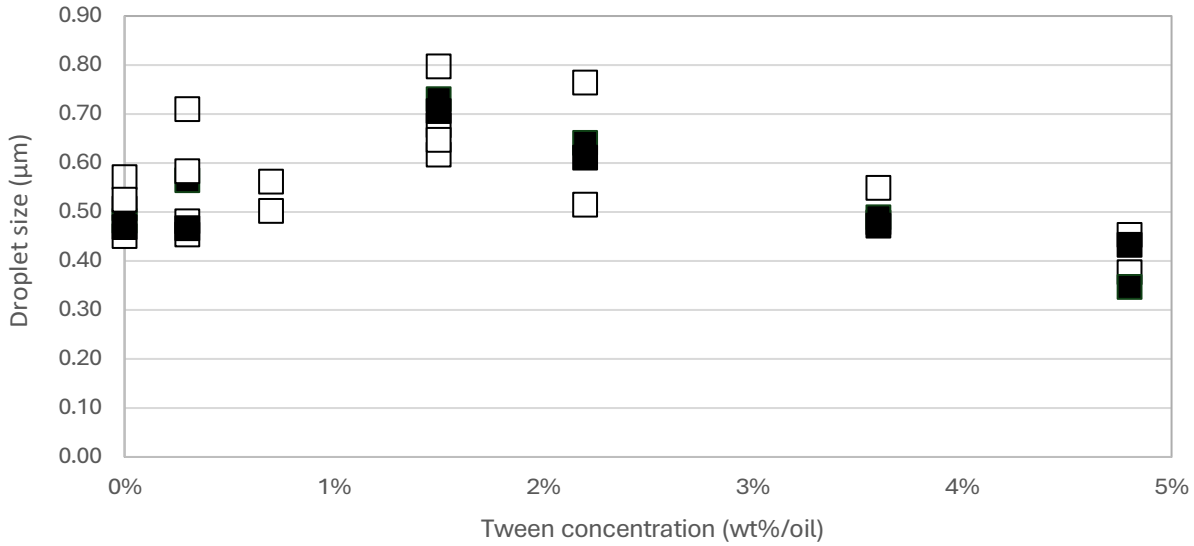

Figure 4.4 The effect of Tween 60 concentration (wt%/oil) on particle size (D_{43}) and fat globule size of emulsions at 5% WPI (wt%/oil). Square = 30% oil (wt/v). Hollow = Particle size. Filled = Fat globule size.

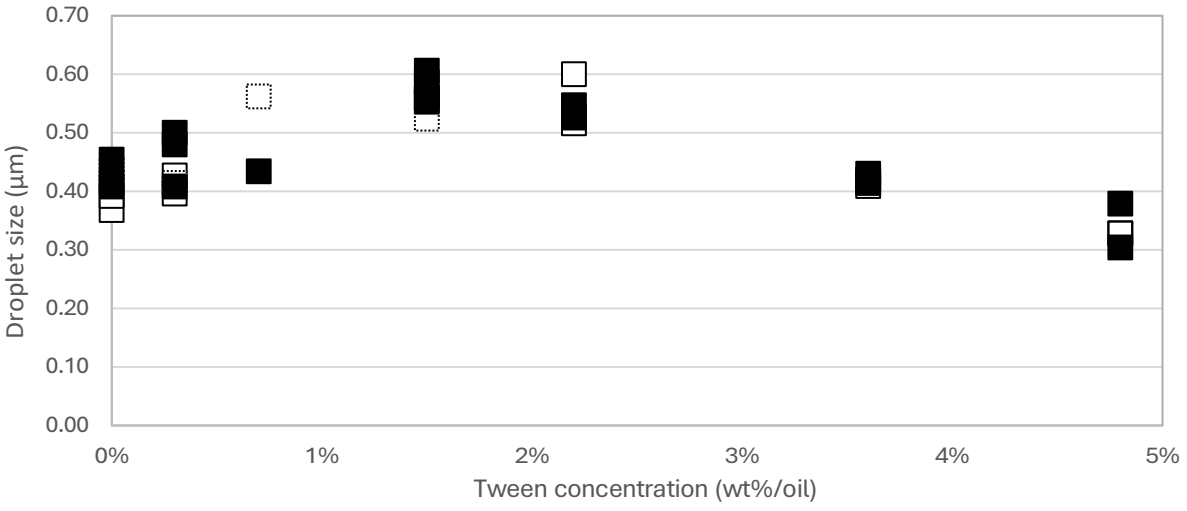

Figure 4.5 The effect of Tween 60 concentration (wt%/oil) on particle size (D_{32}) and fat globule size of emulsions at 5% WPI (wt%/oil). Square = 30% oil (wt/v). Hollow = Particle size. Filled = Fat globule size.

Across replicate experiments, the average droplet diameter ranged from 0.407 to 0.997 μm depending on the concentration of surfactant. At low Tween concentration the volume surface average increases with increasing surfactant concentration from 0-1.5 (wt%/oil). The maximum size occurred at 1.5 and 2.2% Tween. At the highest T60 concentration at 4.8% (wt%/oil) there was a difference between replicate samples in particle size and SSA. One replicate shows a SSA is as high as 17000 m^2/kg and is likely due to experimental error during the homogenization process. Residual surfactants in the homogenizer as well as sporadic increases in homogenizer pressure are some of the factors which may have impacted the particle size.

In the absence of T60, there is enough whey protein to saturate the oil surface as indicated by the specific surface area values for the control (*Figure 4.C*). As Tween was added to the system the particle size increases, and specific surface area decreases. This indicates that Tween (when present) adsorbs more quickly than the whey proteins as new surface area is created in the homogenizer, effectively reducing the ability of the whey proteins to adsorb. At low Tween concentrations there may still be enough whey absorption to stabilize the created surface area and avoid coalescence. At 1.5 and 2.2% Tween the particle size distributions show evidence of coalescence – suggesting that the lower specific surface area arises from inadequate coating of the oil surface and this incomplete coverage results in coalescence or partial absorption from whey proteins.

This observation is different than the demonstration that whey can be displaced from the surface of emulsions if Tween is added after homogenization by other researchers (Courthaudon et al., 1991; Demetriades C McClements, 1998; Euston et al., 1996). In those studies, only whey is present during the creation of the surface area and therefore whey is the primary surfactant which is then replaced over time on addition of other surfactants.

When Tween is added to whey at low relative concentration the specific surface area decreases markedly compared to when whey is used exclusively to stabilize the emulsion (*Figure 4.6*). When compared to the data for Tween 60 alone (dotted line), the emulsion broadly behaves as if the whey proteins are not present.

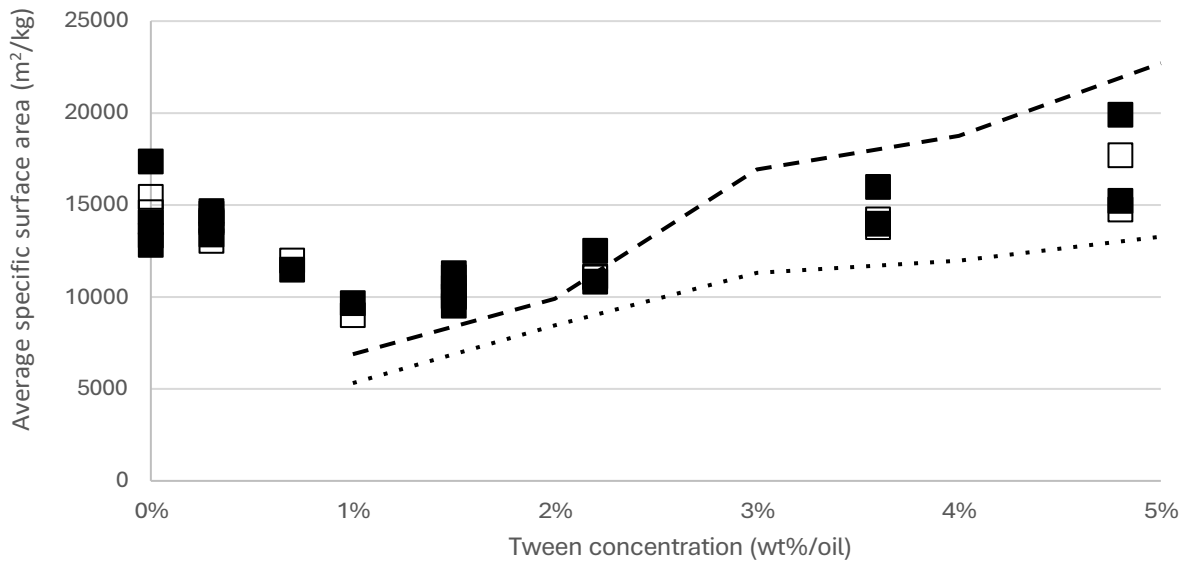

Figure 4.6 The effect of Tween 60 concentration (wt%/oil) on the average specific surface area (m^2/kg) of emulsions at 5% WPI (wt%/oil). Square = 30% oil (wt/v). Hollow = Particle size. Filled = Fat globule size. Dotted line = 30% oil- in water emulsion stabilized by pure Tween 60 (as seen in Chapter 3). Dashed line = 15% oil-in water emulsion.

It is likely that this phenomenon is related to the conformation of competing surfactants and is related to the concentrations used to form the emulsion. During homogenization as collisions occur and new surface area is created, some Tween also adsorbs on the surface (*Figure 4.6*). When whey proteins collide with oil droplets and stabilizes new surface, there must be a rate which facilitates this adsorption. The concentrations used in this study are always above the CMC as determined in Chapter 3. Whey is a globular protein which must unfold to expose their hydrophobic sites to adsorb onto the surface. This means the rate of its adsorption must be slower compared to Tween even when the relative size of whey and Tween micelles are similar. This illustrated when Tween is added at low concentration with 5% (wt%/oil) WPI, where it adsorbs on to the oil droplet surface. Although the concentration is above CMC, the adsorption depletes the number of micelles which facilitates the adsorption of whey to stabilize the droplet. Small droplets may be formed initially, but the depletion in micelles only allows a small fraction of Tween adsorption resulting in a decrease in specific surface area. Above Tween concentration

of 2.2% (wt%/oil), the emulsion could be stabilized entirely by T60 where it completely outcompetes whey onto the oil droplet surface. The availability of Tween micelles results in a higher frequency of micelles and increases the probability of Tween collisions over whey protein.

No significant change in particle size was seen from 3.6% (wt%/oil) and above which indicates the limits of the energy that can be produced by the homogenizer under the applied conditions. The effect of increasing T60 concentration when the results in no significant change in particle size as observed in Chapter 3. This agrees with study by Mackie et al. (1993), where at low relative homogenizer pressure, the effect of increases in surfactant concentration results in a plateau in specific surface area. A similar trend was observed in Chapter 3, where the specific surface area plateaued when the concentration exceeded 3% (wt%/oil).

To explore this further, emulsions were made with a fixed 1% (wt%/oil) Tween 60 concentration, but with varying WPI concentration (0 to 5%). The effect of WPI concentration on particle size distribution is shown in *Figure 4.7*.

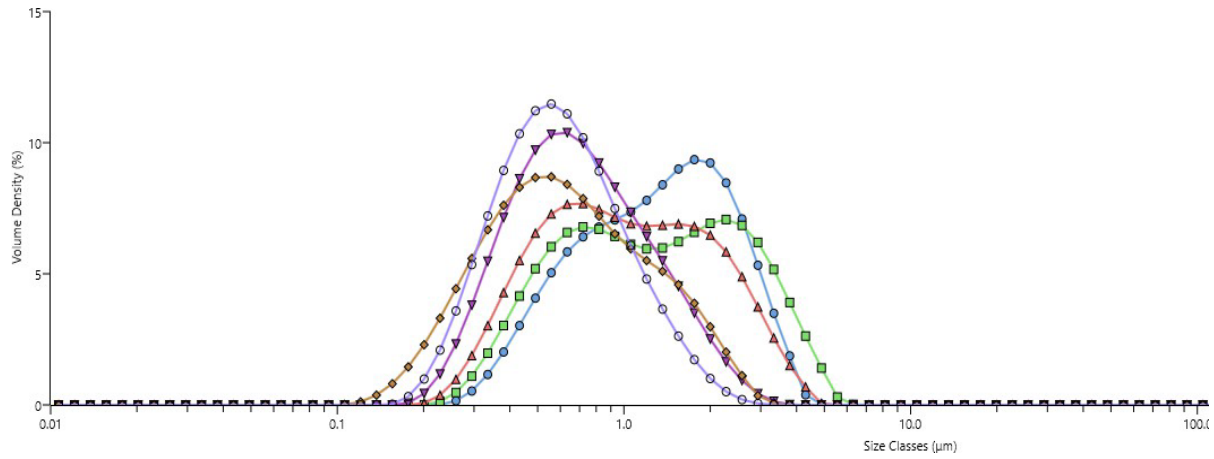

Figure 4.7 PSD after three homogenizer passes for 30% oil emulsions stabilized with T60 (1% wt%/oil) at different WPI concentration (1-5% wt%/oil). Blue circles = 0% WPI. Green line = 1% WPI. Red triangles = 2% WPI. Purple inverted triangles = 3% WPI. Brown diamonds = 4% WPI. Circle= 5% WPI.

In the absence of WPI at 1% T60 there is a shouldering effect where there is likely not enough Tween or whey surfactant to stabilize the oil droplet surface area initially created by the homogenizer. Similar observations are seen in the distribution with the addition WPI up to 2% (wt%/oil). Beyond this concentration the distribution becomes uniform indicating there is enough surfactant to saturate the oil surface. The reduction of particle size with increasing WPI suggests that whey is likely to be stabilizing the surface of oil droplets at levels above 3% WPI. *Figures 4.8 and 4.S* show this trend for the surface average and volume average droplet size distributions. It is clear that there is no difference between FGSD and PSD, indicating that the bimodal nature of the PSD at low WPI is not due to flocculation. The average droplet size steadily decreases with increasing WPI concentration until 3%, where the size does not reduce any further.

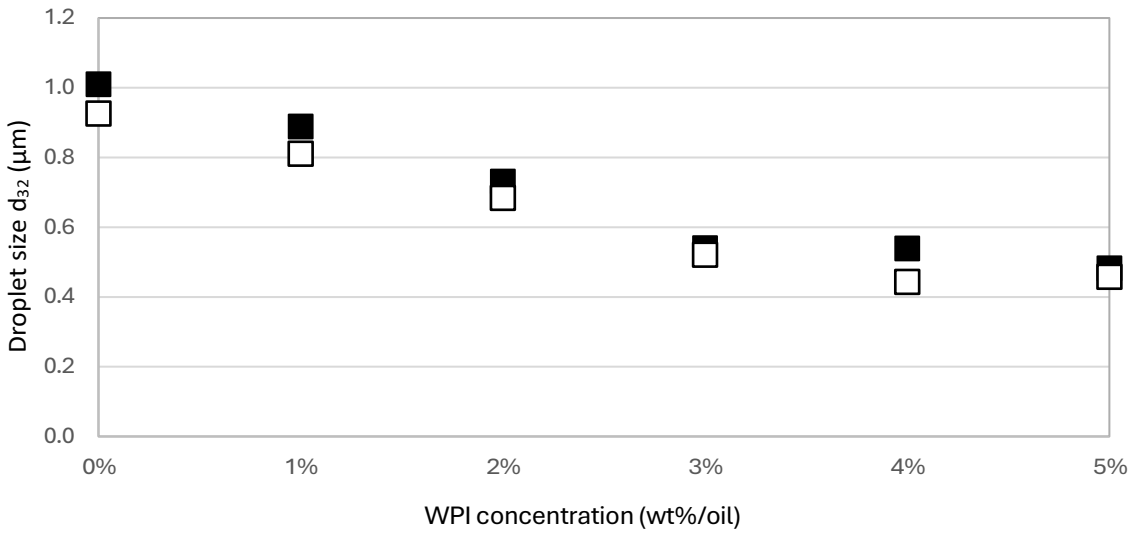

Figure 4.8 The effect of WPI concentration (wt%/oil) on surface average (d_{32}) particle size (µm), fat globule size (µm) of 30% (wt/v) oil emulsions at 1% Tween 60 (wt%/oil). Hollow square PSD. Filled square = FGSD.

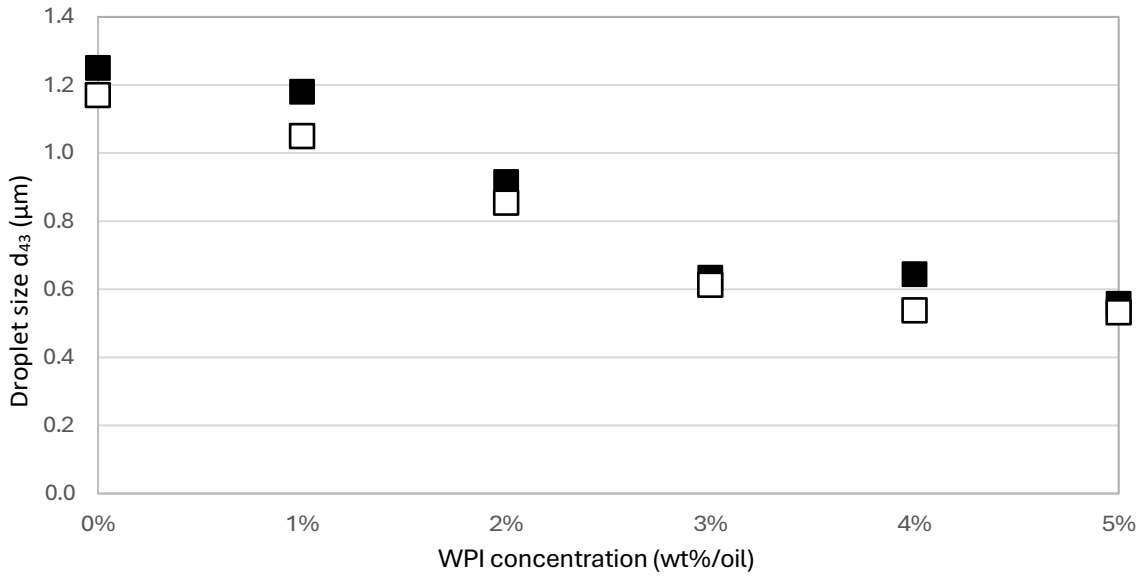

Figure 4.S The effect of WPI concentration (wt%/oil) on volume average (d_{43}) particle size (µm), fat globule size (µm) of 30% (wt/v) oil emulsions at 1% Tween 60 (wt%/oil). Hollow square PSD.Filled square = FGSD.

Figure 4.10 shows that although the average droplet size decreases as WPI increases, the specific surface area stays approximately constant at the level that the 1% Tween 60 can achieve without WPI (i.e. 0% WPI) and increases only at 4 and 5% WPI.

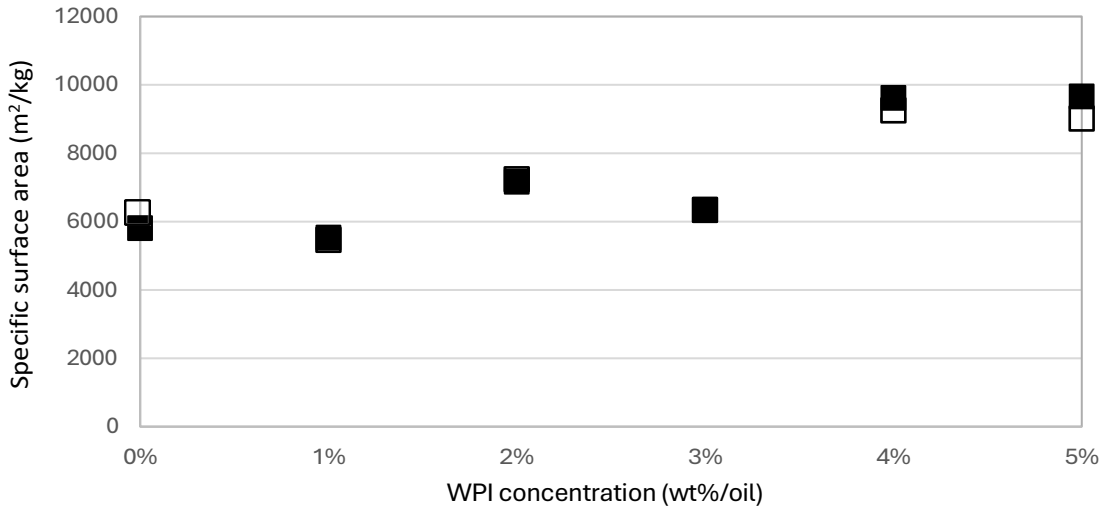

Figure 4.10 The effect of WPI concentration (wt%/oil) on specific surface area (SSA) of 30% (wt/v) oil emulsions at 1% Tween 60 (wt%/oil). Hollow square PSD. Filled square = FGSD.

This appears counter intuitive until the bimodal nature of the size distributions is accounted for. As discussed above for *Figure 4.7*, there is evidence of coalescence in the emulsions made at WPI concentrations below 3%. Even though the average size is decreasing, the emulsion surface area is approximately constant as the area is dependent on the availability of the Tween surfactant. Only at high WPI concentrations can the primary droplets created by the homogenizer be stabilized without subsequent coalescence. This is further evidence that Tween dominates the adsorption behaviour and the ability for whey protein adsorption. It also supports the idea that whey protein adsorption is slower than adsorption of Tween unless the concentrations are high.

To confirm these hypotheses the changes in the surface loading of whey protein were measured. It was expected that the whey protein loading in the emulsions would decrease as the Tween concentrations increase until at Tween to oil ratios above 3%, no whey protein adsorption would occur. In emulsions made with varying WPI levels at 1% Tween, it is expected that protein loading would be approximately constant and possibly lower at low concentrations.

Determination of WPI protein concentrations and protein loadings.

Protein loadings were calculated from the measured reductions in aqueous protein concentrations after separating the serum phase from the emulsions by centrifugation. This assumes the decrease in concentration is proportional to amount adsorbed onto the fat droplet surface. Serum phase protein concentrations were measured using HPLC using known concentration WPI solutions as standards. In WPI, the three main whey proteins measured are α- Lactalbumin (α-Lac), Beta Lactoglulin A and B (βlg A/βlg B). The chromatogram measures the intensity of light absorption because of eluting compounds passed through the column. This is plotted against residence time of which each analyte is passed through the column.

Figure 4.11 shows the relevant peaks from the chromatograms found for a series of dilutions of a 2.14% WPI solution. This concentration is the aqueous phase WPI concentration used in a 5% WPI to oil ratio emulsion at 30% oil content. The undiluted peaks correspond to the peak areas that would be observed in a serum phase if there was no adsorption. Any reduction in peak height will be proportional to the amount of adsorbed on the oil droplet surface during the creation of the emulsion.

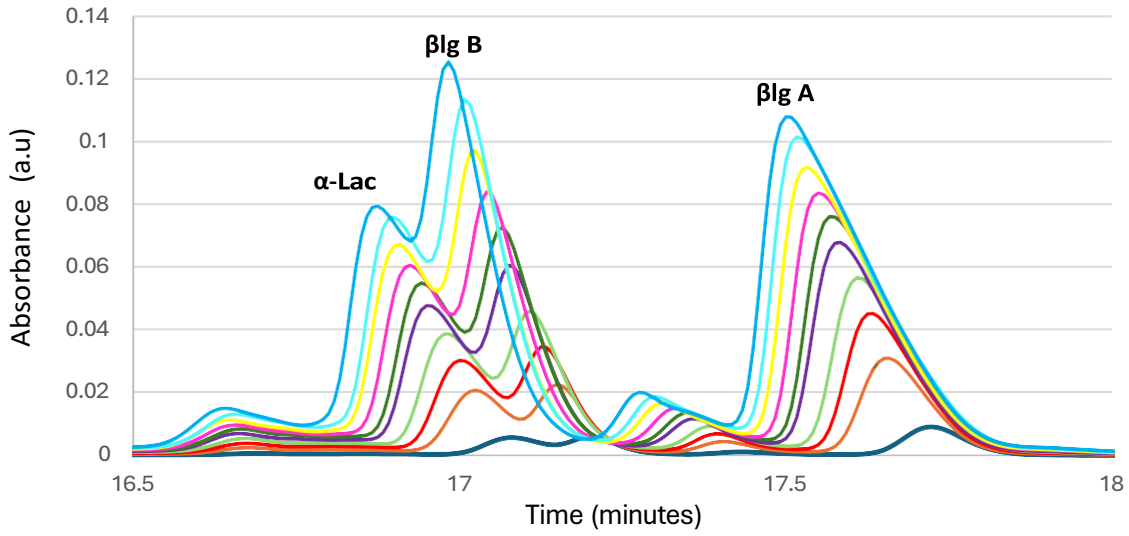

Figure 4.11 Chromatograms of a dilution series of a 2.14% WPI solution. Dark blue = 0.107% WPI. Brown = 0.43% WPI. Red = 0.64% WPI. Green = 0.86% WPI.
Purple = 1.07% WPI. Dark green = 1.28% WPI. Pink = 1.5% WPI. Yellow = 1.71% WPI. Turquoise = 2.03% WPI. Blue = 2.14%. Note: Early and later time series data has been omitted to focus on the relevant peaks.

The residence time of which whey proteins are passed through the HPLC column is approximately 17 minutes. The separation of whey proteins normally follows in the order of α-Lactalbumin> β-lg B > β-lg A. The WPI standard solution shows overlapping of the β- lg B and α-Lac peaks (*Figure 4.11*). In HPLC, the separation of analytes depends on their molecular weight and are passed through the column by order of magnitude, i.e. small molecular weight analytes are measured first and are followed by analytes increasing of increasing molecular weight. The molecular weight of β-lg A/B and α-Lac are very similar which means they are separated through a narrow window resulting in the "shouldering effect" when their peaks overlap (*Figure 4.11*).

Across identical experiments conducted on separate days, there is also variation between samples of pure WPI solution standards (2.14%). Adjustments were made to the flow rate of the columns which for initial experiments were 0.8 mL/min where the separation between β-lg B and α-Lac was poor causing the overlapping effect. In subsequent experiments the flow rate was reduced to 0.4 mL/min to extend the residence time of each protein and improve analyte separation and peak resolution. For this reason, the residence times on *Figure 4.16* (below) are longer than for *Figures 4.11 and 4.13*.

To convert the peak areas to concentration of each protein, the proportion of each protein in WPI must be known. The mass fraction of total protein in WPI895 is 95.4%w/w. Based on product composition information the β-lg and α-Lac concentrations were estimated to be 71.2% β-lg and 14% α-Lac makes up 14% (on a total dry solids basis). The ratio of β-lg A and β-lg B in WPI895 was assumed as the ratio typically found in skim milk which was 0.51 to 0.49 respectively. Therefore, of the total β-lg composition (71.2%) were divided into β-lg A (36.12%) and β-lg B (35.08%).

Figure 4.12 shows the effect of concentration over peak area attained for the dilution series of 2.14% WPI895. As expected for all whey protein fractions (βlg A and B + α-Lac), when the WPI concentration increases, the respective peaks areas also increase. WPI895 contains a low concentration of α-Lac relative to βlg and above 80% dilution the concentration of α-Lac begins to plateau. This effect is most likely due to the overlap of the peaks of α-Lac and βlg B. A small overallocation of area attributed to βlg B would result in slightly less attributed to α-Lac. Despite this small error, because all measurements are expected to fall within the range of the standard curve (as the maximum concentration expected in any serum phase corresponds to 2.14% WPI), the gradients shown in *Figure 4.12* can be used to calculate serum phase concentrations of each protein.

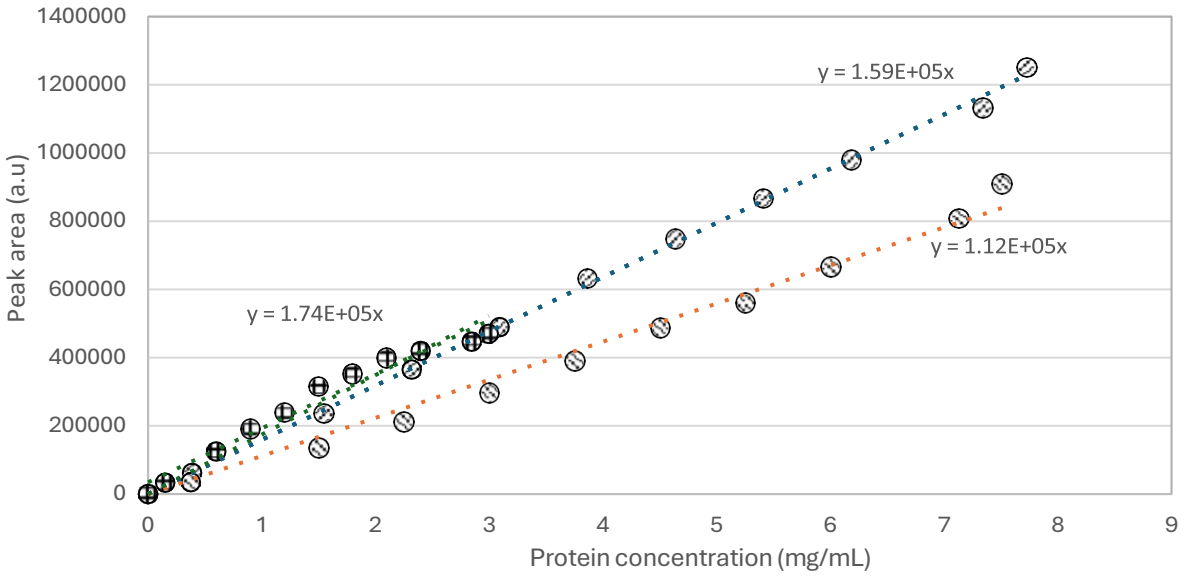

Figure 4.12 The standard curve for a dilution series of 2.14% WPI solution (wt/v). Circle with cross hatch = α-Lac. Circle with up-right skewed stripes = βlg A. Circle with up-left stripes = βlg B.

Figure 4.13 shows example chromatograms for the proteins in the serum phase of emulsions made with 5% WPI and varying Tween 60 concentrations. These show increasing amounts of proteins in the serum as the level of Tween increases, indication less absorption onto the fat droplet surface therefore T60 displaced whey proteins.

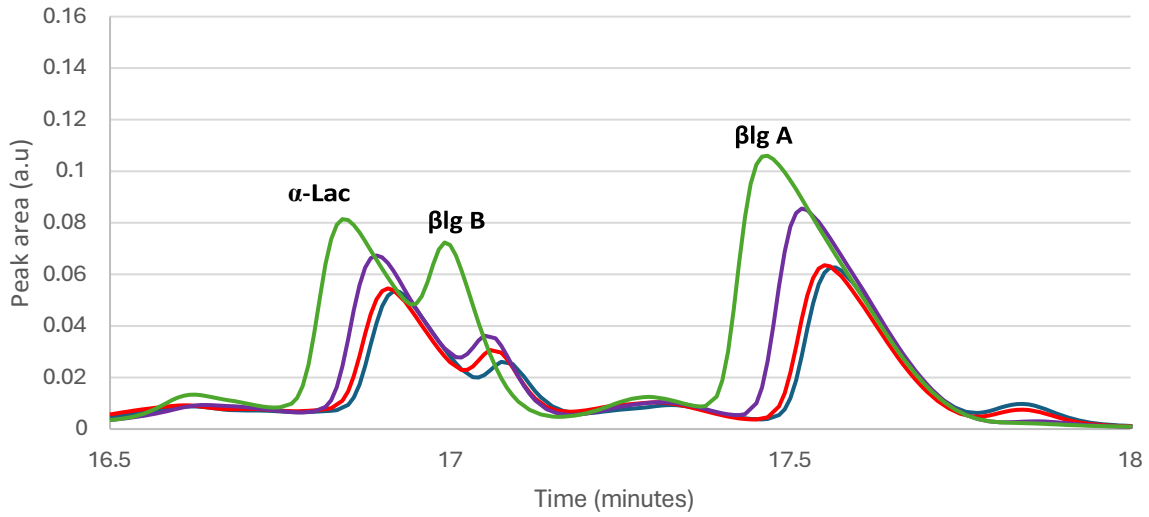

Figure 4.13 Example chromatogram of whey proteins in emulsion serum at various T60 concentrations (0-1.5 wt%/oil) at 2.14% WPI. Blue = 0% (wt%/oil). Red = 0.3% (wt%/oil). Purple = 0.7% (wt%/oil). Green = 1.5% (wt%/oil). Note: Early and later time series data has been omitted to focus on the relevant peaks.

Table 4.1 summarises the residual protein concentrations in the serum phase for each emulsion estimated from the chromatogram areas (*Figure 4.13*) and standard curve (*Figure 4.12*), compared to the total amount of each protein in the emulsion. Generally, whey protein in WPI895 is highly undenatured and in its natural state. To suppress the effects of protein aggregation or denaturation of residual whey protein, the reducing agent DTT was used to prevent disulfide bonding. As indicated in the PSD and FGSD (*Figure 4.8 and Figure 4.11*) there was no evidence of either protein aggregation or coalescence. Regardless, the use of DTT was employed as a control to account for any aggregation which may have occurred between particle size measurements and HPLC analysis.

The total WPI concentration in all these emulsions was 5% of the oil concentration for a 30% (w/w) of the emulsion. This equates to 2.14% (w/w) WPI, and the total βlg and α-Lac protein concentration was 18.80 mg/mL. The measured values for the aqueous phase were close to the expected value, although some measurement for emulsion serum phases are close or higher than expected at high Tween concentrations. This is not possible in the system and this level of deviation shows extent of inaccuracies in measurement. Despite this, there is a clear trend of when whey protein adsorption occurs.

Table 4.1 Concentration of proteins in serum after separation of emulsions made at various T60 concentration (wt%/oil) and 5% WPI (wt%/oil) after homogenization at 200 bar after 3 passes. (Bracketed numbers represent the fraction of protein in solution that was recovered in serum).

T60 (wt%/oil)	βlg B (mg/mL)	βlg A (mg/mL)	α Lac (mg/mL)	Total whey concentration (mg/mL)
0	1.17 (16%)	2.74 (38%)	2.29 (60%)	6.53 (33%)
0	1.37 (18%)	3.42 (47%)	3.02 (79%)	7.98 (43%)
0.3	1.46 (20%)	3.61 (50%)	2.89 (76%)	8.37 (43%)
0.3	1.83 (24%)	3.50 (48%)	2.98 (78%)	8.47 (45%)
0.7	1.90 (25%)	5.31 (73%)	3.90 (102%)	11.63 (89%)
1.5	4.12 (55%)	6.80 (94%)	4.35 (114%)	16.08 (82%)
1.5	4.39 (59%)	7.59 (105%)	4.42 (105%)	16.63 (89%)
2.2	7.14 (96%)	7.40 (102%)	3.91(103%)	19.42 (100%)
3.6	8.12 (109%)	7.67 (106%)	3.82 (100%)	20.64 (106%)
4.8	8.61 (115%)	7.97 (110%)	3.81 (100%)	21.47 (110%)
2.14% WPI	7.47	7.25	3.81	19.51
2.14% WPI	7.47	7.25	3.81	18.73

Figure 4.14 also shows these results graphically. Overall, it shows that at 5% WPI to oil ratio, there is considerable whey absorption but there is also some protein still free in solution. As the Tween levels increase, the residual WPI in solution is approximately equal to the total amount present, suggesting no whey protein absorption. The results also show that β-lg absorbs preferentially over *α*-lac as there is less β-lactoglobulin (A or B) in the serum phase after creation of the emulsion even though there is more of it present.

This idea is more clearly seen by calculating the protein loading on the emulsions based on the mass of each protein lost and the surface area created.

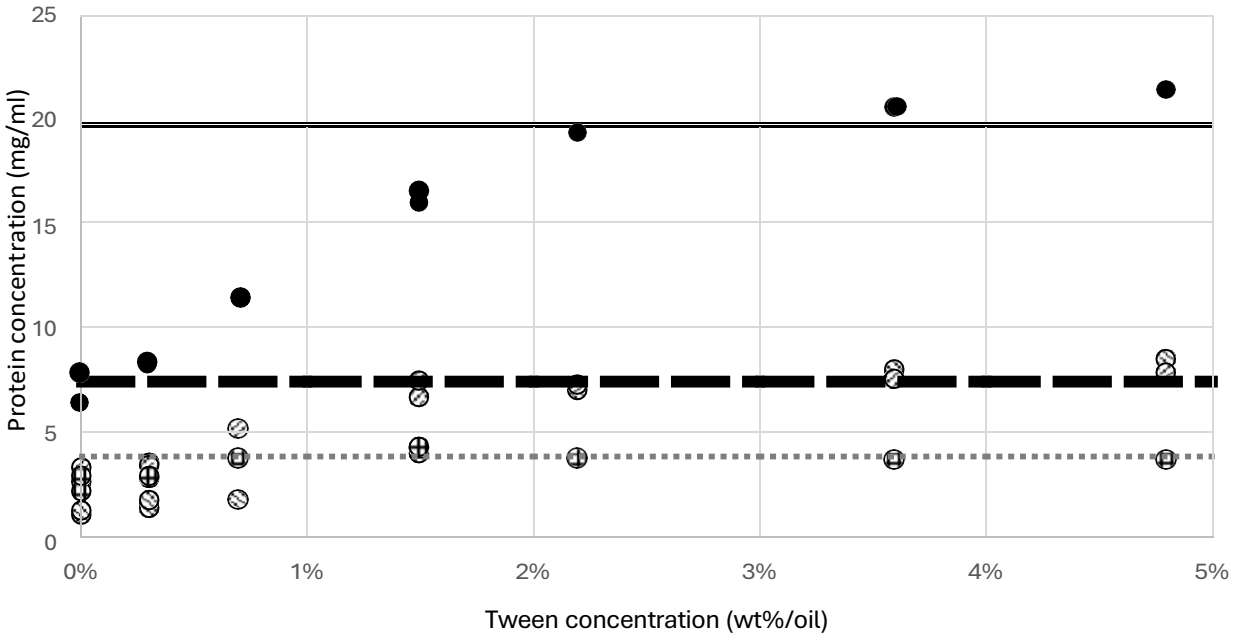

Figure 4.14 Concentration of WPI in emulsion serum from a 30% o/w emulsion at various T60 concentration (0-4.8% wt%/oil) with 5% WPI (wt%/oil) after homogenization at 200 bar after 3 passes. Solid line = Whey concentration in 2.14% WPI. Black dashed line = βlg B concentration in 2.14% WPI. Grey dashed line = βlg A concentration in 2.14% WPI. Grey dotted line = Total α-Lac concentration in 2.14% WPI. Filled circle = Total whey concentration. Circle with cross hatch = α-Lac concentration. Circle with up-right skewed stripes = βlg A concentration. Circle with up-left stripes = βlg B concentration.

Using a mass balance, the surface loading for the concentration of the WPI for emulsions at various T60 concentrations.

$$Surface\ loading\ (\frac{g}{m^2}) = \frac{Aqueous\ phase\ volume\ (L)\ \times\ (Initial\ concentration\ -\ Serum\ concentration\ )(\frac{g}{L})}{Oil\ phase\ volume\ (L)\ \times\ Oil\ density\ (\frac{g}{L})\ \times\ Specific\ surface\ area\ (\frac{m^2}{g})}$$

Figure 4.15 shows the protein loading for each emulsion. The effect of T60 on the surface loading of T60-WPI emulsions showed that as surfactant concentration increased, the surface loading decreased. At low concentration of T60, where there is protein adsorption and not enough tween to outcompete or displace whey.

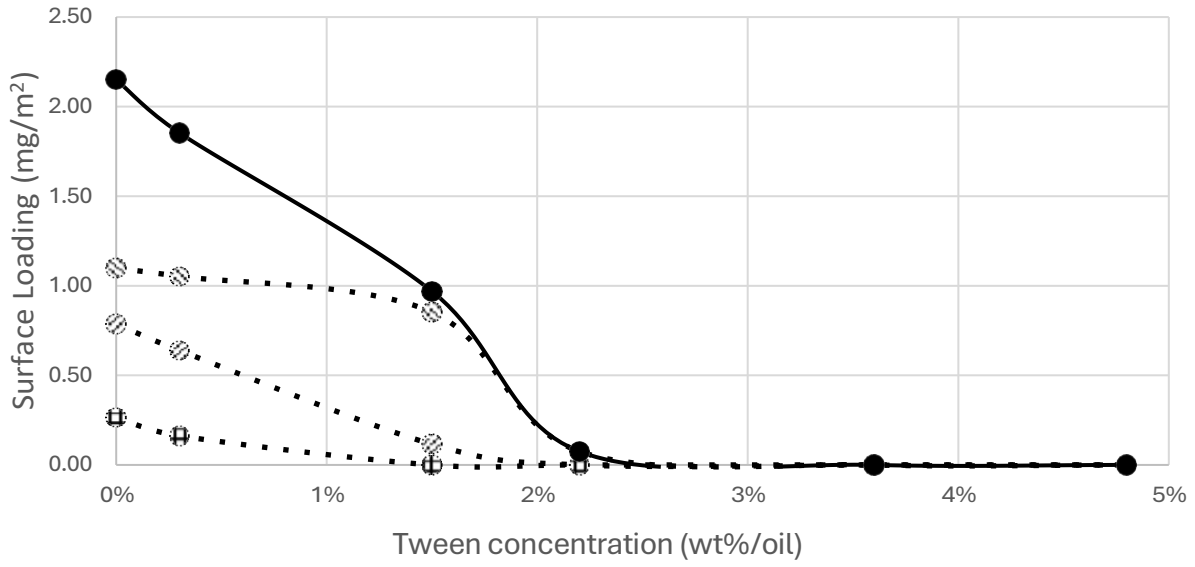

Figure 4.15 Surface loading of WPI at various T60 concentrations (0-4.8 wt%/oil) at 2.14% WPI. Filled circle = Total whey protein. Circle with cross hatch = α-Lac. Circle with up-right skewed stripes = βlg A. Circle with up-left stripes = βlg B.

The total protein loading without Tween 60 was estimated to be approximately 2mg/m^2 (*Figure 4.15*). In studies by Hunt and Dalgleish (1994) the protein loading required to stabilize a 20% oil-in-water emulsion using WPI was 1.5 mg/m^2. The surface loading for pure βlg and a-Lac (1:1 molar ratio) in o/w emulsion is approximately 1.5 mg/m^2 (Courthaudon et al., 1991). In this experiment we report a value of 2.09 mg/m^2, which differs, but is in within the same range as the reported value in literature. However, the difference could be due to the composition of βlg and α-Lac in WPI 895 is 71.2% and 14% respectively or the uncertainty in the experimental results. This does not foreshadow the overall effect of the competitive adsorption between whey and Tween which has been discussed.

Figure 4.16 shows the chromatograms of the serum phases of the emulsions with fixed 1% Tween 60 at increasing WPI concentrations (wt%/oil).

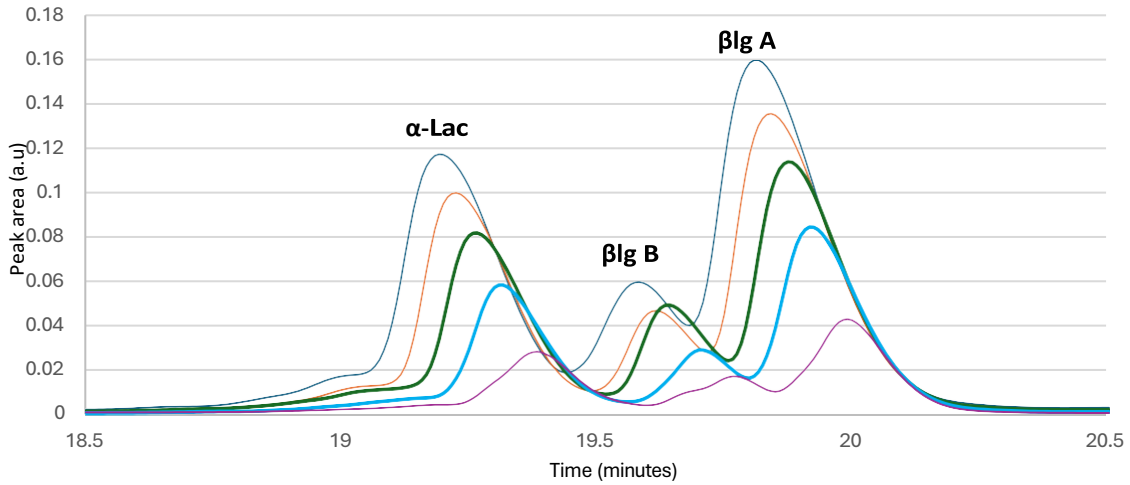

Figure 4.16 Chromatograms of whey proteins in emulsion serum at various 1 wt%/oil T60 and variable WPI concentrations (1-5 wt%/oil). Blue = 5% (wt%/oil). Red = 4% (wt%/oil). Green = 3%. Indigo = 2% Purple = 1% (wt%/oil). Note: Early and later time series data has been omitted to focus on the relevant peaks.

By application of the standard curves, the residual protein concentrations were estimated together with the fraction of available proteins that remain in the aqueous phase of the emulsion. These are shown in Table 4.2 and graphically on *Figure 4.17*.

Table 4.2 Concentration of proteins in serum after separation of emulsions made at 1% T60 concentration (wt%/oil) and varying WPI concentrations (wt%/oil) after homogenization at 200 bar after 3 passes. (Bracketed number represent the fraction of protein in the solution that was recovered in the serum).

WPI (wt%/oil)	βlg B (mg/mL)	βlg A (mg/mL)	α Lac (mg/mL)	Total whey concentration (mg/mL)
1	0.24 (16%)	0.31(21%)	0.07 (9%)	0.63 (16%)
2	0.94 (31%)	1.29 (44%)	0.22 (14%)	2.50 (32%)
3	2.15 (48%)	2.85 (66%)	0.57 (25%)	5.74 (49%)
4	3.52 (59%)	4.83 (83%)	0.72 (24%)	9.44 (60%)
5	5.62 (75%)	7.61 (105%)	1.30 (34%)	15.29 (78%)
2.14% (pure WPI solution	7.47	7.25	3.81	19.51

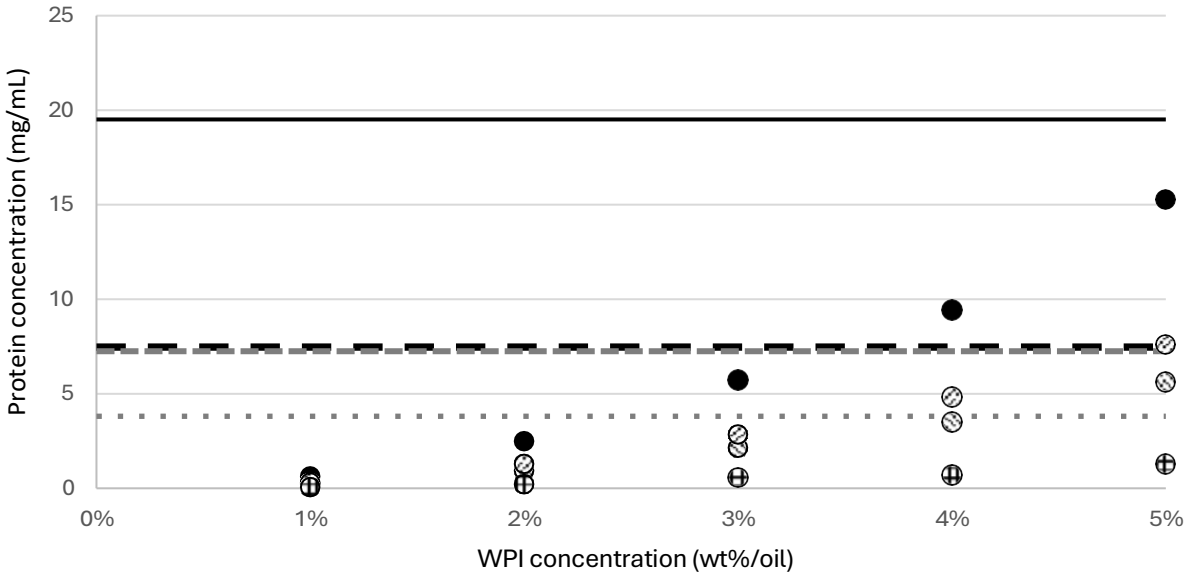

Figure 4.17 Serum phase protein concentrations from 30% o/w emulsions at various WPI concentration (1-5% wt%/oil) with 1% T60 (wt%/oil) after homogenization at 200 bar after 3 passes. Solid line = Total aqueous whey. Black dashed line = Total aqueous βlg B. Grey dashed line = Total aqueous βlg A. Grey dotted line = Total aqueous α-Lac. Symbols = Serum phase concentration. Filled circle = Total whey. Cross hatched circle = α-Lac. Circle with up-right skewed stripes = βlg A. Circle with up-left stripes = βlg B.

These results show that at 1% WPI, most of the available whey proteins were adsorbed during formation of the emulsion. As WPI concentrations increased, there was an accumulation of excess proteins not adsorbed by the fat. This trend is easier to see in Figure 4.18, in terms of the surface loading of each protein.

From 1-2% WPI (wt%/oil) there was a small increase in surface loading. Beyond 2% WPI (wt%/oil) the surface loading decreased. β-lg shows high surface loading relative to α-Lac up to 2% WPI (wt%/oil). Above this concentration, the adsorption of α-Lac gradually increases as WPI concentration increases to 5% (wt%/oil). In WPI895, the protein composition of Blg is 71.2% as compared to 14% of α-Lac which explains greater concentrations of βlg, although the surface loading of α-Lac is disproportionate to relative concentration at 4 and 5% WPI. This suggests a stronger affinity of α-Lac over βlg when both are in excess (Ye, 2008). This finding is contrary to what was reported by Ye (2008) and Closs et al. 1993, who found that there was a slight preference for the adsorption of βlg and others who found no preferential adsorption between the two proteins (Dickinson et al 1989, Hunt and Dalgleish 1994 and Euston et al. 1996).

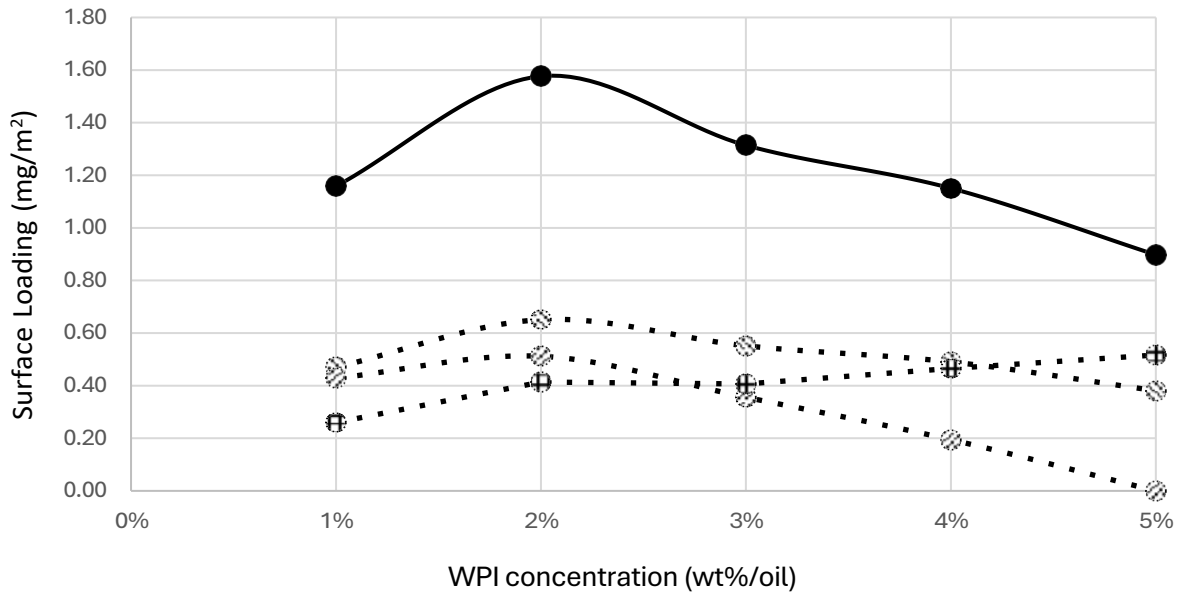

Figure 4.18 Surface loading of WPI at various WPI concentrations (1-5 wt%/oil) at 1% T60 (wt%/oil). Filled circle = Total whey protein. Circle with cross hatch = α-Lac. Circle with up-right skewed stripes = βlg A. Circle with up-left stripes = βlg B.

The surface activity of α-Lac is greater than βlg due to differences in relative molecular weight. In the competitive adsorption between whey protein, complete displacement of βlg occurs before α-Lac and remaining proteins (Suttiprasit et al., 1992). Due to compositional differences in WPI we do not observe the complete displacement of βlg before α-Lac. In addition, the overlapping effect of βlg B and α-Lac as indicated in the chromatograms means we cannot be conclusive on the order adsorption of each protein in WPI emulsions (*Figure 4.13* & *4.16*). The overlap of peaks in the chromatograms may contribute to the apparent increase in adsorption of α-Lac, although for the latter trials the flowrate through the HPLC had been reduced and there was clearer separation between peaks (*Figure 4.16*).

In the literature there are several conditions that influence particle size and specific surface area in the competitive adsorption of protein and chemical surfactants. The adsorption of whey in the presence of Tween may be enhanced through heat treatment at/above the unfolding temperature of βlg, where the availability of hydrophobic sites is greatest (Demetriades & McClements, 1998). At the lower temperature studied in this work the degree of unfolding is low and therefore the concentration of whey proteins in a conformational state to facilitate rapid adsorption may be low or the unfolding in order to adsorb is relatively slow. At higher temperature (70-80°C), βlg is mostly unfolded which may increase the adsorption rate and could outcompete Tween. Experiments of this nature would need to control temperature carefully to avoid denaturation and aggregation of whey which could give rise to emulsion instability (Euston et al., 2000; Euston et al., 2001).

Changes in both temperature and pH on particle size have implications for emulsion properties. Near the isoelectric point of whey, the increase in particle size is due to loss of electrostatic forces causing oil droplets to move closer and form flocculates (Teo et al., 2016). In addition, to flocculation this results in an increase in apparent viscosity and creaming. This is characterized by an increase in particle size and decrease in specific surface area as aggregates do not spread efficiently on the oil droplet surface compared to individual protein molecules. Aggregation of whey may be regulated by Tween when the concentration is high enough and is attributed to the complete displacement of βlg (S. G. Anema, 2020). This agrees with the observation in our study where bimodal

distributions in the PSD are less evident when the concentration of T60 is high relative to WPI (*Figure 4.9* and *4.10*).

Mineral interactions also affect the surface adsorption of whey protein in o/w emulsions. The changes in surface loading are related to changes in the ionic strength of whey when emulsions are formed with calcium and/or sodium chloride (Ravindran et al., 2018; Teo et al., 2016; Ye & Singh, 2000). Stabilization of emulsion by whey protein is primarily through electrostatic repulsion. Ionic interactions through the addition of calcium on WPI emulsions causes a reduction in the electrostatic repulsion forces, inducing droplet flocculation (Ye & Singh, 2000). Mineral addition to emulsions using calcium chloride at high enough concentration would likely induce depletion flocculation with free protein in aqueous solution (Dickinson et al., 2003). Ye and Singh (2000), found that at low concentrations of WPI, ionic calcium induced bridging between oil droplets saturated with WPI although these effects were reduced when WPI concentration was increased. Since Tween is non-ionic the interaction of minerals is unlikely to affect its adsorption behavior (Teo et al., 2016).

Further changes in emulsion properties occur because of mineral interactions with whey proteins above their denaturation temperature. The addition of calcium chloride to heated whey emulsions at 90°C leads to the formation of protein gels (Lin et al., 2021; Riou et al., 2011). Liang et al. (2020), found that calcium ions bind to anionic components of denatured whey forming a network of cross-linked proteins.

Since the emulsion in this study was prepared below denaturation temperature (40°C), it is unlikely that they would form gels due to the lack of exposed hydrophobic sites for the binding of calcium. In this study the emulsions made at low Tween 60 concentration may have exhibit flocculation behaviour if calcium was added due to the loss of electrostatic repulsion. The surface loadings in our study show no whey adsorption at concentrations above 2.2% (wt%/oil) T60 (*Figure 4.18*). The potential for flocculation due to minerals or protein aggregation in these systems would be low. It would, however, allow the formulation of stable emulsions in high mineral and temperature conditions. In addition, the amount of whey is reduced significantly at 0.3%-1.5% T60 (wt%/oil) which may also be enough to reduce the effect of flocculation despite the lower specific surface area than using whey without Tween (*Figure 4.6*).

4.4 Conclusion

The results presented in this chapter identified the limiting factors in the competitive adsorption of whey protein and non-ionic surfactants in the surface loading and particle size using model emulsions. The minimum droplet size is related to how well surfactants saturate the oil droplet surface during homogenization. In the competitive adsorption of whey and T60 in o/w emulsions, the presence of either emulsifier can be influenced emulsion structure. In all cases, whey protein cannot outcompete Tween (if present) on to the surface even at high relative concentration. Even with a high frequency of collisions in the high shear homogenizer, the rate of whey adsorption may not be quick enough to rearrange and align its hydrophobic regions onto the oil surface and coat the surface of oil droplets before the next collision allowing the adsorption of Tween. This seems to be related to both the molecular size and the rate of adsorption of T60.

At all Tween concentration in this study the concentration is above CMC. At low T60 concentration, Tween micelles adsorbed initially but are depleted in solution where larger polymers of whey tend to stabilize the surface preferentially. The partial adsorption of Tween reduces the ability of whey adsorption and results in a decrease in specific surface area. Insufficient adsorption occurs due to surfactant scarcity, leading to coalescence. When T60 concentration increases above 2.2% (wt%/oil), the availability of micelles increases in solution and outcompetes whey entirely for the surface. Beyond this region the adsorption is linear until the limit of the homogenizer for droplet break up is reached, and the oil droplet surface is saturated entirely by T60. This preferential adsorption of Tween, particularly above the CMC, may reduce the risk of flocculation upon the addition of minerals in high protein emulsions.

Chapter 5: Conclusion & Recommendations

5.1 Conclusions

This research has investigated the limiting factors in the competitive adsorption of dairy protein and non-ionic surfactants under various homogenization conditions and formulations in oil-in-water emulsions.

At the same surfactant to oil ratio, there were no significant changes in specific surface area, even at different oil concentrations or Tween type (T60 vs T80). This adsorption behaviour follows the expected trends predicted by simple surface coverage of oil droplets based on molecular size.

At high surfactant concentrations the surface area of emulsions can be influenced by both homogenization pressure and to a less extent temperature used to form emulsions. In emulsions stabilized by Tween, the stabilised surface area is limited by surfactant availability up to 3% (wt%/oil). In surfactant rich emulsions the effect of increasing the concentration results in little to no change in specific surface area (m^2/kg) as under these conditions, droplet break up is limited by the hydrodynamics of the homogenizer.

Tween dominates adsorption and the resulting particle size distribution when the homogenization is carried out in the presence of whey proteins. In the competitive adsorption with whey Tween adsorbs onto the surface irrespective of concentration. Similar to the Tween only emulsions studied in Chapter 3, at high surfactant concentrations, the specific surface area increases until it is limited by the hydrodynamics of the homogenizer and its inability to break particles down any smaller.

Above 2.2% (wt%/oil) Tween concentrations, the specific surface area created was similar to what occurs in the absence of whey protein. This suggests that Tween adsorption is faster than the whey proteins present, even when at relatively high concentrations. Above the critical micelle concentration (~0.06%wt/oil in a 30% oil emulsion), Tween exists as micelles. These are of similar or slightly larger size than whey proteins which may allow it to outcompete for the surface. Collisions in the high shear

environment of the homogenizer increase with concentration and size. This hypothesis is further evidenced by the absence of any whey protein adsorption.

At low relative Tween concentrations, even though the initial Tween concentrations are above the CMC, surfactant adsorption will deplete the number of micelles present, resulting in a much higher relative concentration of whey proteins compared to the remaining Tween micelles. As the amount of Tween in the system reduces, more and more whey proteins can compete for the surface, even if adsorption is slower.

Less surface area is stabilized when a little Tween is present than with just whey alone. Bimodal particle size distributions are observed, suggesting that smaller droplets are initially formed and some Tween is adsorbed until it is depleted in the solution. The partial adsorption reduces the ability of whey adsorption and because there is not enough surfactant to fully stabilise the droplets, coalescence occurs. This results in a decrease in specific surface area compared to emulsions stabilized by whey exclusively.

The work shows that the surfactant concentrations required to achieve a required specific surface area can be estimated by considering the molecular size. It also shows than when present, Tween 60 dominates the adsorption over the whey, to a point where coalescence can occur if there is not enough present, even if there is enough whey to stabilise the emulsion without Tween being present. It is possible that the globular structure of whey proteins means even if collision frequency with the oil droplet surface is high during homogenization, the chances of its orientation being conducive to spreading over the surface is low. Unfolding must occur to allow the hydrophobic portions of the molecule to access the oil surface. Because of the micellar structure of the Tween, any collision is likely to result in successful adsorption.

5.2 Recommendations

Investigations need to explore how the adsorption of dairy proteins can be improved when stabilizing emulsions with nonionic surfactants. The adsorption of whey protein may be enhanced if the temperature is increased to around 70°C where the protein unfolds, and the hydrophobic components becomes available. In addition, non-globular serum proteins such as Beta casein may compete with Tween if caseinates are used instead of whey protein.

Emulsions such as those created in this work at concentrations where whey is outcompeted by the Tween, should also be investigated for mineral stability where they are less likely to flocculate. Since dairy emulsions are often high protein and minerals, these areas of studies may be useful to understand how the functional properties of emulsions can be improved.

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