



DESIGN OF COMPLEX EMULSION INTERFACES FOR SUGAR REDUCTION APPLICATION

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Abstract

A low carbohydrate and free sugar diet is generally recommended by nutritionists and clinicians for losing weight, reducing risk of diabetes, cardiovascular diseases and metabolic syndrome. The aim of this research was to design complex emulsion interfaces for sugar reduction in emulsion-based food. A strategy was applied to evaluate whether O/W emulsions can be stabilised at a lower emulsifying starch content by utilising protein as co-emulsifier. It was hypothesised that oil-in-water (O/W) emulsions stabilised using octenyl succinic anhydride (OSA) starch do not require a closely packed starch layer at the interface for breakdown in the oral cavity by saliva α -amylase, also the interfacial starch can be reduced by utilising whey protein (WP) or sodium caseinate (NaCAS) as co-emulsifier a co-emulsifier when compared to using starch alone. If this emulsion was successfully stabilised with starch/protein mixed emulsifier, it can be developed into $W_1/O/W_2$ emulsion in which a highly concentrated sucrose solution can be encapsulated as a requirement of the taste enhancement approach. When the $W_1/O/W_2$ emulsion breaks down in the oral cavity and releases the sucrose, this will give consumer satisfaction in terms of sweet taste, without being a high carbohydrate-digestion product. By using this strategy, emulsion-based food products can be produced with an overall low carbohydrate content and whilst maintaining a satisfying level of sweet taste.

Three O/W emulsions comprised of 20% w/w oil and 80% w/w aqueous phase were formed, which were stabilised independently with three emulsifiers; 0.8% octenyl succinic anhydride (OSA)-starch, 1% whey protein (WP) and 1% sodium caseinate (NaCAS) w/w. Stability against coalescence of the three emulsions was observed by measuring their droplet size distributions. Next, a mixed

emulsifier strategy was used - the quantity of OSA starch was halved and combined with half of the quantity of either WP or NaCAS to make two mixed emulsifier systems: 0.4% OSA/0.5% WP and 0.4% OSA/0.5% NaCAS. Both mixed emulsifiers showed a comparative ability to reduce the interfacial tension at the oil/water interface compared with using OSA starch alone. The mixed emulsifiers used to produce two O/W emulsions were characterised by droplet size measurements, Zeta potential, the microstructure and interfacial composition analysis. These all demonstrated that the emulsions showed high stability against coalescence. There were no significant changes found in the volume based mean droplet size ($p > 0.05$). Zeta potential was (39.0 ± 2.6) and (57.3 ± 3.9) mV for emulsions stabilised with OSA/WP and OSA/NaCAS, respectively. The microstructure and continuous phase analysis before and post-emulsification revealed the presence of both starch and protein at the oil/water interface.

The two O/W emulsion stabilised with the mixed emulsifiers were developed to create $W_1/O/W_2$ emulsions, where 50% and 5% w/w sucrose solution was used as the inner and the outer aqueous solution, respectively. The multiple emulsions were designed to breakdown in the oral cavity and release high concentrations of encapsulated sugar close to the taste receptors during consumption, allowing the overall levels of sugar used in the bulk of the food to be reduced without compromising the sweet taste. These emulsions were stabilised with 0.57% PGPR as the inner emulsifier and with the mixed emulsifiers in the outer phase. They were characterised by measuring the stability against coalescence, the encapsulated efficiency (EE) of the dissolve sugar in the internal aqueous phase and the release of the encapsulated sugar during an enzymatic digestion before and post fortification with different mineral

salts. Both emulsions were stable against coalescence over a two-week storage period at ambient temperature with an average droplet size of $(47.8 \pm 0.8) \mu\text{m}$ and $(46.0 \pm 2.4) \mu\text{m}$. The EE of these emulsions, which was defined as the mass of the sugar entrapped in the internal aqueous phase immediately after the emulsification process divided by the total mass of sugar added to this phase, was between $(78.6 \pm 2.7) \%$ and $(76.7 \pm 1.2) \%$, respectively, at day one. Amylase mediated sucrose release from the internal aqueous phase of both $W_1/O/W_2$ emulsions was measured *in-vitro* at day 1 of the storage period, showing the release of $(66.92 \pm 6.5) \%$ and $(58.8 \pm 4.6) \%$ of the encapsulated sugar, respectively. The emulsions' behaviour at the typical levels of mineral salts in a close-to-market approach and the release of sugar, after fortification with these minerals was investigated. $W_1/O/W_2$ emulsions were created using a solution of 0.111% NaCl, 0.146% CaCl_2 , 0.224% KCl and 0.016% MgCl_2 w/w in both aqueous phases. The emulsions containing OSA/WP were stable and there was no change observed in the sugar release behaviour after mineral fortification. On the contrary, emulsions containing OSA/NaCAS did exhibit stability and maintained the release behaviour but only when fortified with NaCl and KCl. However, these emulsions were unstable against coalescence when they were fortified with CaCl_2 and MgCl_2 , mostly because caseinate can strongly bind divalent cations like Ca^{2+} and Mg^{2+} .

Double emulsions stabilised with OSA/WP showed the best EE, sugar release and stability against coalescence after mineral salts fortification comparing with the same emulsions when stabilised with OSA/NaCAS. This research shows that the interfacial starch of $W_1/O/W_2$ emulsions can be partially replaced by milk proteins for sugar reduction as final product of starch digestion with keeping stability against coalescence. Depending on the outcomes of emulsion digestion

(*in-vitro*) by α -amylase enzyme at 37.0 °C, sugar can be encapsulated in these emulsions to release in the mouth cavity keeping a level of sweet taste. The benefit of replacement of the starch by protein was that the amount of starch in both O/W and W/O/W emulsions has been reduced up to 50%. Therefore, carbohydrate and then glucose as final product of digestion is be reduced without effect on consumer satisfaction in terms of sweet taste.

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Symbols and Abbreviations

AUC	Area under curve
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BS	Back scattering
%BS	Back scattering percentage
CLSM	Confocal laser scanning microscopy
CMC	Critical micellar concentration
EE	Encapsulation efficiency
%D	Destabilisation percentage
GOPOD	Glucose oxidase peroxidase
IEP	Isoelectric point
LCMS	Liquid chromatography mass spectrometry
M	Molarity
M _w	Molecular weight
NaCl	Sodium chloride
NaCAS	Sodium caseinate
OSA	Octenyl succinic anhydride
O/W	Oil-in-water
PGPR	Polyglycerol polyricinoleate
UV	Ultraviolet
WP	Whey protein

W/O	Water-in-oil
$W_1/O/W_2$	Water-in-oil-in-water
W_1	Inner water phase
W_2	Outer water phase
ζ - Potential	Zeta potential

Chapter 1: Introduction and Literature Review

1. Introduction and Literature Review

1.1. Introduction

A low carbohydrate and free sugars diet is generally recommended by nutritionists and clinicians for losing weight, reducing the risk of diabetes, cardiovascular diseases and metabolic syndrome. A high intake of free sugars increases the overall energy intake and might reduce the intake of nutritionally more adequate foods calories; leading to an unhealthy diet and weight gain. It also increases the risk of noncommunicable diseases such as dental caries and dental diseases (WHO, 2015). For athletes, high-fat diets have recently gained popularity rather than high-carbohydrate e.g. it is becoming more and more clear that in training regimes and diet for trained athletes are not necessary to achieve optimal glycogen in muscles (Jeukendrup, 2003). Food that control sugar release and prevents surges and drops in blood glucose (due to an increase in insulin) could have health benefits (Norton et al., 2015). Indeed, low calorie and kinetically stable food-grade complex emulsions are highly sought (Leal-Calderon et al., 2012). Recently the role of fortified and functional foods in general wellbeing has gained more importance among the consumers (Aditya et al., 2015).

Several approaches have been made to attempt to achieve the delivery and release of functional ingredients at specific parts of the human body. These include altering the composition, redesigning the specific products at the microstructure level and the use of nano/micro carriers (Aditya et al., 2017). However, adding functional ingredients in complex food products faces substantial challenges such as unpleasant tastes and the degradation of these ingredients when exposed to (temperature, pressure, presence of oxygen and light, etc.) during processing and storage (McClements, 2015). In addition, the

addition of new ingredients to an existing product can lead to unwanted changes in the product's physio-chemical stability and organoleptic properties e.g. colour and appearance of the product (Velikov and Pelan, 2008). The advancements of the nano/micro delivery technology is recently investigating to overcome these challenges (Matalanis et al., 2011).

An emulsion consists of two immiscible liquids, usually oil and water, with one of them dispersed in the other one as small droplets with dimeters between 1 – 100 μm in most foods. When oil is dispersed in water, the emulsion is named oil-in-water emulsion, and when water is dispersed in oil, the emulsion is named water-in-oil emulsion. It is possible to form double emulsions like water-in-oil-in-water emulsions when water droplets are dispersed in larger oil droplets that themselves are dispersed in an aqueous phase (McClements, 2005). Double emulsion is one of the lipid based delivery systems and the most advanced functional microstructures used to obtain the controlled release of entrapped ingredients (Aditya et al., 2017). This type of microstructure is required for the taste enhancement approach to reduce sugar in liquid based foods, as sugar dissolves in water and encapsulation in an oil phase is the only means to deliver changes in tastant concentration to the taste buds during oral processing, provided the sugar is released. Sucrose is hydrophilic, it gives fast sweet taste, faster than being a high amount of carbohydrates that is digested to produce glucose for the sweet taste. For example, xylitol was encapsulated in the core of a multiple emulsion which was incorporated into chewing gum to obtain release of xylitol giving a sweet taste for a longer time (Santos et al., 2015). In addition, vitamin B12 was protected from degradation during cheese manufacture when it was encapsulated in double emulsions (Giroux et al., 2013). These emulsions were also used to decrease salt and fat in liquid and

semi-solid foods without changing the sensory properties of these products (Frasch-Melnik et al., 2010a, Lad et al., 2012). However, using multiple emulsions as lipid based delivery systems also creates challenges such as poor stability and a lack of food-grade emulsifiers that can be used for their stabilisation. In addition, both hydrophilic and hydrophobic emulsifiers are required for the formulation of multiple emulsions because of the presence of two different interfaces in a single structure (Aditya et al., 2017, de Souza Simões et al., 2017).

Among the ingredients that can be employed to fabricate carrier structures for the delivery of the functional ingredients are proteins and polysaccharides (Matalanis et al., 2011, Yao et al., 2015). These are used as functional ingredients for the formulation of various food emulsions. They have the ability to change the shelf life of the emulsions by varying the textural and rheological properties (Dickinson, 2008). Their interactions have been characterised in several pH conditions, although no structure-activity correlation has been established yet (Ghosh and Bandyopadhyay, 2012). Therefore, for this research a mixed protein/starch interface was formed to produce emulsions with low starch content that can be broken down in the oral cavity. The object of this research was to produce emulsions for the encapsulation of sucrose solutions to contribute to keep a normal sweet taste, whilst reducing the actual content of carbohydrate in liquid and semi-liquid emulsion based consumer foods. This is important as the amount of total sugars according to the nutrient values and weights for edible portions of thick chocolate milk shakes is 20.85% (USDA, 2017a) and thick vanilla milk shakes is 17.75% (USDA, 2017b); these percentages need to be reduced by up to 20% by 2020 according to the Public Health in England plan (PHE, 2017). However, it needs more research such as

sensory studies and applying these designs W1/O/W2 emulsion interface in emulsion based foods, to decide how much the level that can be achieved.

This thesis was divided into the following chapters: introduction and literature review, materials used and analytical methods, three chapters of results and discussion (single emulsions stabilised with single emulsifier, single emulsions stabilised with starch/protein mixed emulsifier systems, programmed complex emulsions for sugar reduction), and finally conclusions and recommendations for future work.

This thesis is divided into six different chapters starting with the introduction and literature review as first chapter and then the second chapter is materials and methods followed by three chapters of results as follows: the third chapter covers results about single O/W emulsions stabilised with single emulsifier, the fourth chapter covers results about O/W single emulsions stabilised with starch/protein mixed emulsifier systems and the fifth chapter covers results about the production of programmed complex emulsions for sugar reduction applications in emulsions based food. The final chapter covers the conclusions and recommendations for future work.

1.2. Research hypothesis and overall aim

1.2.1. Hypothesis

It was hypothesised that O/W emulsions stabilised using starch emulsifier, like OSA starch, and designed for amylase mediated oral breakdown, do not require a closely packed starch layer at the interface for breakdown in the oral cavity. This breakdown is as the result of the combined action of mixing with salivary α -amylase and the mechanical action impart by teeth and tongue. If this was

proven, these emulsions can be developed into $W_1/O/W_2$ emulsions in which a high concentration sucrose solution can be encapsulated inside the internal aqueous phase of the emulsions, which is a requirement of the taste enhancement approach. These stable emulsions will give consumer satisfaction in terms of sweet taste during the oral breakdown by releasing the encapsulated sucrose, without being a high glucose production as a result of carbohydrate digestion. By using this strategy, emulsion-based food products can be produced with an overall low carbohydrate content and whilst maintaining a satisfying level of sweet taste. Therefore, the overall aim of this research was to improve the design of O/W emulsion interfaces in food intended for amylase mediate oral breakdown and to reduce glucose as breakdown product.

1.2.2. Objectives

- To select a protein as co-emulsifier to commercial OSA starch and aqueous phase conditions (pH, ionic strength).
- To develop an experimental plan based on the outcome of the first objective to assess the emulsifying ability of OSA starch (NC46) at range (0.4-3.2) % w/w /protein (either whey protein or sodium caseinate) mixtures at range (0.5-1.0) % w/w, in which half the minimum amount of OSA starch needed for stabilising an oil-in-water emulsion will be replaced by half the quantity of protein that is needed to stabilise the same emulsion.
- To evaluate whether O/W emulsions can be stabilised at a lower overall starch content in the emulsion by utilising protein as a co-emulsifier with the starch, when compared to using starch alone, therefore, starch concentration can be reduced to up to 50%.

- To develop O/W emulsions stabilised with mixed emulsifier systems to $W_1/O/W_2$ emulsions with encapsulation of a concentrated sucrose solution in the internal aqueous phase.
- Digestion of the $W_1/O/W_2$ emulsions *In-vitro* to release the sugar from the internal to external aqueous phase to deliver a change in the sugar concentration (the concentration depends on interfaces structure encapsulation efficiency and level of sugar release) available for taste perception during consumption of the emulsion allowing to reduce overall sugar content and minimise delivering sugar as a digestible product.
- To investigate the emulsions' behaviour at the typical levels of mineral salts (e.g. the maximum levels of mineral salts in two specific commercial milk shakes; thick chocolate milk shakes and thick vanilla milk shakes) and manufacture conditions in a close-to-market approach and the release of sugar post-mineral salts fortification.

1.3. Literature review

Octenyl succinic anhydride (OSA)-starch can be used as emulsifier to ensure starch digestion and breakdown of the emulsion in the oral cavity. It can be used instead of the native starches because the latter have limited solubility in water. In addition, the amount of the modified starch needed at the interface should be lower than the native starch, which is good for reducing the overall starch content in emulsion-based food. Furthermore, to reduce the starch more, protein can be used to partially replace the OSA starch at the interface. OSA starch used in this research was initially refined from waxy maize which contains $(4.9\% \pm 0.4)$ amylose. The modification involves esterification of OSA at free

hydroxyl groups and is not directly related to the content of amylose and amylopectin. This modification is limited to 3% of OSA groups for food use because of its unpleasant taste. In both O/W and W/O/W emulsions stabilised with OSA starch the amount of starch can be reduced up to 50% by replacing half the amount of starch used by milk proteins, which leads to reducing glucose as final product of starch digestion. The literature section covered the description, the modification, the digestion of starch and using milk proteins as co-emulsifiers to OSA-modified starch, it also describes the composition, the molecular weights and the functional properties of milk proteins, and the modification of caseins. In addition, this section talked about the surface and interfacial tension of oil/water interfaces, obtaining of protein/polysaccharide stabilised O/W emulsions, and then how emulsions characterisation using light and confocal laser scanning microscopy, particle size analysis and Zeta potential. The choice of PGPR in W/O and $W_1/O/W_2$ formation, using complex emulsions for encapsulation of functional ingredients, delivery and release of these ingredients and also tongue and amylase types.

1.3.1. Description of starch macromolecule and the meaning of 'waxy'.

Starch are odourless, tasteless white polysaccharides occurring widely in plant tissue, exist in granules, obtained mainly from cereals and potatoes, function as a carbohydrate store and are an important constituent of the human diet. Most starches contain about 20-25% w/w amylose and 75-80 % w/w amylopectin, depending on their origin. However, some waxy starches contain very small amount of amylose lower than 1%, while some starches such as amylo maize contains 65% of amylose (Parker and Ring, 2001). The amylose content of OSA

starch dependent on the type of starch. Kasprzak et al. (2018), have reported that OSA starch initially refined from waxy maize contains ($4.9\% \pm 0.4$) Amylose. These polysaccharides consist of a large number of glucose units joined by glycosidic bonds and both are based on chains of 1→4 linked-D-glucose. Essentially, amylose is linear and helical, while amylopectin is highly branched containing on average one branch point, which is 1→4→6 linked, for every 20–25 straight chain residues, as shown in Figure 1-1. High amylose starches are highly hydrated and open structure, consist of double helices packed in a hexagonal array (Parker and Ring, 2001). Waxy maize starch is a complex, long-chain and high molecular weight carbohydrate. This starch has a high percentage of amylopectin and a low percentage of amylose.

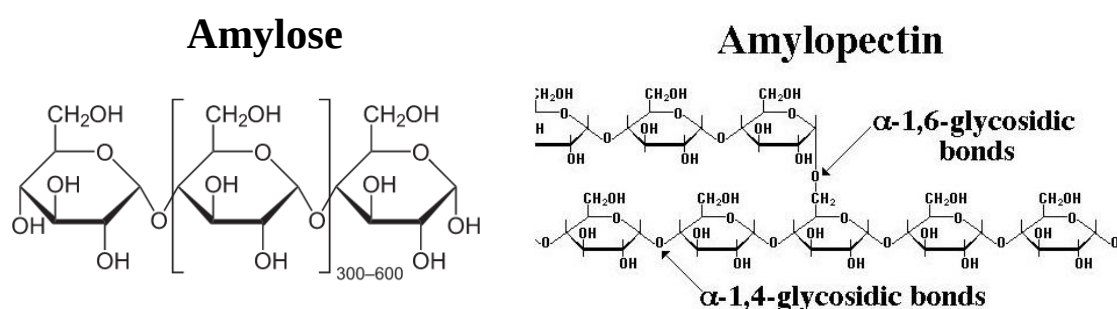


Figure 1-1. The structure of amylose and amylopectin consist of a large number of glucose units joined by glycosidic bonds and both are based on chains of 1→4 linked-D-glucose. The amylose is linear and helical, while amylopectin is highly branched containing 1→4→6 linked -D-glucose.

1.3.1.1. Starch modification with octenyl succinic anhydride

Native starches have limited solubility in water. Therefore, the modification of starch to improve its solubility in water could be via (1) changing starch structure by hydrolysing the starch into small molecules chain of D-glucose such

as gelatinisation using heating to convert the starch to aqueous dispersion and (2) adding chemically substituted groups such as octenyl succinic anhydride (OSA) to generate hydrophobic starch as shown in Figure 1-2, to give a chemical structure of octenyl succinic anhydride starch (OSA starch) with negative charges on the molecules as shown in Figure 1-3. The OSA groups react and covalently link with any available hydroxyl groups of accessible glucose, thereby improve the emulsifying functionality of the starch (Magnusson and Nilsson, 2011, Sweedman et al., 2013). The modification with octenyl succinic anhydride (OSA), making the starch surface active by giving its molecules an amphiphilic nature (Krstonošić et al., 2011, Magnusson et al., 2011, Marefati et al., 2015).

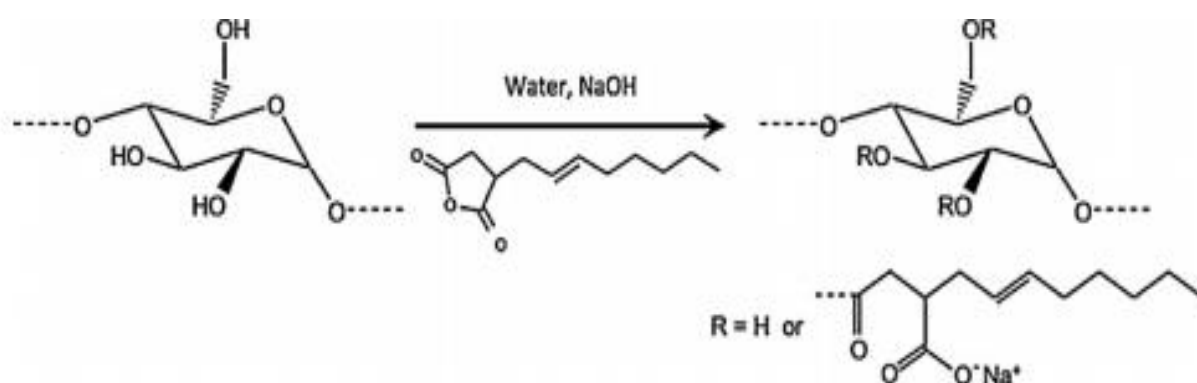


Figure 1-2. Formulation of OSA starch by adding chemically substituted groups “octenyl succinic anhydride” after the hydroxylation of starch into small molecules chains in the presence of water and sodium hydroxide (Sweedman et al., 2013).

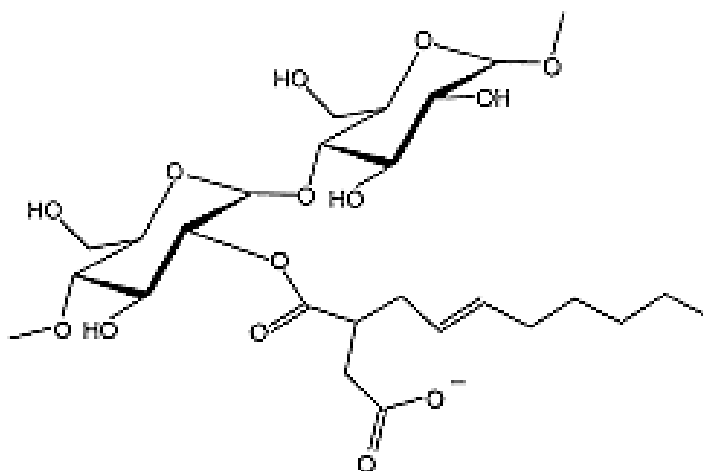


Figure 1-3. The chemical structure of open of octenyl succinic anhydride starch (Magnusson and Nilsson, 2011).

The modification involves esterification of OSA at free hydroxyl groups at the surface of starch granules; this process is spatially heterogeneous on the granules surface as well as among the granule population and this modification is limited to 3% of OSA group for food use because of its unpleasant taste. The timescale of digestibility of interfacial OSA starch used is appropriate to the consumption of food emulsions (Chiu et al., 2015). Increasing levels of OSA modification leads to increased stability of O/W emulsions (Chiu et al., 2017). OSA starches are chemically modified food starches; they are surface active substances able to adsorb at oil/water interfaces. In this way, OSA starches are able to stabilise droplets against coalescence with their surface tension being comparable to that of proteins. Steric hindrance is the main stabilising mechanism of OSA starches (Tesch et al., 2002). In addition, OSA starches have a degree of hydrophobicity, emulsifying ability and digestion properties better than the native starch (Miao et al., 2014, Chiu et al., 2017). When starch is modified by adding octenyl succinic anhydride, the optimum degree of

substitution (ds) to give the best emulsification is about 0.07 (Viswanathan, 1999). OSA starch in the dissolved state is more surface active than in the granular state but emulsions that are formed using the dissolved state have less stability, the stability of OSA starch stabilised emulsions is dependent on the form of starch used (Matos et al., 2016). Dissolved state means starch milled or pre-gelatinized and the OSA substitution was done. The OSA starch (NC 46) used in this research was in its dissolved state because no granules have been seen under an optical microscope (EVOS fl, Life Technologies Ltd., Paisley, UK) in a suspension of this starch.

There has been increasing interest for using native and modified starches in entrapping functional ingredients, starches in granular or amorphous forms are modified by chemical, physical, and/or enzymatic means to obtain the desired properties for targeted encapsulation. Other polymers are also employed in combination with starch to facilitate some encapsulation systems, which can play a positive role in controlling the release of functional ingredients in nutritional applications (Zhu, 2017). Cooked starch increases the oral digestibility of starch but does not influence the taste rate or taste time (Lapis et al., 2017). During consumption, some carbohydrates undergo a partial digestion process that begins in the oral cavity by the amylase enzyme produced by the salivary glands, however, the enzyme activity is quickly inhibited by gastric acid (Fathi et al., 2014). In $W_1/O/W_2$ emulsions stabilised with OSA starch as external emulsifier, the starch microstructure will breakdown in the mouth cavity by the α -amylase enzyme during consumption and the emulsion will take the initial form of oil droplet coalescence (Chiu et al., 2015), therefore, it could be hypothesised that during this coalescence process if sucrose was encapsulated in the internal aqueous phase can be released from the

internalised aqueous phase and became available for perception. In the current research a systematic process was followed in which OSA starch was used in the first place to decide the minimum amount that can stabilise an O/W emulsion, and then half this amount was replaced by protein for starch reduction as well as maintaining breakdown of the emulsion in the mouth by digesting this starch by saliva α -amylase enzyme.

1.3.2. Proteins as emulsifiers

Proteins are the most commonly used emulsifying agents in the food industry because they are natural, cheap and widely available. Proteins have a complex structure, stabilise the oil/water interface by forming a viscoelastic adsorbed layer. Their adsorption at the interface influenced by surface hydrophobicity and charge. In addition, the stability of protein stabilised oil/water interfaces can be influenced by the mechanical properties of the adsorbed layer, which depends on the structure of proteins and the strength of the interactions between them. (Wilde et al., 2004). Proteins coat the droplets of an emulsion and provide an energy barrier to droplet' coalescence (Vanessa Cabra, 2008). The formation of a protein stabilised emulsion firstly requires arriving of protein molecules to the interface. At the interface, they unfold and rearrange their secondary structure to expose their hydrophobic residues to the hydrophobic phase (Vanessa Cabra, 2008, Wilde et al., 2004) and any part of proteins which have charged groups at the surface of the molecule arrange so they are in contact with water molecules (Vanessa Cabra, 2008). Emulsions can be formed using proteins, polysaccharides or protein-polysaccharide complexes. The stability of these emulsions is governed firstly by the repulsion between two closely approaching liquid droplets and secondly by Ostwald ripening (Ghosh

and Bandyopadhyay, 2012). In most food emulsions Ostwald ripening is negligible because the shared-soluble components between triacylglycerol and water are so low, however in case of flavour oils and alcohol it is important (McClements, 1999). High repulsion between the two neighbouring droplets leads to a maximum stability due to minimization of the chance for coalescence (Ghosh and Bandyopadhyay, 2012).

1.2.2.1. Milk proteins

Milk protein (whey proteins and sodium caseinate) was used independently as co-emulsifiers to commercial OSA starch in this research. These proteins consist of two major categories: caseins represent about 80%, which exist naturally as a casein micelle, and whey proteins which represent about 20%. Caseins contain α_s1 -, α_s2 -, β - and κ -casein which interact with each other and with calcium phosphate to form the casein micelle (Dissanayake, 2011, Surel et al., 2014). Casein micelles, representing 95% of the caseins and are spherical colloidal particles with an average diameter of 150 nm. These micelles comprise about 94% protein and 6% colloidal calcium phosphate (Livney, 2010). Whey proteins include β -lactoglobulin, α -lactalbumin and small amounts of bovine serum albumin, lactoferrin, immunoglobulins, lysosome, lactoperoxidase and proteose peptones (Dissanayake, 2011, Euston et al., 2000, Surel et al., 2014). Additionally, Whey proteins consist of antibodies and a long list of enzymes, hormones, growth factors, nutrient transporters and disease resistance factors are present. Function of α -lactalbumin is important factor in the milk synthesis process, while function of β -lactoglobulin is not known (Hurley, 2009). Lactoferrin is a monomeric globular glycoprotein and has anti-microbial activity

(Livney, 2010). Table 1-1 shows the general milk proteins and their molecular weight.

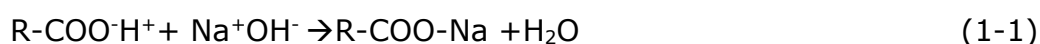
Table 1-1. General milk proteins, their content in milk and their molecular weight (Livney, 2010)

Proteins	Content in milk (g/l)	Molecular weight (kDa)
Caseins	24-28	
α_{S1} -casein	12-15	22.1-23.7
α_{S2} -casein	3-4	25.2-25.4
β -casein	0-11	23.0-24.1
K-casein	3-4	19.0
Whey protein	5-7	
β -lactoglobulin	2-4	18.3
α -lactalbumin	1-1.5	14.2
Bovine serum albumin	0.1-0.4	66
Immunoglobulins	0.6-1.0	146-1.030
Lactoferrin	≈ 0.1	80
Milk fat globule membrane (MFGM) proteins	≈ 0.4	13-200
Total milk proteins	30-35	

The emulsifying capacity of protein is affected by several factors such as its concentration, the homogeniser type and homogenisation degree (Masson and Jost, 1986). Consequently, in the previous studies the concentrations of protein were varied to that are optimal to stabilise O/W emulsions e.g. Cornacchia and Roos (2011) used a ratio of protein (WP or NaCAS): sunflower oil of 1:10, pH 7. Akhtar and Dickinson (2003), used 1.5 % w/w WP for stabile O/W emulsions containing 20% v/v oil phase. Frsch-Melnik et al. (2010b) used 1% w/w NaCAS and Panagopoulou et al. (2017) used 1% w/w WP to stabilise the outer interface of $W_1/O/W_2$ emulsions where 20% w/w primary W/O emulsion.

1.2.2.2. Modification of caseins

An acid casein is produced by adding acid to pasteurised skimmed milk, which is insoluble in water. This acid casein is formed at the pH of 4.7 at which the charged net on casein is equal to zero. Neutralising acid casein with sodium hydroxide leads to sodium caseinate formation, which is soluble in water. Equation (1-1) shows the neutralisation of acid casein with sodium hydroxide in which (R) represents casein.



Sodium caseinate is a soluble salt of casein, which contains all the essential amino acids; alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine (Pandit, 2014).

1.2.2.3. Functional properties of whey protein and sodium caseinate

Whey protein (WP) is a natural amphiphilic polymer used as a hydrophilic emulsifier (Mun et al., 2010). It exhibits good solubility in water over a wide range of pH from 2 to 9. One challenge for the use of whey protein is maintaining solubility during heat processing. Most foods are heat processed in some way, and whey proteins are susceptible to changes during heating, such as denaturation (Burrington, 2012). Whey protein is composed of 70% β -lactoglobulin, 20% α -lactalbumin, small amounts of serum albumin and other proteins. These proteins are globular and adsorb slowly and unfold partially at

an emulsion interface (Surel et al., 2014). Euston et al. (2000) have suggested that at the oil/ water interface whey protein unfolds partially at the oil droplet surface and, as a result, some hydrophobic amino-acid residues remain directed towards the water phase favouring droplet aggregation. Whey protein is a highly effective stabilising agent making a thinner and denser layer than that by casein at the oil/water interface, it stabilises emulsions by electrostatic repulsion (Dickinson, 2008). Dickinson et al. (1993), have stated that surface-active substances present in typical food oils are unlikely to have an effect on the competitive adsorption of milk proteins in food emulsions. The isoelectric point of WP is pH 5.2; therefore, whey protein has a net negative charge at pH 7.0 (Burrington and Agrawal, 2012). Near the value pH 5.2 emulsions stabilised with WP exhibit poor stability (Masson and Jost, 1986).

The large molecular weight of caseinate and its hydrophilicity preclude this protein from diffusion across the oil phase during emulsion processing (Bonnet et al., 2010). Sodium caseinate has an isoelectric point of pH 4.6, emulsions stabilised with this protein are unstable at pH values close to 4.6 due to the reduction in electrostatic repulsion between the oil droplets. Sodium caseinate (NaCAS) carries a negative charge at pH values higher than its isoelectric point (Zinoviadou et al., 2012). Caseins are globular phosphoproteins that make a colloidal complex with calcium phosphate and other minerals, and constitutes about 80% of cow milk proteins (Tahmaz and Operta, 2010). Liu et al. (2013), have stated that the highest solubility of caseins was at pH 8.0, the best emulsification was at pH 10, and the greatest thermal stability was at pH 9.0 and concluded that alkali conditions between pH 8.0–10.0 should be selected for optimum production. Van Hekken et al. (1996), noted that none of the modified caseins were as soluble in water at pH 7.0 as the unmodified control.

The loops and tails of casein protrude from the oil droplet surfaces leading to steric stabilisation against coalescence and flocculation (Dickinson, 2008).

1.2.2.4. Thermal effect on milk proteins

Whey proteins may undergo a structural change when they are subjected to thermal processing, this structural change is commonly known as denaturation, which is depending on the heating conditions (Qian et al., 2017). Under a given chemical environment, heat treatment of WP prior to emulsion formation reduces its ability to stabilise emulsion droplets while heat treatment of NaCAS solutions significantly improves its emulsifying properties (Raikos, 2010). Casein seems to have a protective effect against denaturation. Heat treatment leads to WP denaturation and the formulation of whey protein-casein polymer (Qian et al., 2017). Heat treatment affects the structure milk of proteins at the interfaces of O/W emulsions. In WP stabilised emulsions, the heat affects the emulsion stability, possibly because of flocculation instability as a result of protein-protein interactions. However, heat-stable caseins dominate during the O/W emulsion formation. The denaturation degree of milk proteins is the key factor that determines their interfacial functionality, which in turn, effects the emulsions properties in terms of stability. Controlling the heat during processing of emulsion is beneficial to prevent the negative impact of protein structure changes at the molecular level on the properties of milk protein-based emulsions (Raikos, 2010).

1.2.3. Polysaccharide-protein interfaces

Polysaccharides /proteins can improved delivery systems of function ingredients over protein-based interfaces or polysaccharide-based interface in terms of protecting emulsions from environmental stresses, resilience to pH adjustments, controlling of interface structure, thickness, charge and permeability with controlled release and stability (Ghosh and Bandyopadhyay, 2012). The combination of proteins and polysaccharides can improve the delivery systems of functional ingredients in terms of stability and encapsulation efficiency when compared with those obtained by involving a single material (de Souza Simões et al., 2017). In addition, Benichou et al. (2004) stated that compatible blends of biopolymers are excellent amphiphilic candidates that can serve both as stability and enhance release controllers for water-in-oil-in-water emulsions. Proteins play a key role in emulsion stabilisation aided by the presence of polysaccharide (Ghosh and Bandyopadhyay, 2012).

Formation and solubility of polysaccharide-protein complexes depend on the nature, concentration, ionic strength and charge of these biopolymers. They also depend on the pH, the temperature of the medium and the presence or absence of surfactants. Therefore, many changes can be applied to affect the stability of food emulsions (Ghosh and Bandyopadhyay, 2012). For example, strong attractive complexes of polysaccharide/protein are typically formed with mixtures of negatively charged polyelectrolytes and positively charged proteins $\text{pH} < \text{IEP}$. Weaker complexes tend to be formed between polyelectrolytes and proteins carrying zero charge $\text{pH} = \text{IEP}$, or a net negative charge $\text{pH} > \text{IEP}$. On adjusting the pH and/or ionic strength of the aqueous phase, the strength of the polysaccharide/protein interaction may vary, even moving from net attractive to net repulsive or vice versa (Dickinson, 2008). Very dilute solutions

of proteins-polysaccharides are co-soluble, which means that the entropy of mixing is dominated and the system is stable (Magnusson and Nilsson, 2011). The same charged biopolymers repel each other at high concentrations making the system separate to two distinct phases (Grinberg and Tolstoguzov, 1997, Magnusson and Nilsson, 2011).

The driving forces for noncovalent protein-polysaccharide interactions are electrostatic, hydrophobic and hydrogen bonding and Van der Waals interactions. Electrostatic interaction and hydrogen bonding depend on the solution parameters and type, molecular weight, charge density, and hydrophobicity of the biopolymers. Also, few non-polar parts are present on the biopolymers that are responsible for the hydrophobic interaction between them (Ghosh and Bandyopadhyay, 2012). The better ratio of protein/polysaccharides to stabilise O/W emulsions was 3:1, in which 3.2 % w/w of each covalent conjugate of protein/polysaccharides was used as emulsifier and the focus was on the stability of emulsions against creaming (Akhtar and Dickinson, 2003). Table 1-2 shows some polymers that were used as mixed emulsifier to stabilise oil/water interfaces using electrostatic complexes mechanisms versus conjugates or both together and the related list of references.

Table 1-2. Some polymers that were used as mixed emulsifier to stabilise oil/water interfaces using electrostatic complexes mechanisms versus conjugates or both together.

Polymers		interactions	Reference
Carboxy methyl dextran	WP	Covalent linked conjugates	(Nagasawa et al., 1996)
Inulin or starch	WP	Electrostatic repulsion	(Herceg et al., 2007)
Polysaccharide	Casein-acidic	Synergistic complexes gel-like structure	(Tokaev et al., 1987)
Xanthan	WP	Steric & electrostatic contributions	(Benichou et al., 2002)
Xanthan gum, succinoglycan or carboxymethylcelluloses (ionic)	NaCAS	Steric stabilisation (complex)	(Cao et al., 1990)
Dextran (non-ionic)	NaCAS	Conjugates	Muschiolik (2007)
Dextran sulfate (ionic)	BSA	Electrostatic (conjugates)	(Larichev et al., 1983)
t-carrageenan	BSA	Electrostatic complex	(Dickinson and Pawlowsky, 1997)
Monomeric emulsifiers (non-ionic)	BSA	Blended steric stabilizers	(Gaiti et al., 1994)
Pectin	pea protein	Conjugate	(Tamnak et al., 2016)
Sodium alginate	Soy proteins	Steric stabilisation (complex)	(Wang et al., 2011)
Sodium dodecylsulfate (anionic)	Chitosan	Complex	(Schulz et al., 1998)
Gum arabic	Gelatin	Complex	(Benichou et al., 2004)
Cyclodextrins (alpha, beta and gamma)	Oil components	Complex	(Yu et al., 1999)
OSA starches: Gum 539-E or Purity Gum 2000	-	Steric hindrance	(Tesch et al., 2002)
OSA starches in granular state	-	Steric hindrance	(Yusoff and Murray, 2011)
OSA starches in dissolved state	-	Electrostatic repulsion	(Matos et al., 2016)
Native starch Quinoa granules	-	Pickering stabilisation	(Matos et al., 2016)
OSA starch in dissolved state	Native starch Quinoa granules	Instability	(Matos et al., 2016)
Starch	Global proteins	Multi-layered edible films or Coatings	(Weiss et al., 2006)
Glycerol or sorbitol	WP	Complexes	(Burrington, 2012).

Chitosan	NaCAS	Electrostatically formed complexes	(Zinoviadou et al., 2012)
OSA starches	Xanthan gum	Hydrodynamic interaction	(Krstonošić et al., 2011)
Soy polysaccharide	Soy protein	Electrostatically formed complexes	
Bacterial cellulose	WP	Instable Interface	(Panagopoulou et al., 2017)
Xanthan gum, galactose or mannose polysaccharide	WP	Strong complexes	(Benichou et al., 2007)
Pectin, gum arabic or carboxy methylcellulose	Proteins Egg yolk or WP	Complexes Steric stabilisation	(Ghosh and Bandyopadhyay, 2012) (Schuch et al., 2015)
Cherry gum, in combination with acacia and	Gelatin	crystal-bearing interfacial films	(Vaziri and Warburton, 1995)

1.2.3.1. Obtaining protein/polysaccharide stabilised O/W emulsions

There are two methods for obtaining protein-polysaccharides interfaces. One is the *Mixed Emulsions Method*, which involves the addition of a bulk aqueous solution containing the protein-polysaccharide mixture before the homogenisation. The alternative method is the *Layer-by-layer Method* or *Bilayer Method*, where an emulsion is prepared with the addition of a solution of a charged polysaccharide to a protein stabilised emulsion to produce droplets having a polysaccharide/protein bilayer surface. This latter technique is important due to its use in nano-encapsulation and protecting emulsions from environmental stresses. However, the problem with this method is emulsion droplet flocculation resulting in bridging flocculation and depletion flocculation (Dickinson, 2008). Depletion flocculation occurs when the concentration of unabsorbed polysaccharide exceeds a critical value. Therefore, the *Mixed*

Emulsions Method is more appropriate for making emulsions and leads to better stability than the *Layer-by-layer Method*, it could also be rather more resilient to pH adjustments (Ghosh and Bandyopadhyay, 2012). However, when considering the encapsulation of functional ingredients during the production of protein-polysaccharides interfaces, *the Layer-by-layer Method* is better than the mixed emulsions method. *The Layer-by-layer Method* has a better interface structure control, thickness, charge and permeability with controlled release of actives and improved stability (Ghosh and Bandyopadhyay, 2012, McClements, 2012). Proteins and polysaccharides can be combined as layer-by-layer at emulsion droplets (Weiss et al., 2006, Muschiolik, 2007). OSA starch, WP and NaCAS have a negative charge at pH 7.0 (Burrington, 2012, Zinoviadou et al., 2012). The adsorption at the interface of these polymers can be affected by their different molecule size and charge repulsion. Small concentrations of WP or NaCAS proteins compete with OSA starch for the oil/water interface. Krstonošić et al. (2011) stated that at a certain emulsifier concentration the surface is saturated and no more emulsifier can enter the boundary layer, above this concentration molecules aggregate to form micelles, the minimum concentration above which micelles are formed is CMC. Therefore, viscosity of OSA starch solution increased by adding another polymer because it led to a higher CMC. Benichou et al. (2004) reported that protein/polysaccharides blends can serve emulsions stability. In addition (Ghosh and Bandyopadhyay, 2012) stated that the role of proteins in emulsion stabilisation is aided by the presence of polysaccharide, and de Souza Simões et al. (2017) reported that a combination of proteins/polysaccharides can improve emulsions stability. Tamnak et al. (2016) found that a $W_1/O/W_2$ emulsion stabilised with mixed (polysaccharide/protein) had a relative stability higher than those stabilised with

only polysaccharide. Panagopoulou et al. (2017) reported that the droplet size of $W_1/O/W_2$ emulsion stabilised with polysaccharides/protein is highly dependent on the concentration and type of the emulsifiers used. Benichou et al. (2007) and Tamnak et al. (2016) stated that mixtures of polysaccharides and proteins can be used as stability enhancers when preparing double emulsions. Proteins play a key role in emulsion stabilisation aided by the presence of a polysaccharide (Ghosh and Bandyopadhyay, 2012). In addition, Schuch et al. (2015) found that the addition of an exclusively selected polysaccharide in the external aqueous phase of $W_1/O/W_2$ emulsions stabilised with only WP leads to better stability.

Creation of consistent interfaces of mixed polysaccharide/protein inhibiting transfer of phases play an important role in improving the stability of the $W_1/O/W_2$ emulsions (Vaziri and Warburton, 1995). Muschiolik (2007) reported that stability of $W_1/O/W_2$ emulsions was enhanced using NaCAS/dextran instead of using protein alone. Tamnak et al. (2016) found that a $W_1/O/W_2$ emulsion stabilised with mixed (pectin/pea protein) as the external emulsifier had relative higher stability than those stabilised with only pectin. Significant improvements in the stability of $W_1/O/W_2$ with a more controlled release were made when macromolecules (bovine serum albumin) were blended in the external interface, in combination with a large excess of non-ionic monomeric emulsifiers (Benichou et al., 2004). The type of emulsifier determines the kind of emulsion that can be made, and for water-in-oil emulsions formulation the water needs to be added slowly with continuous stirring to the oil phase Which must contain the emulsifier (Williams, 2003). In the present research the *Mixed Emulsions Method* was chosen because it is easier and more appropriate for making

emulsions than the *Layer-by-layer Method*. As it also leads to better stability, it could also be rather more resilient to pH adjustments.

1.2.4. Analysis methods for an emulsion stability

1.2.4.1. Surface and interfacial tension

The forces between the molecules in a liquid are responsible for the surface tension phenomenon. At the surface, the molecules do not have the same number of neighbouring atoms on all sides therefore, molecules at the surface bond more strongly to the atoms associated with them. A film 'surface' is formed making it more difficult for an object to move through this surface comparing with the object movement when the molecules is completely immersed away from the surface as shown in Figure 1-4. The same situation also happens at the interface between two immiscible liquids such as oil/water interface. In this case the term interfacial tension is used instead of surface tension (Stauffer, 1965).

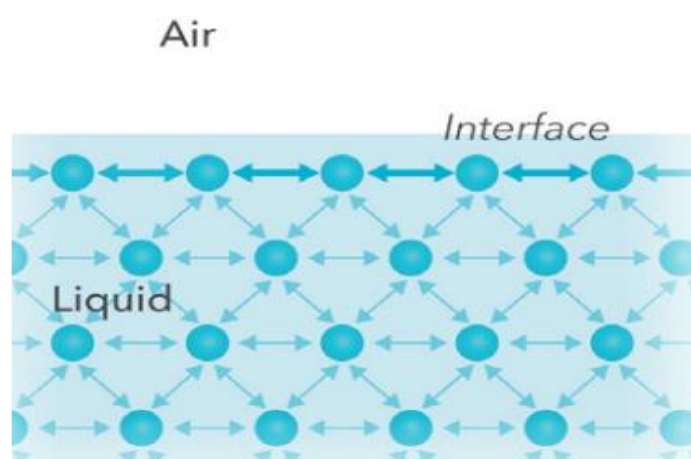


Figure 1-4. Force distribution between molecules in a liquid when they are at the interface between the liquid and air, and when they are completely immersed away from the surface (Biolinscientific, 2018).

1.2.4.1.1. Measurement of surface and interfacial tension

Measurements of surface and interfacial tension can be done by reversed pendant drop (or pendant drop shape) analysis. The shape of the drop suspended from a needle is determined by the balance of forces at the drop surface including the surface or interfacial tension. The surface or interfacial tension is related to the drop shape by using the Equation (1-2) as follows:

$$\gamma = \Delta\rho g (R_0^2)/\beta \quad (\text{Biolinscientific, 2018}). \quad (1-2)$$

Where γ is the surface tension, $\Delta\rho$ is the density difference between the two liquids, g is the gravitational acceleration, R_0 is the radius of the drop curvature at the apex and β is a shape factor. The shape factor can be defined through the Young-Laplace equation (Biolinscientific, 2018) expressed as 3 dimensionless equations as shown in Figure 1-5.

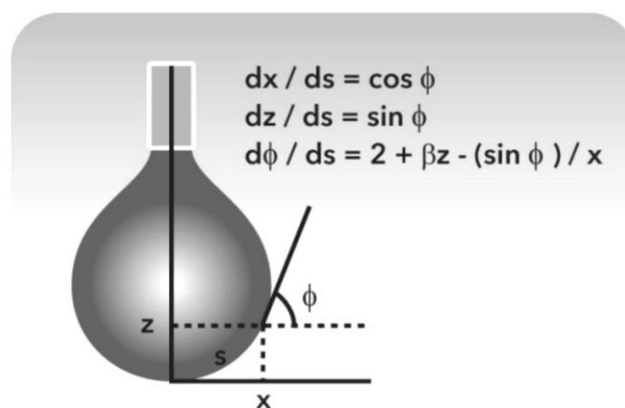


Figure 1-5. The shape of a drop hang on a needle, β is the shape factor which is determined by the Young-Laplace equation expressed as 3 dimensionless equation (Biolinscientific, 2018).

The software of the tensiometer uses iterative approximations to allow the solution of the Young-Laplace equation to be performed to find the value of the shape factor. Therefore, for any two liquids, or liquid and air, with known-densities which are in-contact, the surface or interfacial tension can be calculated using Young-Laplace equation. The size of the droplet used needs to be considered during measuring the surface and interfacial tension, to ensure that the droplet has the suitable pendant shape to achieve reliable results (Stauffer, 1965). Measuring the interfacial tension has been applied in the present research to identify whether OSA/WP and OSA/NaCAS mixed emulsifier systems can reduce the interfacial tension at oil/water interface as strong as using single emulsifier or not. Reducing the interfacial tension by the mixed emulsifier systems means that these emulsions can be used to stabilise oil/water interface.

1.2.4.2. Particle size analysis and measurement of the mean diameter

Droplet size usually are reported as droplet size distributions (DSDs) and mean droplet size based on either the volume distribution ($d_{4,3}$) or the surface area distribution ($d_{3,2}$) (Horiba, 2016). A droplets size analyser usually determines the size distribution of suspended particles using the principles of light scattering and provides a range of measuring. The scattering pattern is formed by light intensity as a function of scattering angle, particle shape and particle size. The optical system of the droplet size analyser consists of a source of light and a sample chamber. The sample interacts with the light beam and scatters it and then the scattered light is focused by a Fourier lens system. A system of photodetectors records the patterns of the scattered light intensity (Beckman

Coulter, 2003). Equation (1-3) defines the mean droplet size, based on the volume distribution, as follows:

$$D[4,3] = \frac{\sum_{i=1}^n D_i^4 v_i}{\sum_{i=1}^n D_i^3 v_i} \quad (\text{Horiba, 2016}) \quad (1-3)$$

Where (D_i) is the geometric mean for each channel which is the square root of upper x lower diameters. The numerator is the sum of the geometry of each channel D_i in the fourth power and multiplied by the percent of that channel (V_i), the denominator is the sum of the geometry of each channel D_i in third power multiplied by the percent of that channel (V_i) (HORIBA, 2016). The geometric mean for each channel is the square root of 'upper x lower' diameters (Yusoff and Murray, 2011). Channels are formed when the incident light is scattered by the particles to different lengths of light waves of scattered light (Coulter, 2011). Coalescence instability of an emulsion occurs when two or more droplets merge together to make a single larger droplet, and extensive coalescence leads to phase separation (McClements, 2005). Therefore, the particle size analysis over a storage period of an emulsion gives information to identify whether the size of droplets changed or not. Márquez et al. (2010) stated that the mean droplet size based on the volume distribution ($d_{4,3}$) is more affected by the presence of small amounts of large particles than the mean droplet size based on the surface area distribution ($d_{3,2}$).

1.2.4.3. Liquid chromatography mass spectrometry based method

This method depends on a liquid chromatography mass spectrometry based method. The liquid chromatography (LC) is defined as separation of a mixture by passing it as a solution, suspension or vapour through a medium in which the components of this mixture move at different flow rates. The LC is connected with mass spectrometry (MS), which is defined as an instrumental procedure for identifying the chemical characteristics of a component using the separation of gaseous ions depending on their differing mass and charge. Liquid Chromatography Mass spectrometry (LCMS) is an analytical technique that combines the resolving speciality of liquid chromatography with the detection of a mass spectrometry (Primer, 1998). The chromatography separates the sample substances using specific columns, different mobile phase gradients e.g. acetonitrile (C_2H_3N), controlled flow rates and temperature, and then introduces them to the spectrometer. The software of the LC is programmed to detect the molecular mass of each component presented in the sample. LC uses a high pressure for separating a liquid phase and producing a high gas load, while Mass spectrometry requires a vacuum and limited gas load (Primer, 1998). This method is the best for sugar measurement because it depends on detecting the molecule weight of sucrose and ignoring any other components presented the sample.

1.2.4.4. Zeta potential

The charged of the droplets surface attracts a layer of opposite charged ions; the electric potential at the boundary of this double layer of ions is named the zeta potential (ζ), typically, ζ -potential ranges from +100 mV to -100 mV

(nanoComposix, 2012). The ionic distribution at the electrical double layer is divided into two layers; (1) the stern layer is the layer of ions near the particle surface where ions are strongly attracted to the surface of the particle, (2) the diffuse layer surround the stern layer where the ions are diffused. It is assumed that the particles in a liquid move not only with the ions in the stern layer but also with part of the diffuse layer due to Brownian motion. The field where this movement happens is the "slipping plane" and the electrical potential at the slipping plane is named zeta potential. The potential is defined as zero at a position far from the particle surface. At high zeta potential, the particles are stable as result of high electrostatic repulsion, however, a low zeta potential (approaching zero) increases the probability of particle aggregation. Therefore, zeta potential is an index for the stability of the dispersion of particles (Beckman-Coulter, 2011).

When an electric field is applied in a suspension, charged particles move toward the electrode that is opposite to their charge. The velocity of particle movement is proportional to the amount of charge on the particles, therefore zeta potential can be estimated by measuring the particles' velocity using electrophoretic light scattering. Particles are irradiated with a laser and the scattered light emitted from them is detected. The electrophoretic mobility of the particles can be determined by measuring the frequency of the scattered light. The scattered light from the particles is detected by combining incident light (reference light) with the scattered light. Since the fluctuation of the combined light intensity is equivalent to the difference of frequency between the scattered and incident light, it is possible to measure small frequency shifts. The amount of frequency shift V_D of scattered light is related to the mobility of particles (U), which is calculated using the equation (1-4) (Beckman-Coulter, 2011) as follows:

$$v_D = \frac{Uq}{2\pi} \cos \frac{\theta}{2} = \frac{Un}{\lambda} \sin \theta \quad (1-4)$$

where scattering vector (q) = $4\pi n \sin(\theta/2)/\lambda$, n is the refractive index of the medium, θ is the scattering angle and λ is the incident light wavelength. In many solutions containing an electrolyte, zeta potential can be calculated from the Smoluchowski equation (1-5) (Beckman-Coulter, 2011) as follows:

$$Z = \frac{\eta}{\varepsilon_0 \varepsilon_r} U \quad (1-5)$$

Where ε_0 is dielectric constant in vacuum and ε_r is dielectric constant of the solvent. Droplets with a zeta potential higher than +30 mV or lower than -30 mV are considered highly charged and lead to an emulsion which is stable against coalescence because the droplets will repel each other, while droplets with a zeta value within this range can aggregate leading to emulsion instability (Clogston and Patri, 2011, Wongsagonsup et al., 2005).

1.2.5. The choice of PGPR in complex emulsion formulation

Polyglycerol polyricinoleate (PGPR) is a synthetic hydrophobic emulsifier, known to be one of the most efficient and powerful emulsifiers used for water-in-oil (W/O) emulsions preparation (Knoth et al., 2005, Mun et al., 2010, Su et al., 2006). In most publications about edible water-in-oil-in-water ($W_1/O/W_2$)

emulsions, the low-HLB (Hydrophile-Lipophile Balance) emulsifier (PGPR) is used to stabilise the W/O-interface (Balcaen et al., 2017). The high emulsifying properties of PGPR are attributed to the high water-binding capacity of the hydrophilic polyglycerol chain (Márquez et al., 2010). PGPR is prepared by the esterification of concentrated castor oil fatty acids with polyglycerol, and is soluble in fats and oils and insoluble in water (Mun et al., 2010, Leal-Calderon et al., 2012).

PGPR has gained generally recognized as safe (*GRAS*) from the Food and Drug Administration (FDA) (Andrade and Corredig, 2016). The digestibility of polyglycerol polyricinoleate is 98.0% and it has been demonstrated that the substance is tolerated by humans at high doses with no clinical symptoms. PGPR does not interfere with the metabolism of lipids and there are no evidence for its adverse effects on growth and reproduction nor was it shown to be a carcinogenic risk in rats (Márquez et al., 2010). In addition, Wilson et al. (1998) stated that use of PGPR in chocolate couverture does not constitute a health hazard for human. Nonetheless, the use of this emulsifier in food is highly regulated because of its unpleasant taste (Dickinson, 2015, Raoufi et al., 2012). Figure 1-6 shows the chemical structure of PGPR in which black dots denote polyricinoleic acid chains (Bastida-Rodríguez, 2013).

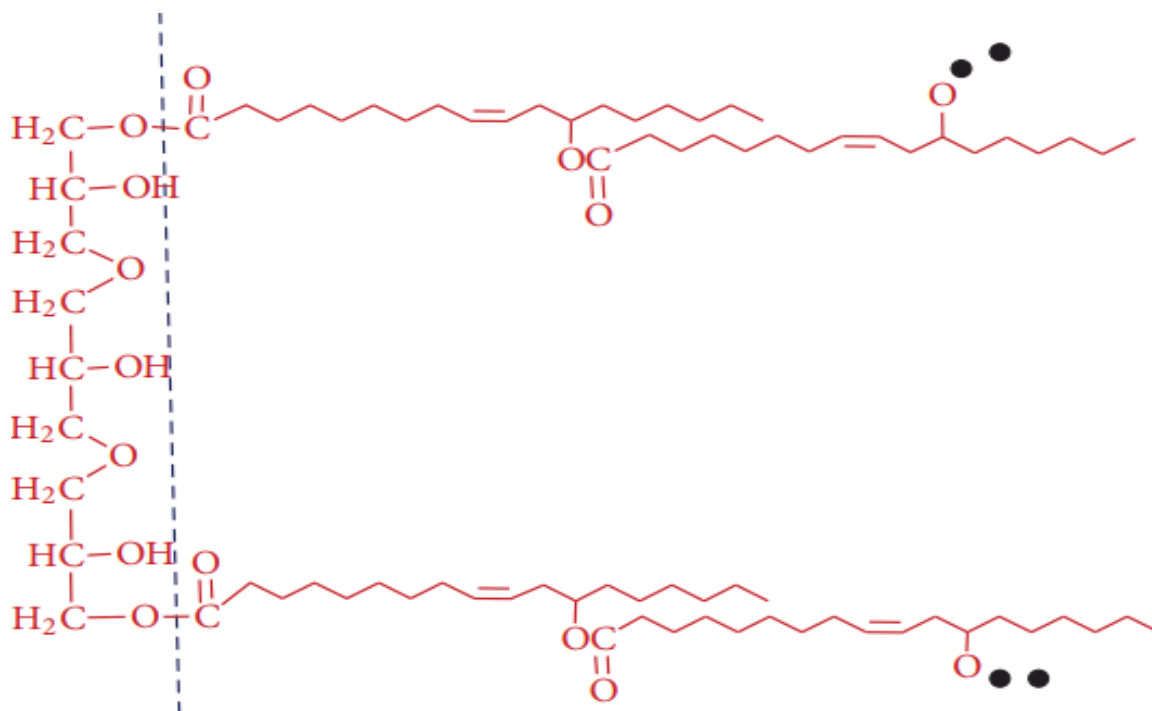


Figure 1-6. Chemical structure of PGPR, black dots denote polyricinoleic acid chains (Bastida-Rodríguez, 2013).

The balance of hydrophilic and lipophilic parts at the emulsifier molecule (HLB value) determines the functional behaviour of an emulsifier at the oil/water interface. At a high HLB value the hydrophilic part dominates and the emulsifier typically stabilises O/W emulsions, whereas at a low HLB values the lipophilic part dominates and the emulsifier typically stabilises W/O emulsions (Bastida-Rodríguez, 2013). The HLB value of PGPR is (1.5 ± 0.5) which is very low (Taylor, 2011), the number of hydrophilic hydroxyl groups is low and the degree of esterification is high therefore, it is a typical emulsifier for W/O emulsions. The mechanism of lipophilic emulsifiers to stabilise a W/O interface has been investigated by Kanouni et al. (2002) who found that the high elasticity of interfacial film formed by these emulsifiers allowed the film to withstand stress without breaking and, therefore, produced stable W/O emulsions. Where the

hydrophilic parts is presented in water while the lipophilic parts is presented in oil.

In food manufacture, it is commonly employed along with lecithin to control yield behaviour of chocolate (Bastida-Rodríguez, 2013, Schantz and Rohm, 2005). PGPR is also used as an emulsifier in high or low lipid content products (Márquez et al., 2010). The choice of hydrophobic emulsifiers is critical to control the mean droplet size and encapsulation efficiency of $W_1/O/W_2$ emulsions (Zhang and Reineccius, 2016). In $W_1/O/W_2$ emulsions some technological issues can be solved using PGPR, for instance diffusion of an emulsifier across the oil phase, which makes a gel like structure at the interface of (W_1/O) emulsion (Aditya et al., 2017).

1.2.6. Complex emulsions as lipid-based delivery systems

Complex emulsions have great potential for food science applications as a means to reduced fat content or for controlled encapsulation of functional ingredients and delivery of these components at specific parts of the human body (Clegg et al., 2016). $W_1/O/W_2$ emulsions can be used to isolate nutrients in the aqueous phase of the primary water-in-oil emulsion (Laugel et al., 1996, Tedajo et al., 2005). However, using these emulsions as delivery systems and functional ingredients to complex food products facing substantial challenges making them not widely separated (McClements, 2015). These challenges such as degradation of these ingredients during processing and storage, which could affect the physio-chemical stability of these systems (Velikov and Pelan, 2008). Typically, complex emulsions are prepared via two emulsification steps; preparation of Water-in-oil (W/O) emulsion as primary stage and then homogenisation of this emulsion into an external aqueous phase (Frasch-Melnik

et al., 2010b). W/O emulsions can be used as an oil phase substitute in food emulsions to obtain water-in-oil-in-water ($W_1/O/W_2$) emulsions, which contain same quantity of dispersed phase but with lower oil content (Márquez et al., 2010). The stability of a W/O emulsion is an important parameter when considering the applicability of these emulsions to produce more complex emulsions. The emulsion needs to have a relatively high stability in order to achieve a product of invariable quality during its shelf life. Instability of W/O emulsions is usually is due to coalescence or sedimentation (Márquez et al., 2007). This destabilisation should not be reduced by using high concentrations of PGPR as this cannot be in excess in food systems because it is highly regulated (Bonnet et al., 2009). Márquez et al. (2010) stated that a W/O emulsion has a liquid appearance, because they worked at a relatively low dispersed phase however, if it inverts into an O/W emulsion, its consistency is thicker because the system has a high concentration of oil as the dispersed phase. Table 1-3 shows the use of either PGPR or span 80a as hydrophobic emulsifier to stabilise the primary water-in-oil emulsion and some polymers that have been used as external mixed emulsifiers to stabilise $W_1/O/W_2$ and the relevant references.

Table 1-3: Some of the hydrophobic and hydrophilic emulsifiers that have been used to stabilise W/O/W emulsions and the relevant list of references.

Emulsion	Polymers	References
W/O/W	(PGPR /Pectin /pea protein isolate conjugate)	Tamnak et al. (2016)
W/O/W	(PGPR/Xanthan gum, locust bean gum (LBG), guar gum, konjac gum & Fenugreek gum /WP)	Benichou et al. (2007)
W/O/W	PGPR /Tween 20	(Vladislavljević et al. 2014)
W/O/W	(PGPR /either OSA starch NC 46 or pea protein)	(Chiu et al., 2015)
W/O/W	(PGPR/ either Egg yolk or WP)	(Schuch et al., 2015)
W/O/W	(PGPR/cellulose),(PGPR/WP) and (PGPR/cellulose/WP)	(Panagopoulou et al., 2017)
W/O/W	(PGPR/pectin/pea protein) and (PGPR/Tween 20)	(Tamnak et al., 2016)
W/O/W	(PGPR/xanthan/WP)	(Benichou et al., 2002)
W/O/W	PGPR/skim milk or NaCAS	(Giroux et al., 2013)
W/O/W	(PGPR/WP or NaCAS), (Ammonium phosphatide/WP or NaCAS) or (sucrose ester O-170/ WP or NaCAS)	(Balcaen et al., 2017)
W/O/W	PGPR/NaCAS	(Bonnet et al., 2009)
W/O/W	(BSA/Span 80), (Span 80/ Tween 80) or (BSA)	(Gaiti et al., 1994).
W/O/W	fat crystals, monoglyceride & triglyceride/NaCAS	(Frasch-Melnik et al., 2010b)
W/O/W	PGPR/gelatin	(Santos et al., 2015)
W/O/W	PGPR/Tween 80	(Aditya et al., 2015)
W/O/W	(PGPR/waxy maize starch) or (PGPR/OSA starch)	(Chiu et al., 2017)

These challenges such as degradation of these ingredients during processing and storage, which could affect the physio-chemical stability of these systems (Velikov and Pelan, 2008) Typically, complex emulsions are prepared via two emulsification steps; preparation of Water-in-oil (W/O) emulsion as primary stage and then homogenisation of this emulsion into an external aqueous phase (Frasch-Melnik et al., 2010b). W/O emulsions can be used as an oil phase

substitute in food emulsions to obtain water-in-oil-in-water ($W_1/O/W_2$) emulsions, which contain same quantity of dispersed phase but with lower oil content (Márquez et al., 2010). The stability of a W/O emulsion is an important parameter when considering the applicability of these emulsions to produce more complex emulsions. The emulsion needs to have a relatively high stability in order to achieve a product of invariable quality during its shelf life. Instability of W/O emulsions is usually is due to coalescence or sedimentation (Márquez et al., 2007). This destabilisation should not be reduced by using high concentrations of PGPR as this cannot be in excess in food systems because it is highly regulated (Bonnet et al., 2009). Márquez et al. (2010) stated that a W/O emulsion has a liquid appearance, because they worked at a relatively low dispersed phase however, if it inverts into an O/W emulsion, its consistency is thicker because the system has a high concentration of oil as the dispersed phase.

1.2.6.1. Encapsulation of functional ingredients

The encapsulation efficiency (EE) of $W_1/O/W_2$ emulsions is defined by Vladislavljević et al. (2014) as the mass of a component (X) entrapped in the double emulsion droplets immediately after the emulsification process at (time = 0) divided by the total mass of this component added. One requisite for a high EE is high stability in $W_1/O/W_2$ emulsions characterised by very small internal water droplets W_1 with a mean diameter about 0.5 μm and with an extremely thin oil film between them. The phase behaviour of polysaccharide/protein aqueous two-phase systems was investigated in the presence of sucrose by Spyropoulos et al. (2010) who stated that the miscibility of these mixtures was increased by adding sugar. An emulsion-like microstructure of these water-in-

water emulsions had been seen in polysaccharide/protein aqueous two-phase systems, protein-rich equilibrium and the polysaccharide-rich phases, resulting phase separation behaviour similar to that of the water and oil phases of a usual emulsion (Spyropoulos et al., 2010). In addition, bi-continuity in water-in-water systems undergoing phase inversion has been stated for other aqueous two-phase systems, for instance mixed polymers by Wolf et al. (2000) and for biopolymer surfactant mixtures by Spyropoulos et al. (2008).

1.2.6.2. Delivery and release of functional ingredients

In $W_1/O/W_2$ emulsions, the release of ions depends on the reverse micelle release rather than direct diffusion, but if the oil film becomes extremely thin, the transport by reverse micelles is impeded (Cheng et al., 2007). The extremely thin oil films between W_1 droplets, small W_1 droplets and high W_1 yield are to be expected in $W_1/O/W_2$. Release in $W_1/O/W_2$ can be verified by changing the average oil droplet size as well as the behaviour of the W_1 phase (changing droplet size) (Muschiolik, 2007). A high performance liquid chromatography was used by Lapis et al. (2017) for quantifying the hydrolysis products of starch. Therefore, this equipment could be used in measuring sucrose in the serum of $W_1/O/W_2$ emulsions. Salts of sodium, calcium, potassium, magnesium and iron have been encapsulated in W/O emulsions (de Souza Simões et al., 2017, Márquez et al., 2010, Dubey and Windhab, 2013). Since starch is an important part of human diet, the oral detection of starch would be highly beneficial (Lapis et al., 2017). As a potential procedure for calcium fortification and the controlled the release of sugar in foods, formulation based on $W_1/O/W_2$ emulsions was considered. The influence of the addition of

calcium on the stability W/O and $W_1/O/W_2$ emulsions was measured by analysing different potential factors such as droplet size distribution and interfacial tension. In theory, the digestion of $W_1/O/W_2$ emulsion is show in Figure 1-7.

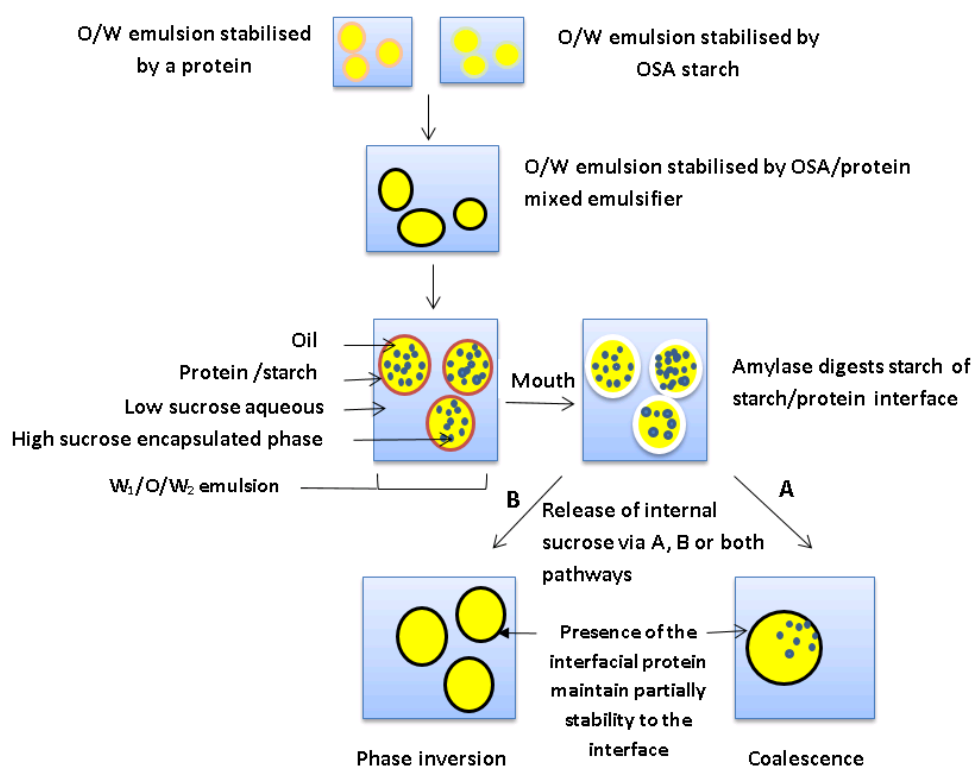


Figure 1-7. Schematic of the design of $W_1/O/W_2$ emulsion and the anticipated pathway of its oral destabilisation. Single emulsions stabilised with either OSA or protein were prepared to decide the quantity of each emulsifier is needed, and then an emulsion with low-starch content was formed by mixing half the quantity of each emulsifier. The single emulsion stabilised with mixed OSA/protein was developed to $W_1/O/W_2$ emulsion with encapsulation of sugar. The double emulsion can partially break down in the mouth following via A, B or both pathways.

Many mechanisms have been suggested to reduce the amount of glucose as final digestion of starch e.g. consuming of foods that release glucose more slowly (dietary fibre) or resistant starches which have a relative low glycaemic

index. In addition, the biopolymer gellan gum has been shown to slow the release of glucose to the body in acidic conditions (Norton et al., 2015).

During chewing salivary α -amylase hydrolyses up to 43% of the total starch in foods to glucose and dextrans (short chains of partly digested starch) depending on the food type and duration of salivary exposure (Woolnough et al., 2010). Sugar released from foods during *in-vitro* digestion by adding pancreatin do not differ significantly regardless of pre-exposure to saliva (Woolnough et al., 2010). Therefore, the relative profiles for sugar of either salivary exposure during chewing *in vivo* or *in-vitro* digestion by pancreatin are similar. The fraction of starch that is hydrolysed to glucose and dextrans by salivary action during the oral phase may be termed as “extremely rapidly digestible starch,” which contribute to the rapid initiation of glycaemic response following ingestion of food. There is an inconsistency between the current methods of *in-vitro* digestion with respect to exposure to α -amylase exposure during the oral phase (Woolnough et al., 2010).

1.2.6.3. Tongue and amylase types

α -amylase is an enzyme found in human saliva, pancreatic juice, serum, breast milk and liver tissue which is responsible for hydrolysing the α (1-4) linkages of starch. Salivary α -amylase function is the first step of digestion which begins with the chewing of food in the mouth in the presence of salivary α - amylase in order to breakdown the starch in food into maltose and glucose (Enemchukwu et al., 2013). *In-vitro*, α -amylase is also able to hydrolyse α -(1, 4) linkages but has no activity on α -(1, 6) linkages which are responsible for the more highly branched structure of starch. In the food industry, yeast produces α -amylase

to breakdown starch in flour into simpler sugars, causing bread to rise and imparting flavour (Enemchukwu et al., 2013). Salivary α -amylase activity can be active for at least six months at 25.0 °C. It requires a neutral or slightly alkaline medium between pH 7.0 to pH 7.4 and the human body temperature is optimal for its activity (Sampson et al., 1981).

Salivary α -amylase activity and the rate of starch degradation have potential roles in the oral digestion and perception of starch (Lapis et al., 2017). Chiu et al. (2017), stated that native salivary amylase hydrolyses emulsifying starch in the external aqueous phase of $W_1/O/W_2$ resulting in destabilisation of the external O/W_2 of the multiple emulsion and the releasing of the inner W_1/O phase. Therefore, the α -amylase enzyme could be added *in-vitro* to digest OSA starch at the external interfaces of $W_1/O/W_2$ emulsions stabilised with this starch. Starches which are the substrate for ptyalin ' α -amylase' and their products (short chains of glucose) are able to partially prevent the enzyme against inactivation by gastric acid when the food proceeds the stomach to be digested in acid environment of about pH 3.3 (Enemchukwu et al., 2013).

Chapter 2: Materials and Methods

1. Materials and Methods

The initial stage of this research involved the design of a single oil-in-water emulsion (O/W) with a protein/starch mixed interface. In the second stage, this single emulsion was used to form water-in-oil-in-water ($W_1/O/W_2$) emulsions. The multiple emulsions were designed to break down in the oral cavity, releasing encapsulated sugar close to the taste receptors. This allows to test the hypothesis that the overall content of sugar used in the bulk of the food could be reduced without compromising the sweet taste. All materials used in emulsion preparation were food grade and used as received except for the addition of sodium azide which was used as a preserving agent in samples destined for stability trials.

2.1. Materials

The preparation of oil-in-water emulsions was for designing two types of a starch/protein mixed emulsifier system. These mixed starch/protein emulsifiers were used in the formulation of $W_1/O/W_2$ emulsions in the later stages of this research. Sunflower oil was locally sourced (Sainsbury's Supermarket Ltd, London, UK) and used at 20 % w/w as the dispersed phase of the O/W emulsions. Three emulsifiers were used independently to stabilise the O/W systems and then were mixed together to obtain protein/starch mixed interfaces. These emulsifiers were:

- OSA starch (NC 46, Univar B. V., Netherlands), moisture 0 -10%, a chemically modified food ingredient: initially refined from waxy maize and then modified by adding octenyl succinic anhydride to make it

hydrophobic. Based on dry weight, it contains $\geq 97\%$ carbohydrate, protein $\leq 0.5\%$, 0.15% fat, 0.27% minerals.

- Whey protein (WP) (The Organic Whey Protein Company, West Sussex - UK). 100% organic whey protein with purity equal 75.5% prepared from organic milk, contains 4% fat, 5% carbohydrate (sugars), 2.11% salt and 0.67% calcium and phosphate.
- Sodium caseinate (NaCAS) (1-800-Acros-01, New Jersey, USA), protein $\geq 92\%$ of dry substance, water $\leq 6\%$ and ash ≤ 4.5 .

Sodium azide (NaN_3) was obtained from Sigma-Aldrich (Gillingham, UK) and used as the preserving agent for samples destined for stability trials, at a concentration of 0.02% w/w. Magnesium silicate 4% (w/w) from Sigma-Aldrich, (Dorset, UK) was used to purify sunflower oil of any surface active impurities. Polyglycerol polyricinoeleate (PGPR Grindsted 90, DANISCO, Denmark) was used to stabilise the internal water phase (W_1) the of $W_1/O/W_2$ emulsions in the next stage of this research but in the single emulsion experiment was used to see the effect of its addition on emulsion stability. Disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) and sodium dehydrogenase phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) were used together as a sodium phosphate buffer preparation; both were obtained from Fisher Scientific Limited (Loughborough, UK). Coulter Latron 300Ls (Galway, Ireland) solution was used for preparing the calibration solution during zeta potential measurements.

Acetic acid (2.0M) (Scientific Laboratory Supplies, UK) was used for the sodium acetate buffer preparation, sodium hydroxide (Honeywell, Fluka™, UK) was used for the acetate buffer pH adjustment. Nile Red (0.01 g/L in polyethylene glycol), Fast Green (Merck, 0.1 g/L in water) and Fluorescein

isothiocyanate (FITC) (1g/L in water) stains were purchased from (Sigma Aldrich - Steinheim, Germany) and used for confocal laser scanning microscopy (CLSM) investigation.

Sucrose was locally sourced (ALDI stores Ltd, Warwickshire, UK) and was used for complex emulsions formation. Glucose was obtained from Thornton & Ross Ltd. (Huddersfield, England) and was used instead of sucrose in some stage of this research. The Megazyme starch assay kit K-TSTA-100A (Megazyme International Ireland Ltd., Wicklow, Ireland) was used for detecting glucose content in the outer aqueous phase of the $W_1/O/W_2$ in measurement of glucose encapsulation and glucose release. α -amylase enzyme from porcine pancreas, Cat. No. A3176 type VI-B, with concentration ≥ 10 units/mg of the solid weight, was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). This enzyme was dissolved in sodium phosphate buffer (0.01M, pH 7.0) and used for enzymatic digestion (*in-vitro*) to quantify release of encapsulated sugar from $W_1/O/W_2$.

Silver nitrate ($AgNO_3$) with concentration $\geq 99.0\%$ was purchased from (Honeywell, Fluka [®], UK) was used to inactivate the α -amylase enzyme, which was used for an enzymatic digestion. A 00G-4378-B0, 5 μ m liquid chromatography column (Phenomenex, UK) was used with a mobile phase gradient of acetonitrile C_2H_5N (Fisher Scientific – UK) for sucrose detecting using the liquid chromatography mass spectrometry (LCMS) based method.

Sodium chloride (anhydrous, purity $\geq 99\%$) and calcium chloride (dihydrate, purity $\geq 99\%$) were purchased from (Honeywell/ Fluka[®], UK). Potassium chloride (anhydrous, purity $\geq 99.5\%$) and magnesium chloride (anhydrous) were purchased from (Sigma-Aldrich, Dorset, UK). All these salts were used

for fortification of the $W_1/O/W_2$ emulsions with mineral salts. Deionised water was used throughout all research stages.

1.3. Emulsion preparation methods

2.2.1. Oil-in-water emulsions using single emulsifier systems

The oil-in-water emulsions were prepared in which each emulsion consisted of 20 % w/w oil and 80 % w/w water including the water soluble emulsifier. A high shear overhead mixer (L5M Series fitted with emulsor screen, Silverson, Chesham, UK) was used at a speed of 8000 rpm for 5 min for the formulation of the emulsions. Prior to homogenisation (i.e. before adding the oil), emulsifiers were dissolved in the water using the high shear overhead mixer at a speed of 4000 rpm for 5 min to ensure homogeneous distribution and complete dissolution.

A processing protocol using single emulsifiers was established to optimise their concentration in the system to produce stable O/W emulsions. Three groups of emulsions were prepared using either OSA starch, whey protein (WP) or sodium caseinate (NaCAS). In the first group, O/W emulsions were stabilised with OSA starch prepared according to the method used by Chiu et al. (2015) with some modifications. The aqueous phase of emulsions in this group started with 4 % w/w OSA as emulsifier and stepwise decreased by 0.5 % steps until 0.4% w/w. In the previous studies the concentrations of protein were varied to be optimal to stabilise O/W emulsions e.g. Frasch-Melnik et al. (2010b) used 1% w/w NaCAS and Panagopoulou et al. (2017) used 1% w/w WP to stabilise the outer interface of $W_1/O/W_2$ emulsions where 20% w/w primary W/O emulsion. Therefore, in the second group of single

emulsions, six concentrations of WP were implemented to optimise protein concentration for mixed starch/protein systems. The emulsions in this group contained 0.5, 0.6, 0.7, 0.8, 0.9 and 1% w/w whey protein dissolved in the aqueous phase. In the third group of emulsions the same procedure was followed but NaCAS was used instead.

During the emulsion processing, the formulation beaker was placed in a container with ice water to keep the temperature during the formulation below 50 °C. The formulation temperature was measured during processing using a thermometer (Hygiplas multipurpose stem thermometer, Bristol, UK). The isoelectric point of WP is at pH 5.2 (Masson and Jost, 1986, Pelegrine and Gasparetto, 2005) and of NaCAS is at pH 4.6 (Zinoviadou et al., 2012). Therefore the pH value was measured immediately after formulation and after 14 days of storage at ambient temperature to ensure the pH did not decrease to the isoelectric points of the proteins used. A pH Meter (inoLap pH level1, Weilheim, Germany) was used for the pH measurements.

The different concentrations of emulsifier would impact on the pH value in the system and affect O/W emulsion stability. Therefore, to keep the pH approximately constant and to know whether pH had an effect on emulsions stability or not, the effect of using buffer as the aqueous phase of O/W emulsions was investigated. The sodium phosphate buffer (0.1 M, pH 7) was prepared as follows: Sodium azide (NaN_3) solution was prepared at concentration of 0.02 % w/w and used to prepare two solutions A and B as follows. To prepare solution (A), 17.8 g disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) was dissolved in 1 L of distilled water. Solution (B), 15.6g of sodium dehydrogenase phosphate dehydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) was dissolved

in 1L of distilled water. 610 mL of solution (A) was added to 390 mL of solution (B) to produce 1 L of 0.1M pH 7.0 sodium phosphate buffer.

Using this buffer and water in the formation of the emulsions, five pairs of O/W emulsions were prepared each containing a different concentration of one of the three emulsifiers selected from the initial experiments based on water as aqueous phase as it shown in Table 2-4. In the case of OSA starch, 0.8 % w/w OSA starch was chosen because it was the lowest concentration which stabilised the O/W emulsion as discussed in section 3.1.1. In addition, using the lowest possible concentration of OSA was important because the aim of this PhD research was to perform starch replacement as well as to reduce the sugar content, which is the final stage of starch digestion. In the case of whey protein, the second and third pairs of emulsions were stabilised with 0.5% and 1.0 % w/w respectively. In the case of sodium caseinate the fourth and fifth pairs of emulsions were stabilised with 0.5% and 1.0 % w/w respectively.

Table 2-4. Compositions of five pairs of O/W emulsions stabilised with different concentrations of emulsifiers were two types of aqueous phase were used either water or phosphate buffer.

Emulsion pairs	Weight (% w/w)			Oil	Water	Buffer
	OSA	Emulsifier WP	NaCAS			
OSA	0.8			20	79.2	
						79.2
WP		0.5			79.5	
						79.5
		1			79	
						79
NaCAS			0.5		79.5	
						79.5
			1		79	
						79

2.2.2. Mixed interface O/W emulsions

In the present study, proteins and polysaccharides that display a negative charge under the solvent pH conditions of 7.0 have been chosen. Low concentrations of OSA starch/milk protein were used in such a way so that they do not repel each other in the droplet surface. Each of these emulsifiers alone, at the concentration used, cannot stabilise an O/W emulsion for the required period of storage (14 days). An experimental plan was systematically developed to assess the emulsifying ability of OSA starch/protein mixtures. Whey protein and sodium caseinate were selected as co-emulsifiers with commercial OSA starch depending on the aqueous phase conditions like pH and ionic strength. Both proteins have a high stabilising efficiency and have a negative charge at pH 7.0 (Burrington, 2012, Zinoviadou et al., 2012).

Based on the formulation of stable O/W emulsions using either 0.8% w/w OSA starch, 1% w/w whey protein or 1% w/w sodium caseinate in the previous experiment, new concentrations of mixed interfaces were selected. OSA starch was partly replaced by either whey protein or sodium caseinate, in which the mixed interfaces were prepared by making the aqueous phase first with both the OSA starch and protein (*mixed method*). Half of the emulsifier quantity needed for starch stabilised O/W emulsions was replaced by half of the emulsifier quantity needed for protein stabilised O/W emulsions. Consequently, O/W emulsions containing (0.4% OSA/0.5% WP) w/w mixed emulsifier or (0.4% OSA/0.5% NaCAS) w/w were formulated. Each one of the new emulsions was formulated using either water or 0.1M sodium phosphate buffer, and as result four different O/W emulsions were created as shown in Table 2-5.

Table 2-5. Composition of four O/W emulsions stabilised two different mixed emulsifier systems and formulated with either water or 0.1 M pH 7 sodium phosphate buffer.

Emulsion	Weight % w/w				
	Emulsifier			Oil	Buffer
	OSA	WP	NaCAS		
1	0.4	0.5		20	79.1
2	0.4	0.5			79.1
3	0.4		0.5		79.1
4	0.4		0.5		79.1

The effect of adding the oil-soluble surfactant polyglycerol polyricinoleate (PGPR) on stability against coalescence of O/W emulsions stabilised with the mixed emulsifier systems was investigated. The previous studies varied in the concentration of PGPR involved as emulsifier in food emulsion e.g. Su et al. (2008) used 2% w/w PGPR to stabilize W/O emulsions containing 25% aqueous phase for 7 days of storage at 20.0 °C. In this research, 2% w/w PGPR was used during the formulation W/O emulsion as a primary stage of water-in-oil-in-water ($W_1/O/W_2$) emulsions processing see section 2.4.1. However, in the stage of the research only the secondary stage O/W of the $W_1/O/W_2$ emulsion was investigated therefore, depending on the final $W_1/O/W_2$ emulsion calculation only 0.57% w/w PGPR was added to O/W emulsions. The stability against coalescence of emulsions stabilised with mixed emulsifier either OSA/WP or OSA/NaCAS was investigated with and without the addition of 0.57% w/w PGPR based on total emulsion. Two different aqueous phases were used for formulating these emulsions: water or 0.1M sodium phosphate buffer, emulsions were investigated during two weeks of storage at ambient temperature.

2.2.3. Preparation of Water-in-oil-in-water

Water-in-oil-in-water ($W_1/O/W_2$) emulsions were prepared following the procedure of Frasc-Melnik et al. (2010b) some with modification. Initially, a water-in-oil (W_1/O) emulsion consisting of 30% w/w aqueous phase was prepared as primary stage and then incorporated into the external aqueous phase (W_2) as secondary stage to formulate a $W_1/O/W_2$ emulsion. Water containing 0.02% w/w sodium azide to prevent microbial spoilage was used throughout the emulsions formulation.

2.2.3.1. Primary stage of preparation

The water-in-oil (W/O) emulsions were prepared with 70% w/w oil phase including polyglycerol polyricinoleate (PGPR) and 30% w/w aqueous phase. The aqueous phase consisted of a 50% w/w sucrose solution. The procedure stated by Chiu et al. (2017) was followed for W/O formulation as primary stage of $W_1/O/W_2$ emulsions preparation. The PGPR and sunflower oil were combined in which 2% of PGPR and 68% oil w/w of the total emulsion weight were mixed by a high shear overhead laboratory mixer (Silverson L5M fitted with emulsor screen, supplier, Chesham, UK) at 4000 rpm for 5 min. Subsequently, the aqueous phase was emulsified into the oil phase also using the high shear overhead mixer (Silverson). Processing conditions were 8000 rpm for 5 min. The final composition based on emulsion was 70% oil including 2% PGPR and 30% w/w aqueous phase. To ascertain that indeed an oil continuous emulsion had been prepared, the emulsions were diluted in oil. The microstructure of the emulsion was observed using a digital inverted transmission light microscope (EVOS fl, Life Technologies Ltd., Paisley, UK), fitted with a 100 x bright field objective, which indicated to microstructure of

W/O emulsion. The emulsion kept its structure of very small droplets in the oil continues phase after dilution.

2.2.3.1.1. Droplet stability

The stability against coalescence of the W/O emulsions was assessed by light scattering measurements using a scan analyser (Turbiscan Lab®, Formulaction - TurbiSoft-Lab-2.2.0.82-2, Bristol, UK) over a 15-day storage period at ambient temperature. This equipment measures the intensity of a monochromatic light source at 880 nm wavelength. Samples were placed in the equipment tubes (diameter: 27.5 mm, height: 44 mm). the scan analyser scans along the tube giving a profile of the back scattering percentage (%BS) and transmission percentages as a function of length tube and time (Pan et al., 2002). The resultant profiles depicting the emulsion's condition over time determine curves of the light back-scattering percentage as a function of the sample tube height (Domian et al., 2015). The back-scattering of monochromatic light is a function of the dispersed phase volume and droplet diameter (Mengual et al., 1999). Emulsions that have a higher %BS value have a lower water droplet size (Márquez et al., 2007).

According to the procedure applied by Márquez et al. (2010) the middle part of the tube was used to analyse the coalescence process in W/O emulsions, the tube length (42mm) was divided into thirds. The average of %BS was calculated from the 14–28mm zone (%BS_{14–28}) which corresponded to the middle part of the tube. The %BS_{14–28} was used as a measure that relates to droplet size of the W/O emulsions to allow comparison with emulsions at different measuring points during their storage period (Márquez et al., 2007).

The %BS value was used to estimate the destabilisation percentage (%D) but not droplet size or its distribution. The %D value indicates whether there is instability against coalescence and it is defined by Equation (2-6) following Márquez et al. (2010):

$$\%D = [(BS_{in14-28} - BS_{f14-28}) / BS_{in14-28}] \times 100 \quad (2-6)$$

Where: $BS_{in14-28}$ is the initial %BS₁₄₋₂₈ value, BS_{f14-28} is the %BS₁₄₋₂₈ value after 15 days. Measurements were performed in triplicate, and results are reported as mean with standard deviation.

2.2.3.2. Secondary stage of preparation

In secondary stage, the W_1/O emulsion prepared in primary stage was homogenised into the external aqueous phase (W_2) using the high shear overhead mixer for 5 min at 4000 rpm. In the first stage of preparation (section 2.2.3.1) a sucrose solution with a concentration of 50% w/w was encapsulated in the W_1/O emulsion. In the secondary stage, a sucrose solutions (W_2) with a concentration of 5% w/w was prepared and then homogenised with the W_1/O emulsion for forming the $W_1/O/W_2$ emulsion. A diagram about the formulation stages for 100 g of the multiple emulsions ($W_1/O/W_2$) stabilised with a starch/protein mixed emulsifier system is provided in Figure 2-8. According to the method used by Chiu et al. (2015) with some modification 28.6% of the W_1/O emulsion was added to 71.4% w/w of the external aqueous phase W_2 , which includes the external emulsifier. The secondary emulsification is usually carried out in a lower shear

condition compared to the primary emulsification to avoid the rupture of the primary (W/O) emulsion (Aditya et al., 2017).

Formulation of $W_1/O/W_2$ emulsion

Preparation of aqueous phase (W_2)

*Preparation of sucrose solution 5% w/w

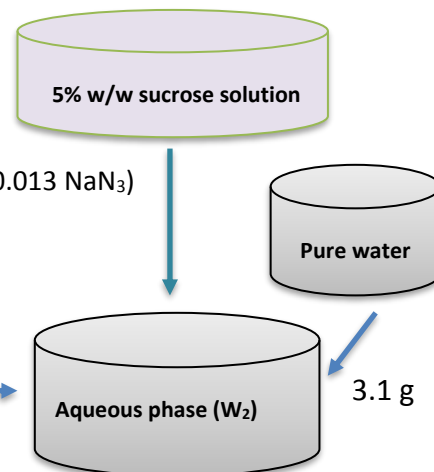
95 g water contains 0.019 Sodium Azide (NaN_3) + 5 g sucrose

Mixing by magnetic stirrer at 500 rpm for 15 min

67.4 g (3.37 g sucrose, 64.017 g water and 0.013 NaN_3)

*Addition of the emulsifiers

(0.4 g OSA + 0.5 g WP) or (0.4 g OSA + 0.5 g NaCAS) →



Preparation of aqueous phase (W_1) (sucrose solution 50 % w/w)

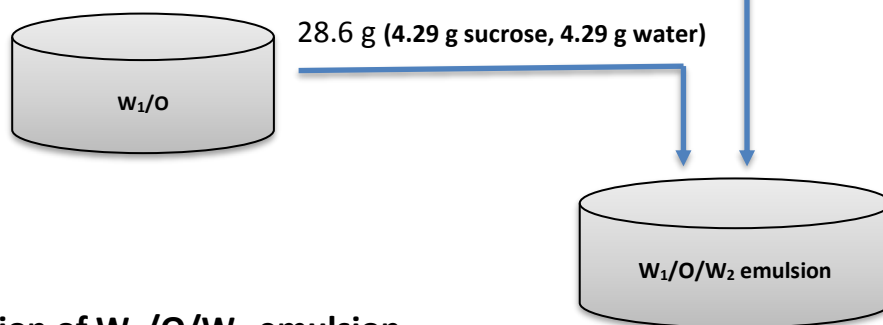
50 g sucrose + 50 g water

Mixing by a magnetic stirrer at 500 rpm for 2 hours

Preparation of W_1/O emulsion

68 g Oil + 2 g PGPR + 30 g of W_1 (15 g sucrose + 15 g water)

Mixing by a high shear overhead mixer at 4000 rpm for 5 min



Preparation of $W_1/O/W_2$ emulsion

Mixing by a high shear overhead at 8000 rpm for 5 min

Figure 2-8 . The formulation stages for 100g of multiple emulsions $W_1/O/W_2$ stabilised with 0.57% w/w PGPR in the internal aqueous phase, and stabilised with mixed emulsifier system either 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS in the external aqueous phase.

Milk proteins are susceptible to changes during heating such as denaturation especially the whey protein which is more sensitive to heat than the sodium caseinate (Burrington and Agrawal, 2012). Therefore, the formulation temperature was kept below 50 °C to avoid denaturation of the proteins used as emulsifier. The effect of osmotic pressure gradients between the aqueous phases of the $W_1/O/W_2$ emulsions on the stability against coalescence and the release of enclosed sugar were taken to account. Therefore, the emulsion system contained 7.6% w/w sugar in total, of which 4.2% was entrapped. The emulsion was stored at ≈ 24.0 °C for investigation over a 14-day storage period. The final composition of the $W_1/O/W_2$ emulsion was as presented in Table 2-6.

Table 2-6. The composition of the $W_1/O/W_2$ emulsions

Component	Weight (%)
Water	71.420
Oil	19.431
Sugar	7.660
OSA starch	0.400
Protein (WP or NaCAS)	0.500
PGPR	0.570
Sodium Azad (NaN3)	0.019

Each 100 g of $W_1/O/W_2$ emulsion contained 8.58 g of 50% sucrose solution and 67.4 g of 5% sucrose solution. Therefore, it contains $4.29\text{g} + 3.37\text{g} = 7.66$ g of sucrose. Sucrose rich $W_1/O/W_2$ emulsions have been added to ingredients of beverages by 1% by Zhang and Reineccius (2016) who demonstrated that $W_1/O/W_2$ emulsion-based beverage could be made successfully.

The emulsion was stored at ≈ 24.0 °C for investigation over a 14-day period storage. The composition of the external aqueous phase (W_2) was varied to

make five different emulsions; three of them were used as reference samples and two were used as the main samples as shown in Table 2-7. In the reference emulsions, the water phase (W_2) contained a single emulsifier of either 0.8% OSA, 1.0% WP or 1.0% NaCAS w/w. In the main samples, the external water phase (W_2) contained mixed emulsifiers of either (0.4% OSA/0.5% WP) or (0.4% OSA/0.5% NaCAS) w/w.

Table 2-7. Composition of five different $W_1/O/W_2$ emulsions which were prepared with 50% sucrose solution as the internal aqueous phase and 5% sucrose solution as the external aqueous phase, stabilised with inner emulsifier (PGPR) and five different external emulsifier.

Emulsion type	Emulsion	Weight (%) w/w							
		Emulsifier				Sugar solution		water	
		WP	NaCAS	OSA	PGPR	Oil	5%		50%
Reference	WP	1			0.571	19.429	67.4	8.58	3
Reference	NaCAS		1		0.571	19.429	67.4	8.58	3
Reference	OSA			0.8	0.571	19.428	67.4	8.58	3.2
Main	WP/OSA	0.5		0.4	0.571	19.429	67.4	8.58	3.1
Main	NaCAS/OSA		0.5	0.4	0.571	19.429	67.4	8.58	3.1

These emulsions were processed at controlled temperature below 50 °C, and were subjected to pH measurements immediately after the processing and after 14 days. 50 mL of each emulsion was placed into a glass test tube "100 mm height, 16 mm internal diameter" and creaming stability was measured on day 1, 7 and 14 using creaming index defined by the Equation (2-7).

$$\text{Creaming index (\%)} = \frac{\text{height of serum}}{\text{total height of the emulsion}} \times 100 \quad (2-7)$$

The emulsions were subjected to droplet size measurements using the same method used in single O/W emulsion see section 2.3.2. In addition, the

emulsions were subjected to microstructure investigation using the same method used in single O/W emulsion see section 2.3.4.

2.2.4. Fortification of $W_1/O/W_2$ emulsions with mineral salts

The presence of salts and minerals could affect the properties of $W_1/O/W_2$ emulsions such as stability and pH value. Therefore, taking a close-to-market approach and checking the behaviour of $W_1/O/W_2$ system at the typical levels of mineral salts used in food was tested. $W_1/O/W_2$ emulsions were subjected to stability against coalescence investigations after mineral salts fortification by measuring the mean droplet size using the same method mentioned in section 2.3.2. Measurements were performed at the day 1, 3, 7, 14 and 28 of storage at room temperature (≈ 24.0 °C). The addition of mineral salts to the system was performed according to the procedure of Márquez et al. (2010) with some modification. The amount of mineral salts was chosen according to the nutrient values and weights for edible portions of thick chocolate milk shakes (USDA, 2017a) and thick vanilla milk shakes (USDA, 2017b). Sodium chloride (NaCl), calcium chloride ($CaCl_2$), potassium chloride (KCl) and magnesium chloride ($MgCl_2$) were added to the $W_1/O/W_2$ emulsions either independently or all combined. The same $W_1/O/W_2$ emulsions as used throughout this research were evaluated.

2.2.4. Independent mineral salts fortification

Four mineral salt solutions of either NaCl, $CaCl_2$, KCl or $MgCl_2$ with concentration 0.111, 0.146, 0.224 or 0.016% w/w respectively, were prepared by dissolving these salts in water using a vortex stirrer at 500 rpm

for 30 min. Each mineral salt solution was used to prepare two different sucrose/salt solutions with sucrose concentrations of 5% and 50% w/w, the solutions were stirred at 500 rpm overnight to dissolve the sugar properly. A group of five different $W_1/O/W_2$ emulsions were prepared for each mineral salt alone. Each group of emulsions formulated using the 50% sugar/salt solution in the internal water phase and the 5% sugar/salt solution in the external water phase of each emulsion as shown in Table 2-8. In the external water phases of the first and second emulsions of each group, OSA starch/protein mixed emulsifiers were used, whereas OSA starch alone was used as emulsifier in the third one. WP and NaCAS were used independently as external emulsifiers in the fourth and the fifth emulsion.

Table 2-8. Composition of four groups of $W_1/O/W_2$ emulsions containing sucrose and either sodium chloride, calcium chloride (CaCl_2), potassium chloride (KCl) or magnesium chloride (MgCl_2) in the inner and outer water phase of each group.

Emulsifier			Weight% w/w				NaCl	water
WP	N-CAS	OSA	PGPR	Oil	Sucrose solution			
					5%	50%		
0.500		0.400	0.570	19.428	67.420	8.431	0.111	2.989
	0.500	0.400						2.989
		0.800						3.084
1.00								2.889
	1.00							2.889
CaCl ₂								
0.500		0.400	0.570	19.428	67.420	8.431	0.146	2.954
	0.500	0.400						2.954
		0.800						3.054
1.00								3.854
	1.00							2.854
KCl								
0.500		0.400	0.570	19.428	67.420	8.431	0.224	2.876
	0.500	0.400						2.876
		0.800						2.976
1								2.776
	1							2.776
MgCl ₂								
0.5		0.4	0.571	19.428	67.4	8.43	0.016	3.084
	0.5	0.4						3.084
		0.8						3.184
1								2.984
	1							2.984

2.2.5. Combined mineral salts fortification

A solution of the four mineral salts comprised of 0.111% NaCl, 0.146% CaCl₂, 0.224% KCl and 0.016% of MgCl₂ was prepared by dissolving these mineral salts together in water using a vortex stirrer for 30 min. This solution was used to prepare 5% and 50 % w/w sucrose/salt solutions, which were used in the formulation of the inner aqueous and the outer aqueous phases of five sucrose rich W₁/O/W₂ emulsions. The composition of the different sucrose rich W₁/O/W₂ emulsions fortified with a group of mineral salts in both aqueous phases are shown in Table 2-9.

Table 2-9. Composition of different sucrose rich W₁/O/W₂ emulsions were fortified with a group of mineral salts in both aqueous phases.

Weight % w/w											
Emulsifier					Sucrose solution						
WP	N-CAS	OSA	PGPR	Oil	5%	50%	Sodium chloride NaCl	Calcium chloride CaCl ₂	Potassium chloride KCl	Magnesium chloride MgCl ₂	water
0.5		0.4									2.603
	0.5	0.4									2.603
		0.8	0.572	19.448	67.4	8.43	0.111	0.146	0.224	0.016	2.703
1.0											2.503
	1.0										2.503

The emulsions were kept at room temperature (≈ 24.0 °C) for 28 days in which the particle size data were collected at day 1, 14 and 28 of their storage period. In addition, pH measurements of the W₁/O/W₂ emulsions fortified with the mix of the four mineral salts in the presence of either glucose or sucrose was performed at day 1 and 28 of their storage period. The effect of adding these mineral salts in both aqueous phases of W₁/O/W₂ emulsions on the release encapsulated glucose in the inner phase was investigated during 5

minutes of digestion by α -amylase enzyme using ultraviolet based method as outlined in section 2.3.6.2.

2.3. Analytical methods

2.3.1. Interfacial tension measurements

Interfacial tension measurements were used to confirm the absence of surface active impurities in the purified oil and to determine the ability of the emulsifiers used in reducing the interfacial at oil droplets surface. Purification of sunflower oil was performed by mixing sunflower oil with 4% (w/w) and stirred for 30 min at 600 rpm with a magnetic stirrer (RCT Basic, IKA-Werke GmbH & Co, Staufen, Germany). Afterwards, the magnesium silicate was removed by centrifuging the oil mixture in 50 mL tubes for 30 min at 2700 g (Jouan CR3i multifunction Centrifuge, Thermo Fisher Scientific, USA). The absence of surface active impurities in the purified oil was confirmed by measuring the interfacial tension of the oil against water at 20.0 °C which gave a constant value of $(27.1 \pm 1.7) \text{ mN.m}^{-1}$. The purity remained unchanged during storage at ≈ 24.0 °C in the dark over the experimental period of two weeks. Density of the sunflower oil was 0.91 g/cm^3 at 20.0 °C and for emulsifier solutions, Table 2-10 shows the density of different emulsifier's solutions for which interfacial tension was measured. This data was collected at 20.0 °C using an Anton Paar DMA 5000 density meter[®] (Anton Paar Ltd, Herts, UK).

Table 2-10. Density of different emulsifier's solutions for which interfacial tension was measured.

Sample solution w/w per 80% scale [aqueous phase]		Average density g/cm ³ at 20.0 °C	
0.8 OSA	79.2	Water	1.013±0.000
		Buffer	1.019±0.000
0.5 WP	79.5	Water	1.000±0.000
		Buffer	1.017±0.000
1.0 WP	79.0	Water	1.002±0.000
		Buffer	1.017±0.000
0.5 NaCAS	79.5	Water	1.000±0.000
		Buffer	1.018±0.000
1.0 NaCAS	79.0	Water	1.002±0.000
		Buffer	1.018±0.000
0.4 OSA, 0.5 WP	79.1	Water	1.006±0.006
		Buffer	1.002±0.000
0.4 OSA, 0.5 NaCAS	79.1	Water	1.002±0.000
		Buffer	1.013±0.000

All surface tension and interfacial tension measurements were performed using a drop shape tensiometer (PAT-1, Sinterface Technologies, Berlin, Germany) at 20.0 °C, recording data each second for 600 s after drop formation. Three independent measurements of each sample were performed. The oil phase was placed in a quartz glass cuvette and then a drop of either pure water or aqueous solution was formed in the oil. The choice of drop content depended on the experiment. A drop of 35 mm³ volume was formed inside the oil phase via a straight capillary with an outer diameter of 2 mm.

2.3.2. Droplet size

All emulsions were stored at room temperature ($\approx$ 24.0 °C) and subjected to stability against coalescence investigations. The particle size distributions of the O/W emulsions were acquired with a low angle laser diffraction instrument (Beckman Coulter Particle Sizer LS 13 320, High Wycombe, UK)

fitted with a water dispersion cell (Universal liquid module, Beckman Coulter LS 13 320, High Wycombe, UK). This equipment measures the size distribution of suspended particles using the principles of light scattering and provides a measuring range between 0.04 and 2000 μm (Beckman Coulter, 2003). Measurements were performed at the day 1, 3, 7 and 14 of storage. Each sample was measured at least three times; two replicates in two sample loadings. The samples were continuously flown through the cell during the measurement to ensure homogeneous distribution of the sample through the cell for collecting consistent data. The results are reported as volume based frequency distributions and the volume based mean diameter ($d_{4,3}$) as obtained from the equipment software. Data was analysed using the instrument's software, where three diffraction patterns were averaged and analysed based on the refractive index of 1.333 for water.

2.3.3. Zeta potential measurements

Zeta potential (ζ -potential) of the oil droplets was also measured to infer stability hypothesising that certain values mean electrostatic repulsion thus stability. A zeta sizer (Delsa™ Nano Submicron Particle Size and Zeta Potential Particle Analyzer, High Wycombe, UK) was used for measuring zeta potential. Data was analyzed using the instrument's software (Delsa™ Nano UI Software Versions 3.73). ζ -potential and pH values were measured for O/W emulsions consisting of 20% oil and also measured in their aqueous phases alone, the aqueous phase of these emulsions was either water or 0.1M, pH 7 sodium phosphate buffer, measurements performed at on day 1 of the storage period at 25.0 °C. Dilution for the sample was 2 droplets of

the sample in 40mL of water ($\approx 1:100$). The calibration solution was prepared by adding 2 droplets of Coulter Latron 300Ls in 40ml distilled water. Zeta potential with any mineral salts fortification. Since zeta potential provides information about the droplet surface, stability and aggregation processes, it was measured for solutions containing the single emulsifiers; OSA starch, WP and NaCAS. Zeta potential was also measured for the emulsions stabilised with these single emulsifiers, for solutions of OSA starch/WP and OSA starch/NaCAS and emulsions stabilised with these mixed emulsifier systems. The zeta potential of each sample was measured at least three times and the results are reported as mean and standard deviation.

2.3.4. Emulsion microstructure

2.3.4.1. Optical microscopy

Optical micrographs were taken to inspect for flocculation and consistency. Micrographs were captured immediately and 1, 3, 7 and 14 days after the preparation of each single emulsion. A digital inverted transmission light microscope (EVOS fl, Life Technologies Ltd., Paisley, UK) fitted with a 20x bright field, long working distance objective (AMEP4624, Life Technologies Ltd., Paisley, UK) was used for this microscope analysis.

2.3.4.2. Confocal laser scanning microscopy

A Confocal Laser Scanning Microscope (Zeiss LSM880, Carl Zeiss Microscopy GmbH, Jena, Germany) was used at 20.0 °C to validate the presence of protein and starch at the interface of O/W emulsions. The microstructure was visualized following staining with Nile Red (0.01 g/L in polyethylene glycol),

Fast Green (0.1 g/L in water) and Fluorescein isothiocyanate (FITC) (1g/L in water). Protein was stained with Fast green which excited at 633 nm and absorbed light at 671-677 nm (Kaláb et al., 1995). The oil was stained with Nile red excitation 488-530 nm and emission 575-580 nm (Rumin et al., 2015).

Two O/W emulsions stabilised with the mixed emulsifier systems, either OSA/WP or OSA/NaCAS were prepared, the composition of these emulsions is shown in Table 2-11. A drop of each emulsion was placed onto a glass slide. Afterwards, the drop was carefully mixed at a ratio of 1:2 with Nile Red, Fast Green solution and FITC using a pipette tip followed by sliding over a glass cover slip. The samples were then imaged with the CLSM using an Argon laser which is excited at wavelength 488 nm and with a He-Ne laser which is excited of wavelength 561 and 633 nm. A band pass filter between 512-554 nm was selected for the FITC detection; when excited at 494 nm, 651-710 nm for the Fast Green detection; when excited at 633 nm and 580 - 620 nm for the Nile Red detection; when excited at 488 nm. Images were acquired using a 20x and 75x objectives at 400 scans per min; these were then line averaged over 8 scans. Following the procedure reported by Spyropoulos et al. (2010) samples were stained 24 h prior to inspection to enhance the contrast in the micrographs.

Table 2-11. Composition of two different O/W emulsions were prepared for confocal laser scanning microscopy.

Emulsion	Weight (g/100g)			Oil	water	Stains
	Emulsifier					
	WP	NaCAS	OSA			
WP/OSA	0.5		0.4	20	79.1	Nile red + Fast green+ FITC
NaCAS/OSA		0.5	0.4			

2.3.5. Interfacial composition of mixed OSA/protein interfaces

The interfacial composition of O/W emulsions stabilised with mixed emulsifier systems was inferred from compositional analysis of the continuous phase pre and post emulsification, in which protein and starch quantifications were included in the analysis.

2.3.5.1. Quantification of protein

Protein concentration at the droplet surface was determined using the method reported by Koliandris (2010), in which the bicinchoninic acid (BCA) Protein Assay Kit (Pierce Protein, Thermo Scientific, Rockford, USA) was applied. Samples of O/W emulsions that were stabilised with OSA/WP or OSA/NaCAS mixed emulsifiers were centrifuged to separate any oil droplets from the serum because the kit is not working properly in presence of lipids. Additionally, the presence of hydrophobic ligands could affect the way by which proteins interact with the reactants of the kit. Samples were poured into Eppendorf tubes and centrifuged using a micro centrifuge at 13,000 g for 10 min at 20.0 °C (Heraeus Fresco 21, Thermo-Fisher, Neots, UK). The quantification of protein using the BCA assay was performed following the instructions in the kit. Samples were diluted 1:100 with distilled water prior to the assay to be in the linear working range of the assay (20-2000 µg/mL) where the anticipated protein concentration is in this range. The reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) and Reagent B (containing 4% cupric sulfate) were mixed as indicated in the kit (50:1). 2 mL of the reagent mixture was added to 100 µL of serum in a transparent cuvette with 1 cm

light path and a final volume of 2.27 mL and incubated at 37.0 °C for 30 min. The absorbance at 562 nm was then measured within 60 min of the end of incubation using a spectrophotometer (Genesys 10S Vis Spectrophotometer, USA). The protein was determined based on the obtained standard curve as shown in Figure 2-9.

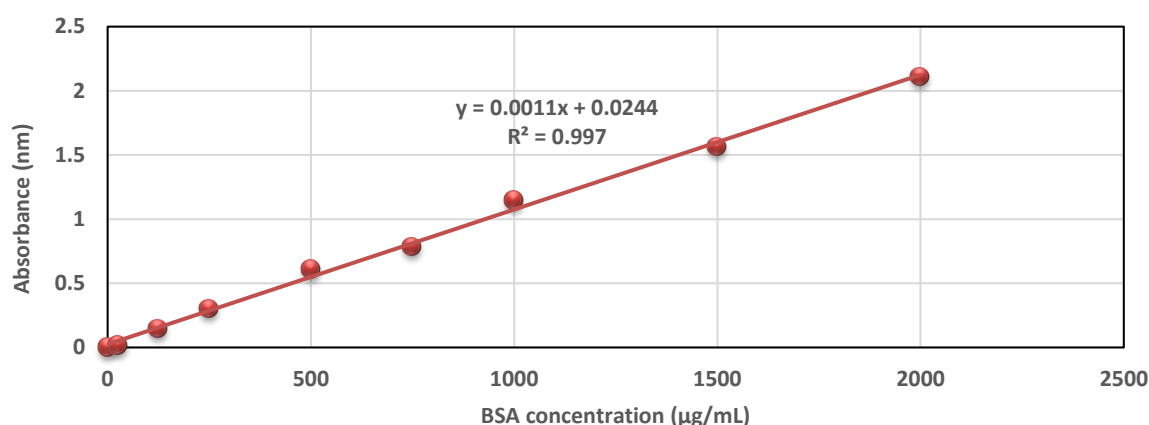


Figure 2-9. Standard curve used for proteins determination in emulsions serum.

2.3.5.2. Quantification of starch

A standard published protocol (AOAC Method 996.11, Megazyme International Wicklow, Ireland) was followed to measure the total starch in the continuous phase pre and post emulsion homogenisation. The principle of this method is that the starch is hydrolysed by α -amylase to maltodextrin by incubation in a medium of pH 5.0 sodium acetate buffer at 100°C for 6 minutes. Afterwards, maltodextrin is hydrolysed by amyloglucosidase to D-glucose by incubation in pH 5.0 acetate buffer at 50°C for 30 minutes (Megazyme-International, 2015).

Preparation of reagent solutions and sodium acetate buffer was performed following the instructions in the assay kit. The Megazyme total starch assay required the initial preparation of sodium acetate buffers (2.0M, pH 5.0). Acetic acid (2.0M) was used for the sodium acetate buffer preparation. The sodium acetate buffer was prepared by adding 11.8 mL glacial acetic acid to 900 mL of distilled water. The pH was then adjusted to 5.0 by the dropwise addition of 1M sodium hydroxide and the volume was adjusted to 1 L.

The procedure for the liquid samples was as follows: 2 mL of the serum was centrifuged twice at 13'000 g for 10 minutes at 20.0 °C, and then 1 mL of the serum was transferred to a 15 mL tube with 8 mL of ethanol (95% v/v) and centrifuged at 1800 g for 10 minutes at 20.0 °C. After that the supernatant was discarded and 3.9 mL of pH 5.0 sodium acetate buffer was added to the pellet and mixed using a Vortex mixer until the pellet was released from the bottom of the tube, and then 0.1 mL of amyloglucosidase, which was supplied in the kit, was added. The mixture was then immediately incubated in boiling water maintained at 100 °C for 6 min while mixing twice.

Standardised regular maize starch sample was provided in the kit containing 96% starch based on dry weight and used as control. The procedure for measuring starch in the control sample (procedure for dry sample) involved mixing 1 mg of sample, 0.2 mL of ethanol (80% v/v) and 3 mL of thermostable α -enzymes solution together and then incubating the mixture for 12 minutes at 100°C while mixing four times using a Vortex mixer during this period of incubation. After that 0.1 mL of the amyloglucosidase supplied was added.

The samples obtained from both the control and sample procedures were incubated in a water bath at 50 °C for 30 min while mixing six times using a Vortex mixer during this period of incubation. After that the samples were transferred to a 100 mL volumetric flask and the volume was made up by adding water. 15 mL of each sample was centrifuged at 2300 g for 10 min at 20.0 °C and then 0.1 mL of each sample was mixed with 3 mL of GOPOD reagent buffer and the resulting mixture was incubated at 50.0 °C for 20 min. After the incubation, the absorbance was measured a spectrophotometer (Genesys 10S Vis Spectrophotometer, USA) at 510 nm. Afterwards, from the measured absorbance values, the concentration of starch was identified using Mega-Clactm software (Megazyme-International, 2016) giving the total starch determination for the control and the liquid samples. Moisture content in samples of OSA starch, WP and NaCAS was measred,these samples were weighed and dried in a oven (Sanyo electric biomedical Co. Ltd, Osaka, Japan) at 105°C/20hour. After that left for 30 min before they were weighted again. The mositure was calculated in the Equesion 2-8 as follows.

$$\text{Moisture \%} = 100 - [(\text{dried sample weight} / \text{fresh sample weight}) * 100] \quad (2-8)$$

2.3.6. Measurements of encapsulation efficiency in $W_1/O/W_2$ emulsions

The encapsulation efficiency (EE) of sugar in the $W_1/O/W_2$ emulsions was defined by Vladislavljević et al. (2014) and Zhang and Reineccius (2016) as follows: the EE as the mass of a component (X) entrapped in the double

emulsion droplets divided by the total mass of this component added to the inner water phase. Therefore, EE was calculated by Equation (2-9) as follows:

$$EE (\%) = [(\text{mass of sugar added} - \text{mass of sugar released}) / \text{mass of sugar added}] * 100 \quad (2-9)$$

Each 100 g of $W_1/O/W_2$ system contained 7.66g sugar in total, 4.29 of them was encapsulated sugar in the core of emulsion whereas 3.37 was the mass of sugar was added into the outer aqueous phase of the emulsion during processing see section 2.2.3. The mass of sugar release is $(X - 3.37 \text{ g})$, where (X) is the mass of sugar in outer aqueous phase measured by the present LCMS method, if the value of (X) was negative this means the sugar diffused from the external aqueous phase to the internal aqueous phase. EE was measured on day 1 and day 14 of the $W_1/O/W_2$ emulsion storage period, and it was inferred from the external aqueous phase W_2 analysis. If sugar that was encapsulated in the W_1 phase was not detected in the W_2 phase, it would mean that it was still encapsulated in the internal aqueous phase W_1 . Encapsulation efficiency (EE) of sugar in the multiple emulsion was measured using two different methods; a liquid chromatography mass spectrometry (LCMS) based method and an ultraviolet (UV) absorption based method (Megazyme, 2017). The UV absorption based method was necessary to validate the results obtained from the LCMS based method.

2.3.6.1. Liquid chromatography mass spectrometry based method

Samples were analysed using high-performance liquid chromatography (Agilent 1100- Japan) connected with a mass spectrometer Quattro Ultima unit VB250 (Micromass UK Limited). A column (00G-4378-B0, 5 μ m LC - Phenomenex, UK) was used with a mobile phase gradient of 80% w/w acetonitrile (C₂H₃N) and 20% w/w water. The flow rate was 0.7 mL/min. The software of the mass spectrometer was programmed to detect the molecular mass of 341.39 g/mol, which is the molecular weight of the sucrose, at 20.0 °C. Nevertheless, it can be a potential error that some of the sucrose that was detected with this method could be came from the nature amylase activity on starch. Two sucrose free emulsions were prepared in which water alone was used as the internal and external phases. These emulsions were made and stabilised as outlined in Table 2-7 for the two 'main' emulsions.

2.3.6.1.1. Preparation of calibration solutions

To obtain the calibration curve for measuring EE of sucrose in the inner aqueous phase of W₁/O/W₂ emulsion a sucrose solution was prepared by mixing 2 g of water, 9 g of the 5% w/w sucrose solution and 1 g of 50% w/w sucrose solution. Consequently, each 12 g of this sucrose calibration solution contained 0.95 g of sucrose. This solution was diluted as follows; 12 g of the solution and 12 g of the sucrose-free W₁/O/W₂ emulsion stabilised with either OSA/WP or OSA/NaCAS as hydrophilic emulsifier system was mixed by hand in a 50 mL glass tube for 15 seconds. Immediately after mixing, 5 g of each mixture was transferred to a 15 mL plastic tube with 2.5 g of distilled water. The tubes were mixed by inverting them twice and then they were

centrifuged at 756 g for 2 min at 20.0 °C to separate the oil droplets from the serum. Afterwards, 0.5 mL of each serum phase was diluted with 9.5 mL of water in a 15 mL plastic tube, and then manually mixed for 15 seconds to give a stock calibration solution, and then the dilution procedure outlined in Table 2-12 was followed.

Table 2-12. Dilutions of a stock calibration solution

Calibration solution no.	Content
1	2.00 mL of the stock calibration solution + 8.00 mL water
2	1.50 mL of the stock calibration solution + 8.50 mL water
3	1.00 mL of the stock calibration solution + 9.00 mL water
4	0.50 mL of the stock calibration solution + 9.50 mL water
5	0.25 mL of the stock calibration solution + 9.75 mL water

Each calibration solution content was then passed through a Millex filter unit (MF-Millipore™ Membrane - Cork, Ireland) before transferring to a 2 mL certified screw thread vial in which the samples were analysed. All solutions were analysed using liquid chromatography mass spectrometry (LCMS). The LCMS detected the molecular mass of 341.39 g/mol as shown in Figure 2-10, which is the molecular weight of sucrose.

Function:1 SIR

Mass (m/z)	Dwell (Secs)	Cone (Volts)
341.39	0.150	40.00
503.40	0.150	40.00

Method

Ionization Mode: ES-

Inter-Channel Delay: 0.02

Inter-Scan Delay: 0.1

Repeats: 1

Span: 1

☐ Use Tune Cone Settings

Retention Window (Mins)

Start: 0

End: 11.7

APCI Probe

☐ Use Tune Page Settings

Probe Temp: 100

Buttons: Add, Change, Delete, Clear All, OK, Cancel

Figure 2-10. Mass spectrometry method for sucrose detection in the external aqueous phase of $W_1/O/W_2$ emulsion, where the mass of 341.39 is the molecular weight of sucrose

The concentration of sucrose was presented in the LCMS data as an area under the curve (AUC). The AUC values were related to their known concentrations using excel software to plot the calibration curve. Two independent calibration curves were acquired for EE measurements on day 1 and day 14. Samples of $W_1/O/W_2$ emulsions were prepared for measurements as follows.

2.3.6.1.2. Preparation of samples solutions

Five sucrose-rich $W_1/O/W_2$ emulsions were prepared described in Table 2-7. To prepare the sample solutions; 12 g of sucrose-rich $W_1/O/W_2$ emulsion

samples was added to 12 g of distilled water in a 50 mL glass tube and mixed by hand, to ensure a homogeneous distribution of the sugar in the sample. The water was added because the emulsions were highly viscous and this would aid in the centrifugation step. 5 g of this homogenous mixture was transferred into a 15 mL plastic tube and 2.5 mL of water was added, again the content was manually mixed. The addition of 2.5 mL of water was used for LCMs in order to reduce the standard deviation between the samples as the dilution was 300 times. The same procedure was performed on three replicates of each type of the five different emulsions. All plastic tubes were centrifuged at 756 g for 1 min at 20.0 °C. This speed was chosen so that the oil droplets would be separated from the serum but to avoid the breakdown of emulsion droplets by the forces of centrifugation. 1 mL of the serum phase was collected into an Eppendorf tube using a syringe to reduce any oil contamination of the serum.

For sample serum dilution, 0.5 mL of each emulsion serum was transferred into a 15 mL plastic tube and then 9.5 mL of distilled water was added. The content of each tube was mixed for 15 seconds by hand and then 2 mL of each homogenised solution was transferred to a 15 mL tube with 8 mL of distilled water. Again, tubes were mixed for 15 seconds to make a sample solution of each emulsion. The same procedure was repeated to produce three replicates of each sample. Each solution was passed through a Millex filter (MF-Millipore™ Membrane - Cork, Ireland) before collection in a 2 mL certified screw thread vial. Sucrose was measured by injecting 5 µL of the emulsion serum into the HPLC system where it was eluted at a flow rate 0.7mL/min at 20.0°C. A mobile phase consisting of 20% w/w distilled water and 80% w/w acetonitrile (C₂H₃N) was used as the ion-exchange guard. An

analytical column (00G-4378-B0, 5 μ m LC -Phenomenex, UK) was used as the stationary phase. An instrumental procedure for identifying the chemical characteristics of the sucrose using the separation of gaseous ions depending on their differing mass and charge was applied by the mass spectrometer. All peaks were integrated by the LCMS computer software. Area under the curve (AUC) of molecular mass of 341.39 g/mol was exported to the excel software. Sugar concentration in the sample was calculated from a standard curve generated from six levels of sucrose 0, 200, 400, 600, 800 and 1000 μ g/mL. Results were thus based on quantification of sucrose corresponding to the retention times of these linear sucrose concentrations.

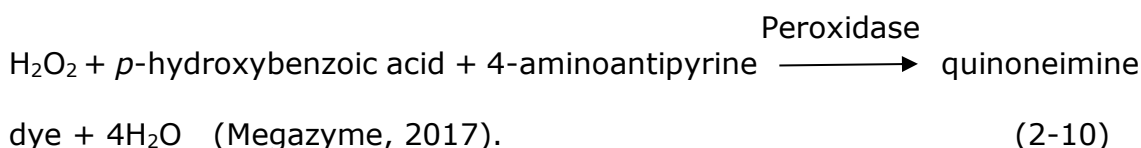
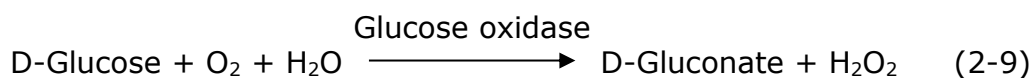
2.3.6.2. Encapsulation efficiency measurements based on UV absorption

The aim of this experiment was to validate the results of the encapsulation efficiency (EE) in W₁/O/W₂ emulsions that were obtained from the liquid chromatography mass spectrometry based method. The EE was analysed in this experiment via an enzymatic digestion assay followed by an ultraviolet (UV) absorption based method. A Megazyme total starch assay kit was used (Megazyme, 2017). Glucose was used in both inner and outer aqueous phases during the formulation of W₁/O/W₂ emulsion instead of sucrose because the final part of the Megazyme total starch assay kit is specifically designed to measure glucose. Other than replacing sucrose with glucose, the emulsions were prepared following the same method see section 2.2.3. Nevertheless, it can be a potential error that the starch assay kit will measure digested starch from naturally present amylase.

The total starch assay kit contains six bottles as follows; bottle 1 and 2 are thermostable α -amylase and Amyloglucosidase, respectively. These enzymes were not used because the procedure started with glucose determination and not starch, therefore, only the final part of the Megazyme starch assay kit was used in this method involving "bottle 3, 4, and 5". The final part of the assay kit includes bottle (3) with glucose oxidase peroxidase (GOPOD) reagent buffer (50mL, pH 7.4) containing of *p*-hydroxybenzoic acid plus sodium azide (0.095% w/v) as a preservative. Bottle (4) contained reagent enzymes containing of glucose oxidase plus peroxidase and 4-aminoantipyrine. Bottle 5 was D-glucose standard solution (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid and was used to assess whether the time for the GOPOD reagent to make maximum colour formation with 100 μ g of D-glucose standard solution was 15 min. Bottle 6 is standardised regular maize starch control, which was not used.

The reagent suspensions prepared as follows contents of bottle 3 were diluted in 1 L of distilled water and then 20.0 mL of this solution was used to dissolve the contents of bottle 4. This solution was then quantitatively transferred to the remainder of solution in bottle 3. The bottle was then covered with aluminium foil to protect the reagent from light. The preparation of the determination reagent, GOPOD, was now complete and the absorbance of this solution was less than 0.05 when it was read against distilled water at a wavelength 510 nm using a spectrophotometer (Genesys 10S Vis Spectrophotometer, USA). The time for the GOPOD reagent to maximum colour formation with 100 μ g of D-glucose standard solution (bottle 5) was about 15 min. The reaction for glucose concentration determination is as follows. D-glucose is oxidised to D-glucose-6-phosphate with the release of

one mole of hydrogen peroxide (H_2O_2), which is quantitatively identified in a colorimetric reaction using peroxidase and the creation of a quinoneimine dye as shown in the following chemical Equations (2-9) and (2-10) as follows:



2.3.6.2.1. Procedure

Duplicate aliquots (0.1 mL) of the diluted sample serum were transferred to the bottom of glass test tubes (16 x 100 mm) and 3.0 mL of GOPOD reagent were added to each tube. The tubes were incubated at 50 °C for at least 20 min and then the samples were placed in a transparent cuvette. The absorbance for each sample was read at 510 nm against the reagent blank at temperature ≈ 25.0 °C within 60 min using a spectrophotometer (Genesys 10S Vis Spectrophotometer, USA). The reagent blank consisted of 0.1 mL distilled water plus 3.0 mL GOPOD reagent. Three independent data points for each concentration were acquired and a graph showing absorbance versus glucose concentration was plotted.

The calibration curve was acquired by preparing a stock solution of 10 % w/w glucose which was then diluted to smaller concentrations. The stock solution was prepared by dissolving 10 g of sugar in 90 g of water using a magnetic stirrer at 500 rpm for 30 min. In a 100 mL volume flask, 1 mL of the mother

solution and 99 mL of water was mixed by a Vortex stirrer at 500 rpm for 2 minutes. In 25 mL volume flasks, 0, 5, 10, 15, 20 and 25 mL of this solution was independently placed, afterwards the flasks filled by 25, 20, 15, 10, 5 and 0 mL of water, respectively. Each solution was mixed using the Vortex stirrer for 2 min to produce five concentrations of glucose; 0, 200, 400, 600, 800 and 1000 $\mu\text{g/mL}$. These solutions were used to calculate the calibration curve for measuring glucose concentration in aqueous solution. Each glucose solution was diluted by 1:100 in order to work with the Megazyme Assay kit. 0.1 mL of each was transferred to glass test tubes (16 x 100 mm) and then 3 mL of the determination reagent GOPOD was added and then each solution was analysed using the same procedure of the samples analyse described at beginning of the section.

2.3.6.2.2. Control emulsions preparations

To assess whether the last part of the Megazyme Assay kit can correctly identified a concentration of glucose in the serum of an emulsion, triplicates of three different O/W emulsions, stabilised with either OSA, WP or NaCAS, were prepared. 5% glucose solution was used as the aqueous phase of each emulsion. 2 mL of each emulsion was put in an Eppendorf tube and centrifuged in a micro centrifuge (Heraeus Fresco 21, Thermo-Fisher, and Waltham, USA) at 1000 g for 1 min at 20.0 °C. Afterwards, 1 mL of the serum was put in a new Eppendorf tube and centrifuged again at 13000 g for 10 min at 20.0°C. The two step method was used to avoid serum contamination by oil droplets. 0.1 mL of serum plus 9.9 mL of distilled water was mixed by a Vortex mixer for 2 min. 100 μL of each serum phase was transferred to glass test tubes (16 x 100 mm) and then 3 mL of the determination reagent

GOPOD was added. A reagent blank consisting of 0.1 mL distilled water plus 3.0 mL GOPOD reagent was used during measurements. The serum phase was incubated and analysed following the procedure described in section 2.3.6.2.

2.3.7. *In-vitro* assay for measuring sugar release in double emulsions

The aim of this experiment was to measure the release of encapsulated sugar from the internal aqueous phase to the external aqueous phase after *in-vitro* enzymatic digestion of the $W_1/O/W_2$ emulsions. The principle of the method was to digest starch in the external interface of $W_1/O/W_2$ emulsion by the α -amylase enzyme leading to a partial breakdown of the $W_1/O/W_2$ emulsion and the release of sugar from the internal aqueous phase to the external aqueous phase. The release was analysed according to the procedure of Chiu et al. (2017) with a slight modification. The quantity of sugar released was inferred from the outer aqueous phase analysis during a period of digestion as a function of time. Increasing level of sugar detected in W_2 indicated that it was released from the internal aqueous phase, W_1 .

Sodium phosphate buffer was prepared as follows: 4.68g of sodium phosphate dibasic anhydrous (NaH_2PO_4) was placed in a 1000 mL volumetric flask. Afterwards, 8.662g of sodium phosphate monobasic (Na_2HPO_4) was added to the flask and then the volume made up to 1 litre with distilled water. The content of the flask was mixed at 500 rpm for 30 minutes using a magnetic stirrer to give 1 litre of sodium phosphate buffer (0.1M, pH 7.0), afterwards this buffer was diluted 10 times getting sodium phosphate buffer with less molarity (0.01M, pH 7.0).

Porcine salivary α -amylase enzyme solution was prepared by suspending porcine pancreatic α -amylase in the (0.01M, pH 7.0) sodium phosphate buffer under continuous stirring at the ratio of 1:99 w/w according to the procedure of Miao et al. (2014) with a slight modification. The enzyme was dissolved and was activated by placing the beaker containing the mixture in a water bath at 37 °C and using a magnetic stirrer set at 500 rpm for 10 minutes, followed by centrifugation for 10 min at 1500 g at 20.0 °C. Afterwards, 54.0 mL portion of the supernatant was transferred into a fresh beaker and kept at 37 °C until used in the digestion procedure. Silver nitrate (AgNO_3) solution with concentration 4 % w/w was prepared by dissolving silver nitrate crystals in water at the ratio of 4:96 w/w using a magnetic stirrer at 500 rpm for 30 min. Preparation was performed in a dark room and the ready solution was transferred to a bottle completely covered with aluminium foil and kept in a dark room. The solution was used to inactivate the α -amylase enzyme after digestion.

2.3.7.1. Digestion and sugar release

Fresh $W_1/O/W_2$ emulsions were prepared see section 2.2.3 for an enzymatic digestion of the interfacial starch of these emulsions and release of sugar measurements. A water bath was set up on a magnetic hotplate stirrer at 500 rpm at 37 °C and a timer was set up for monitoring the time of digestion. Samples of $W_1/O/W_2$ emulsion were mixed by hand and then distributed into glass tubes, 10 g to each tube. A small magnetic stir bar was added to each tube. The silver nitrate solution was placed in 15 mL plastic tubes, 2.5 mL in each tube, in a dark room. These tubes had been covered by aluminium foil

to protect silver nitrate from exposure to the light. The silver nitrate was to be used for stopping the digestion by inhibiting the reaction between the enzyme and OSA starch.

10 mL of α -amylase enzyme solution with activity (calculated from supplier information and the amount used) of 10 units per mL was added, to each glass tube, whilst stirring, and timing was started when the enzyme was added, to monitor the release of sugar. Immediately after addition of the enzyme to the first glass tube, 5 mL of the mixture were withdrawn and transferred to the first tube of silver nitrate tube to stopping the reaction of digestion. In the second tube, the digestive reaction was left for 30 s before transferring 5 mL of the mixture to the second silver nitrate tube. This procedure was performed on all other samples with an increase in digestion time to 60, 90, 120, 150, 180, 210, 240, 270 and 300 s.

After stopping the reaction, the plastic tubes were centrifuged at 1000 g for 1 min at 20.0 °C. The aluminium cover was removed and 2 mL of the serum in each tube was drawn independently into an Eppendorf tube using a syringe and then the Eppendorf tubes were immediately centrifuged at 13'000 g for 2 min at 20.0 °C using a micro centrifuge (Heraeus Fresco 21, Thermo-Fisher, and Waltham, Germany). 1 mL from each serum sample was transferred into new Eppendorf tube and centrifuged again under the same conditions to reduce any contamination by oil droplets.

The emulsions' serum was then diluted by transferring 0.5 mL of each serum into a 15 mL plastic tube and mixed with 9.5 mL of water for 2 minutes using a vortex mixer. 2 mL of each homogenised solution was transferred to another 15 mL tube and mixed with 8 mL of water for 2 minutes to make the sample solution. The amount of sugar released was defined by Chiu et al.

(2017) as the percentage of sugar relates to the external aqueous phase using Equation (2-12) as follows.

$$\% \text{ released sugar} = \frac{[(\text{mass of sugar in the outer aqueous phase} - X) / (\text{mass of sugar was encapsulated in the inner aqueous phase})] * 100}{12} \quad (2-12)$$

Where (x) is the mass of sugar that was originally present in the external aqueous phase of $W_1/O/W_2$. At least three replicates of each type of emulsion and two measuring methods were used to identify the level of the sugar in the serum. Glucose release from the inner to the outer aqueous phases of the $W_1/O/W_2$ emulsions after digestion at different periods of time was measured. This level of either sucrose or glucose after digestion and release was measured via LCMS based method and the UV absorption based method outline see sections 2.3.6.1 and 2.3.6.2.

2.3.8. Statistical analysis

Statistical analysis was performed to identify if there were any statistically significant change in the droplets' diameter in a period of two weeks storage at the room temperature ($\approx 24.0^\circ\text{C}$). Mean droplet diameter and standard deviation are reported based on the measurements of three independent replications of each emulsion. Data was analysed using statistical tests; one way Analysis of Variance (ANOVA) and Bonferroni multiple comparison. The level of significance for both statistical tests was set at $p = 0.05$. Tests were performed using a statistics software (SPSS, IBM, Version 24 - USA).

Chapter 3: Results and Discussion - Single Emulsions Stabilised with Single Emulsifier

3. Single emulsions stabilised with single emulsifiers

The aim of the research was to answer the research question of how much starch can be replaced by protein at the interface to still obtain oral breakdown. In addition, if whether there was any impact of the starch replacement on the release of encapsulated sugar solution or not. The research in this chapter started with using starch alone to stabilise oil-in-water (O/W) emulsions and establishing a processing protocol. A low starch concentration was preferred for given the overall aim of the research to contribute to sugar reduction. In addition, whey protein and sodium caseinate were independently used to stabilise O/W emulsions using the same formulation conditions as were used in OSA stabilised emulsions. The research focused on droplet size and stability against coalescence over storage of O/W emulsions stabilised with single emulsifiers either OSA starch, whey protein or sodium casenate. The initial data on droplet size and stability was plotted as a function of processing parameters, and then processing conditions were selected to optimise the starch concentration in the system. The focus was on the quantity of each emulsifier was needed, in which data was plotted as function of concentration ratios. The temperature was controlled during the processing as well as the change in pH over storage was observed because temperature and pH value are essential parameters affecting the emulsification ability of an emulsifier and emulsion stability e.g. starch gelatinisation at 55-80°C and WP denaturation at 70°C.

3.1. Unbuffered aqueous phase

3.1.1. OSA starch stabilised emulsions

A closely packed layer of starch at the interface is needed to stabilise an oil-in-water (O/W) emulsion (Yusoff and Murray, 2011). However, the hypothesis of this research is that O/W emulsions do not require a closely packed layer of starch at the interface to breakdown in the oral cavity. Therefore, some of the interfacial starch can be replaced by protein to keep the emulsion stability as well as to get easier oral break down of the emulsion. The aim of this experiment was to find out the minimum concentration of starch to be applied in the next processes. Therefore, the concentration of starch was chosen to be as low as possible to stabilise these emulsions at the constant processing conditions used.

Figure 3-11 shows the volume based mean droplet size ($d_{4,3}$) of a group of O/W emulsions stabilised with gradually decreasing concentration of OSA starch. This group of emulsions containing 20% sunflower oil and 80% aqueous phase was prepared. The aqueous phase of these emulsions was a solution of OSA starch with starting concentration at 4% w/w of OSA and the concentration was decreased stepwise by 0.5% w/w. Therefore, the first aqueous phase was a solution containing 3.2 g of OSA starch and 76.8 g see section 2.2.1. The stability against coalescence within the emulsion was observed by measuring the mean droplet size at days 1, 7 and 14 of emulsions shelf life when they was stored at ambient temperature ($\approx 24.0^\circ\text{C}$). No evidence of droplet coalescence because there were no statistically significant differences in the droplet size at different time points in all emulsions stabilised with OSA concentrations equal or bigger than 0.8% w/w. In addition, increasing concentration of OSA led smaller droplet size

therefore, the droplet size decreased from 8 μm at concentration of 1.2% OSA w/w to 4.7 μm at a concentration of 3.2% OSA w/w. Therefore, at the processing conditions used, 0.8% w/w of OSA starch was enough to keep the O/W emulsion stable against coalescence during a 14-day storage period.

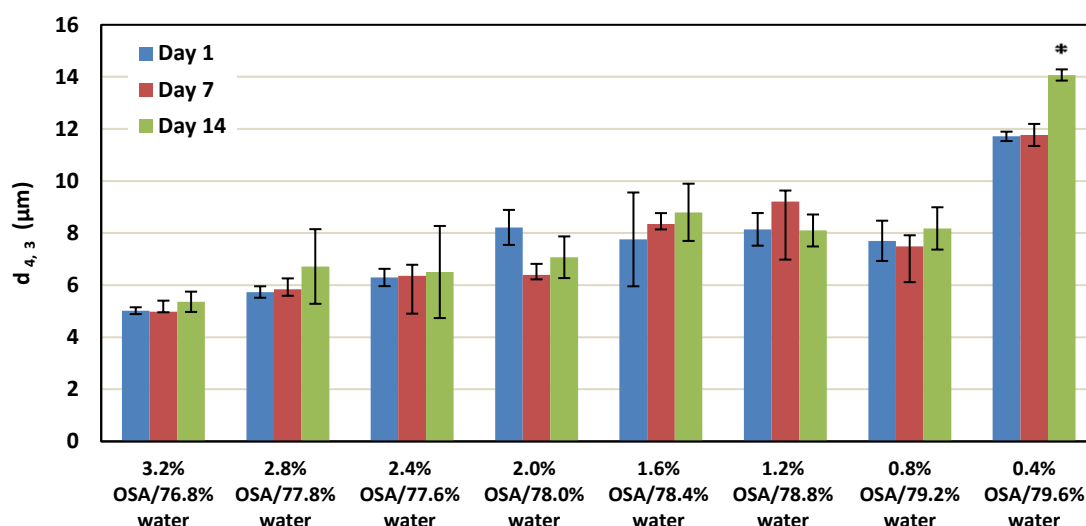


Figure 3-11. Volume based mean droplet size for a group of O/W emulsions containing 20% w/w oil phase, the aqueous phases 80% w/w were solutions comprising of gradually decreasing concentration; 3.2, 2.8, 2.4, 2.0, 1.6, 1.2, 0.8 and 0.4% w/w, respectively, of OSA starch as emulsifier. Data are means of three replicates ($n=3$) $\pm$ standard deviation. * = significant difference in ($d_{4,3}$) between days ($p < 0.05$).

All O/W emulsions with 20% w/w oil dispersed phase were stable against coalescence if they were produced at a concentration of $\geq 0.8\%$ w/w of OSA starch. However, these emulsions with concentration 0.4% w/w of OSA starch were not stable against coalescence. Among the OSA concentrations used, there was gradual decrease in the main droplet size with the increase of the starch concentration. The ability for OSA starch to stabilise O/W emulsions for a long period had been reported in many previous studies e.g. Miao et al. (2014) and Chiu et al. (2017) used OSA to stabilise this emulsion

over 30 days. Therefore, in this research a 14-day period of storage was considered enough for the research purpose especially at early stages of the research. Some of the error bars were big therefor, to identify whether there were any statistical differences in the mean droplet size measurements between days in each emulsion or not, one-way ANOVA and a subsequent Post Hoc statistical test 'Tukey' were applied. The statistical test proved there was no significant differences ($p > 0.05$) between days for each concentration $\geq 0.8\%$ w/w OSA starch as shown in Table 3-13, which confirmed that these concentrations can produce stable O/W emulsions with 20% dispersed oil phase.

Table 3-13. P-values obtained from one-way ANOVA statistical test between days for each OSA starch concentration that were used as emulsifiers.

Concentration%	P-value
3.2	0.17
2.8	0.36
2.4	0.31
2.0	0.19
1.6	0.88
1.2	0.99
0.8	0.71
0.4	0.00

The statistical analysis data for the effect of OSA concentrations at each of the three time points measured, calculated on mean droplet size measurements are shown in Table 3-14.

Table 3-14. The statistical analysis data for the effect of OSA concentrations at each of the three time points measured, calculated on mean droplet size measurements. Groups with different superscript letters indicate to statistical significance within the same column ($p < 0.05$).

Concentration% w/w	Mean $\pm$ STD		
	Day 1	Day 7	Day 14
0.4	11.72 $\pm$ 0.17 ^{a,d}	11.77 $\pm$ 0.42 ^b	14.07 $\pm$ 0.22 ^{d,e}
0.8	7.70 $\pm$ 0.77 ^{a,d}	7.49 $\pm$ 1.38 ^b	8.18 $\pm$ 0.81 ^{d,e}
1.2	8.14 $\pm$ 0.62 ^{a,d}	9.21 $\pm$ 2.23 ^b	8.10 $\pm$ 0.61 ^{d,e}
1.6	7.76 $\pm$ 1.8 ^a	8.34 $\pm$ 0.20 ^{b,c}	8.80 $\pm$ 1.1 ^{c,e}
2.0	8.21 $\pm$ 0.67 ^{a,c}	6.39 $\pm$ 0.17 ^{a,c}	7.07 $\pm$ 0.80 ^{b,e,f}
2.4	6.30 $\pm$ 0.33 ^a	6.36 $\pm$ 1.45 ^a	6.51 $\pm$ 1.76 ^{a,b,f}
1.2	8.14 $\pm$ 0.62 ^{a,d}	9.21 $\pm$ 2.23 ^b	8.10 $\pm$ 0.61 ^{d,e}
2.8	5.74 $\pm$ 0.22 ^{a,b}	5.84 $\pm$ 0.24 ^a	6.72 $\pm$ 1.43 ^{a,b}
3.2	5.02 $\pm$ 0.13 ^a	4.98 $\pm$ 0.02 ^a	5.36 $\pm$ 0.39 ^a

For the concentration of 0.4 % w/w, there were significant differences between days ($p < 0.05$). A subsequent Post Hoc statistical test 'Tukey' was used to identify which of the days showed the significant differences, which proved significant differences between day 1 and 14, and also, between day 7 and 14 as shown in Table 3-15. Consequently, using OSA concentrations $\leq$ 0.4 % w/w cannot lead to stable O/W emulsions that comprise of a 20% oil dispersed phase under the experiment conditions used. Therefore, the OSA concentrations $\leq$ 0.4 % w/w were excluded in this research and 0.8% OSA concentrations was chosen as the minimum concentration used for the emulsion to be stable.

Table 3-15. The statistical significant differences between days for 0.4% OSA concentration using one-way ANOVA and Multiple Comparisons (Tukey HSD) statistic tests.

Multiple Comparisons		
Dependent Variable: value		
Tukey HSD		
day	day	P-value
1	7	0.11
	14	0.00
7	1	0.11
	14	0.00
14	1	0.00
	7	0.00

*The mean difference is significant at the 0.05 level.

The finding that 0.8% w/w concentration, while low, is still enough to stabilise the emulsion and was lower than that was reported by Chiu et al. (2017) where 1.5% OSA starch was enough to stabilise O/W emulsions with an oil phase of 20% w/w. The ability for OSA starch to stabilise O/W emulsions for a long period had been reported in many previous studies e.g. Miao et al. (2014) and Chiu et al. (2017) used OSA to stabilise this emulsion over 30 days and Yusoff and Murray (2011) for over 3 months but they used starch granules. Therefore, in this research a 14-day period of storage was considered enough for the research purpose especially at early stages of the research.

3.1.2. Protein stabilised emulsions

This stage of the research involved the formulation of single O/W emulsions stabilised with proteins in order to choose the optimum concentration to be used as co-emulsifiers with the OSA starch. Whey protein and sodium caseinate were independently used in this experiment. The emulsifying

capacity of protein is affected by several factors such as concentration of protein, homogeniser type, homogenisation degree, volume fraction of oil pH and ionic strength of the water phase (Masson and Jost, 1986). Therefore, Protein concentrations used for O/W stabilisation varied on previous studies; Masson and Jost (1986) formulated O/W emulsions with 1.24% WP and 10% commercial grapeseed oil free from emulsifiers, pH 7. Dickinson et al. (1993), used 10 % soybean oil and 0.45 % WP w/w, pH 7. Koliandris (2011) used 0.5% Bovine Serum Albumen and 30% hexadecane containing 0.85% PGPR 90 w/w for O/W emulsions formulated at range of pH between 5 and 9. Cornacchia and Roos (2011), employed a ratio of protein (WP or NaCAS): sunflower oil of 1:10, pH 7. Akhtar and Dickinson (2003), used 1.5 % w/w whey protein for stabile O/W emulsions containing 20% v/v oil phase. Frasch-Melnik et al. (2010b) used 1% w/w NaCAS and Panagopoulou et al. (2017) used 1% w/w WP to stabilise the outer interface of $W_1/O/W_2$ emulsions where 20% w/w of a water-in-oil emulsion homogenised with the external water phase (W_2). Akhtar and Dickinson (2003) chose 0.8% w/w concentration of whey protein for making protein/polysaccharide complexes.

3.1.2.1. Whey protein

Different concentrations of WP were involved to stabilise a group of O/W emulsions. The volume based mean droplet size ($d_{4,3}$) for six O/W emulsions stabilised with 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% w/w of whey protein, respectively, each emulsion consisted of 20% oil and 80% w/w aqueous phase as shown in Figure 3-12. Measurements of the mean droplet size were performed on three replicates of each concentration at day 1, 7 and

14 of the emulsion storage period at ambient temperature ($\approx 24.0\text{ }^{\circ}\text{C}$). An increase in the protein concentration caused a decrease in mean droplet size and the emulsion stability improved, where the differences between the three measuring data points were less with the 1.0% concentration comparing to smaller ones.

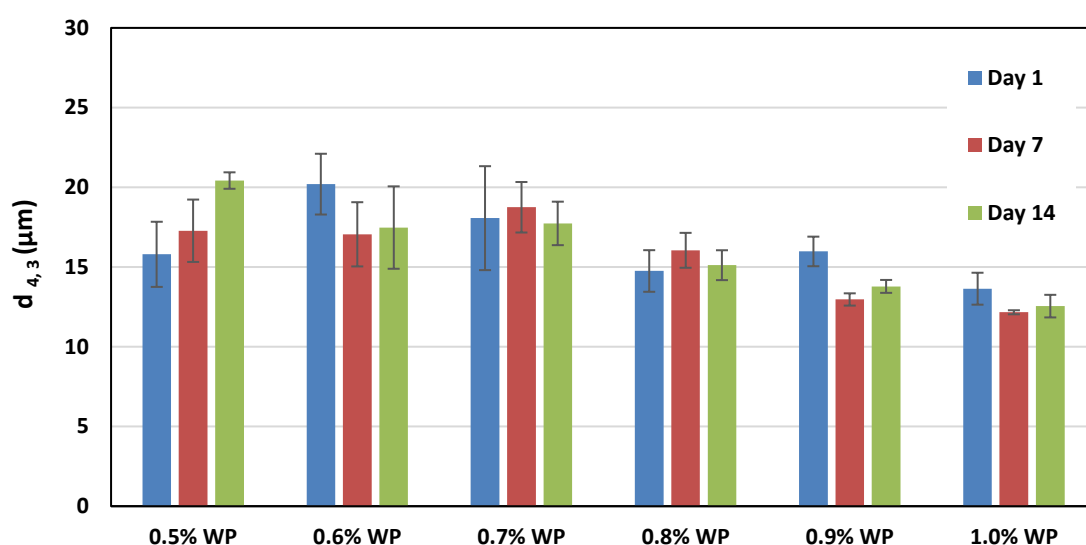


Figure 3-12. Volume based mean droplet size for a group of O/W emulsions that consisted of 20% oil and 80% w/w aqueous phase and stabilised with 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% w/w, respectively, of WP. Measurements were performed at day 1, 7 and 14 of the emulsions' self-life at ambient temperature. Error bars represent $\pm$ standard deviation ($n=3$).

To identify if there were any statistically significant differences in the mean droplet size at day 1, 7 and 14 of storage. One-way ANOVA and Tukey statistical tests were used. The statistical tests verified that there were significant differences ($p<0.05$) in the mean droplet size between the emulsions at each day measured as shown in Table 3-16. However, the differences in the mean droplet size between days for each concentration alone were not significant ($p>0.05$).

Table 3-16. The statistical analysis data for the effect of whey protein concentration at each of the three time points measured, calculated on mean droplet size measurements.

Concentration% w/w	Mean $\pm$ STD		
	Day 1	Day 7	Day 14
0.5	15.80 $\pm$ 2.04 ^a	17.28 $\pm$ 1.96 ^a	20.42 $\pm$ 0.52 ^a
0.6	20.20 $\pm$ 1.91 ^{a,b}	17.05 $\pm$ 2.02 ^a	17.48 $\pm$ 2.58 ^{a,b}
0.7	18.07 $\pm$ 3.26 ^a	18.75 $\pm$ 1.58 ^a	17.74 $\pm$ 1.36 ^{a,b,f}
0.8	14.75 $\pm$ 1.31 ^{a,c}	16.05 $\pm$ 1.10 ^{a,c}	15.12 $\pm$ 0.94 ^{b,e,f}
0.9	15.98 $\pm$ 0.93 ^a	12.96 $\pm$ 0.39 ^{b,c}	13.79 $\pm$ 0.41 ^{c,e}
1.0	13.64 $\pm$ 1.00 ^{a,d}	12.16 $\pm$ 0.13 ^b	12.55 $\pm$ 0.71 ^{d,e}

*Groups with different superscript letters represent statistical significance within the same column ($p < 0.05$).

The present results attributed to the fact that the increase in the whey protein concentration leads to smaller droplet size and improves emulsion stability. However, this does not mean that the smaller concentrations used in this experiment led to unstable emulsions. This result corresponds with the finding of Akhtar and Dickinson (2003) who demonstrated a decrease in the mean droplet size with increasing concentration of whey protein. Also, Sun and Gunasekaran (2009) reported that a higher concentration of WP is likely to facilitate the interfacial whey protein adsorption, which subsequently increases the stability of the emulsion.

3.1.2.2. Sodium caseinate

Sodium caseinate was also independently used as co-emulsifier to OSA starch to stabilise O/W emulsion in this research. The volume based mean droplet size ($d_{4,3}$) of six O/W emulsions stabilised with 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% w/w sodium caseinate was measured and the results are as shown in Figure 3-13. Each emulsion consisted of 20% w/w oil and 80% w/w aqueous phase; the mean droplet diameter was measured at day 1, 7 and

14 of storage at room temperature. Using one-way ANOVA statistical test indicated that no significant differences were observed in the mean droplet diameter either between the concentrations on each time point or between the time points for each concentration ($p>0.05$). Therefore, no significant changes in droplet diameter during the storage period indicated stable emulsions in this study. However, increasing concentrations of sodium caseinate led to slight decrease in the mean droplet diameter of the emulsion stabilised with NaCAS, but this decrease was not statistically significant.

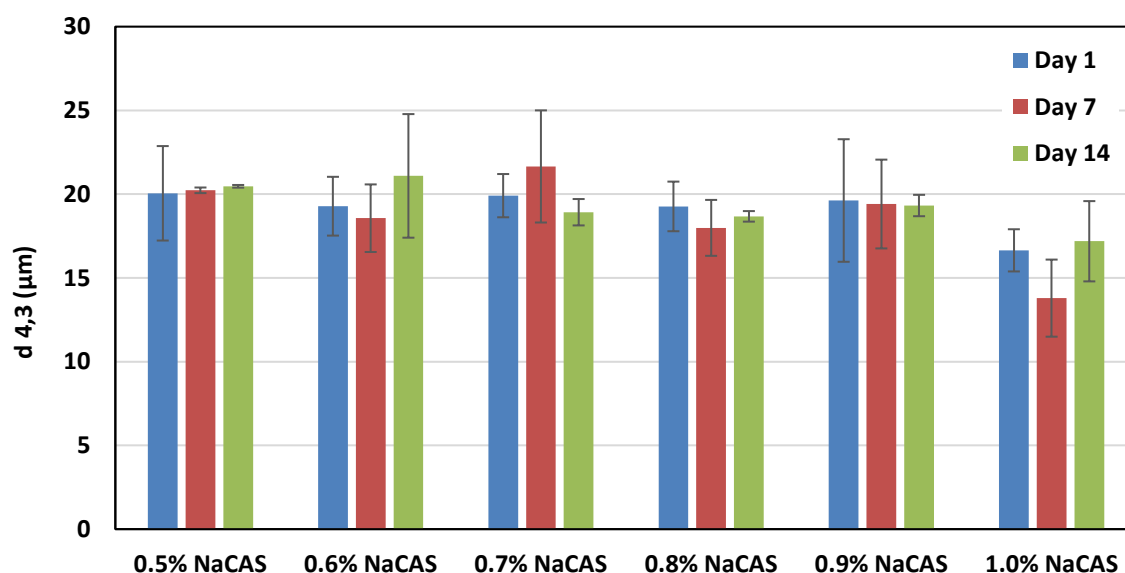


Figure 3-13. Volume based mean droplet size for a group of O/W emulsions consisted of 20% oil and 80% w/w aqueous phase and stabilised with 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0% w/w, respectively, of NaCAS. Measurements were performed at day 1, 7 and 14 of the emulsions' self-life at ambient temperature. Error bars represent $\pm$ standard deviation ($n=3$).

Although the same concentrations of both WP and NaCAS were used, the increase of the former during the experiment led to a statistically significant decrease in the droplet diameter between emulsions at each day alone. However, the opposite happened with the latter. The reason for this

phenomenon could be the formulation of dense multilayers of WP at the droplets surface during the processing produced smaller droplets. Hunt and Dalgleish (1994) reported that WP at the oil/water interface exhibits adsorption in distinct multilayers behaviour. Also Dickinson (2008) reported that whey protein is a highly effective stabilising agent forming a thinner and denser layer than that formed by casein at the oil/water interface. The thickness of these multilayers of WP compared to the droplet size is shown in Figures 4-23 of the next chapter. Therefore, it can be confirmed that the behaviour of each protein at oil/water interface is different and as a result forming different droplets size even though the same concentration and formulation conditions were used.

3.2. Buffered aqueous phase

Emulsions processed using sodium phosphate buffer (0.1 M, pH 7.0) as aqueous phase were stable against coalescence with excluding of the emulsion stabilised with 0.4% OSA w/w, which was not stable. Table 3-17 shows the composition of some of these emulsions, temperature and pH value immediately after formation, when either water or 0.1M sodium phosphate buffer pH 7.0 were used as aqueous phase. The temperature during the homogenisation maintained to be below 50 °C to avoid any negative effects of temperature on WP or OSA starch. Using OSA, WP and NaCAS in water aqueous phase made variation in the emulsion in terms of the pH as shown in the Table 3-17, e.g. OSA reduced the pH below from 7.0 to 5.8. As it mentioned in the research hypothesis some of the starch would be replaced by protein. Therefore, this reduction in the pH could affect the stability of the emulsions against coalescence emulsifier. In addition, the

different concentrations of the used emulsifier would affect the pH values in the system and impact on O/W emulsions stability. To keep the pH approximately constant and to know whether pH had an effect, the effect of using buffer as the aqueous phase of O/W emulsions on the stability of these emulsions against coalescence was investigated.

Table 3-17. Composition of O/W emulsions, temperature and pH values immediately after formulation, and also pH values after 14 days of storage at ambient temperature.

Weight per 100g					Immediately after formulation		After 14 days	
WP	N-CAS	OSA	Oil	H ₂ O	Buffer	pH	Temperature °C	pH
0	0	0.8	20	79.2		5.8	31	5.9
0	0	0.8	20		79.2	7.1	34	7.1
0.5			20	79.5		7.0	36	7.1
1			20	79		6.5	34	6.8
0.5			20		79.5	7.2	32	7.1
1			20		79	6.2	33	7.2
	0.5		20	79.5		7.5	33	7.5
	1		20	79		7.2	30	7.1
	0.5		20		79.5	7.4	28	7.3
	1		20		79	7.2	31	7.1

3.2.1. OSA starch stabilised emulsions

A group of O/W emulsions were prepared with aqueous phases contained 0.8g OSA starch and 79.2 g of either water or 0.1 M sodium phosphate buffer pH 7. The volume based mean droplet size ($d_{4.3}$) of three replications of these emulsions was measured during a 14-dys storage at ambient temperature and the results are shown in Figure 3-14. The change in the mean droplet size of the emulsions was statistically not significant over 14 days of storage when the continuous phase was water. However, when the continuous phase was phosphate buffer the mean droplet diameter increased from 9.8 to 25.1

μm over the storage period, and across the measuring points, there were statistically significant differences ($p < 0.05$).

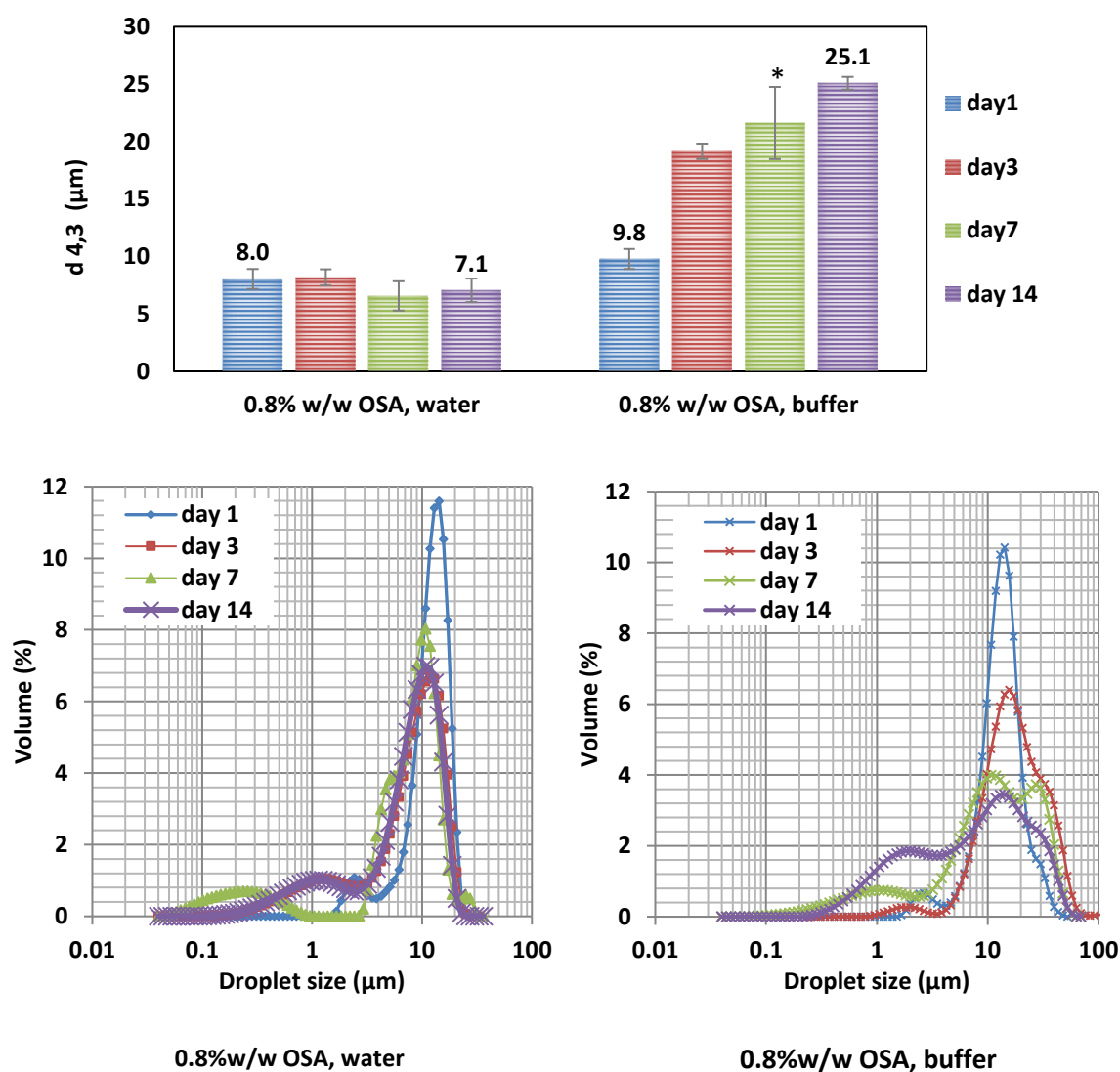


Figure 3-14. Volume based mean droplet size ($d_{4,3}$) and volume fraction distribution of two O/W consisting of 20% oil phase emulsion, stabilised with 0.8% w/w OSA starch as emulsifier. Either water or 0.1 M sodium phosphate buffer pH 7.0 were used as the aqueous phase. Error bars are mean of three replicates ($n=3$) $\pm$ standard deviation. * = significant difference in between days ($p < 0.05$).

The mean diameter of the emulsion produced using the buffer increased over time; indicative of coalescence. This could be because the buffer reduced the

ability of OSA starch to keep stable emulsion during both the processing and the storage period by screening some of the negative charge on its molecules. OSA starch can be present in two forms, in the granular state or the dissolved state (starch milled or pre-gelatinized and then substituted by OSA) (Matos et al., 2016). In the present research the OSA starch (NC 46) used in this research is dissolved state because no granules have been seen under an optical microscope, therefore the presence of buffer or ions can have impact on emulsion stability.

The volume fraction distribution curves of both emulsions that stabilised with the lowest starch concentration that marked stable emulsion using water continuous phase are also included in Figure 3-14. At day one, the distribution with water was different from other measuring days, this is could be due to a mistake related the measuring background in the particle analyser during day one. However, in the other 3 measuring days, the distribution curves showed that emulsions stabilised with 0.8% w/w OSA starch were stable against coalescence if the aqueous phase was water, whereas when the aqueous phase was 0.1M sodium phosphate buffer pH 7.0, the emulsion was not stable. This phenomenon is known as buffer capacity, which is the ability of a buffer to resist the change in the pH value (Urbansky and Schock, 2000). The same behaviour of the buffer has been reported by Su et al. (2008) when 0.1 M sodium phosphate was used as aqueous phase of O/W emulsions stabilised with gum Arabic as emulsifier. These emulsions were unstable with using buffer; therefore, it necessitated the formulation of emulsions in water systems.

3.2.2. Whey protein stabilised emulsions

The stability of O/W emulsions stabilised with six concentrations of WP with using distilled water as aqueous phase was already investigated, these emulsions were stable with all concentrations used. In this experiment only the smallest and highest concentrations “0.5% and 1% w/w” of WP were used for investigating the effect of using buffer instead of water on emulsion stability against coalescence. Therefore, the stability of two O/W emulsions prepared with 0.1M sodium phosphate buffer pH 7.0 was compared with the other two emulsions prepared with water used. Figure 3-15 shows the volume based mean droplet size of these emulsions.

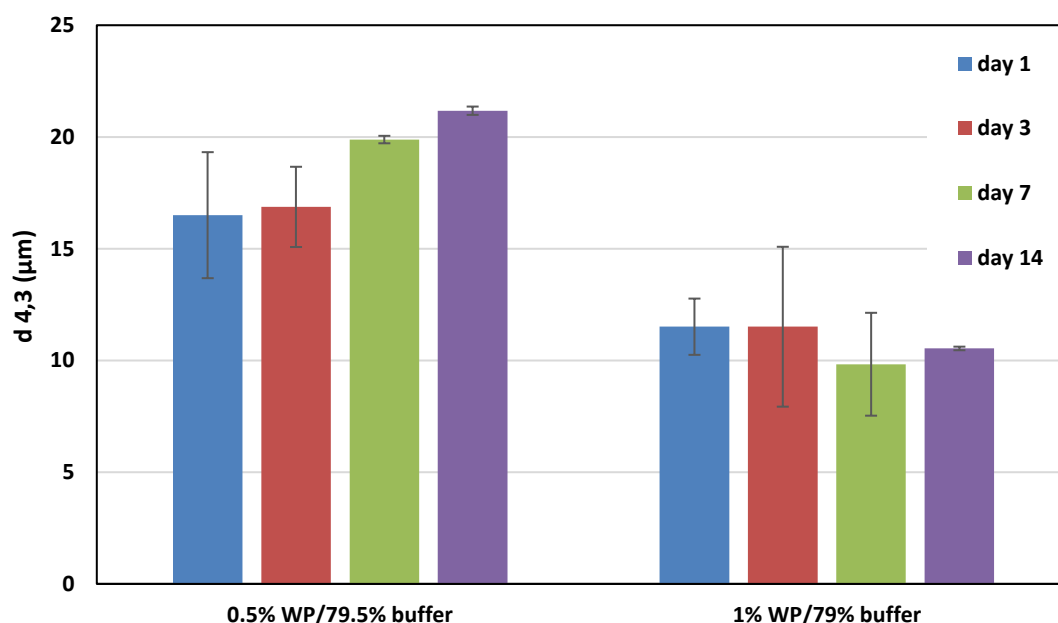


Figure 3-15. Effect of using 0.1 M sodium phosphate buffer pH 7.0 as the aqueous phase, on the mean droplet size in volume distribution ($d_{4,3}$) of two emulsions stabilised with either 0.5% w/w or 1% w/w whey protein. Error bars represent $\pm$ standard deviation (n=3).

One-way Analysis of Variance (ANOVA) was used to identify if there are any significant changes in the mean droplet size in the volume distribution ($d_{4,3}$). The test showed that, statistically no significant differences ($p > 0.05$) were found in ($d_{4,3}$) of these emulsions over the two week period. Although the change in the mean diameter of the emulsion stabilised with 0.5% w/w whey protein in the presence of buffer was statistically not significant. Using the 1.0% w/w concentration gave better stability than using 0.5% w/w; this could be as result of the formulation of droplets with smaller diameters. Using either water or 0.1 M sodium phosphate buffer pH 7.0 as aqueous phase in W/O emulsions stabilise with WP has no impact on stability against coalescence and they gave similar results. This implying that even the ability of the buffer on screening some the charge on the protein molecules they have enough negative charges to stabilised the emulsion by repulsion between droplets. Adsorption of proteins at the interface influenced by surface hydrophobicity and charge (Wilde et al., 2004). Therefore, high repulsion between each two neighbouring droplets leads to a maximum stability due to minimization of the chance for coalescence (Ghosh and Bandyopadhyay, 2012). In addition, proteins coat the droplets of an emulsion by an adsorbed layer and provide an energy barrier to droplet coalescence (Vanessa Cabra, 2008), and stability of the emulsion is influenced by the mechanical properties of this layer (Wilde et al., 2004).

3.2.3. Sodium caseinate stabilised emulsions

The behaviour of sodium caseinate in the presense of sodium phosphate buffer as the aqueous phase of O/W emulsions was investigated during a

two week period as shown in Figure 3-16. Two concentrations of NaCAS, 0.5 and 1.0% w/w, were used as emulsifiers and two aqueous phases were used either water or (0.1M) sodium phosphate buffer pH 7.0. One-way ANOVA statistical test was used to identify if there were any significant changes in the mean droplet size based on the volume distribution ($d_{4,3}$). Similar to the whey protein, using either water or sodium phosphate buffer as the aqueous phase had also no statistical impact on the mean diameter ($p > 0.05$). Using 1.0% w/w of NaCAS in the formation of droplets with smaller diameters than that when only 0.5% w/w was used, led to a better stability.

Large droplets coalesce into larger droplets therefore lead to faster phase separation; this will be delayed with smaller droplets, but not prevented (McClements, 2005). Liu et al. (2013), have stated that the highest solubility and best emulsification of caseins was at alkali pH close to 7.0. Therefore, the results were similar because both aqueous phases used in the processing of the emulsion have $pH \approx 7$. In addition sodium caseinate stabilise O/W emulsions with high electrostatic repulsion between the oil droplets (Zinoviadou et al., 2012), and the heads and tails of casein protrude from the oil droplet surfaces leading to steric stabilisation against coalescence and flocculation (Dickinson, 2008). Caseins are globular phosphoproteins their large molecular weight and hydrophilicity preclude this protein from diffusion across the oil phase during an emulsion processing (Bonnet et al., 2010, Tahmaz, 2010). It can be concluded that the high electrostatic repulsion between the droplets and the steric stabilisation against coalescence and flocculation both mechanised led to the stability of the W/O emulsions, even the buffer, which may screen some the charge on NaCAS was used.

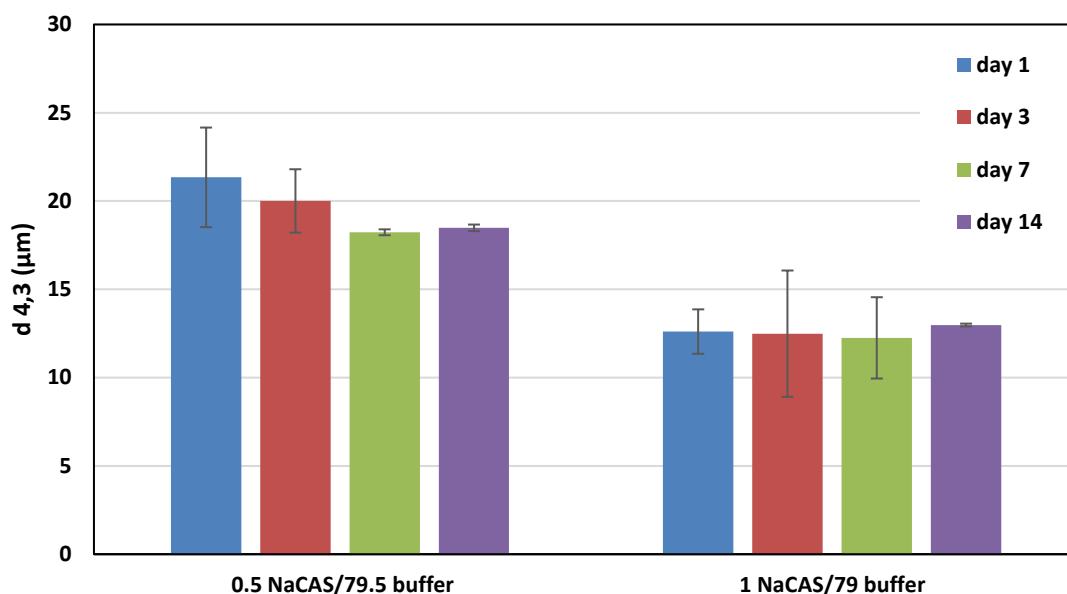


Figure 3-16. Impact of using 0.1 M sodium phosphate buffer pH 7.0 as the aqueous phase on the mean droplet size distribution ($d_{4,3}$) of two emulsions stabilised with either 0.5% w/w or 1% w/w sodium caseinate. Mean droplet size was measured during a period of storage 14 days. Error bars represent $\pm$ standard deviation ($n=3$).

ζ - Potential measurement was carried out for these emulsions as shown in the next chapter section 4.3. The emulsification of OSA starches is independent from the type of starch, pH value, ion valence and the starch concentration above the lower limit of starch needed for stabilisation, therefore, steric hindrance is the main stabilising mechanism (Tesch et al., 2002). OSA starch as emulsifier stabilises O/W emulsions by steric hindrance (Yusoff and Murray, 2011). However, OSA starch can be in a dissolved state (starch milled or pre-gelatinized and then substituted by OSA) or in a granular state. The dissolved state is more surface active but it forms O/W emulsions with stability less than the granule state, therefore the stability of these emulsions depends on the form of starch used (Matos et al., 2016). In the present research the OSA starch (NC 46) used in this research is dissolved state because no granules have been seen in a suspension of this starch,

therefor the formulation of O/W using OSA starch is dependents on the type and the concentration of this starch. In contrast, proteins stabilised emulsions by electrostatic repulsions by forming a viscoelastic adsorbed layer coat the droplets of an emulsion and provide an energy barrier to droplet' coalescence.

3.3. Conclusions for single emulsions stabilised with a single emulsifier

Oil-in-water emulsions with 20% w/w oil dispersed phase were stable against coalescence if they were produced at a concentration of $\geq 0.8\%$ w/w of OSA starch in deionised water as aqueous phase. However, the stability of these emulsions using 0.1M sodium phosphate buffer pH 7.0 instead of water was poor. A concentration of 0.8% w/w OSA starch was chosen to fit with the research aim of reducing the starch concentration in emulsion-based foods. In addition, O/W emulsions with 20% w/w oil dispersed phase can be stabilised with either whey protein or sodium caseinate with a range of concentrations from 0.5 to 1.0% w/w and it did not matter if the aqueous phase was water or sodium phosphate buffer pH 7.0. However, the ability of whey protein on making stable O/W emulsions was better when water was used as the aqueous phase, while the ability of sodium caseinate was better with sodium phosphate buffer.

Using the concentration of 1.0% w/w of both WP and NaCAS produced an O/W emulsion with a smaller droplet size than the other concentrations used. Furthermore, the stability of the emulsion regarding the consisting data between the measuring points was better even though there was no significant statistical difference between days in each concentration alone.

This concentration was therefore the favoured option for both proteins when formulating mixed starch/protein emulsifier systems in the next stage of the research.

**Chapter 4: Results and Discussion - Single Emulsions
Stabilised with Starch/Protein Mixed Emulsifier
Systems**

4. Single emulsions stabilised with mixed emulsifier systems

The research in this chapter was focused on using protein as co-emulsifier to the OSA starch. Therefore, it moved on to the partial replacement of the OSA starch by protein to formulate O/W emulsions stabilised with the mixed starch/protein emulsifier systems. In addition, the impact of using buffer solution instead of deionised water as continuous phase of the O/W emulsion was investigated because the different concentrations of the emulsifiers would affect the pH value in the system.

An experimental plan was systematically developed to assess the emulsifying ability of OSA starch/protein mixtures. Whey protein (WP) and sodium caseinate (NaCAS) were selected as co-emulsifiers with commercial OSA starch depending on the aqueous phase conditions like pH and ionic strength. OSA starch and both proteins have been used as emulsifiers and have a negative charge at pH 7.0 (Burrington, 2012, Zinoviadou et al., 2012). The adsorption kinetics and diffusion to the interface of these polymers can be affected by their different molecule size, also charge repulsion can be accrued because all are negatively charged. Small concentrations of both proteins can independently compete with OSA starch for the oil/water interface.

Based on the formulation of stable O/W emulsions using single emulsifiers in the previous experiments sections 3.1 and 3.2, mixed emulsifiers systems were selected. OSA starch was partly replaced by either WP or NaCAS. Based on the processing condition used, half of the quantity of OSA used and half of the quantity of either WP or NaCAS were combined to make two mixed emulsifier systems: 0.4% OSA/0.5 WP and 0.4% OSA/0.5 NaCAS w/w. These mixed emulsifiers were firstly tested for their ability to reduce the interfacial tension at the oil/water interface. To identify if these mixed emulsifiers will

exhibit competitive adsorption behavior at the droplets surface preventing droplets from aggregation. Secondly, the mixed emulsifiers systems were tested regarding to their ability in stabilisation O/W emulsions comparing to the use of single emulsifiers as control emulsifiers. Thirdly, the zeta potential of the O/W emulsions stabilised with these mixed emulsifiers was also measured to infer stability. Fourthly, to validate the presence of protein and starch at the interface of O/W emulsions, the interfacial microstructure was investigated. Finally, the interfacial composition was inferred from compositional analysis of the continuous phase pre and post emulsification.

4.1. Interfacial tension

The interfacial tension at the interface of the emulsifiers solutions used and oil was measured to assess whether the mixed emulsifier system contributes to reduced interfacial activity or not, as starch alone and protein alone are known to facilitate emulsion formation by reducing the interfacial tension. Therefore, if these mixed emulsifier able to reduce the interfacial tension at the oil/water interface then can be used to stabilise O/W emulsions. In addition, to find out whether there was consecutive adsorption would could be due to differences in molecular size and any other reasons. The interfacial tension of the aqueous dispersions of the emulsifiers was analysed during a 6000 s long measuring period, after this time no more reduction in the interfacial tension was observed in all cases used. Figure 4-17 shows the interfacial tension for 0.8g OSA/79.2g water compared to a control of water alone.

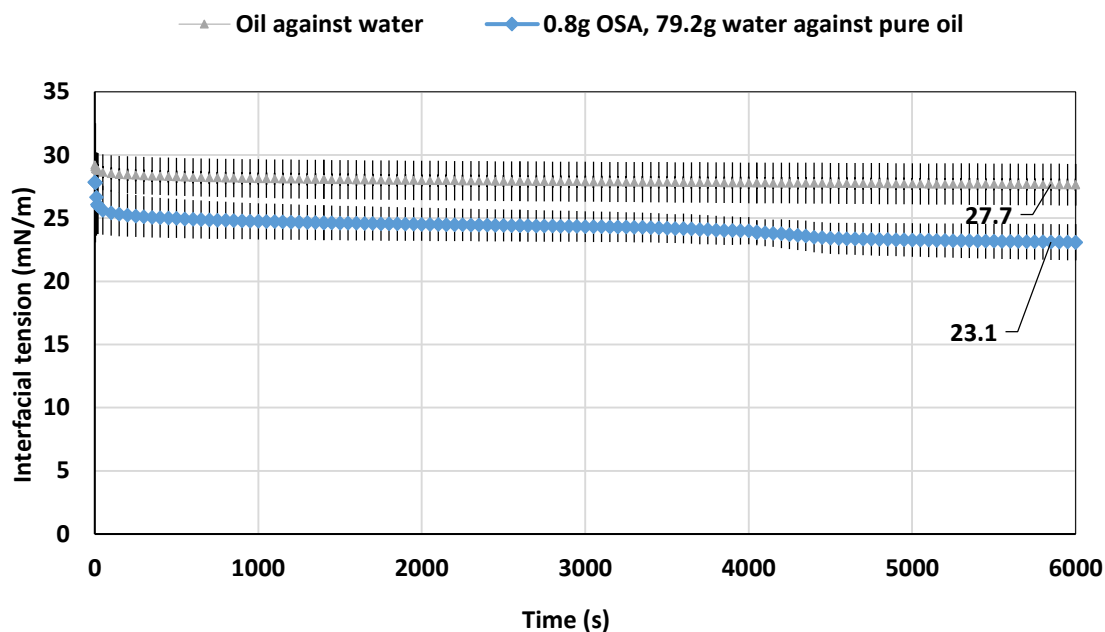


Figure 4-17. Interfacial tension for water against oil as control and 0.8g OSA, 79.2g water against oil, performed during 6000 s at 20.0°C. Error bars represent $\pm$ standard deviation ($n=3$).

The interfacial tension was reduced during 6000 s from (27.7 ± 1.6) for the control against oil interface to (23.1 ± 1.4) mN.m^{-1} for 0.8g OSA, 79.2g water against oil. Therefore, these results confirmed that the interfacial tension reduction until the concentration is above the critical micellar concentration (CMC) when interfacial tension is not further affected by concentration. Krstonošić et al. (2011) stated that at the certain emulsifier concentration the surface is saturated and no more emulsifier can enter the boundary layer, above this concentration molecules aggregate to form micelles, the minimum concentration above which micelles are formed is CMC. Interfacial tension for the aqueous solutions of the mixed emulsifier OSA/WP was also tested and compared with the aqueous phases of WP alone and OSA alone. Figure 4-18 shows the results of the interfacial tension measurement for water as control, 0.4g OSA/0.5g WP/79.1g water and 1.0g WP/79.0g water against oil. It seems to be that even the lower concentration

of WP was above the CMC because both concentrations give much closed values after the limit of time used.

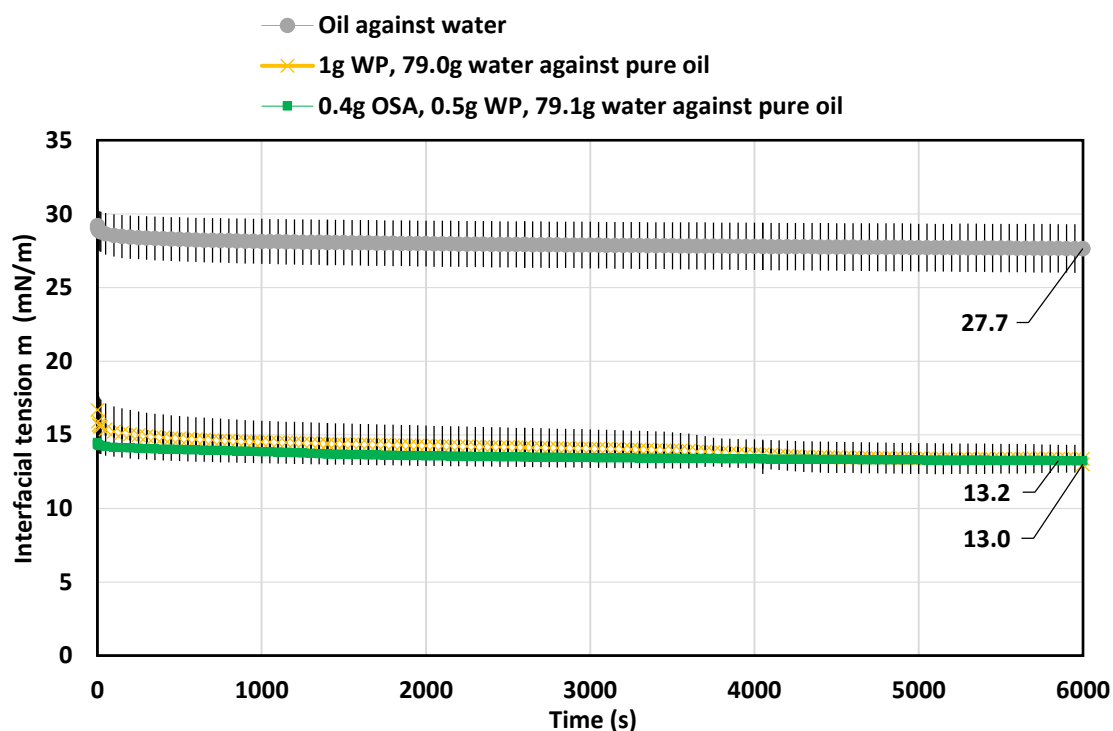


Figure 4-18. Interfacial tension for water dispersion against pure oil as control, 0.4g OSA/0.5g WP/79.1g water dispersion against oil and 1.0g WP/79.0g water dispersion against oil, performed during 6000s at 20.0°C. Error bars represent $\pm$ standard deviation ($n=3$).

The interfacial tension reduction at the oil/water interface was similar with using either OSA/WP system or WP alone during 6000 s measuring time. It was reduced from (27.7 ± 1.6) mN.m⁻¹ for the control to (13.2 ± 0.94) and (13.0 ± 0.2) mN.m⁻¹ with using WP alone and OSA/WP dispersion, respectively. These results indicate that the presence of protein either alone or with the starch clearly contributed in the interfacial tension reduction. It seems to be one of two potential happened; firstly even the lower concentration of WP was above the CMC because both concentration give

much closed values after the limit of time used. Secondly the combination of OSA/WP contribute in interfacial tension better than using OSA alone because using OSA in Figure 4-17 did not reduce the interfacial tension up to $(13.0 \pm 0.2) \text{ mN.m}^{-1}$. This happen could be as a result of increasing the viscosity of the solution, Krstonošić et al. (2011) stated that the viscosity of OSA starch solution increases by adding another polymer because it led to higher CMC point . Higher CMC points leading to more adsorption of the emulsifier at the interface and the more reduction in the interfacial tension. The corresponding results based on NaCAS instead of WP are presented in Figure 4-19. Data indicates that the OSA/NaCAS mixed emulsifier system reduced the interfacial tension for the oil/water interface better than using each emulsifier alone.

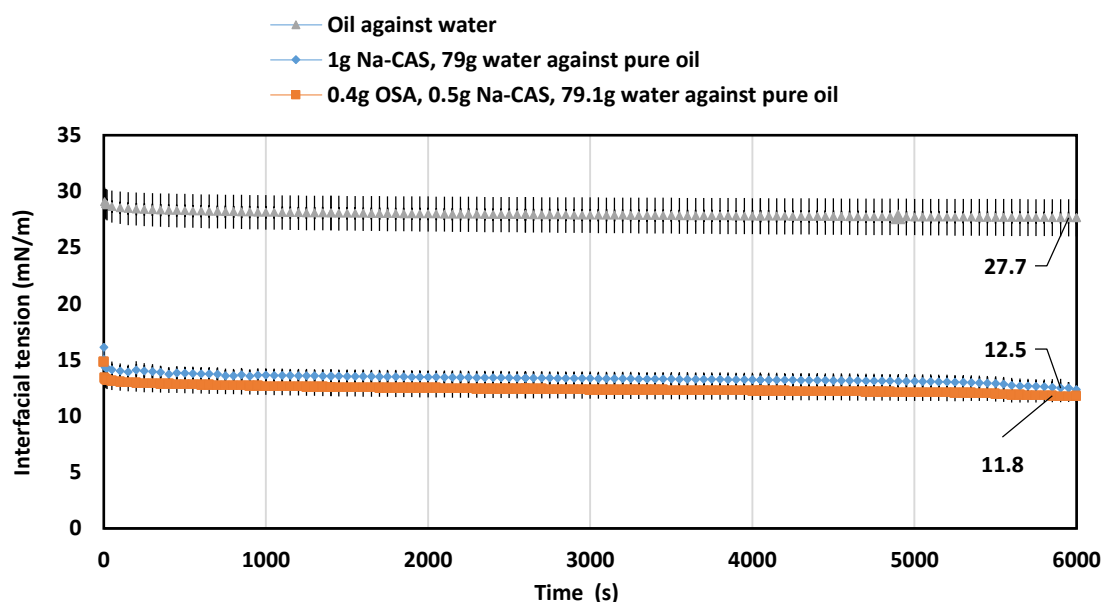


Figure 4-19. Interfacial tension measurements for water dispersion against oil as control, 1.0g NaCAS/79.0g water against oil and 0.4g OSA/0.5g NaCAS/79.1 water against oil, performed during 6000s at 20.0°C. Error bars represent $\pm$ standard deviation ($n=3$).

The interfacial tension reduced from (27.7 ± 1.6) mN.m⁻¹ for the control to (12.5 ± 0.4) and (11.8 ± 0.5) mN.m⁻¹ with using NaCAS alone and OSA/NaCAS solutions, respectively.

Generally, using WP or NaCAS independently reduce the interface better than OSA starch. Combining starch and protein led to lower interfacial tension values than that for OSA starch alone. Therefore, mixed emulsifier systems by reducing the interfacial tension can play important role on emulsions formulation. These outcomes confirmed the finding of Benichou et al. (2004) that protein/polysaccharides blends can serve emulsions stability. In addition (Ghosh and Bandyopadhyay, 2012) stated that the role of proteins in emulsions stabilisation is aided by the presence of polysaccharide , and de Souza Simões et al. (2017) reported that combination of proteins/polysaccharides can improve emulsions stability. In light of the interfacial tension data, the present results indicated that proteins can adsorbed faster than OSA starch to oil/water interface because protein had reduced the the interfacial tension bigger than OSA starch. However, the differences in molecular size and other properties of each polymer also could make difference in the adsorption at the oil/water interface. therefor the present results suggested that mor invigtigation was required to identifie whether both polymers at the droplet surface or not. The interfacial tension experiments were done not to confirm the prescence of both OSA/protein at the interface because this was confirmed by the interfacial composition analysis in section 4.5.1 but to assess whether the mixed emulsifier system contributes to reduced interfacial activity or not.

4.2. Droplet size

Three replicates of four emulsions were observed to identify the emulsifying ability of the mixed emulsifier systems developed in this research. OSA/WP and OSA/NaCAS mixed emulsifiers were used to stabilise O/W emulsions that contained 20% w/w dispersed oil phase and 80% w/w water phase. The water phase consisted of 0.9% of the mixed emulsifier and 79.1% w/w of either water or 0.1M sodium phosphate buffer. Measurements of the mean droplet size were performed at four measuring time points at day 1, 3, 7 and 14 of the emulsions storage period at room temperature ($\approx 24.0\text{ }^{\circ}\text{C}$). Figure 3-20 shows the mean droplet diameter ($d_{4,3}$) and the volume fraction distribution of two O/W emulsions stabilised with 0.4% OSA/0.5% WP mixed emulsifier system. Storing the OSA/WP stabilised emulsions for a two-week period had no impact on the mean droplet diameters; when either water or buffer aqueous phases were used. One-way ANOVA statistical test verified that there were no significant differences ($p > 0.05$) in the mean droplet size in volume distribution ($d_{4,3}$) of these emulsion through 14 days of storage. The volume fraction distribution on the left was when the emulsion was formed with water, whereas the distribution on the right was when the emulsion was formed with buffer. Distributions at day 1, 3, 7, and 14 were very close to each other for both emulsions indicating that emulsions were stable against coalescence.

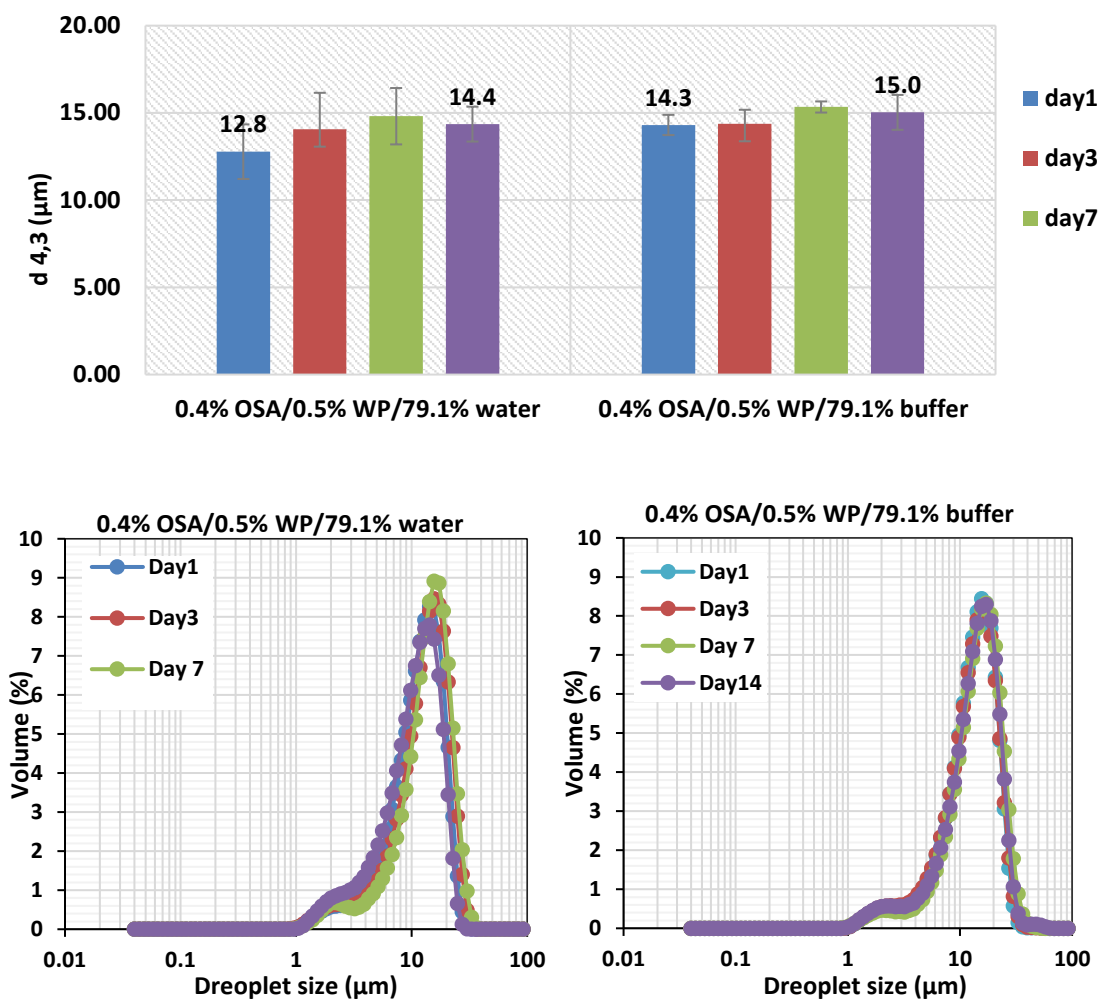


Figure 4-20. The mean droplet diameter ($d_{4,3}$) and the volume fraction distribution of two emulsions comprising of 20% w/w oil, stabilised with 0.4% OSA/0.5% WP mixed emulsifier system. Water or 0.1 M sodium phosphate buffer pH 7.0 was used as aqueous phases. Measurements were performed at day 1, 3, 7 and 14 of the storage period at ambient temperature. Error bars represent $\pm$ standard deviation ($n=3$).

By comparing mixed interfaces in Figures 4-20 and 4-21 and single emulsifier in Figures 4-15 and 4-16 of chapter 3 it can be concluded that adding 0.4%w/w OSA starch to 0.5% w/w of either WP or NaCAS, improved stability of all emulsions by reducing the droplet size and giving more consist data points. OSA/NaCAS stabilised emulsions, whether the aqueous phase was distilled water or buffer, the mean droplet and one-way ANOVA statistical test diameter showed no statistically significant differences ($p > 0.05$) over

storage duration as shown in Figure 4-21. The distributions of these emulsions were very close distributions at all measuring points and there was no indication for coalescence implying that emulsions were stable over the two-week storage period.

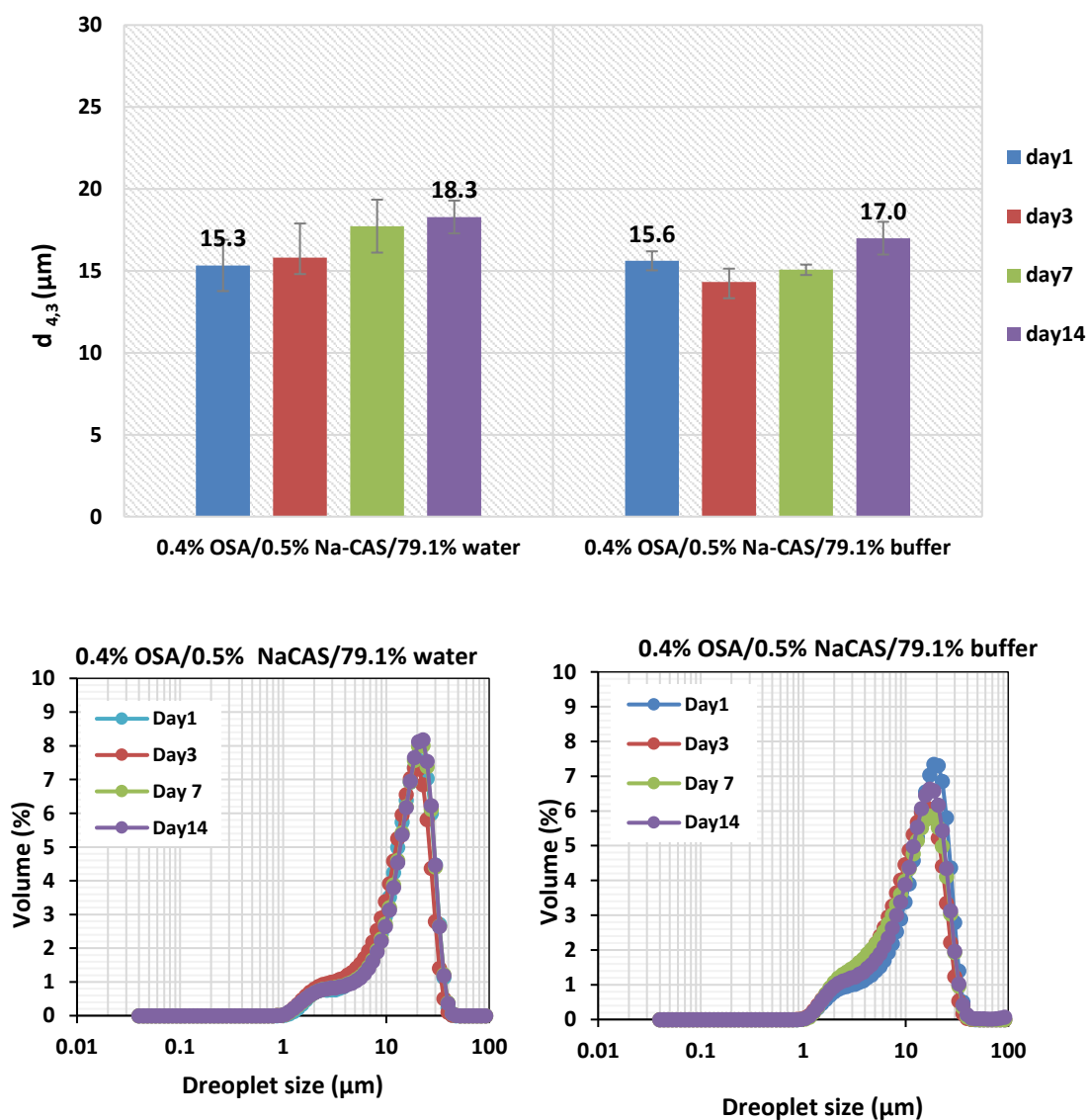


Figure 4-21. The mean droplet diameter ($d_{4,3}$) of two emulsions comprising of 20% w/w oil, stabilised with 0.4% OSA/0.5% NaCAS mixed emulsifier system and the volume fraction distribution of these emulsions. Either water or 0.1 M sodium phosphate buffer pH 7.0 was used as aqueous phase. Measurements were performed at day 1, 3, 7 and 14 of the storage period at ambient temperature. Error bars represent $\pm$ standard deviation ($n=3$).

By comparing the mean diameter of emulsions stabilised with OSA starch and emulsions stabilised with either protein alone or OSA starch/proteins, it can be seen that the mean size of the droplets increased when proteins were included see Figures 3-14, 4-20 and 4-21. There were two potentials for these results firstly could be as a result of the change in the viscosity of the aqueous phases post adding the protein, which in turn affect on the formulation conditions. Secondly, could be due to thickness of the absorbed layer at the interface of the droplets; this property of protein has been observed (Dickinson, 2008, Koliandris et al., 2010). The present results about droplet size for emulsions stability against coalescence have been agreed with zeta potential (ζ -potential) measurements which can be an indication that coalescence was unlikely due to charge repulsion for all emulsions as discussed below.

4.3. Zeta potential

Droplets with a ζ -potential between -10 mV and +10 mV are considered approximately neutral, whereas droplets with zeta potentials bigger than +30 mV or smaller than -30 mV are considered strongly cationic and strongly anionic, respectively (Clogston and Patri, 2011). Therefore, the magnitude of the ζ -potential provides an indication of the potential stability of a colloidal system. Generally, when all the droplets have a ζ -potential of lower than -30 mV or greater than +30 mV, they will repel each other and the dispersion is stable. On the other hand, when the droplets have ζ between -30 mV and +30 mV there will be no force to prevent the droplets from coalescence (Wongsagonsup et al., 2005). Zeta potential and pH of two O/W emulsions

consisting of 20% oil stabilised with 0.8% OSA and their aqueous phases alone are shown in Table 4-18.

Table 4-18. ζ -potential and pH values of two O/W emulsions stabilised with OSA, and their aqueous phases alone, the aqueous phase of these emulsions was either water or 0.1M, pH 7 sodium phosphate buffer, measurements performed at 25.0°C on day 1 of the storage period.

	ζ -potential (mV)	STDEV.	pH
Emulsion 0.8g OSA/79.2g water/20g oil	-31.51	± 0.83	5.63
Emulsion 0.8g OSA/79.2g buffer/20g oil	-24.26	± 1.08	7.07
Solution 0.8g OSA/79.2g water	-37.84	± 8.86	5.27
Solution 0.8g OSA/79.2g buffer	-14.93	± 2.13	7.07

Data in the Table 4-18 seems to be not accurate depending on the standard deviation even though 0.1M, pH 7 sodium phosphate buffer was used for diluting samples that were already prepared with buffer. This variation could be due to occasional mistake in number of the sample droplets used which was usually two droplets in 40mL of either distilled water or the buffer. The aqueous phase of these emulsions was either water or 0.1m sodium phosphate buffer, measurements were performed at day 1 of the emulsions' storage period at 25.0°C. The instability of the O/W emulsion that was formed using buffer see section 3.2.1 was confirmed by the ζ -potential data. The ζ -potential value of this emulsion was -24.26 mV, which corresponds to a diminished electrostatic repulsion because it was between -30mV and +30mV, while when the same emulsion was formed using water instead of the buffer the value was -31.51 mV, which is out of this range. The presented results of ζ -potential for either OSA water solution or OSA buffer solution in Table 4-17 confirmed the fact that the buffer have the ability to screen the

charge on OSA starch molecules up to a specific limit depending on the buffer capacity. The negative charge of the OSA starch molecules has been reduced from -37.84 mV for OSA in water solution to -14.93 mV for OSA in the buffer solution. Nevertheless, the charge reduction was less at the emulsion interface comparing to the case of solution could be because the emulsifier was adsorbed at oil/water interface. Figure 4-22 shows the negative charge on the chemical structure of OSA starch molecule and the electric double layer surrounding a colloid. The charges on either OSA molecules or droplets of an emulsion can be reduced by the buffer up to a limit depends on the buffer capacity, which in turn depends on the buffer molarity.

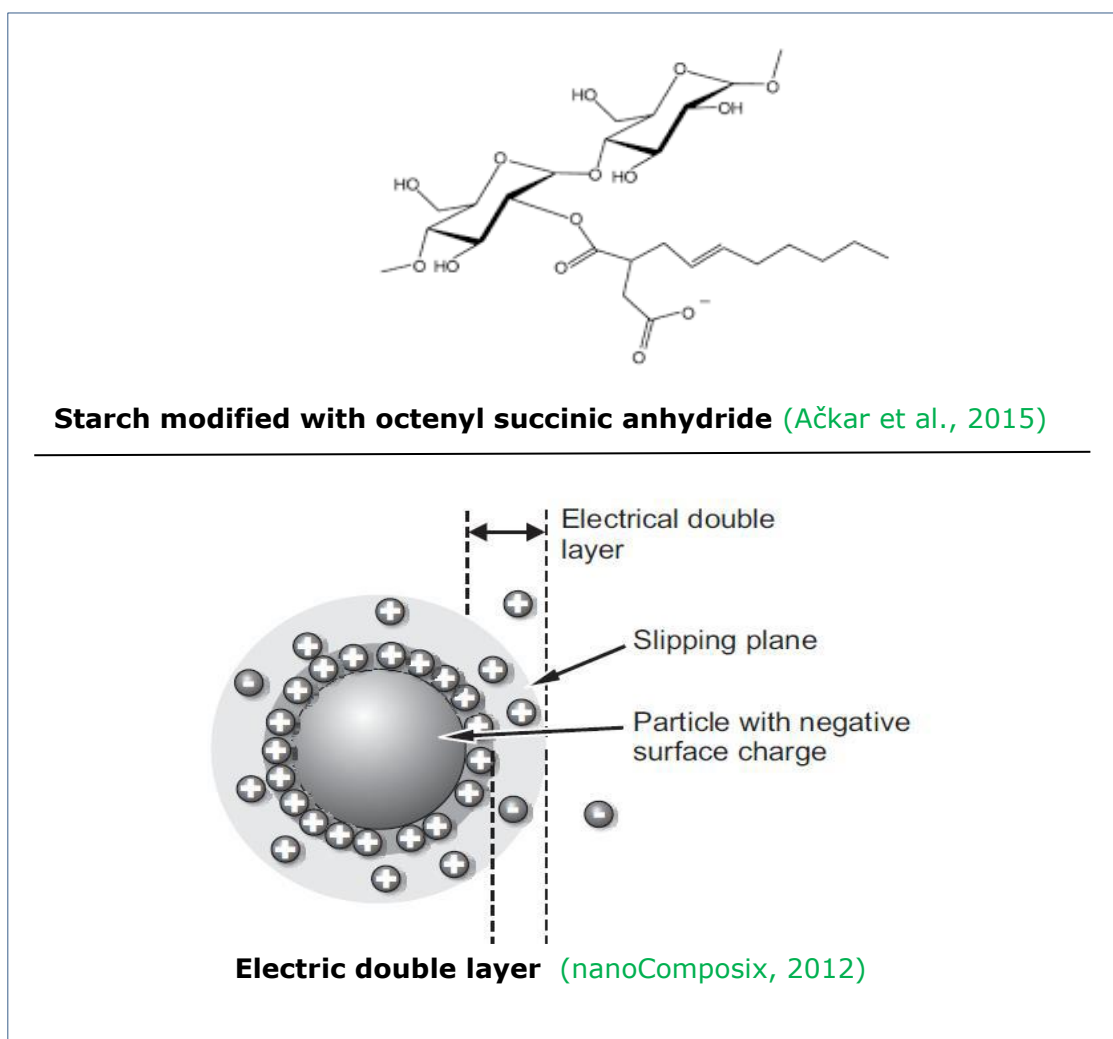


Figure 4-22. Molecule of starch modified with octenyl succinic anhydride and electric double layer surrounding particle of a colloid. The native starches has limited water solubility, not hydrolysed into small molecules chains or gelatinised as OSA starch, do not have negative charge and starch on its molecule and it is not hydrophobic starch. While OSA starch has chemically substituted groups of octenyl succinic anhydride (OSA) to generate hydrophobic starch.

According to Miao et al. (2014), the ζ -potential can be used to identify the negative charge at the surface of OSA starch to measure the emulsion stability. The research work agrees with Miao et al. (2014) regarding the type of charge 'negative charge' but it was different in the ζ value. The quantity of charge that can be measured in OSA solution depends on the degree

modification. The use of OSA starch in food characterise a low degree of substitution (DS) value and they could be considered as a weakly charged polyelectrolyte (Krstonošić et al., 2011). Therefore, zeta potential has the lowest values when OSA starch was used as single emulsifier, which was attributed to the emulsifying ability of OSA starch that is lower than that of sodium caseinate and whey protein as it was discussed below.

The emulsions stabilised using mixed emulsifiers were also subjected to zeta potential measurements. Table 4-19 shows the ζ -potential and pH values of four O/W emulsions consisting of 20% oil stabilised with either 0.4% OSA/0.5% WP or 1% w/w WP alone in two aqueous phases, which were either water or buffer. Measurements performed at 25.0°C on day 1 of the storage period at ambient temperature. Emulsions stabilised with OSA/WP mixed emulsifier systems showed zeta potential values that implied high stability, which were unrelated to whether the aqueous phase was water or buffer.

Table 4-19. ζ -potential and pH values of O/W emulsions stabilised with either OSA/WP mixed emulsifier or WP alone, and ζ -potential and pH for two aqueous phases alone, the aqueous phase of the emulsions was either water or 0.1M, pH 7 sodium phosphate buffer.

	ζ -potential (mV)	STDEV.	pH
Emulsion 0.4g OSA/0.5g WP/79.1g water/20g oil	-39.04	± 2.56	6.77
Emulsion 0.4g OSA/0.5g WP/79.1g buffer/20g oil	-36.64	± 2.27	6.73
Emulsion 1g WP/79g water/20g oil	-51.50	± 0.45	7.17
Emulsion 1g WP/79g buffer/ 20g oil	-39.94	± 0.64	7.03
Solution 1g WP/79g water	-27.06	± 5.30	7.10
Solution 1g WP/79g buffer	-31.93	± 3.20	7.00

The value of ζ -potential when mixed OSA/WP mixed emulsifier was used to stabilise O/W emulsion refers to stability but does not provide any evidence

of the presence of both polymers at the interface. Even though using buffer reduced the ζ -potential value of both the OSA/WP stabilised emulsions and the WP stabilised emulsions, this value was still high enough to retain repulsion between the droplets, implying that WP molecules have amount of charges bigger than that can be screened by the buffer capacity. The ζ -potential of WP in water was relatively low. This was not expected and this could be due to a measurement mistake because the standard deviation was relatively high. WP in buffer gave a high ζ -potential value out of the limit -30 mV or and +30 mV, implying that using this solution as aqueous phase of O/W emulsion should make droplets repel each other and the emulsion will be stable. The present results agreed with the particle size data for the emulsions as well as the conclusion that emulsions stabilised with the mixed system of "OSA/WP" had a higher stability with the water aqueous phase than that with buffer aqueous phase. The zeta potential of emulsions stabilised with WP alone was higher than that in emulsion stabilised with OSA/protein, which was expected because the charges on protein alone was bigger than that on OSA starch alone. Similar outcomes have been reported by Tamnak et al. (2016) who found that a $W_1/O/W_2$ emulsion stabilised with mixed (pectin/pea protein) as external emulsifier had a relative zeta potential higher than those stabilised with only pectin, and had smaller droplets.

The zeta potential data in Table 4-20 indicate that emulsions stabilised with sodium caseinate alone or mixed with starch had higher ζ -potential than emulsions processed in the presence of WP; this could be due to the chemically modified that has been done on sodium caseinate to improve its emulsifying ability see section 1.2.2.2. The ζ -potential values of emulsions stabilised with OSA/NaCAS mixed emulsifier system were indicative of higher

emulsion stability with either water or buffer as the aqueous phase and they explain the droplet size data. However, the performance of sodium caseinate for making a stable O/W emulsion tended to be better in the presence of sodium phosphate buffer as the aqueous phase compared to water. This result is compatible with the droplet size data for these emulsions.

Table 4-20. ζ -potential Zeta potential and pH of four O/W stabilised with either OSA/NaCAS mixed emulsifier or NaCAS alone, and two aqueous phases alone, the aqueous phase of the emulsions was either water or 0.1M sodium phosphate buffer pH 7, measurements performed at day 1 of the storage period at 25.0 °C.

	ζ -potential (mV)	STDEV	pH
Emulsion 0.4g OSA/0.5g NaCAS/79.1g water/20g oil	-57.35	± 3.92	6.6
Emulsion 0.4gOSA/0.5g NaCAS/79.1g buffer/20g oil	-64.39	± 0.68	6.9
Emulsion 1g NaCAS /79g water/20g oil	-63.14	± 0.93	7.2
Emulsion 1g NaCAS/79g buffer/20g oil	-71.15	± 1.20	7.1
Solution 1g NaCAS/79g water	-59.64	± 1.42	7.1
Solution 1g NaCAS/79g buffer	-23.38	± 1.29	7.0

Similarly, for the OSA/WP stabilised emulsions the high value of ζ -potential when mixed OSA/NaCAS was used as mixed emulsifier does not provide any evidence of the presence of both polymers at the oil/water interface. In conclusion, emulsions stabilised with mixed emulsifiers were highly negatively charged limiting the likelihood of coalescence. These results are compatible with the interfacial tension and droplet size data for these emulsions, and indicated that using buffers as aqueous phase could reduce the charge on the emulsifier depending on its molarity (Urbansky and Schock, 2000). In addition, the ζ -potential data confirmed the difference between NaCAS and WP as emulsifiers. The presence of the former as emulsifier alone or mixed with the starch in O/W emulsions in water led to ζ -potential values

higher than those obtained in the presence of the latter. Molecules of both proteins have made considerable increase in ζ -potential when they presence independently at the interface of O/W emulsions comparing to OSA starch alone, because they have more negative charge. For more investigation about the presence of these proteins in the interface the microstructure of the emulsion was observed in the next experiment.

4.4. Emulsion microstructure

Confocal laser scanning microscopy (CLSM) was used to confirm that both starch and protein were indeed at the interface of the O/W emulsion droplets. Two emulsion stabilised with the mixed emulsifier systems either OSA/WP or OSA/NaCAS were subjected to interfacial microstructure characterisation. A drop of each emulsion was mixed with fluorescein isothiocyanate (FITC), Fast green and Nile red to stain starch, protein and oil respectively. A CLSM image of the O/W emulsion stabilised with 0.4%OSA/0.5%WP is shown in Figure 4-23.

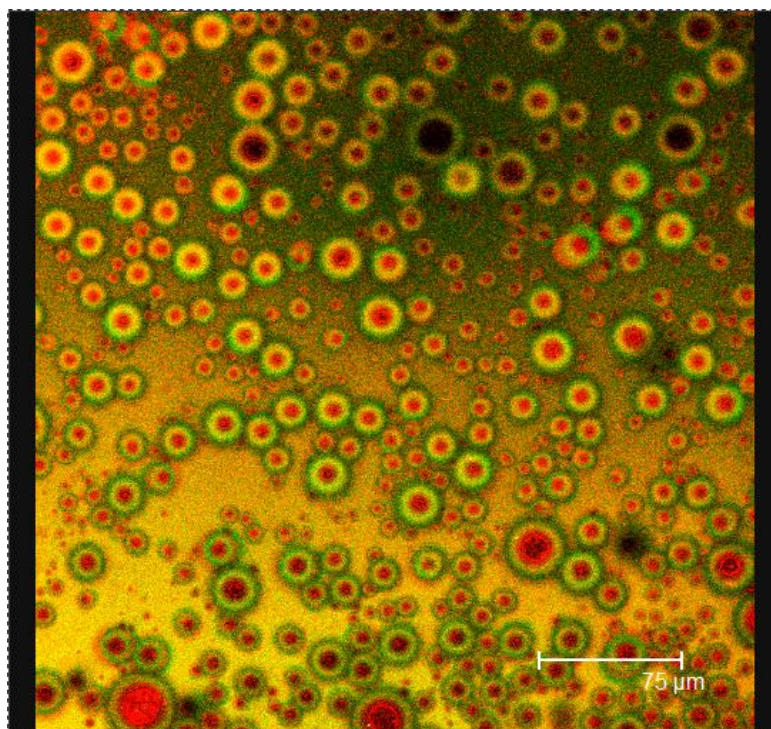


Figure 4-23. A CLSM Image with scale bar 75μm of O/W emulsion stabilised with mixed emulsifier OSA/WP. Starch was stained with fluorescein isothiocyanate (yellow), protein with Fast green and oil with Nile red. Oil appears as red droplets surrounded by yellow and yellow- green mixed layers, which are likely to be starch/protein and the green layer, which is protein. As limitations of the image there is not an exact distinction of which is the starch and which is the protein but there are two different colours.

The oil appeared as red droplets with each of them possessing halos of either yellow, green-yellow or green. The yellow fluorescence of FITC which labelled OSA starch was around the oil droplets. Starch thus adsorbed at the oil/water interface to form a film around the drops, which impeded coalescence. These results agreed with the statement of Tan et al. (2014) that OSA starch can be covalently linked to fluorescein isothiocyanate. The yellow-green fluorescence showed the mixed adsorbance of both Fast green and FITC that stained the mixed OSA/WP system. The green halos should be the Fast green, which identifies WP, adsorbed at the droplet surfaces.

The CLSM Image of O/W emulsions stabilised with OSA/WP indicated to the presence of both polymers at droplet surfaces. The interfacial composition of the big cover layer of the OSA/WP mixed emulsifier at the oil droplet surface appeared as layer on layer which agrees with the finding of Hunt and Dalgleish (1994) in which the behaviour of WP at the oil/water interface exhibited absorption in multilayers. These results agreed with Dickinson (2008) who stated that whey protein makes a dense layer at the oil/water interface. In addition, proteins and polysaccharides can be combined as layer-by-layer at emulsion droplets (Weiss et al., 2006, Muschiolik, 2007). The CLSM was also used to explore the interfacial composition of O/W emulsion stabilised with a mixture of OSA/NaCAS as shown in Figure 4-24. The emulsion was stained with the same stains used for the OSA/WP stabilised emulsions. Some areas of the droplet interface appear to have been covered by the protein (green), while some of them were covered by the starch (yellow). The tentative results of the interfacial composition analysis show that there was a presence of both protein and starch at the droplets surface.

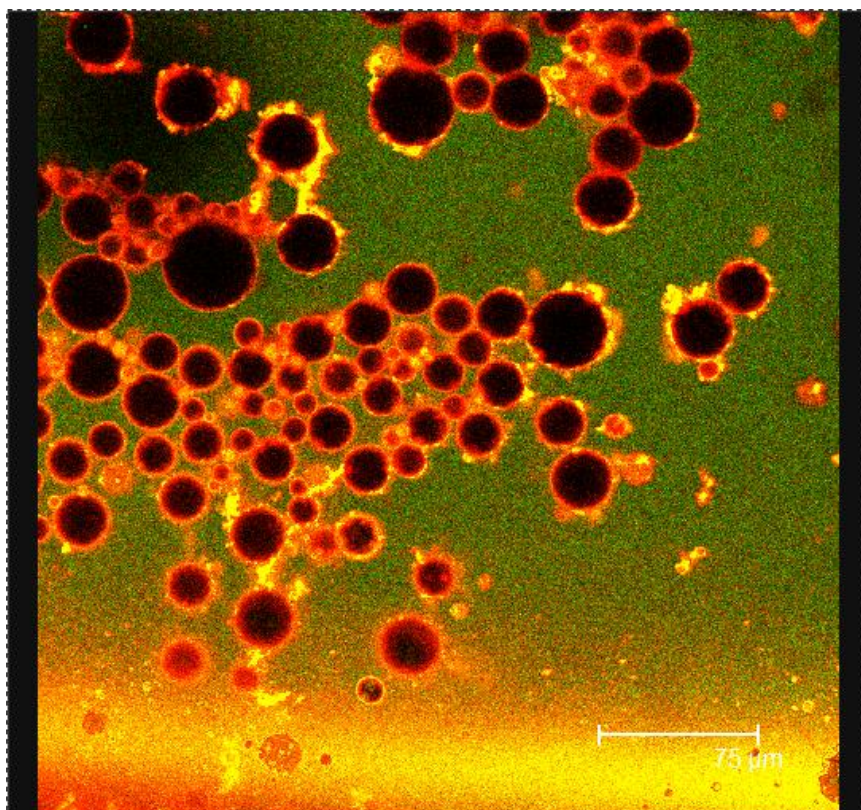


Figure 4-24. A CLSM image (scale 75μm) of an O/W emulsion stabilised with mixed emulsifier (OSA/NaCAS). Starch was stained with fluorescein isothiocyanate (FITC), protein was stained with Fast green and oil was stained with Nile red. The protein covers some areas of the droplets' interface while the starch covers some of them.

In order to validate the patchy surface coverage of the O/W emulsion interface stabilised with 0.4% OSA/0.5% NaCAS w/w, another CLSM image is shown in Figure 4-25. This image shows more detail of the oil droplet surfaces in which the adsorption of OSA/NaCAS emulsifier at the oil droplet surface was different from the adsorption of OSA/WP emulsifier. It appears that the starch randomly covered some areas of the oil droplet surface while the protein covered other areas and the formation of multilayers was not apparent. The tick areas of the interface as mentioned in Figure 4-25 should be protein and small areas should be starch because used was in the dissolved state not granule state. Dickinson (2008), stated that tails and loops

of casein protrude from the droplet surfaces resulting in steric stabilisation against flocculation and coalescence.

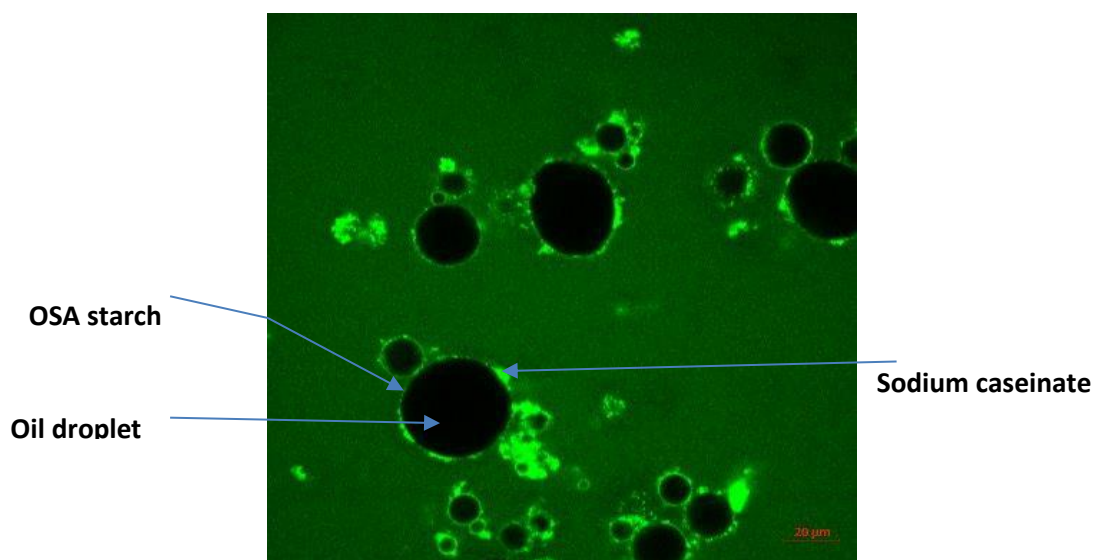


Figure 4-25. CLSM image (scale 20 μ m) for a sample of o/w emulsion stabilised with OSA/NaCAS mixed emulsifier. As limitations of the image there are not exact distinction of which the starch is and which is the protein but seems to be big parts of the interface is protein.

To identify whether OSA starch and protein were indeed presented at the interface or not, O/W emulsions stabilised with either OSA/WP or OSA/NaCAS mixed systems more investigation was done by the interfacial composition analyses as follows.

4.5. Interfacial composition

Three replicates for each one of the O/W emulsions stabilised with starch/protein mixed emulsifiers systems were subjected to two procedures of analysis one to measure the proteins and the other to measure the starch at the interface as follows.

4.5.1. Protein content

The interfacial composition of the emulsions was inferred from continuous phase analysis (emulsion serum) performed post emulsification. Based on the assumption that any proteins not detected in the serum were adsorbed at the oil/water interface, the protein concentration in the serum was measured and the concentration adsorbed was calculated. This method is valid provided that the separation of the serum from the droplet does not lead to partial droplet break-up and there are no reverse micelles within the oil droplets (Koliandris, 2010). This technique is based on the Biuret reaction (reduction of Cu^{2+} into Cu^{+} by proteins), with a colorimetric detection of the Cu^{+} ions by a reaction involving biocinchoninic acid (Smith et al., 1985). The protein concentration was measured in the continuous phase before emulsification as well as in the serum post emulsification as shown in Figure 4-26. The protein was also measured in a solution of 0.4% w/w OSA starch and it was 0.0%. However, this is different with the finding of Kasprzak et al. (2018) who reported that the protein content in the same OSA starch is $(0.28\% \pm 0.01)$. The difference could be due to the difference in the measurement method used.

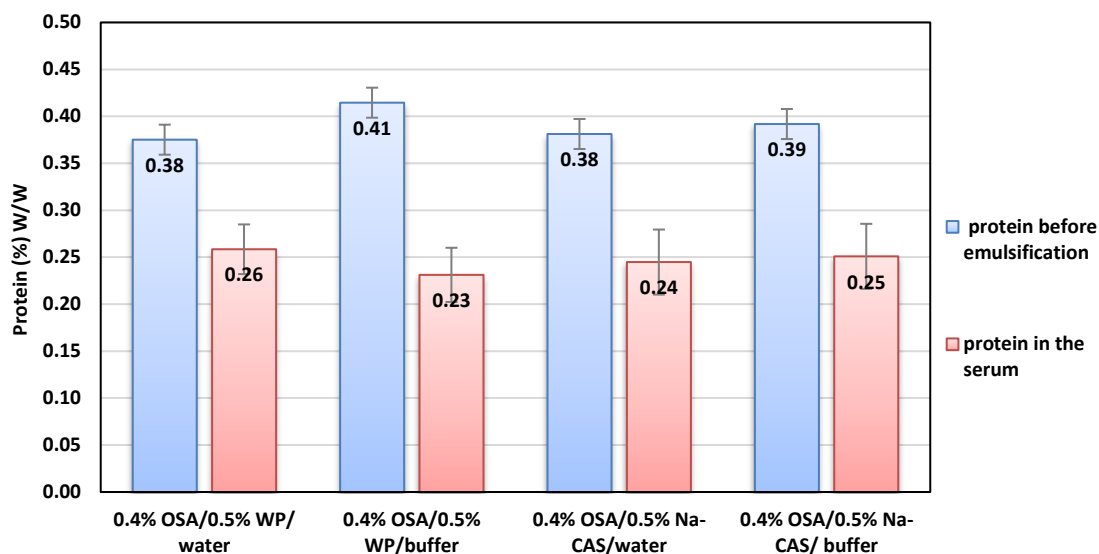


Figure 4-26. Protein content in both the continuous phase before emulsification and in the serum post emulsification of O/W emulsions stabilised by OSA starch/protein mixed emulsifiers systems. Either water or 0.1 M sodium phosphate buffer pH 7.0 was used as aqueous phase. Error bars represent $\pm$ standard deviation ($n=3$).

The concentration of protein before emulsification in the emulsions that contained 0.4% OSA/0.5% WP/water and 0.4% OSA/0.5% WP/buffer was 0.38% and 0.41%, respectively, as indicated in Figure 4-26. The interfacial composition of O/W emulsions stabilised with mixed emulsifier systems was inferred from compositional analysis of the continuous phase pre and post emulsification, in which protein and starch quantifications were included in the analysis. The concentration of WP in the serum (post emulsification) of these emulsions was 0.26% and 0.23%, respectively. Therefore, it was deduced that 0.12% and 0.18% protein, respectively, should be at the interfaces. In emulsions that contained 0.4% OSA/0.5% NaCAS/water and 0.4% OSA/0.5% NaCAS/buffer as shown in Figure 4-26, the protein concentration before adding oil and emulsification was 0.38% and 0.39%, respectively, while in the serum it was 0.24% and 0.25%. Therefore, more

than 50% of the protein was in the serum of these emulsions. Since only 0.5% w/w sodium caseinate was used, the values of protein concentration measured before emulsification using the assay were very close to the purity labelled by the supplier "90% on dry substance". In emulsions stabilised with OSA/WP/water, OSA/WP/buffer, OSA/NaCAS/water and OSA/NaCAS/buffer 0.12%, 0.18%, 0.14% and 0.14%, respectively, of the proteins used were adsorbed at droplet interface of these emulsions. These concentrations are similar to what had been labelled by the supplier that the purity of the WP equal 75.5% and the rest would be fat, carbohydrate, sugars, mineral salts see section 2.1, which endorses the accuracy of the assay.

4.5.2. Starch content

The starch quantity in the serum of the emulsions was measured using the Megazyme Total Starch Assay (Megazyme-International, 2015). This assay allows total starch measuring where starch is hydrolysed in two phases. Firstly, starch is totally solubilised and partially hydrolysed to maltodextrin. Secondly, maltodextrin is quantitatively hydrolysed to glucose using amyloglucosidase (Saunders, 2015). The incubation of both thermostable α -amylase and amyloglucosidase at a pH 5.0 simplifies the assay procedure and minimises the maltulose (4- α -glucopyranosyl-D-fructose) production, which is resistant to hydrolysis by these enzymes see section 2.4.5.2. Starch was measured in the continuous phase before the creation of the emulsions and in the serum phase post the emulsification. Starch concentration before emulsification was measured using the procedure of the assay kit related to measuring the starch in solid samples, in which starch was measured in standardised samples of solid starch was provided in the kit.

Moisture content measurement was required to take that in the account during the intercial composetion analysys. It was in OSA starch, WP and NaCAS was measured and found to be 1.1%, 2.4% and 3.9% respectively. Therefor, weight of water in 0.4% OSA starch, 0.5%, WP and 0.5% NaCAS samples used in the emulsions processing was 0.004, 0.012 and 0.0195%, respectively. Starch content in starch in the ingredients as used was also measured, in which starch was measured in OSA, WP and NaCAS solid samples and it was 84%, 2% and 2%, respectively. Since 0.4%w/w OSA starch was added during emulsion formulation, the actual OSA starch added was calculated as follows:

$$\text{The actual mass of starch in 0.4\% w/w OSA starch} = [(84 \times (0.4 - 0.004)) / 100] = 0.33\text{g} \quad (4-12)$$

$$\text{The actual mass of starch in 0.5\% w/w whey protein} = [(2 \times (0.5 - 0.012)) / 100] = 0.01\text{g} \quad (4-13)$$

$$\text{The actual mass of starch in 0.5\% w/w sodium caseinate} = [(2 \times (0.5 - 0.0195)) / 100] = 0.01\text{g} \quad (4-14)$$

From Equations (4-12), (4-13) and (4-14), and because only 0.5%w/w of each protein was used, the actual concentration of starch before emulsification was $[0.33 + 0.01] = 0.34\%$. Figure 4-27 shows the starch concentration in the serum of emulsions processed with 0.4% OSA/0.5%

WP/water, 0.4% OSA/0.5% WP/buffer, 0.4% OSA/0.5% NaCAS/ water and 0.4% OSA/0.5% NaCAS/buffer it was 0.07%, 0.06%, 0.08% and 0.07% respectively. The starch had only 84% starch as identified with the assay and the rest should be moisture, protein, fat and minerals as mentioned by the OSA starch supplier see section 2.1.

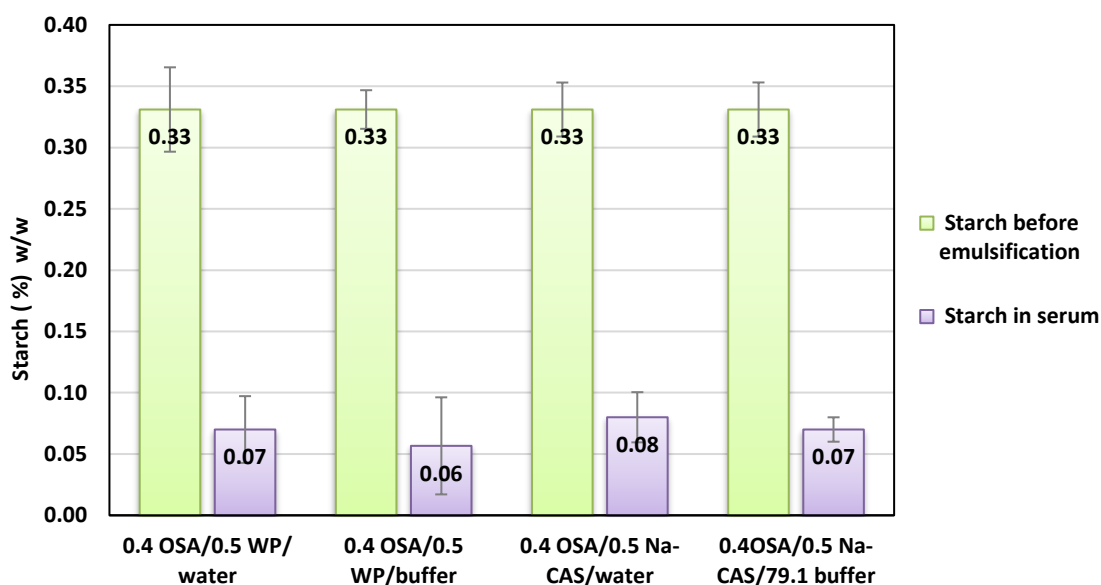


Figure 4-27. Starch concentrations in both the continuous phase before emulsification and in the serum post emulsification of O/W emulsions stabilised by OSA starch/protein mixed emulsifiers. Either deionised water or 0.1 M sodium phosphate buffer pH 7.0 was used as aqueous phase. Error bars represent $\pm$ standard deviation (n=3).

Comparing the starch concentration in the continuous phase before and after emulsification, it can be seen that most of the starch had adsorbed to the droplet interface. From emulsions stabilised with 0.4% OSA/0.5% WP/water, 0.4% OSA/0.5% WP/buffer, 0.4% OSA/0.5% NaCAS/water and 0.4% OSA/0.5% NaCAS/buffer in Figure 4-27, 0.26%, 0.27%, 0.25% and 0.26% w/w respectively, of starch should be adsorbed at the droplets interfaces of these emulsions.

It can be concluded that the ratio of starch: protein in the adsorbed phase of OSA/WP/water, OSA/WP/buffer, OSA/NaCAS/water and OSA/NaCAS/buffer stabilised emulsions were as follows: 0.26:0.12, 0.27:0.18, 0.25:0.14 and 0.26:0.14, respectively. In general, the ratio of starch: protein in formulation was 0.4:0.5, while the ratio of adsorption at the interface was about 2:1. Therefore, about 2/3 of the surface coverage was starch and 1/3 of it was converted by protein. Indeed, these results were confirmed the previous suggestions regarding the emulsions microstructure that starch and protein were presented in the interface.

4.6. Single emulsion processing in the presence of PGPR.

The effect of the presence of polyglycerol polyricinoleate (PGPR) during the processing on the stability of O/W emulsions stabilised with the mixed emulsifier systems developed in this research was investigated. Figure 4-28 shows the mean droplet size based on the volume disruption ($d_{4,3}$) of four O/W emulsions stabilised with OSA/WP mixed emulsifier. Two of these emulsions comprising of 0.57% w/w (PGPR) soluble in the oil phase which present 20% w/w of the total emulsion and the aqueous phases were either water or 0.1M sodium phosphate buffer. Slightly increase in the mean droplet size of emulsions containing the PGPR was happen comparing to that without PGPR (control emulsions). To identify the significance of this increase one-way ANOVA statistic test was used, which indicates that there was no statistical significant differences between day 1 and 14 for each emulsion ($P > 0.05$). Koliandris (2011), suggested that slight increase in the mean droplet size could be due to some replacement of the interfacial WP by PGPR. The

protein surface coverage depends on the treatments of the protein before homogenisation (Das and Kinsella, 1990). In addition, Dickinson et al. (1993) have stated that in O/W emulsions stabilised with WP, high concentrations of oil-soluble surfactants led to a small increase in the droplet size and a lower protein surface coverage, whereas the opposite effect happened at low surfactant concentrations.

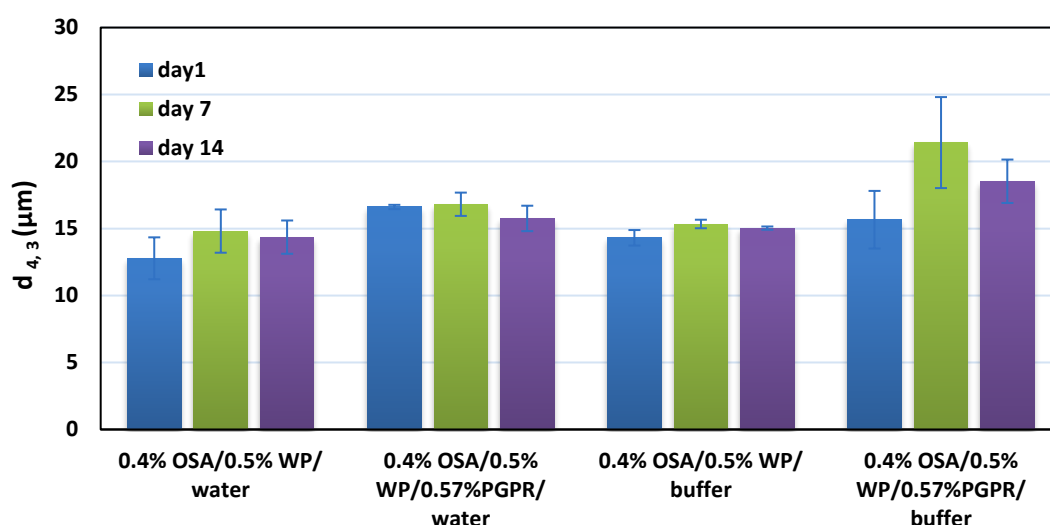


Figure 4-28. The mean droplet size based on volume distribution of O/W emulsions stabilised by 0.4% OSA/0.5% WP w/w mixed emulsifier and consisted of 20% w/w oil phase, two of them contain 0.57% w/w PGPR of total weight. The aqueous phase was either water or 0.1M sodium phosphate buffer pH 7.0. Error bars represent $\pm$ standard deviation ($n=3$).

Emulsions stabilised with OSA/NaCAS mixed emulsifier were also subjected to the same observation in terms of the effect of adding PGPR before homogenisation into the oil phase in the presence buffer and unbuffered aqueous phases. Figure 4-29 shown the mean droplet size based on volume distribution ($d_{4,3}$) of the four O/W emulsions stabilised by 0.4% OSA/0.5% NaCAS w/w. Two different oil phases were used with and without 0.57% w/w PGPR and two aqueous phases were use either water or 0.1M, pH 7.0 sodium

phosphate buffer. The results of this group of emulsions indicate a decrease in the mean droplet size and more constant measuring data points which refers to a better stability comparing to the emulsion without PGPR (control emulsion).

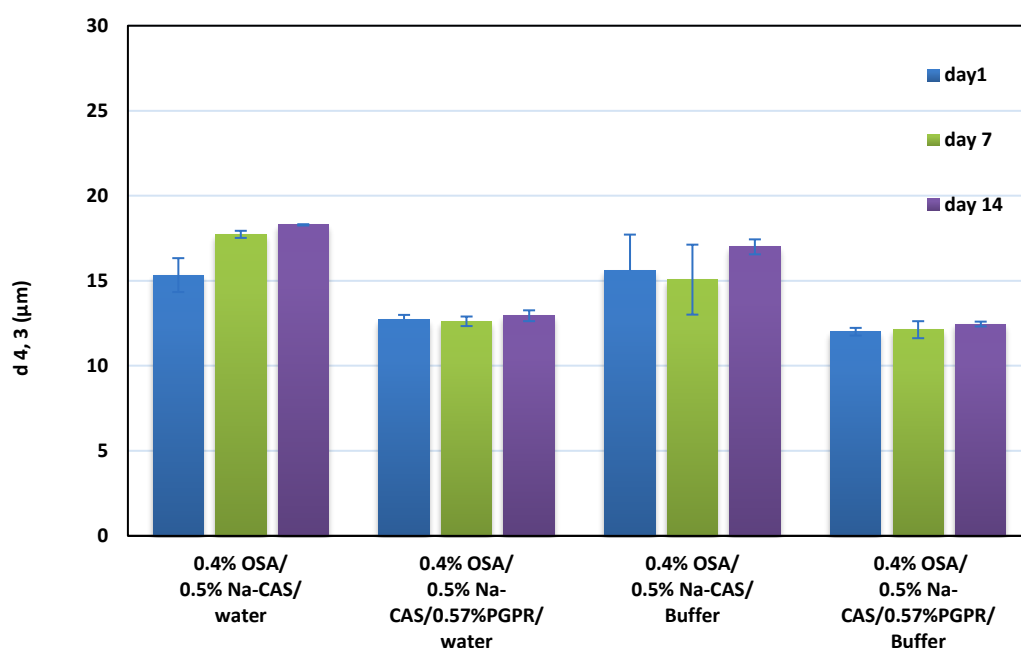


Figure 4-29. The mean droplet size based on volume distribution of O/W emulsions stabilised by 0.4% OSA/0.5% NaCAS w/w mixed emulsifier and consisted of 20% w/w oil phase, two of them contain 0.57% w/w PGPR of total weight. The aqueous phase was either water or 0.1M sodium phosphate buffer pH 7.0. Error bars represent $\pm$ standard deviation ($n=3$).

One-way ANOVA statistical test was used to identify if there any significant change in mean droplet size followed the presence of PGPR, this test indicated that statistically there was no significant differences ($P>0.05$) in the mean droplet size between days in each emulsion alone. The emulsion stabilised with OSA/NaCAS/water in absence of PGPR was an exception ($P<0.05$). A Post Hoc statistic test 'Tukey' was performed on this emulsion, significant differences were in day 1 but no significance differences between day 7 and

14. This could be merely a measuring error in day 1 particularly as in previous experiments this emulsion was stable in the same conditions. Adding PGPR to the oil phase of OSA/NaCAS stabilised emulsions led to decrease in droplet size and improved emulsions stability. Large droplets coalesce into larger droplets therefore lead to faster phase separation; this will be delayed with smaller droplets, but not prevented (McClements, 2005), therefore the stability of the present emulsions could be due to the small droplet formation. This finding agreed with the finding of Dickinson and Tanai (1992) who reported that the addition of oil soluble surfactants prior to emulsification lead to a decrease in droplet size. Addition of oil soluble emulsifiers to an O/W emulsion stabilised with β -casein improved the emulsification properties of the β -casein (Cornec, 1998). Adding oil-soluble surfactants before emulsification decreased droplet size associated with a decrease in protein surface concentration (Koliandris, 2011, Courthaudon et al., 1991). Therefore, the PGPR molecules adsorb at the interface in small domains and then expand to displace the protein from the interface (Wilde et al., 2004). The displacement depends on many factors such as the age of the adsorbed layer, the order of exposure at the interface (Courthaudon et al., 1991).

In the present emulsions, the age of the adsorbed layer was two weeks and during this period no coalescence was observed. Therefore, proteins were not replaced by the PGPR. Dickinson et al. (1993), stated that the competitive adsorption of WP and NaCAS in food emulsions is unlikely to be affected by surface-active impurities present in typical food oils (e.g. PGPR). For contrast, Koliandris (2011) stated that the addition of PGPR to oil phase of O/W emulsion reduces the bovine serum albumin (BSA) surface load and the

droplets size, while significantly increase the BSA concentration in the emulsion serum.

It can be summarised that the processing of the O/W emulsions stabilised with the OSA/WP mixed emulsifier system in the presence of 0.57% w/w PGPR led to slightly increase in the in the mean droplet size. This increase statically was not significant wheatear the aqueous phase was water or buffer. However, adding the same amount of PGPR to these emulsions when they was stabilised with OSA/NaCAS mixed emulsifier led to decease in the mean droplet size, which was also non-significant. Both O/W emulsion systems were developed into $W_1/O/W_2$ emulsions by adding internal aqueous phase into the oil phase of these emulsions. This addition required the formulation of a W/O emulsion as the primary stage of the double emulsion processing, so, the next experiment was focused on the formation and characterisation of the W/O emulsion. Nevertheless, all future data reported in the next chapter of results and discussion are based on unbuffered systems because using water or buffer in O/W emulsions stabilised with the mixed emulsifier systems did not make any statistical significant differences.

4.7. Conclusions for O/W emulsions stabilised with a mixed emulsifier

The results presented in this thesis chapter indicate that the mixed emulsifier systems reduced the interfacial tension to about two times larger than using OSA starch alone and similar to using either WP or NaCAS alone. Proteins adsorbed faster than OSA starch at the oil/water interface because the protein had reduced the interfacial tension to a larger extend than the OSA

starch. There was an inverse relationship between the concentration of these emulsifier and interfacial tension reduction until CMC point. The mean droplet size data indicated that emulsions stabilised with OSA/WP or OSA/NaCAS were stable against coalescence over two weeks of storage at ambient temperature with no matter whether the aqueous phase was water or buffer. Results of interfacial tension and droplet size were agreed with ζ -potential measurements. These emulsions showed a high ζ -potential which indicates to high stability.

Confocal Laser Scanning Microscopy (CLSM) was used for emulsions' microstructure investigation. The micrographs of OSA starch/protein mixed stabilised emulsion showed that both starch and protein were at the interface and the interfacial composition analysis confirmed that. In emulsions stabilised with OSA/WP or OSA/NaCAS; 0.26% and 0.25% of the starch used and 0.12% and 0.14% of protein used were adsorbed at the droplets interface, respectively. Therefore, O/W emulsions could be stabilised at lower emulsifying starch content using mixed WP or Na-CAS as a co-emulsifier compared to using starch alone. The ratio of starch: protein in the emulsions stabilised with mixed emulsifier systems during the formulation was 0.4:0.5, while the ratio the interfacial coverage at was about 2:1. There was no significant effect of the presence of PGPR during the processing on the stability of O/W emulsions stabilised with the mixed emulsifier systems.

Chapter 5: Results and Discussion - Programmed Complex Emulsions for Sugar Reduction

5. Programmed Complex Emulsions for Sugar Reduction

In this chapter, the mixed emulsifier systems designed in the previous research were used to produce $W_1/O/W_2$ emulsions in which 50% w/w sucrose solution was encapsulated as the inner water phase (W_1). Afterwards, measurements of the encapsulation efficiency of sugar in these multiple emulsions were performed. In addition, the release of sucrose post *in-vitro* enzymatic digestion of the interfacial adsorbed OSA starch was investigated. Furthermore, for applying the research strategy about sugar reduction in emulsion based food, the research focused on enriching the multiple emulsions with mineral salts as a close-to-market approach. Therefore, behaviour of the $W_1/O/W_2$ emulsions at typical levels of mineral salts in both aqueous phases as well as the impact of adding these salts on the sugar release were observed. The formulation of W/O emulsions is the primary stage of the formulation $W_1/O/W_2$ emulsions. Therefore, the formulation of $W_1/O/W_2$ started with the processing and characterisation of stable W/O emulsions as discussed below.

5.1. Sucrose enriched W/O emulsions

Triplicates of W/O emulsions consisting of 30% w/w distilled water and 70% w/w oil phase including 2% w/w (of the total emulsion) hydrophobic emulsifier (PGPR) were prepared. The processing was performed at a controlled temperature ($\approx 38^\circ\text{C}$). Back-scattering percentage (% BS) profiles acquired on these emulsions are presented as a function of time and tube height in Figure 5-30. These profiles were acquired immediately after the

formulation and again at day 2, 6, 13 and 15 of storage at room temperature ($\approx 24.0^\circ\text{C}$).

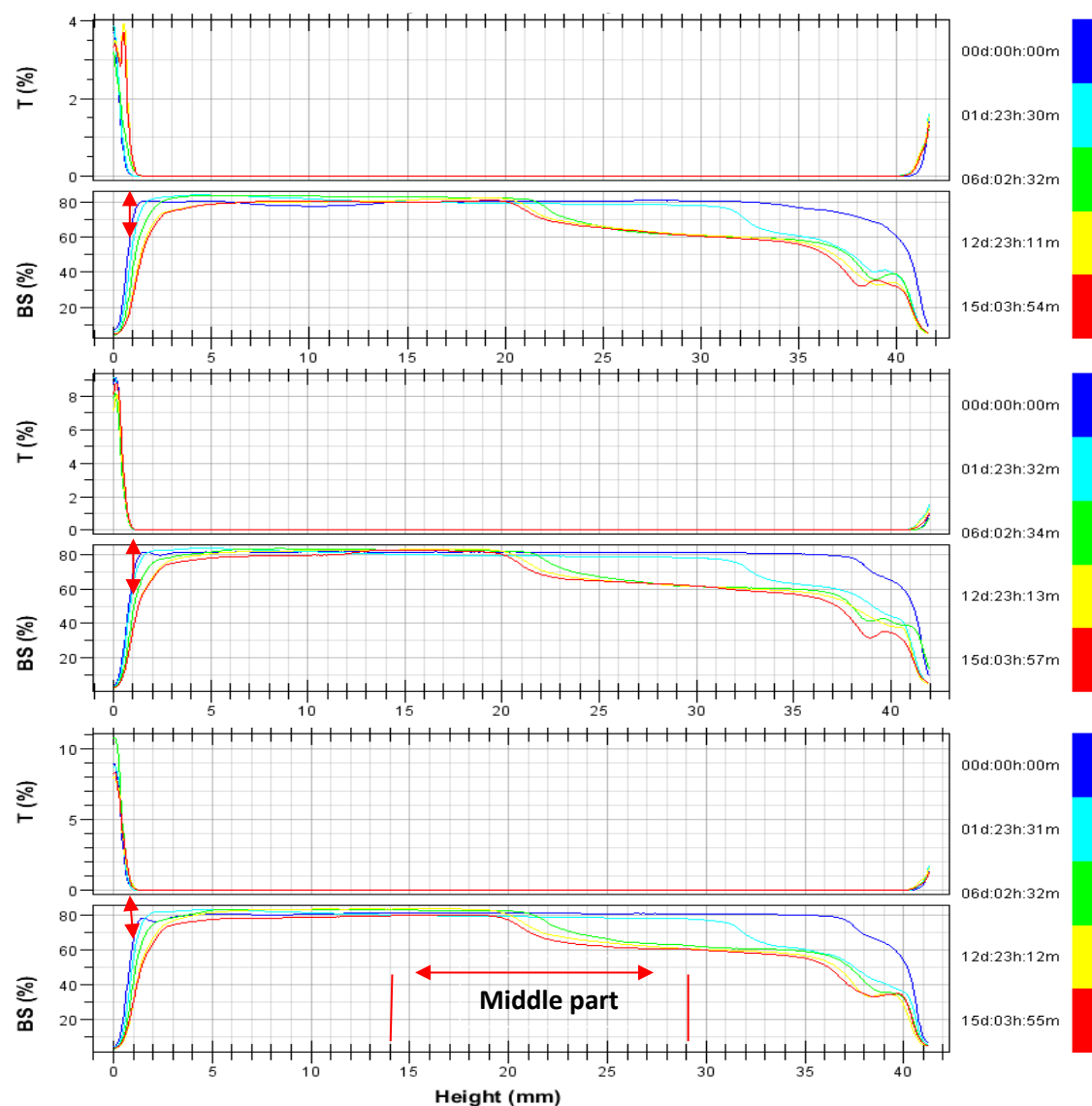


Figure 5- 30. Back-scattering (% BS) profiles as a function of time and tube height of three replications of W/O emulsion containing 30% aqueous (water alone) and 2% w/w PGPR. Measurements were performed at 20°C immediately after the formulation “blue” and at day 2 “light blue”, 6 “green”, 13 “yellow” and 15 “red”.

A stability analyser was employed to measure the emulsion stability against coalescence. The scan analyser measures the intensity of monochromatic

light at 880 nm wavelength after scanning the emulsion along the length of the tube in which it is held, giving profiles of the back scattering and transmission percentages as a function of the length tube, and time (Pan et al., 2002, Domian et al., 2015). The %BS value was used as a relative mean droplet size of W/O emulsion see section 2.2.3.1.1.

The destabilisation percentage (%D) was calculated from the back-scattering profiles in Figure 5- 29 and it was %D = 24.18. The value of %D indicates there was increase in the droplet size by 24.18% between day 1 and day 14 due to coalescence. The emulsions exhibited a slight sedimentation process due to the small increase of %BS at the tube bottom at the expense of a %BS decrease on the top part. These results agreed with Márquez et al. (2010) who stated that W/O emulsions are usually destabilised by sedimentation and coalescence, the decrease of the %BS along the tube containing the emulsion sample indicates an increase of water droplet size due to the coalescence. However, an increase of %BS values at the bottom of the tube at the expense of %BS decrease on the top part evidences a sedimentation of the water droplets.,. Su et al. (2008), found that W/O emulsions stabilised with 2% w/w PGPR were stable after storage for 7 days at 20 °C whereas coalescence was observed with even 2% w/w PGPR in this research. The reason for this difference could be that Su et al. used 25% w/w aqueous phase and PGPR (Palsgaard 4150) whereas 30% w/w aqueous phase and PGPR (Grindsted 90) were involved in the present research. Incremental change of BS light intensity is directly proportional to the dispersion phase concentration and is inversely proportional to the droplets' size (Cerimedo et al., 2008, Mengual et al., 1999). This destabilisation percentage (%D) of the W/O emulsion, which does not contain sucrose was compared with the %D

of a W/O emulsion containing sucrose. Triplicates of second W/O emulsion was produced at the same formulation conditions as the first emulsion consisting of 30% w/w distilled water and 70% w/w oil phase including 2% w/w (of the total emulsion) hydrophobic emulsifier (PGPR). A sucrose solution (50% w/w) was used as the aqueous phase instead of using only water in the first emulsion. The back-scattering profiles of the second emulsion are shown in Figure 5-31; the %D of this emulsion corresponds to the central part of the tube and it was is %D = 0.0%. Even though there was a small decrease of the %BS in the top part of the tube, there was no increase in the bottom, which does not fulfil the definition of an unstable emulsion as suggested by Márquez et al. (2010). Where the decrease of %BS along the tube means coalescence and increase at the bottom with decrease on the top part means sedimentation. Therefore, it can be said that there is no clear coalescence or sedimentation in this emulsion. A difference in %BS over the entire height compared with the central part of the test tube for samples of emulsions that have the same dispersed phase concentration may indicate differences in the formation of droplet composition of the emulsions (Domian et al., 2015).

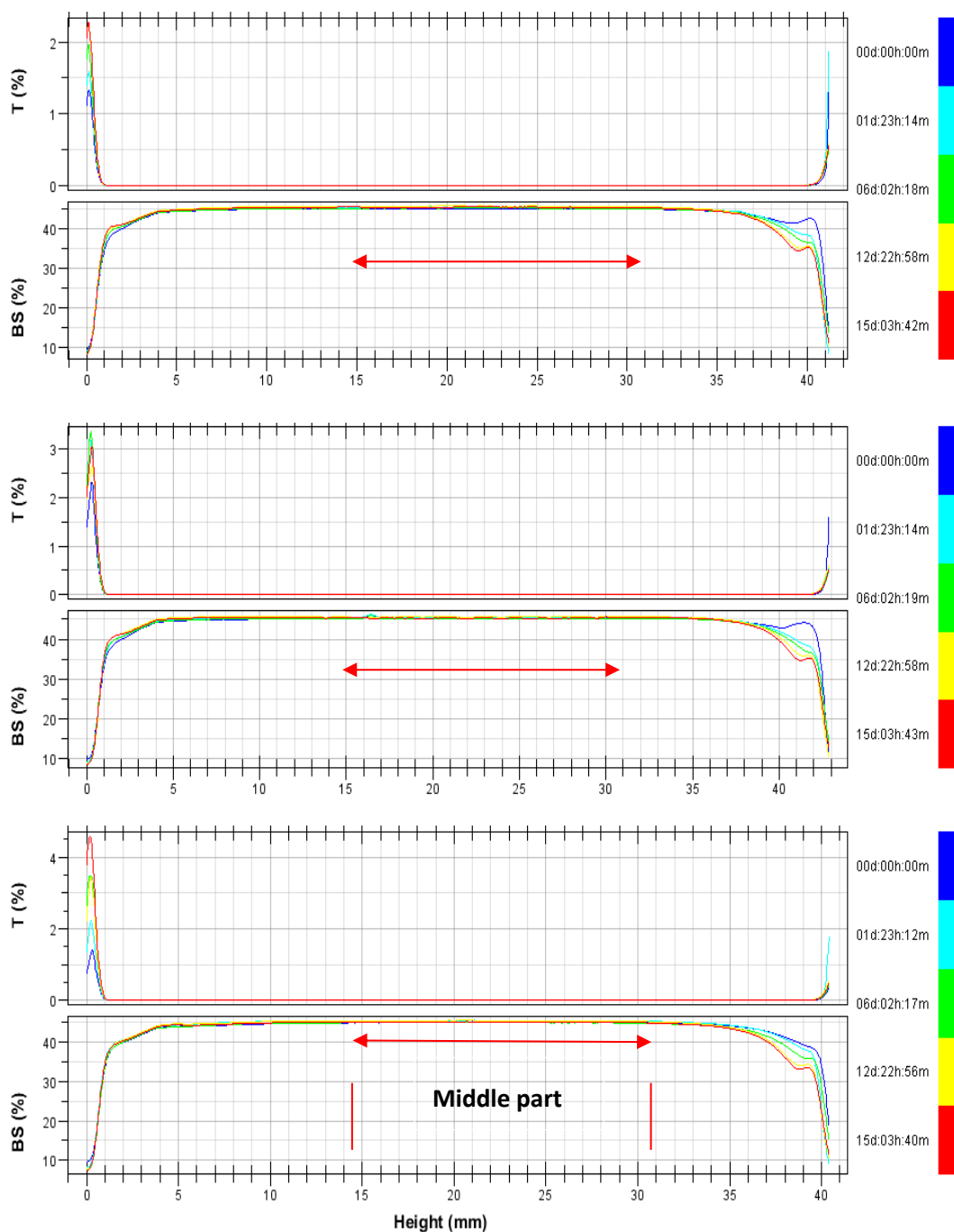


Figure 5-31. Back-scattering (% BS) profiles as a function of time and tube height of three replications of W/O emulsion containing 30% aqueous phase of "50% w/w sucrose solution" and 2% w/w PGPR. Measurements were performed at 20.0°C immediately after the formulation "blue" and at day 2 "light blue", 6 "green", 13 "yellow" and 15 "red".

Figures 5-30 and 5-31 show the %BS for the W/O emulsions formulated with water alone and sucrose solution, respectively. Each figure shows three profiles for three replications of one emulsion. The %BS in Figure 5-30

reduced from 80% to 60% after 15 days in the three profiles; this reduction was mainly after day 3, while it remained the same in Figure 5-31 during 15 days. The %BS of the "50% sucrose" emulsion indicates higher stability of this emulsion compared to the "water only" emulsion. Therefore, the destabilisation %D was reduced from 24.18% in the "water only aqueous phase" O/W emulsion to 0.0% in the "50% sucrose as aqueous phase" O/W emulsion due to the use of 50% w/w sucrose solution instead of water as well as the presence the hydrophobic emulsifier (PGPR) at relatively high concentration. Sucrose could lead to more adsorption of PGPR at the interface and smaller droplet formation giving stable emulsions.

Figure 5-32 shows an optical micrograph of W/O emulsion consisting of 30% aqueous phase and 2% PGPR, the aqueous was 50% w/w sucrose solution. The micrograph at scale bar 100µm was taken of the emulsion after 14 day of storage at ambient temperature and then the emulsion was diluted by oil at the rate of 1:1. The optical micrograph characterised by the presence of small water droplets, which would improve the stability of these systems and explain the higher stability of this emulsions. The micrograph showed the characteristic absence of coalescence between emulsion droplets, this demonstrated that the PGPR was able to emulsify high sucrose and therefore lower water aqueous phases. The high efficiency of PGPR at high levels of concentration in stabilising W/O emulsions has been stated by a number of authors (Márquez et al., 2010, Zhang and Reineccius, 2016). The mechanism of lipophilic emulsifiers to stabilise a W/O interface has been investigated by Kanouni et al. (2002) who found that the high elasticity of interfacial film formed by these emulsifiers allowed the film to withstand stress without breaking and, therefore, produced stable W/O emulsions. The droplet size of

W/O emulsions decreased with increasing concentrations of PGPR (Zhang and Reineccius, 2016).

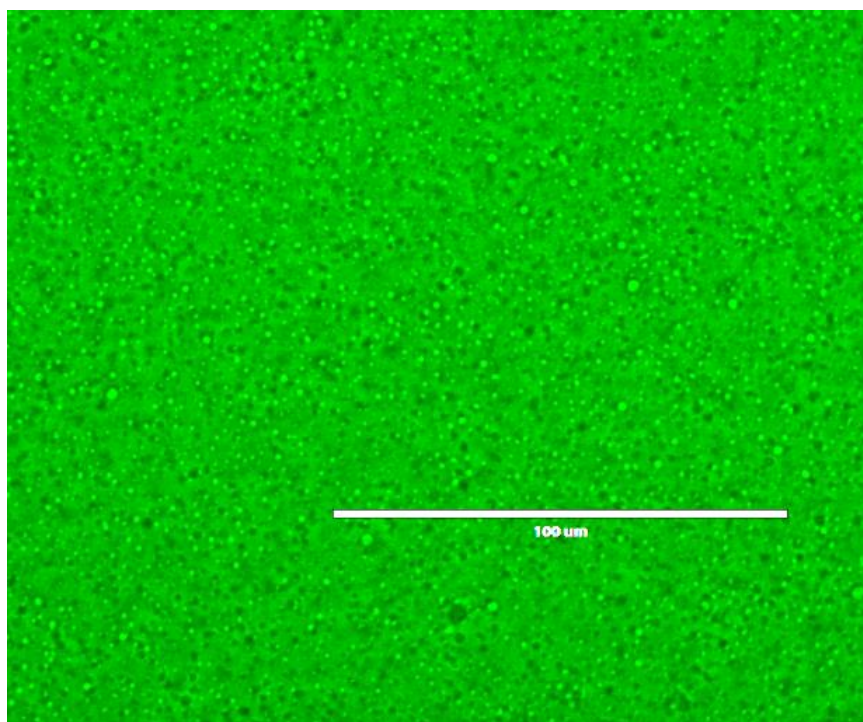


Figure 5-32 . Optical micrograph at scale bar 100 μ m of a W/O emulsion consisting of 30% aqueous phase and 2% PGPR was dissolved in the oil phase, a 50% w/w sucrose solution was used as the aqueous phase, the emulsion was diluted by oil at the rate of 1:1 and after 14 days of storage at ambient temperature.

5.1. Complex emulsions with sucrose enriched internal aqueous phase

The impact of the mixed aqueous phase emulsifier systems (in chapter 4) on the characteristics and stability of five different $W_1/O/W_2$ emulsions was investigated. These emulsions were formulated with 50% sugar solution as the internal phase and PGPR as the lipophilic emulsifier. The selection of hydrophilic emulsifiers is critical in controlling the droplet size and EE of

$W_1/O/W_2$ emulsions (Zhang and Reineccius, 2016), therefore, the five multiple emulsions were stabilised with 0.57% PGPR w/w as internal emulsifier. The 0.57% was calculated based on the amount of the PGPR used in the primary stage of these emulsions, which was the W/O emulsion discussed in the previous section that contains 2% PGPR w/w. The external aqueous phase was stabilised with either 0.8% OSA, 1% WP, 1% NaCAS, 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS w/w. Composition, temperature of formulation, creaming index see Equation 2-7 and pH values at day 1 and day 14 of the five different multiple emulsions investigated in this research are shown in Table 5-21.

Table 5-21. Composition, formulation temperature, creaming index and pH values on day 1 & day 14 of five different types of $W_1/O/W_2$ emulsions.

W ₁ /O/W ₂ emulsion	Weight (%)							At formulation		Visual Observation			pH after 14 days	
	Emulsifier				Oil	Sugar solution				water	Creaming (%)			
	WP	N-CAS	OSA	PGPR							Day			
											1	7		14
OSA			0.8	0.57	19.43	67.4	8.58	3.2	6.0	36	0±0.0	13±1.0	26±0.1	6.1
WP	1							3	6.9	38	15±0.3	24±0.1	24±0.1	6.9
NaCAS		1						3	6.9	34	14±0.7	22±0.3	23±0.1	7.0
OSA/WP		0.5	0.4					3.1	6.7	33	17±1.0	24±0.3	25±0.5	6.7
OSA/NaCAS	0.5		0.4					3.1	6.3	37	16±0.9	25±1.0	23±0.6	6.2

This research does not focus on stability against creaming, but as an additional characterisation of these emulsions, the averaged cream index of all emulsion at day 14 of the storage period was (24.2 ± 0.03) % see Table 5-21. There were no changes to the pH values of the $W_1/O/W_2$ samples during the 14-day storage period as shown in Table 5-21. The pH was measured as a parameter that, if there was a change in the pH would signify some

instability. The pH value is important parameter especially in emulsions stabilised by proteins, because reducing this value up to the IEP of these protein will lead to the denaturation of proteins, which in turn reduces the ability of them to stabilise the emulsions. In addition, knowing the pH will indicate how the emulsion could be used in food industry, therefore pH measurements were applied. A higher concentration of sucrose inside was important as a requirement of the taste enhancement approach as well as the hypotonic conditions which was also important for stability. Balcaen et al. (2017), found that the release from the core of $W_1/O/W_2$ emulsion happened only when the concentration was higher in the inner phase (hypotonic conditions), whereas when the concentration was equal (isotonic conditions) or lower (hypertonic conditions) the release did not occur, regardless of the internal lipophilic emulsifier used. The hypotonic conditions help to deliver high sugar to the mouth taste receptors after digestion of the interfacial OSA by saliva α -amylase during consumption of the product.

5.1.1. Droplet stability

Stability against coalescence of the five emulsions mentioned in the previous section was investigated by acquiring volume based mean droplet size ($d_{4,3}$) at day 1, 7 and 14 of storage at room temperature ≈ 24.0 °C as shown in Figure 5-33. Emulsions stabilised with a single emulsifier were used as references to the mixed emulsifier systems. One-way ANOVA statistical test indicated that there were no significant differences in the mean droplet diameter between the time points of measurements for each emulsion ($P > 0.05$) during the 14-day storage period. It was found that 0.8% w/w

concentration of OSA starch, while low, is still enough to stabilise the $W_1/O/W_2$ emulsion, whereas Chiu et al. (2017) found that 4 % w/w OSA starch to stabilise $W_1/O/W_2$ emulsion that used for sodium delivery. This result was good for the aim this research which was sugar reduction in emulsion based food. $W_1/O/W_2$ emulsions containing protein and stabilised with either single or mixed emulsifier all had similar mean droplet sizes.

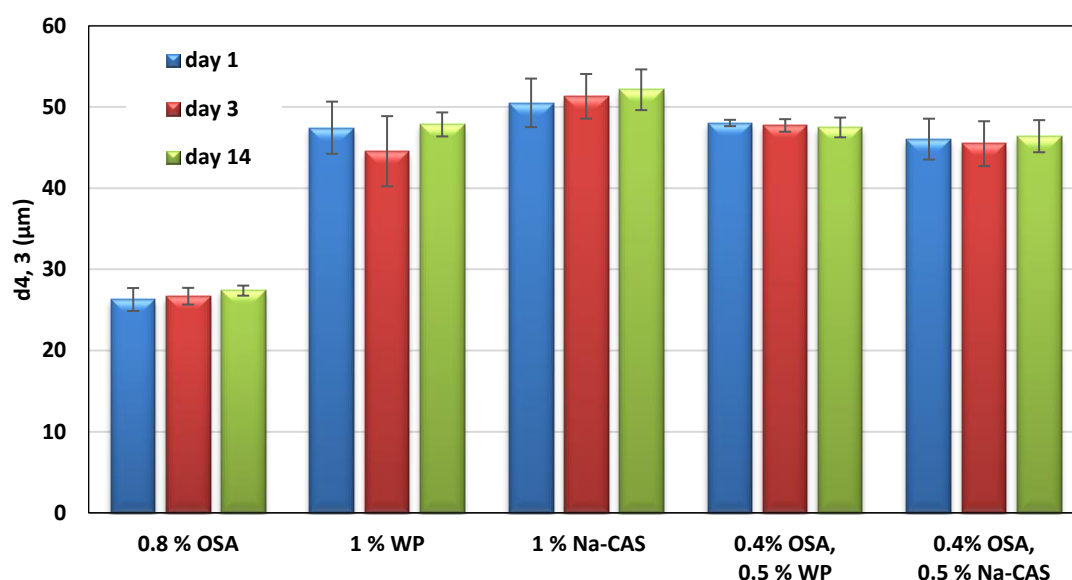


Figure 5-33. Volume based mean droplet size of $W_1/O/W_2$ emulsions stabilised with 0.57% PGPR as internal emulsifier. The external aqueous phase was stabilised with either 0.8% OSA, 1% WP, 1% N-CAS, 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS. Error bars represent $\pm$ standard deviation ($n=3$).

The volume based mean droplet size of these emulsions indicates to a similar distribution between the emulsions that were stabilised with protein alone or with mixed emulsifier systems. In addition, these results indicate to an increase in the mean droplet size by about double when protein is present in the interface. Panagopoulou et al. (2017), reported that the droplet size of $W_1/O/W_2$ emulsion stabilised with polysaccharides/protein is highly

dependent on the concentration and type of the emulsifiers used. Figure 5-34 shows the volume fraction distributions of the five $W_1/O/W_2$ emulsions. Each emulsion had very similar distributions at the four measuring data points which indicates to high stability against coalescence.

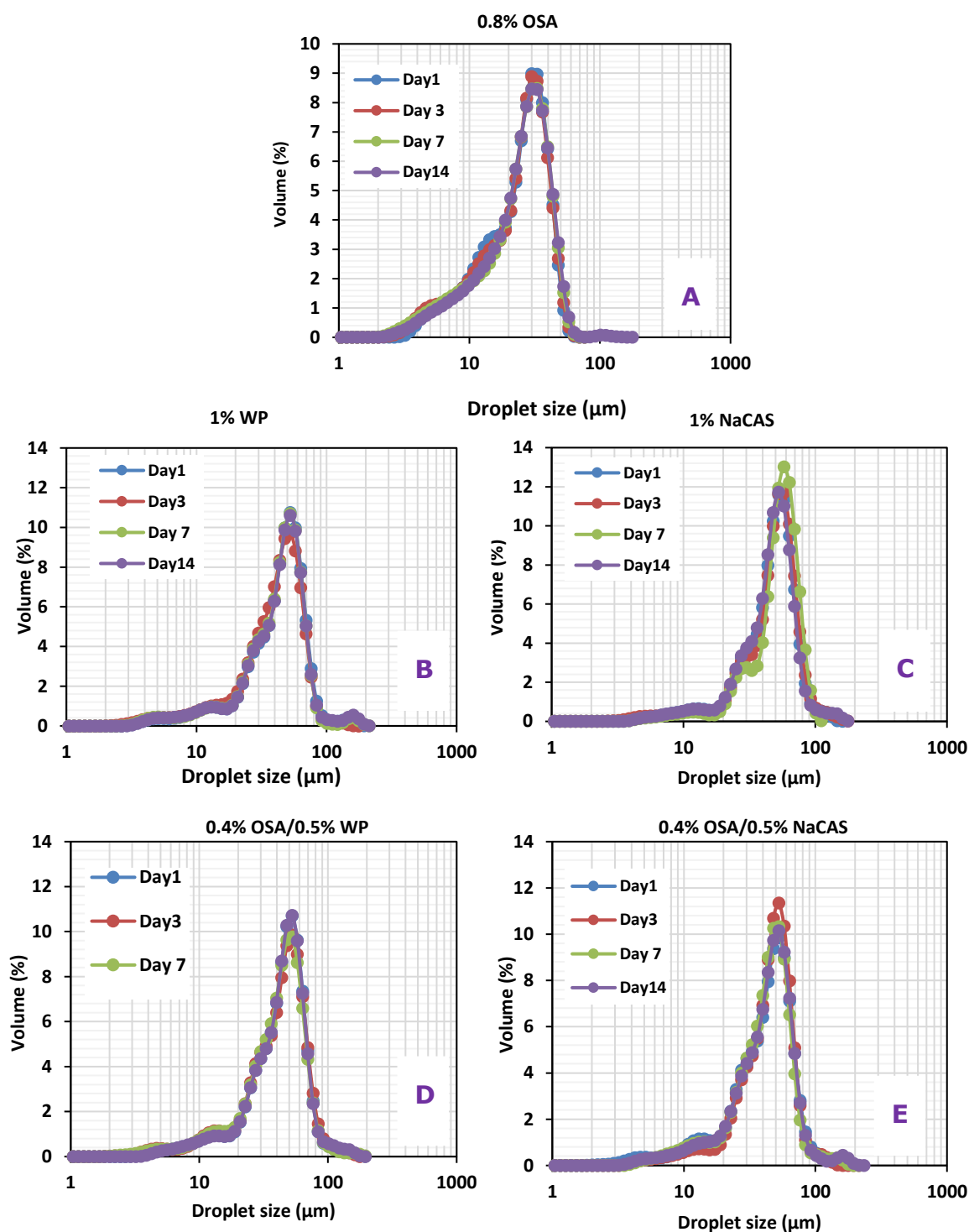


Figure 5-34. Volume fraction distribution of $W_1/O/W_2$ emulsions stabilised with 0.57% PGPR in the internal aqueous phase. The external aqueous phase was stabilised with either A: 0.8% OSA, B: 1% WP, C: 1% N-CAS, D: 0.4% OSA/0.5% WP or E: 0.4% OSA/0.5% NaCAS. Measurement performed at day 1, 3, 7 and 14 of storage at room temperature ($\approx 24.0^\circ\text{C}$).

Mixed emulsifier systems improved the stability of the main $W_1/O/W_2$ emulsions when compared to the single emulsifiers used in the control emulsions, because they led more consistent measuring data points and smaller droplet sizes. These outcomes agree with the report of Benichou et al. (2007) and Tamnak et al. (2016) who stated that mixtures of polysaccharides and proteins can be used as stability enhancers when preparing double emulsions.

Similar to single emulsion that were reported in chapter 4, the mean droplet sizes in these multiple emulsions were double when using protein alone or the mixed emulsifier systems compared to that found when using OSA alone under identical processing conditions. This results agrees with the finding of (Panagopoulou et al., 2017) that incorporation of polysaccharides/protein resulted in an increase of particle sizes compared to using polysaccharides alone. Proteins have been shown to coat the droplets of an emulsion and provide an energy barrier to the droplets' coalescence (Vanessa Cabra, 2008) and they play a key role in emulsion stabilisation aided by the presence of a polysaccharide (Ghosh and Bandyopadhyay, 2012). In addition, Schuch et al. (2015) found that the addition of an exclusively selected polysaccharide in the external aqueous phase of $W_1/O/W_2$ emulsions stabilised with only WP leads to better stability and encapsulation efficiency. Creation of more coherent interfaces (consistent interfaces of mixed polysaccharide/protein) inhibiting transfer of phases play an important role in improving the stability of the $W_1/O/W_2$ emulsions (Vaziri and Warburton, 1995). The mechanical properties of the protein layer at the interface depends on the structure of the proteins and the strength of the interactions between them, the properties influence the stability of protein-stabilised emulsions (Wilde et al.,

2004). Using OSA alone led to smaller drops compared to protein and OSA/protein mixed. This phenomenon was noted during the formulation of the O/W emulsions see section 3.1.1 and 3.1.2 when were stabilised by either the protein or OSA/protein mixed the size of oil droplet was double that when OSA alone was used. The reason for this could be due to the protein layer at the interface or the protein affected the homogenisation conditions such as increasing the viscosity of the continuous phase, which led to formation of larger droplets size.

The microstructure of the five W₁/O/W₂ emulsions acquired on an optical microscope are shown in Figure 5-35. These emulsions were stabilised with 0.57% w/w PGPR in the internal aqueous phase and with different emulsifiers in the external aqueous phase as follows: A = 0.8% OSA, B = 1% WP, C = 1% NaCAS, D = 0.4% OSA/0.5% WP and E = 0.4% OSA/0.5% NaCAS. The size of these droplets remained similar over a 14-day storage period. All droplets were filled with smaller drops and the dark appearance of the oil droplets were clearly recognisable, which means the formulation of W₁/O/W₂ emulsions. The present results agreed with the finding of Chiu et al. (2015) regarding the microscopic evidence of the droplet-in-droplet microstructure. they also agreed with Pawlik et al. (2010) as no appreciable difference in the morphology of the duplex emulsions or visible increase in the emulsions droplet size of was seen between day 1 and 14 of the storage period. All emulsions showed no sign of flocculation during the storage period. The droplets in case of the emulsion stabilised with OSA only (image C) appear smaller while they appear bigger in case of the emulsions stabilised with protein or mixed emulsifiers systems. These results were agreed with the droplet size data that the main droplet size was about 25 - 27µm in case of

OSA stabilised emulsions and about 47 - 52 μm in of using protein alone or OSA/protein mixed emulsifier. The partial replacement of the OSA starch in $W_1/O/W_2$ by either WP or NaCAS doubled the size of the oil drop.

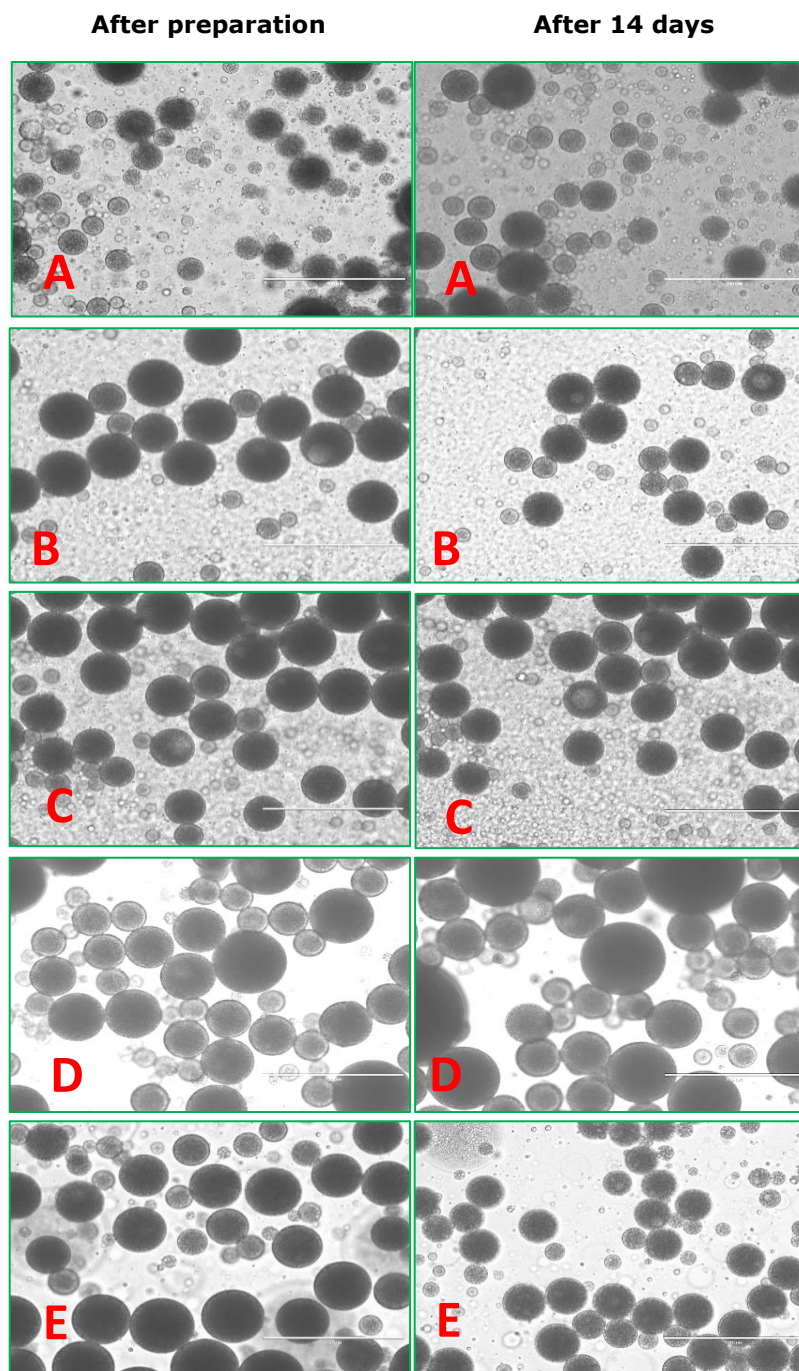


Figure 5-35. Optical microscopy images of $W_1/O/W_2$ emulsions stabilised with 0.57% w/w PGPR in the internal aqueous phase and different emulsifiers in the external aqueous phase as follows: A = 0.8% OSA, B = 1% WP, C = 1% NaCAS, D = 0.4% OSA/0.5% WP and E = 0.4% OSA/0.5% NaCAS. The imaging was performed using an EVOS microscope at scale bar 100μm immediately after preparation and after a 14-day storage period at ambient temperature.

The increase in the droplet size could be because the proteins varied the texture properties of the emulsion (Dickinson, 2008), which consequently affected the homogenisation conditions. Proteins coat the droplets of an emulsion in a relatively wide layer compared to that created by the OSA stabilised emulsions. After adsorption at the interface, the proteins infold and rearrange in their secondary and tertiary structures to expose their hydrophobic groups to the lipid phase (Vanessa Cabra, 2008, Wilde et al., 2004). Therefore, the increase in the droplet size when protein presented in emulsions could be due the formation of this layer at the interface. In order to understand whether the continuous phase was water or oil Márquez et al. (2007) stained samples of $W_1/O/W_2$ emulsions with a hydrophilic dye which gives a reddish colour to the aqueous phases, so oil droplets were seen isolated and surrounded by a red continuous aqueous phase with little droplets of dispersed aqueous phase inside of them. Throughout the experiment, the size of droplets remained very close to that measured at day 1, which was confirmed by droplet size measurement in the next section.

5.2. Encapsulation efficiency assessed by LCMS

Encapsulation efficiency (EE) of sugar in the multiple emulsion was measured using two different methods a Liquid Chromatography Mass Spectrometry (LCMS) based method and an ultraviolet (UV) absorption based method. The EE of an entrapped substance in the inner phase of $W_1/O/W_2$ emulsions is defined by Vladisavljević et al. (2014) in the equation (5-15) as follows:

$$EE (\%) = \frac{[(\text{mass of the substance added} - \text{mass of the substance released}) / \text{mass of substance added}] * 100}{(5-15)}$$

5.1.2. Encapsulation efficiency measurements using a LCMS based method

Two-calibration curves were used for sucrose detection in the LCMS based method as shown in Figure 5-36, and each curve was obtained from separate data points of two different calibration solutions. The first solution was obtained from a $W_1/O/W_2$ emulsion stabilised with 0.4% OSA/0.5% WP as outer emulsifier, whereas the second one was obtained from a $W_1/O/W_2$ emulsion stabilised with 0.4% OSA/0.5% NaCAS. The curves were prepared for EE measurements at both day 1 and 14. Calibration solutions were prepared following the procedure described in section 2.3.6.1.1.

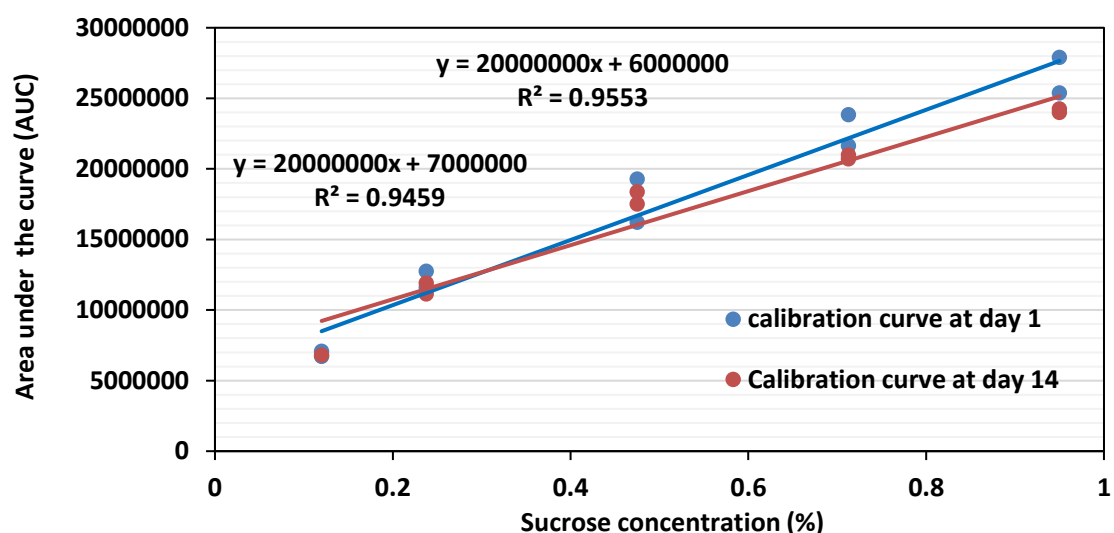


Figure 5-36. Two-calibration curves for sucrose detection in the LCMS based method, each curve obtained from separate data points of two different calibration solutions. The blue and the red curves were prepared for EE measurements at day 1 and 14, respectively.

The EE was inferred from measuring the sucrose concentration in the external aqueous phase based on the assumption that any sucrose not detected in the external aqueous phase was still encapsulated in the internal aqueous phase. At day-14 of the $W_1/O/W_2$ emulsions' storage period, a calibration curve similar to that calibration curve at day 1 was obtained. Similar calibration curves at day 1 and 14 implying that the LCMS detector was working at identical calibration conditions. Figure 5-37 shows the EE of sucrose in two $W_1/O/W_2$ emulsions stabilised with 0.57% PGPR w/w as the inner emulsifier and with either 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS w/w as the outer mixed emulsifier, compared with the three control emulsions stabilised with either 0.8% OSA, 1% WP or 1% NaCAS only. Measurements of EE performed at day 1 and day 14 of storage at $\approx 24.0^\circ\text{C}$.

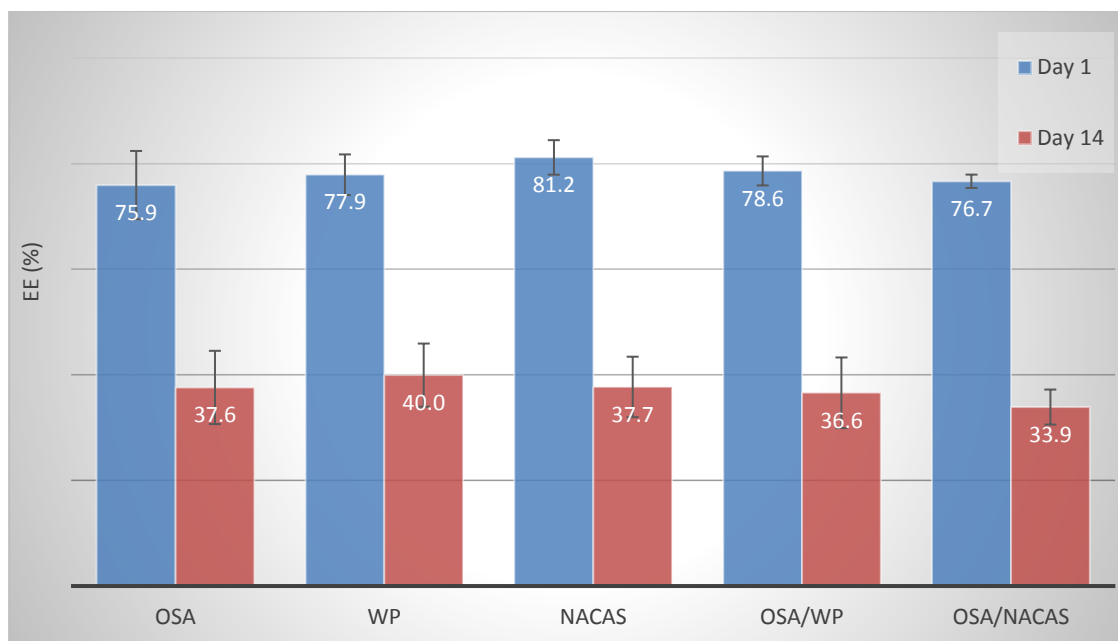


Figure 5-37. Encapsulation efficiency of sucrose in five $W_1/O/W_2$ emulsions stabilised with either OSA, WP, NaCAS, OSA/WP or OSA/NaCAS as outer mixed emulsifier. Emulsions stabilised with single emulsifier were used as controls to those stabilised with mixed emulsifier systems. Measurements performed using the LCMS based method at day 1 and day 14 of storage at $\approx 24.0^\circ\text{C}$. Error bars represent $\pm$ standard deviation ($n=3$).

One-way ANOVA statistical test was independently performed on the EE of emulsions at day 1 and 14 to identify if there were any significant differences in the EE between emulsions stabilised with the five different emulsifiers. This test indicates that there were no significant differences ($P>0.05$) between emulsions in the EE at each time point alone. According to the formulation, the EE should be 100% but some sucrose seems to be released during preparing the samples for the analysis due to the dilutions and centrifugation. The EE of emulsion stabilised with OSA/WP mixed emulsifier system was 78.6% at day 1, which was slightly better than the EE of the reference emulsions that were stabilised with the single emulsifier.

There was a trend that the emulsions containing protein have a slight increase in the EE. The stabilisation mechanism for these could be because

of the big viscoelastic layers of WP around each droplet, which was different from using OSA/NaCAS as confirmed by the microstructure of the emulsion in Figures 4-23, 4-24 and 4-25. However, all emulsions were not stable to sucrose diffusion because at 14 the EE was too lower than that at day 1, even the mean droplet size and microstructure of emulsions were not changed. At day 14, the EE of OSA/WP stabilised emulsion was 36.6%, which is slightly less than that in the reference emulsions stabilised with WP only. This is could be because 1% w/w WP in the external aqueous phase forms a denser layer over time at the external interface, which delays the diffusion of the sugar from the internal to the external aqueous phase. Proteins at the interface infold and rearrange their secondary and tertiary structure to expose their hydrophobic groups to the lipid phase (Vanessa Cabra, 2008, Wilde et al., 2004).

The EE of the emulsion stabilised with OSA/NaCAS at day 1 was 76.7%, which is similar to the EE of the emulsion stabilised by OSA/WP. In addition, the EE of the emulsion stabilised with this mixed emulsifier was very close to the efficiency of the related reference emulsions. Therefore, both WP and NaCAS were used in the partial replacement of the OSA starch in $W_1/O/W_2$ with no impact on the EE of sucrose. However, Muschiolik (2007) reported that the EE of glycine in $W_1/O/W_2$ emulsions was enhanced using NaCAS/dextran instead of using protein alone. On day 14, the EE of the OSA/NaCAS stabilised emulsions was slightly less than the EE of the emulsions when NaCAS was used alone; this is also expected because the concentration of NaCAS was double when it was used alone. High EE (>90%) was achieved with the formulation in $W_1/O/W_2$ emulsions containing 60% sucrose solution in the inner aqueous phase, stabilised with 4% w/w PGPR as the hydrophobic

emulsifier and 14.4% w/w polysaccharides (gum arabic) as the hydrophilic emulsifier, whereas using 10.8% w/w modified starch (Purity Gum™ 1773) showed poor EE < 50% (Zhang and Reineccius, 2016). In the present research, a higher EE ($\approx 76\%$) was achieved in $W_1/O/W_2$ emulsions stabilised with modified starch just 0.8% OSA and 0.57 PGPR w/w. The reason appears to be that Zhang and Reineccius used 10mM citrate buffer pH 3.6 in the inner and outer aqueous phases. This study confirms that buffers screen the negative charge on the modified starch molecule leading to instability against coalescence see table 4-18. (Su et al., 2008) stated that by using distilled water systems, 90% of the EE was achieved in $W_1/O/W_2$ emulsions using a concentration of PGPR 2% w/w in the oil phase and an external emulsifier of 0.5 % NaCAS w/w. The design of this research was based on the formulation of W_1/O emulsion performed by Vladislavljević et al. (2014) however, he used 5% w/w PGPR, while in this research only 2% of the initial W_1/O emulsion was used. Consequently, this could be the reason why the overall EE = 78.1 of all $W_1/O/W_2$ emulsions measured by the LCMS based method, which was lower than that achieved by Vladislavljević et al.

5.1.3. Encapsulation efficiency assessed by UV spectroscopy

In the UV absorption based method, glucose was used instead of sucrose during the formulation of $W_1/O/W_2$ emulsion because the final part of Megazyme total starch assay kit used is specifically designed to measure glucose. The calibration curve for seven concentrations of glucose solution using the last part of Megazyme Assay kit (UV absorption based method) is shown in Figure 5-38. This curve was used for the glucose measurements in the control samples of the next experiment as well as in glucose detection in

the double emulsions' serum during the EE measurements at day-one of the storage period. Another calibration curve was prepared for measuring the EE of $W_1/O/W_2$ at day 14 of the storage, which was identical to the curve calculated at day 1, so it is not reported here.

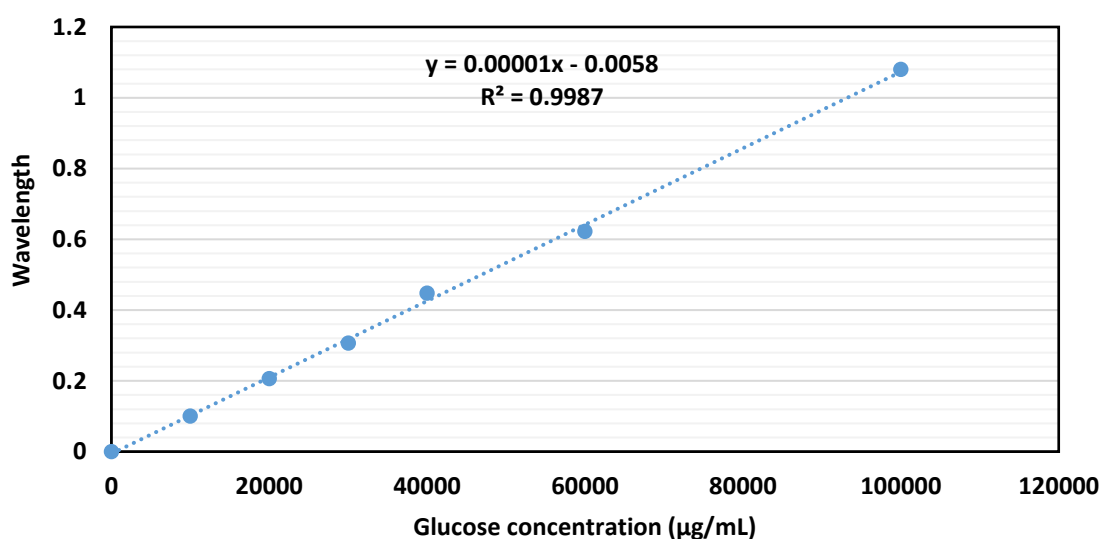


Figure 5-38. Calibration curve for seven concentrations of glucose solution were measured using the last part of Megazyme Assay kit (UV absorption based method) at 20.0 °C.

Three O/W emulsions were stabilised with either 0.8% OSA, 1% WP or 1% NaCAS w/w, and 5% w/w glucose solution was used as the aqueous phase of each emulsion. The glucose concentration was detected in the emulsions stabilised with either OSA, WP or NaCAS as 99%, 98% and 94%, of the original concentration, respectively. Samples were measured three times and data was plotted as independent data points. Three replicates of each emulsion were measured and the serum was diluted by 1:100 before measurement with the assay kit. The last part of Megazyme Assay kit identified the glucose concentration in the serum of three control O/W using

the acquired calibration curve as shown in table 5-22. The outcomes of this experiment validated the UV absorption based method of glucose detection in the serum of different emulsions. Therefore, this method was involved in measuring the EE of $W_1/O/W_2$ as well as measuring the release of glucose as a function of time.

Table 5-22. Measurements of glucose concentration in the serum of three O/W control emulsions containing 20% oil, the aqueous phase was 5% w/w glucose solution. Data obtained using the last part of the Megazyme total starch assay kit. Three replicates of each emulsion were created and each sample measured three times.

O/W emulsion stabilised with:	Glucose (%) $\pm$ STDEV
0.8% OSA	5.000 $\pm$ 0.473
	5.000 $\pm$ 0.001
	4.800 $\pm$ 0.160
1% WP	4.900 $\pm$ 0.051
	4.900 $\pm$ 0.053
	4.900 $\pm$ 0.001
1% NaCAS	4.700 $\pm$ 0.015
	4.700 $\pm$ 0.056
	4.700 $\pm$ 0.002

The fact that there might be some impurity in the proteins used needs to be considered because depending on the supplier information they were not 100% protein. However, if there were some it appears be so little that it did not change the picture much. Figure 5-39 shows the EE of glucose encapsulated in the $W_1/O/W_2$ emulsion stabilised with either 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS w/w in the external water phase. These emulsions were compared to the three other $W_1/O/W_2$ emulsions stabilised with the single emulsifiers 0.8% OSA, 1% WP or 1% NaCAS. Measurements were performed after day 1 and day 14 of storage at ambient temperature ≈ 24.0 °C using the ultraviolet-based method.

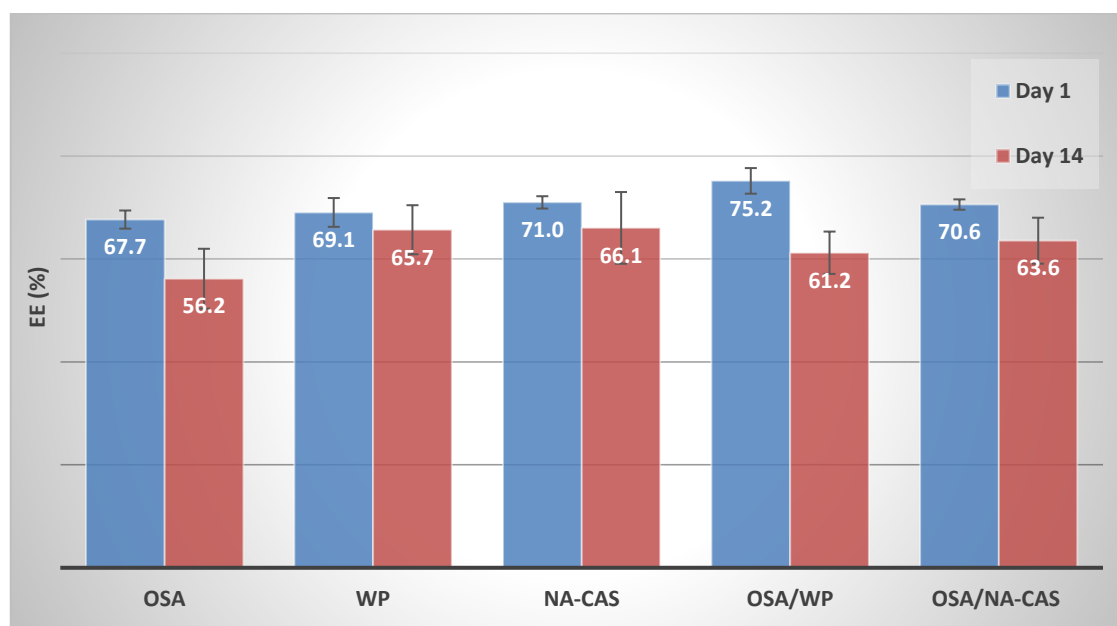


Figure 5-39. Encapsulation efficiency of glucose in five $W_1/O/W_2$ emulsions stabilised with either OSA, WP, NaCAS, OSA/WP or OSA/NaCAS as outer mixed emulsifier. Emulsions stabilised with single emulsifier were used as controls to those stabilised with mixed emulsifier systems. Measurements performed using the LCMS based method at day 1 and day 14 of storage at $\approx 24.0^\circ\text{C}$. Error bars represent $\pm$ standard deviation ($n=3$).

One-way ANOVA and Multiple Comparisons (Tukey HSD) statistical tests were independently performed to identify if there were any significant differences in the EE between the different emulsions. There were significant differences in the EE ($P < 0.05$) between the emulsions at day 1, certainly between OSA/WP, WP and OSA stabilised emulsions, while no significant differences ($P > 0.05$) at day 14. The results of the ultraviolet (UV) absorption based method confirmed the outcomes obtained from the LCMS based method concerning the EE in $W_1/O/W_2$ emulsions. The results of both methods are very similar for day 1, though the values are slightly lower for all emulsions in the UV absorption based method. Emulsions stabilised with NaCAS alone had a higher EE than the other emulsions; this could be related to the ability

of NaCAS as stronger emulsifier in making more stable emulsions. Depending on data in chapter 4 of this thesis NaCAS reduced the interfacial tension and had a zeta potential higher than the other emulsifiers used. In the UV absorption based method, the replacement gave higher EE in the OSA/WP and similar in the OSA/NaCAS stabilised emulsions comparing to the control emulsions.

At day one, the EE of the emulsion that was stabilised with the mixed emulsifier system 0.4% OSA/0.5% WP was 75.2%, which is better than the EE acquired by the single emulsifier stabilised emulsion. These outcomes agreed with the finding obtained by the LCMS based method. In addition, similar outcomes have been reported by Tamnak et al. (2016) who found that a $W_1/O/W_2$ emulsion stabilised with mixed (pectin/pea protein) as the external emulsifier had relative EE higher than those stabilised with only pectin, and they had smaller droplets. In addition, Muschiolik (2007) reported that different proteins (WP, NaCAS, soybean protein and wheat protein) were compared as emulsifiers for $W_1/O/W_2$, the highest EE (tryptophan) were shown by WP isolate; this also had the best emulsification behaviour due to smaller droplets and high stability. The EE of the emulsion stabilised with the mixed emulsifier system 0.4%OSA/0.5% NaCAS was 70.36%, which is less than the EE of the emulsion stabilised by 0.4%OSA/0.5%WP. This was expected because the assay detection for glucose in the control emulsions stabilised with NaCAS was lower than that in the WP stabilised emulsions (see Table 5-22). In addition, the EE of the emulsion stabilised with 0.4%OSA/0.5% NaCAS was very close to the EE of the reference emulsions. Therefore, both WP and NaCAS can be used in the partial replacement of the interfacial OSA starch in $W_1/O/W_2$ without reducing the EE of the glucose. Su

et al. (2008), found that in $W_1/O/W_2$ emulsions stabilised with 0.5% w/w PGPR in internal phase and 0.5% w/w NaCAS, the EE $\approx$ 62%. In this experiment, the EE was $\approx$ 70%, which is expected because 50% sugar was used in the W_1 phase and 0.57 w/w PGPR, while in the work of Su et al., the internal aqueous phase was just water. As it confirmed in W/O emulsions results in Figure 5-31 the presence of sucrose let to stable emulsions.

Even though the EE obtained by the LCMS based method was slightly higher, both analysis methods agreed that emulsion stabilised with protein had a higher EE than emulsions that were stabilised with OSA only. Emulsions stabilised with OSA only had smaller droplet size which would increase of the population of droplets, therefore, smaller droplets have a larger surface area and more release. The statistical results support this conclusion as there was statistically significant difference between OSA, OSA/WP and WP stabilised emulsions. This outcome agreed with the statement of Vladislavljević et al. (2014) who noted that the EE of food-grade $W_1/O/W_2$ emulsions improved where the area was increased, based mean diameter $d_{3,2}$ of the oil droplets. In addition, it agreed with the finding of Schuch et al. (2015) who stated that in $W_1/O/W_2$ emulsions stabilised with WP as hydrophilic emulsifier, the EE is direct proportionality to the oil drop size.

The results of the ultraviolet (UV) absorption based method at day 1 confirmed the outcomes obtained from the LCMS based method concerning the EE in $W_1/O/W_2$ emulsions. The results of both methods are very similar for day 1, though the values are slightly lower for all emulsions in the UV absorption based method. Furthermore, both methods agreed that the EE at day 14 of the emulsions stabilised by the mixed interface systems was slightly

less than that in the emulsions stabilised with WP only. However, at day 14 of the emulsions storage period, the EE acquired from the UV absorption based method was higher by about a third compared to that acquired using the LCMS based method. Although the micro structures at day 1 and 14 were similar for each emulsion in Figure 5-35, and there was no droplet shrinkage. Therefore, these differences in the EE could be because of the difference in the sugar type, which can be different in the diffusion rate from the internal to the external aqueous phase. Another potential, the low EE at day 14 in the LCMS could be due to occasional accident happened in the sample preparation for the analysis.

In addition, the EE of sucrose was higher than the EE of glucose at the first measurement time point. On the contrary, all emulsions measured had a higher EE when glucose was used than that when sucrose was used, at the second measurement time point. This could be because of a difference between in the properties of sucrose and glucose regarding their release from the internal to the external aqueous phase of the multiple emulsion, as glucose is a smaller molecule than sucrose. The EE measured using the LCMS based method and the UV absorption based method was between 70.6 – 78.6% in emulsions stabilised with starch/protein mixed emulsifier systems and varied from 67.6 to 78.6% in all emulsions at the first measurement time point. The present data suggested that the interfacial OSA starch can be partially replaced with protein without influence on the EE of the sugar solution. This replacement gave a similar EE to that achieved in the control emulsions stabilised with single emulsifiers in the LCMS. These outcomes supported the continuation of the research into measuring the release of the

encapsulated sugar after *in-vitro* digestion in the next experiments, using the same principles of measuring analysis.

It can be concluded that mixed or sole use of OSA starch and milk proteins for the stabilisation of $W_1/O/W_2$ emulsions ensured a high EE of sucrose or glucose in the inner phase. This conclusion is compatible with the statement of Benichou et al. (2004) that polymeric amphiphilic molecules are known to improve the interfacial coverage and therefore ensure high EE and controlled release of the function ingredients entrapped in the inner phase of the multiple emulsion. According to the finding of Hino et al. (2001); the presence of sodium chloride (NaCl) of up to 0.2M in the internal aqueous phase of $W_1/O/W_2$ emulsions played an important role in improving the stability as well as increasing the EE of the functional ingredients that do not penetrate into the oil membrane. The outcome suggested that the EE of emulsions used could be improved by adding NaCl into the core of them. However, added NaCl in the outer aqueous phase accelerated the phase separation. The EE in both methods was at least 70.6 %, which is good, but this outcome suggested that during processing some of W_1 was transferred into W_2 .

5.3. Amylase mediated sucrose release from the internal aqueous phase of water-in-oil-in-water emulsions

The starch at the external interface of the $W_1/O/W_2$ emulsion would be partially digested by adding α -amylase enzyme leading to a partial breakdown of the $W_1/O/W_2$ emulsion and the release of sugar from the internal aqueous phase to the external aqueous phase. Digestion and measurement of release of the encapsulated sucrose in $W_1/O/W_2$ emulsions

were performed at day 1 of storage at ambient temperature. These emulsions containing either starch (control emulsion) or starch/protein mixed emulsifiers at the external interface. The sugar release from the inner (W_1) to the outer (W_2) aqueous phase was measured after *in-vitro* enzymatic digestion using porcine pancreas α -amylase enzyme at a level of 10 units/mL. The quantity of sugar released was inferred from the outer aqueous phase analysis as a function of digestion time; the increasing level of sugar detected in the outer aqueous phase would mean that it came from the inner aqueous phase. Nevertheless, it can be a potential error that some of the hydrolysis product of starch could be detected.

5.3.1. Microstructure of the multiple emulsions post enzymatic digestion

The microstructure of three $W_1/O/W_2$ emulsions stabilised with either 0.8% OSA w/w, 0.4% OSA/0.5% WP w/w or 0.4% OSA/0.5% NaCAS w/w at the outer interface is shown in Figure 5-40. Imaging was performed using an optical microscope pre and post *in-vitro* digestion of the emulsions by α -amylase enzyme for 150 seconds at day one of emulsions shelf life.

For all emulsions, there were substantial microstructure changes after digestion indicating to destabilisation of the oil droplet interface and coalescence as much larger oil droplets are seen in the digested emulsions compared to that before digestion. These processes have led to the release of the inner aqueous phase in the digested samples; similar results have been reported by Chiu et al. (2015). In addition, it seems to be that there was breakdown of some oil droplets, therefore, destabilisation of the emulsion

during digestion taken two potential stages, coalescence and then phase inversion. However, Chiu et al. (2015) stated that the enzymes present orally was more effective in digesting OSA starch at the outer aqueous phase of $W_1/O/W_2$ emulsions in-vivo digestion, therefore, it would be the picture better if the digestion was in-vivo. During starch modification, the loading of OSA to starch is inversely proportional to the resistance of starch to digestion in a non-emulsified state therefore, starch modification is limited to 3% OSA for food use (Han and BeMiller, 2007, Viswanathan, 1999). Micrographs of *In-vitro* digestion by porcine pancreas α -amylase has been compared with micrographs of in vivo digestion, the enzymes present orally were more effective in digesting OSA starch at the outer aqueous phase of $W_1/O/W_2$ emulsions (Chiu et al., 2015).

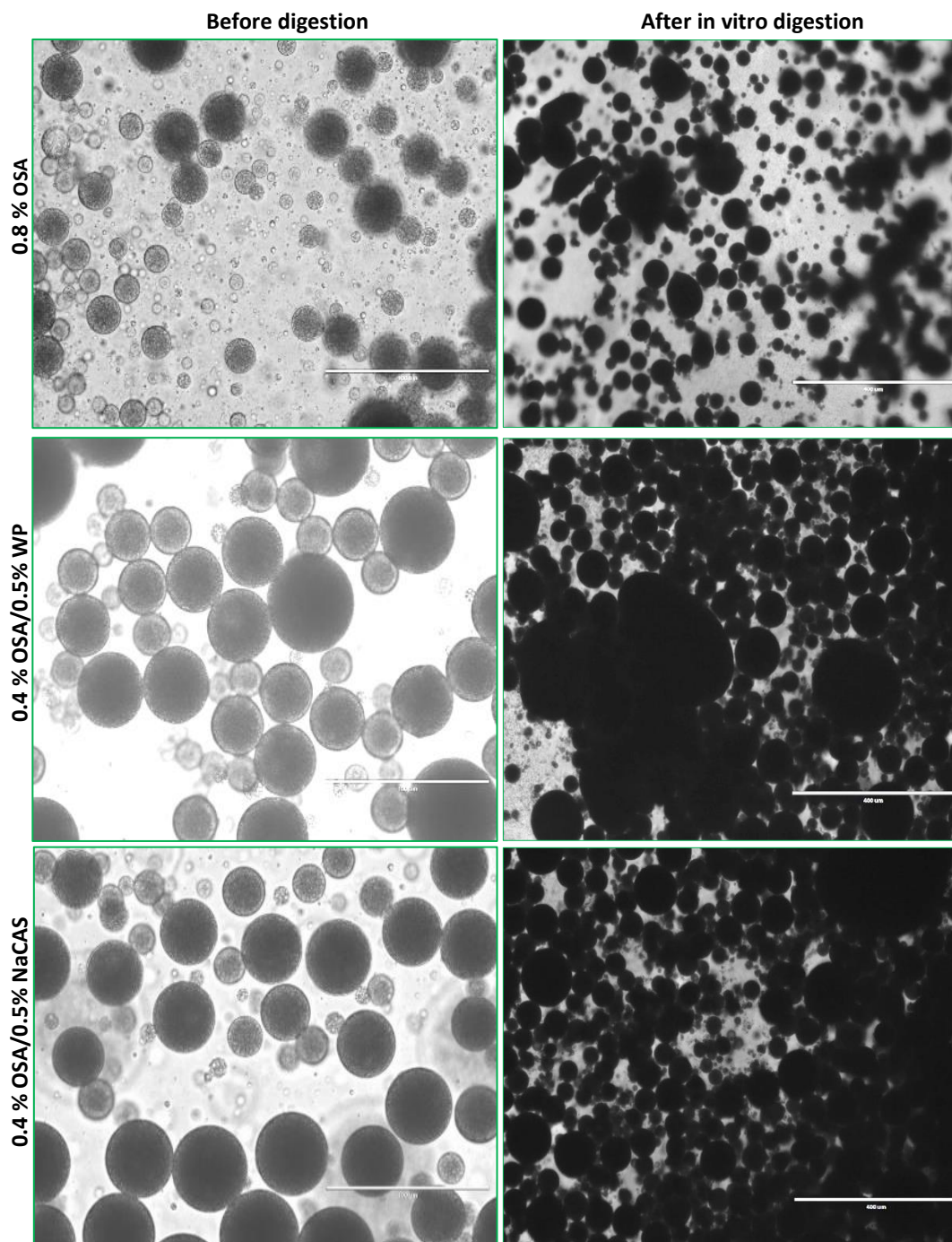


Figure 5-40. Optical microscopy images at scale bar 400 microns of $W_1/O/W_2$ emulsions stabilised with PGPR as internal emulsifier and either OSA, OSA/WP or OSA/NaCAS as external emulsifiers. Imaging was performed before and after *in-vitro* digestion of emulsions by α -amylase enzyme for 150 seconds at day one of emulsions storage period at ambient temperature.

5.3.2. Sucrose release assessed by LCMS

Sucrose release from the inner to the outer aqueous phases of the $W_1/O/W_2$ emulsions at different digestion times were measured with using the Liquid Chromatography Mass Spectrometry (LCMS) based method is shown in Figure 5-41. The y-axis shows the percentage of the encapsulated sucrose released from the inner to the outer aqueous phase. Three different types of $W_1/O/W_2$ emulsion were created, each emulsion stabilised with a hydrophobic emulsifier PGPR in the internal interface. One was the control emulsion stabilised by OSA alone and the other two emulsions (sample emulsions) were stabilised by either OSA/WP or OSA/NaCAS in the external interface, as mentioned in Table 5-20. The measurements performed at day 1 of storage at ambient temperature.

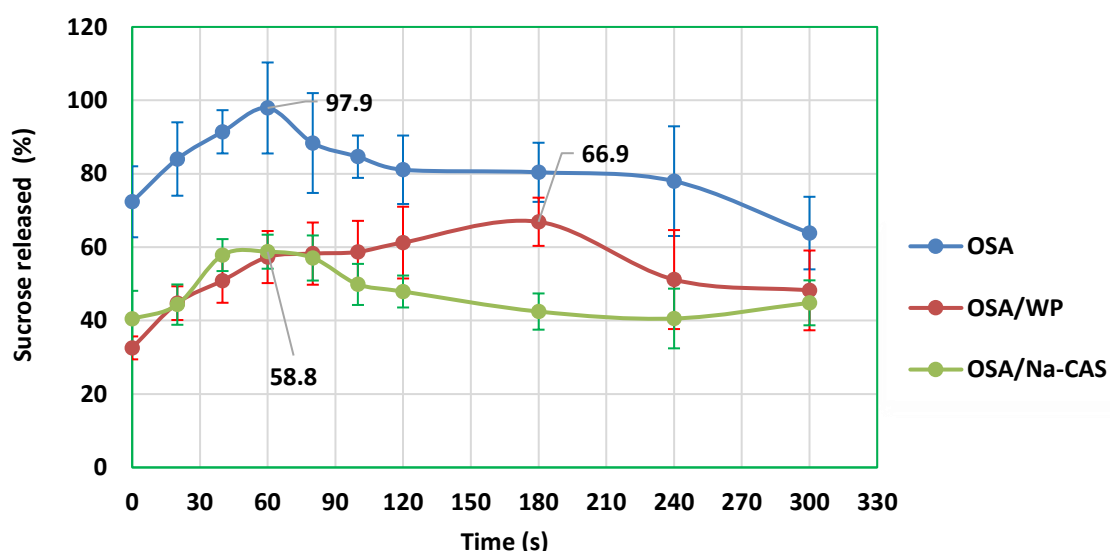


Figure 5-41. Measurements of sucrose release from the inner to the outer aqueous phase of $W_1/O/W_2$ emulsions at different digestion times using the LCMS based method. Three different types of $W_1/O/W_2$ emulsion are shown, each stabilised with (0.57% PGPR) w/w in the internal interface and either 0.8% OSA, 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS w/w in the external interface. Data are presented as means of three replicates ($n=3$) $\pm$ standard deviation.

Time (0) in this experiment refers to the smallest period of time that was possible to be counted. This included the time to add the enzyme to the emulsion and draw out 5 mL of the mixture to be added to the pre prepared 2.5 mL of silver nitrate solution to stop the digestion reaction. The level of sucrose in the external aqueous phase (W_2) at time (0) was 72% in the control emulsion, which is higher than that in the sample emulsions. This behaviour was expected because the chance for the enzyme to work on the interfacial OSA starch was greater in the control than that in the other emulsions, which had mixed starch/protein emulsifiers covering the interfaces. Since the EE of $W_1/O/W_2$ emulsions improved in the presence of the mixed polysaccharide/protein emulsifier comparing to using polysaccharide alone in outer interface results in an enhanced emulsifier (Muschiolik, 2007, Tamnak et al., 2016), it is was expected that the release will be less. Therefore, the release in the control emulsion, which was stabilised with starch only, was higher than that in the mixed-polymer stabilised emulsion. In addition, Benichou et al. (2004) stated that the use of polymeric amphiphilic molecules improve the interface coverage of the multiple emulsion which ensure high EE and controlled release.

In the control emulsion, the level of sucrose release detection increased with time until 97.9 maximum level was reached after 60 s of digestion after which time the level of detection started to decrease. In emulsions that were stabilised by 0.4% OSA/0.5% WP w/w at the external interface, the level of sucrose in (W_2) at time 0 was 33%, which is about half of the starting level of sucrose at the control emulsion. In addition, the maximum level of sucrose release achieved in this emulsion was less than that in the control emulsion by about half but at double the time it took in the control emulsion to reach

its maximum. This means that the digestion and sucrose release procedure where WP was included at the external interface of $W_1/O/W_2$ emulsion was different from the control. This was likely to be because of the presence of the WP at the external interface of the emulsion and making a layer-by-layer starch/protein interface as suggested in the external interface microstructure observation in Figure 4-23, which delays the digestion and subsequently delays the release of sucrose compared to the control emulsion.

The start and maximum levels of sucrose release in the emulsion sample stabilised by OSA/NaCAS were also lower than the level achieved in the control emulsion. However, the time of digestion and the behaviour of this emulsion during digestion is very similar to the control emulsion considering the amount of starch presented in each emulsion and the affecting of this amount on the release. This outcome supports the external interface microstructure observation already in Figure 4-24, which suggested that NaCAS covered some areas of the interface without interaction with the OSA starch. Therefore, the enzyme attacked the starch easily and the digestion procedure was similar to that in the control emulsion where only starch was used. The digestion and sucrose release procedure where WP was included was also different from the OSA/NaCAS stabilised emulsion, which also support the microstructure of these emulsion that refer to different behaviour between these mixed emulsifier at oil/water interface.

There is no clear interpretation why the peak behaviour happened in all $W_1/O/W_2$ emulsions, where the level of sucrose detection decreased after reaching the maximum, but it could be a result of coalescence between oil droplets becoming increasingly larger as the microstructure after digestion of

the emulsions showed. Re-emulsified some of the external aqueous phase into the big oil droplets during the procedure of digestion led to lost sugar. The oil droplets were discarded after centrifuging to separate the external aqueous phase for analysis. In $W_1/O/W_2$ emulsions stabilised with starch as the hydrophilic emulsifier, releasing the inner phase (W_1) because of hydrolysing the emulsifying starch by the salivary amylase had been confirmed by Chiu et al. (2017). The molecular structure of the biopolymers does affect coalescence of internal water droplets with the external water phase in $W_1/O/W_2$ emulsions, consequently it does not affect the release of the function ingredients encapsulated in the inner phase (Schuch et al., 2015).

5.3.3. Glucose release assessed by UV spectroscopy

A calibration curve was used in glucose concentration calculation to show how much glucose was released from the inner to the outer phase of $W_1/O/W_2$ emulsions after *in-vitro* enzymatic digestion are shown in Figure 5-42. The release of glucose was based on the assumption that increased levels of glucose in the serum of $W_1/O/W_2$ came from the internal aqueous phase of these emulsions as a function of the enzymatic digestion period. Nevertheless, it can be a potential error that some of glucose produced in starch digestion.

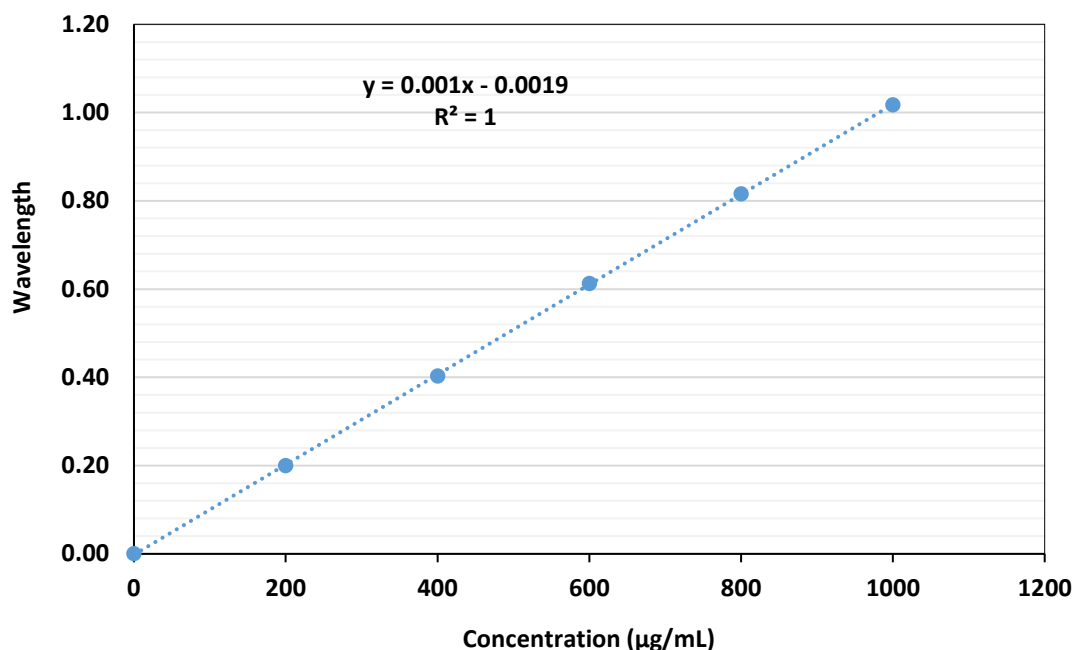


Figure 5-42. Calibration curve for six gradual concentrations of glucose solutions obtained by the last part of Megazyme total starch assay kit.

$W_1/O/W_2$ emulsions stabilised by OSA were tested for amylase-mediated destabilisation. The release of encapsulated glucose in these $W_1/O/W_2$ emulsions during 3 min of *in-vitro* digestion by α -amylase enzyme was measured using the UV absorption based method as shown in Figure 5-43. Three $W_1/O/W_2$ emulsions were stabilised with the hydrophobic emulsifier 0.57% PGPR w/w in the internal interface. In the control emulsion, 0.8% OSA starch w/w was used as the emulsifier in the external interface, and either 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS w/w were used as the external emulsifier in the sample emulsions. The y-axis shows the percentage of the encapsulated glucose released from the inner to the outer aqueous phase.

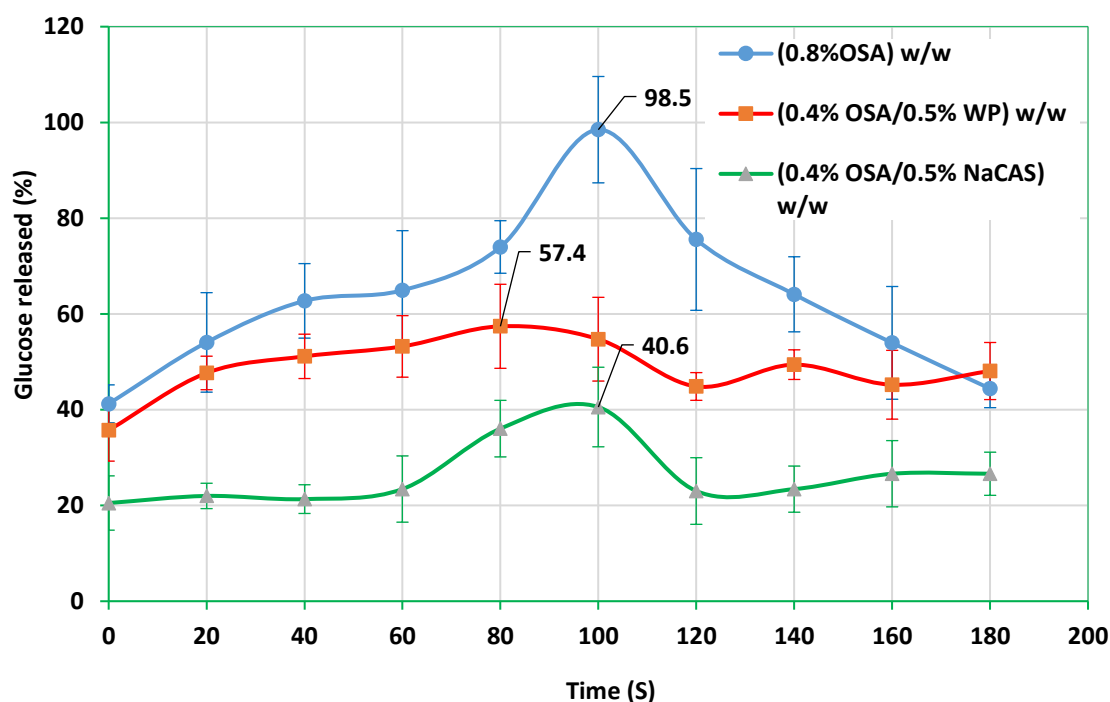


Figure 5-43. Release of encapsulated glucose from the inner to outer in $W_1/O/W_2$ emulsions during 3 minutes of *in-vitro* digestion by α -amylase enzyme using UV based method. Three types of $W_1/O/W_2$ emulsion were involved; stabilised with hydrophobic emulsifier 0.57% PGPR in the internal interface and either 0.8% OSA (control emulsion), 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS w/w in the external interface. Error bars represent $\pm$ standard deviation ($n=3$).

Results obtained for the UV absorption based method confirmed the results of the sucrose release assessed by the LCMS based method. The time of maximum release of glucose in the control emulsion and the OSA/NaCAS stabilised emulsion was the same, and the behaviour of release was similar in both emulsions, considering the amount of OSA starch in each emulsion. This outcome again supports the outer interface microstructure observation that suggested the presence of NaCAS at the outer interface. By comparing the control emulsion with the sample emulsions, it is clear that replacing half the quantity of the OSA starch in the outer interface by 0.5% w/w of WP or NaCAS highly affected the release of the encapsulated sugar from the inner to the outer aqueous phase. In addition, it reduced the starch used and as a

result, less glucose is used in the final product in the emulsion based food. Therefore, manipulation of the percentages of milk proteins used during the emulsion formulation could be considered as a parameter to control the release of sucrose in the oral cavity. Consequently, the level of sweetness experienced by the consumer when the $W_1/O/W_2$ emulsions come into contact with the saliva α -amylase enzyme could be controlled. The use of $W_1/O/W_2$ emulsions as a possible medium with a good potential for the controlled release faces two problems: the relative instability and the relative speed of release of the addenda (Gaiti et al., 1994).

The behaviour of glucose release was different to the sucrose release. Glucose release was slower in the emulsions that was stabilised with either OSA or OSA/NaCAS while it was faster than sucrose release in the OSA/WP stabilised emulsions. It is most likely related to the nature of the sugar rather than the different measuring method. The release of either glucose or sucrose in OSA/WP stabilised emulsions to the outer interface was greater than in those stabilised with the OSA/NaCAS mixed emulsifier system. Figure 5-41 and 5-43 indicated that in all $W_1/O/W_2$ emulsions more than 18.3% of the entrapped sucrose and 20% of the entrapped glucose were released to the outer aqueous phase. Chiu et al. (2017), who encapsulated salt in the inner water phase of $W_1/O/W_2$ emulsions, reported that during consumption of the emulsion the release of the salt from the inner phase to the oral cavity leading to salty taste has been proven. Therefore, the α -amylase enzyme was involved *in-vitro* digestion to breakdown the starch at the external interface of the $W_1/O/W_2$ emulsions used. In addition, Chiu et al. (2015) have confirmed the digestibility of the interfacial OSA starch (NC46) used, on a timescale appropriate to the consumption of food emulsions. Significant

improvements in the stability of $W_1/O/W_2$ with a more controlled release were made when macromolecules (bovine serum albumin) were blended in the external interface, in combination with a large excess of non-ionic monomeric emulsifiers (Benichou et al., 2004). The stability and the controlled release of these emulsions were further controlled when two polymer OSA/protein was used as the outer mixed emulsifier in the present research. The next step of this research was the examination of these multiple emulsions in the environment of milkshakes manufacturing conditions regarding to the mineral salts.

5.4. Mineral modification of $W_1/O/W_2$ emulsions designed for amylase mediated sucrose release

Studying the stability of the multiple emulsions against typical environmental and processing conditions encountered in food manufacture was the objective of this experiment. Therefore, a close-to-market approach and behaviour of water-in-oil-in-water ($W_1/O/W_2$) emulsions at the typical levels of mineral salts were investigated. These emulsions were fortified with different mineral salts in both aqueous phases with the presence of 50% and 5% w/w of sugar solution in the inner and outer water phase, respectively.

5.4.1. Stability of emulsions in the presence of a single mineral salt

Solutions of 0.111% Sodium chloride, 0.146% calcium chloride, 0.224% potassium chloride and 0.016% magnesium chloride w/w were independently used in the formulation of these emulsions. These percentages of salts were

the maximum levels of these mineral salts in two specific commercial milk shakes; thick chocolate milk shakes and thick vanilla milk shakes. Figure 5-44 shows the volume based mean droplet size of three different emulsions fortified independently with each salt. The mean droplet size was used to assess the stability of these emulsions against coalescence day 1, 14 and 28 of a storage period at ambient temperature. One-way ANOVA statistical test indicated that there were no significant differences in the mean droplet diameter between days for each salt alone ($p > 0.05$) in the emulsions made with OSA/WP. Therefore, adding mineral salts to these emulsions did not affect the stability against coalescence during 28 days of storage. However, adding some of these salts either to the 0.4%OSA/0.5%NaCAS stabilised emulsion or the control emulsion stabilised with 0.8% OSA led to coalescence instability, for example, adding CaCl_2 to both emulsions caused statistically significant differences between days ($p < 0.05$). The aggregation of NaCAS as a result of its bonding with Cl_2 could effect on the droplet size measurements.

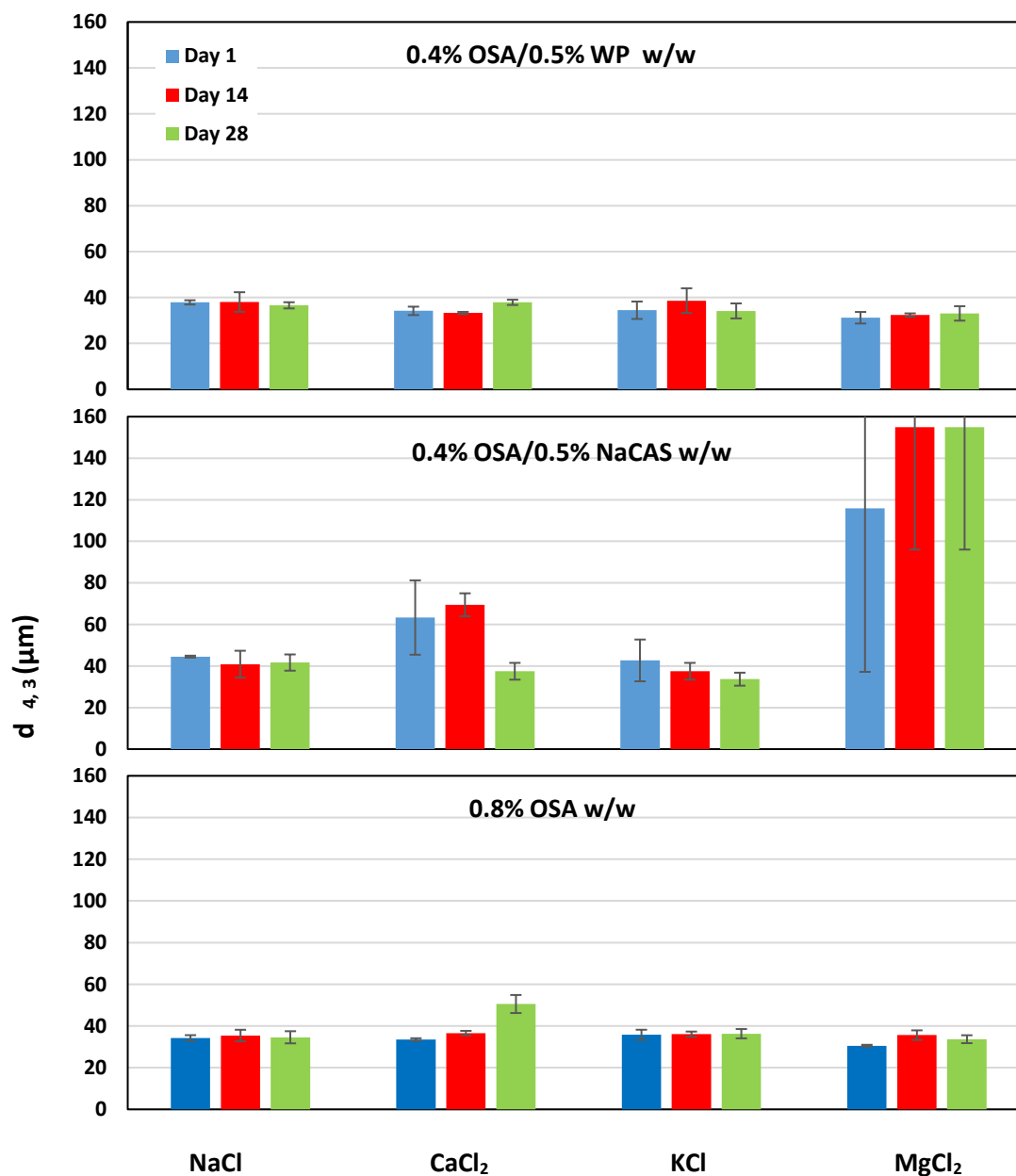


Figure 5-44. Mean droplet size based on volume distribution of sucrose rich $W_1/O/W_2$ emulsion independently made with a solution of either 0.111% NaCl (0.0019 mol), 0.146% $CaCl_2$ (0.0013 mol), 0.224% KCl (0.0033 mol) or 0.016% $MgCl_2$ (0.0002 mol) w/w. Emulsions stabilised with 0.57% PGPR in the internal interface and either 0.8% OSA, 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS w/w in the external interface. Data are presented as means of three replicates ($n=3$) $\pm$ standard deviation. * = significant difference in ($d_{4,3}$) between days ($p < 0.05$).

OSA starch has limited effects in the presence of mineral salts but it has effects in the presence of (0.1M, pH7) sodium phosphate buffer because all buffers have the ability to screen some of the charge on a molecule depending on their molarity, the phenomenon is known as the buffer capacity. Adding MgCl_2 to emulsions stabilised with OSA/NaCAS led to coalescence at the first day of the storage period. Indeed proteins such caseinate can strongly bind divalent cations like Ca^{2+} and Mg^{2+} mostly due to the presence of phosphoryl groups, each caseinate molecule could bind 5 magnesium ions (Bonnet et al., 2010). Therefore, the instability of emulsions that stabilised with sodium caseinate could be attributed to interaction between this protein and the divalent mineral salts.

Adding NaCl and KCl at the concentrations stated above to both aqueous phases of all emulsions did not affect the stability of these emulsions against coalescence. The droplet size in the absence of these mineral salts as show in Figure 5-33 was very similar to that in the presence of them as show in Figure 5-44. It has been shown in literature that the presence of up to 3.5% w/w NaCl in the internal aqueous phase of $W_1/O/W_2$ plays an important role in the suppression of the phase separation, whereas adding NaCl to the outer aqueous phase accelerated the phase separation (Hino et al., 2001). Therefore, the instability of OSA/NaCAS stabilised emulsion and in the presence of CaCl_2 and MgCl_2 , and the instability of OSA stabilised emulsions in the presence CaCl_2 could be avoided by adding these salts to the inner aqueous phase only. However, the presented results confirm adding 0.111% w/w NaCl solution to both aqueous phases did not affect the stability of emulsion used, and this could be attributed to the presence of the high concentration of sucrose in the inner phase as discussed in section 2.4. Zhang

and Reineccius (2016), demonstrated that $W_1/O/W_2$ emulsion stability can be improved by matching the densities of the oil phase and the aqueous phase. In addition, Bonnet et al. (2009) formulated a stable $W_1/O/W_2$ using 1.5% PGPR w/w and 1.2% NaCAS w/w as lipophilic and hydrophilic emulsifier, respectively, and with 0.29 % $MgCl_2$ w/w encapsulated in the internal water phase W_1 , showed that the primary W_1/O and $W_1/O/W_2$ emulsions were quite stable with respect to the size parameters.

It can be concluded that fortification of all emulsions with mineral salts of monovalent ions did not affect stability against coalescence during the storage period. Adding divalent mineral salts to the emulsions stabilised with OSA/WP did not affect their stability, whereas adding these salts to the emulsions stabilised with OSA led to instability only after two weeks and to instability from the first measuring point in emulsions stabilised with OSA/NaCAS. According to the literature, these unstable emulsions would be stable if the salts were added only to inner aqueous phase. After investigating, the presence of single mineral salts in the rich sucrose complex emulsion the next step was to study the effect of adding these salts as group on the stability of these emulsions against coalescence. To know the release after fortification with mineral salts using the LCMS and the UV methods, emulsions were prepared using either glucose or sucrose.

5.4.2. Stability of emulsions in the presence of a group of mineral salts

Measurements of mean droplet size based on volume distribution ($d_{4,3}$) of six $W_1/O/W_2$ emulsions formulated with a mixed salt solution made with

0.111% NaCl, 0.146% CaCl₂, 0.224% KCl or 0.016% MgCl₂ w/w are shown in Figure 5-45. Stability against coalescence of these emulsions was investigated by measuring the mean droplet size.

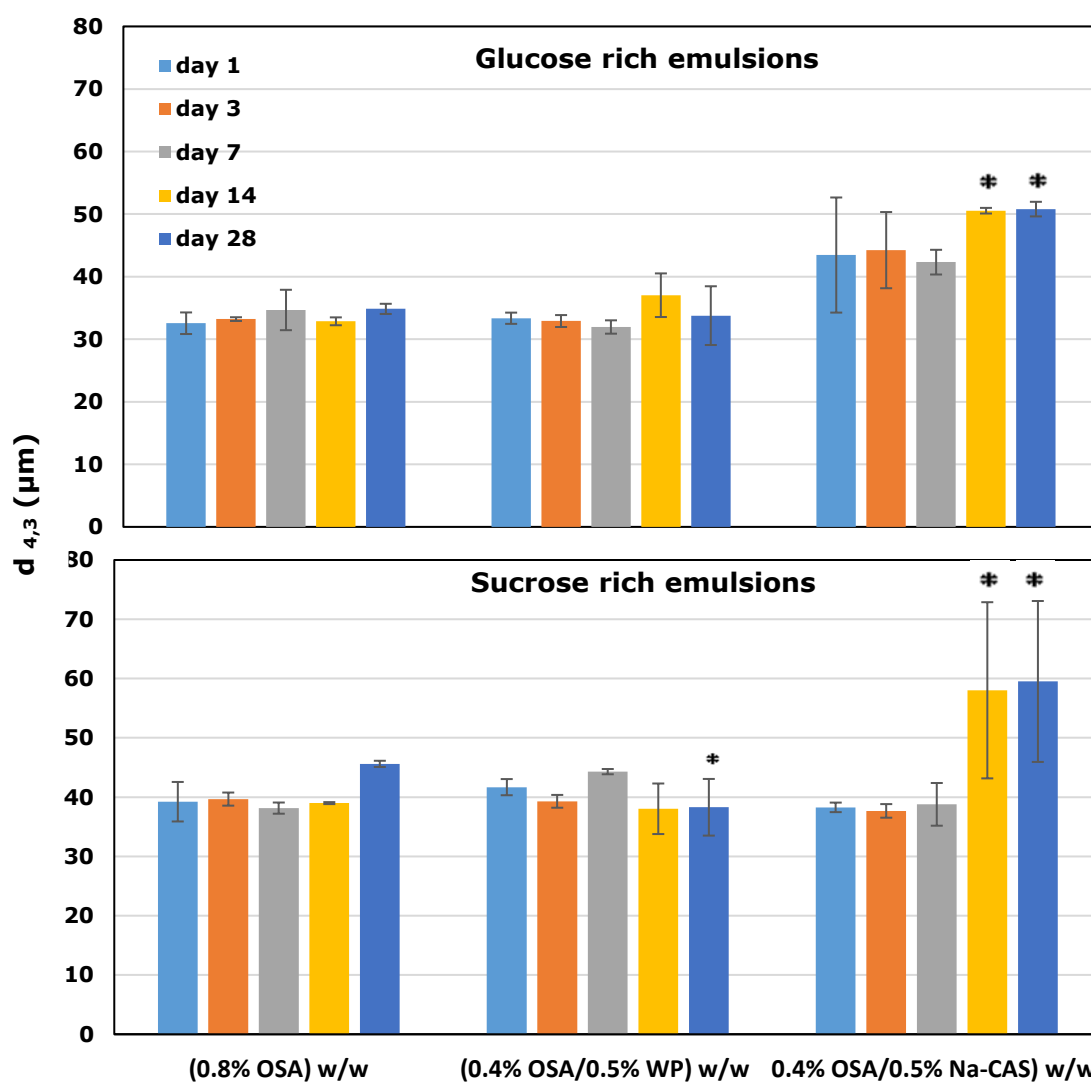


Figure 5-45. Mean droplet size base on volume distribution of six W₁/O/W₂ emulsions were produced using a solution of 0.111% NaCl (0.0019 mol), 0.146% CaCl₂ (0.0013 mol), 0.224% KCl (0.0033 mol) or 0.016% MgCl₂ (0.0002 mol) w/w. The emulsions were stabilised with 0.57% PGPR in the inner interface and either 0.8% OSA, 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS in the outer interface, and stored 28 days at ambient temperature. Data are presented as means of three replicates (n=3) ± standard deviation.

One-way ANOVA and Multiple Comparisons (Tukey HSD) statistical tests were used to identify if there were any statistical differences in the droplet size. The present results indicate that $W_1/O/W_2$ emulsions stabilised with OSA/WP rich in either glucose or sucrose exhibited stability against coalescence post mineral fortification and during the 28-day storage period without any significant changes in the mean droplet size. In addition, the control emulsion stabilised with OSA only showed stability in the glucose rich emulsion, however a significant change ($P < 0.05$) in the mean droplet size was seen at the last measuring point in sucrose rich emulsions. Emulsions stabilised with OSA/NaCAS mixed emulsifier exhibited stability in the first two weeks but there was a significant change in the droplet size at the fourth and fifth measuring points ($P < 0.05$). The instability of emulsions stabilised with either OSA or NaCAS could be attributed to interaction between these emulsifiers and the divalent mineral salts.

It can be concluded that an emulsion stabilised with OSA/WP mixed emulsifier system was the best emulsion for sugar reduction among all different emulsions involved in this research. These results agreed with Muschiolik (2007) where different proteins (WP, NaCAS, soybean protein and wheat protein) were compared as emulsifiers for $W_1/O/W_2$, and showed the best emulsification behaviour was shown by WP due to smaller droplets and high stability. Márquez et al. (2010), confirmed that the presence of calcium chloride, calcium lactate or calcium carbonate, sodium magnesium or potassium chloride at a defined concentration (1g for each 100 g) in the aqueous phase of W/O emulsions allowed the stabilisation at higher water contents. This effect was attributed to the decrease of the size of the water droplets resulting in a decrease in the attractive force between them and the

increase of water content and the emulsifier adsorption. The primary W_1/O emulsion is more stable against coalescence when the water phase contains NaCl. Salt orientates surfactant molecules at the interface and most likely strengthens interactions between them resulting in an increase of the interfacial viscosity and elasticity and thus promotes stability (Pawlik et al., 2010). In $W_1/O/W_2$ emulsions, the release of salt from the inner aqueous phase is proportional to the glucose concentration in the outer phase. When glucose in the external water phase was less than required for balancing the osmotic pressures, the lowest salt release was observed. This release of salt is driven by the chemical potential difference rather than the unbalanced osmotic pressures between the two water phases (Pawlik et al., 2010). Using sugars to match the osmotic pressure alters the system's interfacial tension therefore, sugars act in a similar way to hydrophilic emulsifiers which, by increasing micellar transport, enhances the rate of salt release from the inner aqueous phase (Pawlik et al., 2010).

5.4.3. Measurements of the pH value

PH values of $W_1/O/W_2$ emulsions fortified with different mineral salts in the presence of two types of sugar, either glucose or sucrose, before and after a 28-day storage period at ambient temperature are shown in Table 5-23. A solution containing 0.111% sodium chloride, 0.146% calcium chloride, 0.224% potassium chloride and 0.016% magnesium chloride w/w was used in the formulation of both internal and external aqueous phases of either glucose rich or sucrose rich $W_1/O/W_2$ emulsions. There were not any significant changes in the pH value over storage due to the same mineral

salts concentrations being presented in both inner and outer phase of these emulsions. Since, the pH value changes would signify some instability, these very close pH values between the two days were parameters indicate to stability as well as identify the pH of emulsion at this stage of the research.

Table 5-23. Measurements of pH of either glucose rich or sucrose rich $W_1/O/W_2$ emulsions fortified with different mineral salts, before and after a 28-day storage period at ambient temperature.

External Emulsifier	Encapsulated sugar	Mineral salts	Day 1	Day 28
OSA	glucose	NaCl, KCl, $CaCl_2$ & $MgCl_2$	5.5	5.6
OSA/WP			6.2	6.1
OSA/NaCAS			6.1	6.0
OSA	Sucrose	NaCl, KCl, $CaCl_2$ & $MgCl_2$	5.5	5.6
OSA/WP			6.2	6.1
OSA/NaCAS			6.1	5.9

5.5. The effect of mineral fortification on the glucose release

Glucose release post mineral fortification of the $W_1/O/W_2$ emulsions was investigated. A solution of mineral salts of 0.111% NaCl, 0.146% $CaCl_2$, 0.224% KCl or 0.016% $MgCl_2$ was used for preparing two glucose solutions 50% and 5% w/w. Both glucose solutions were independently use as the inner and outer water phases of three $W_1/O/W_2$ emulsions. Figure 5-46 shows the measurements of encapsulated glucose release in $W_1/O/W_2$ after *in-vitro* enzymatic digestion by α -amylase enzyme. The top part of this figure is a copy of the Figure 5-43 and it is here only for comparison. The release was measured before and after fortification with mineral salts of NaCl, $CaCl_2$, KCl and $MgCl_2$ in both aqueous phases using the UV absorption based method. There was a slight delay in detecting the glucose at the beginning of digestion of OSA/WP stabilised emulsions. This behaviour could be related to how the WP or OSA/WP adsorbed at the interface.

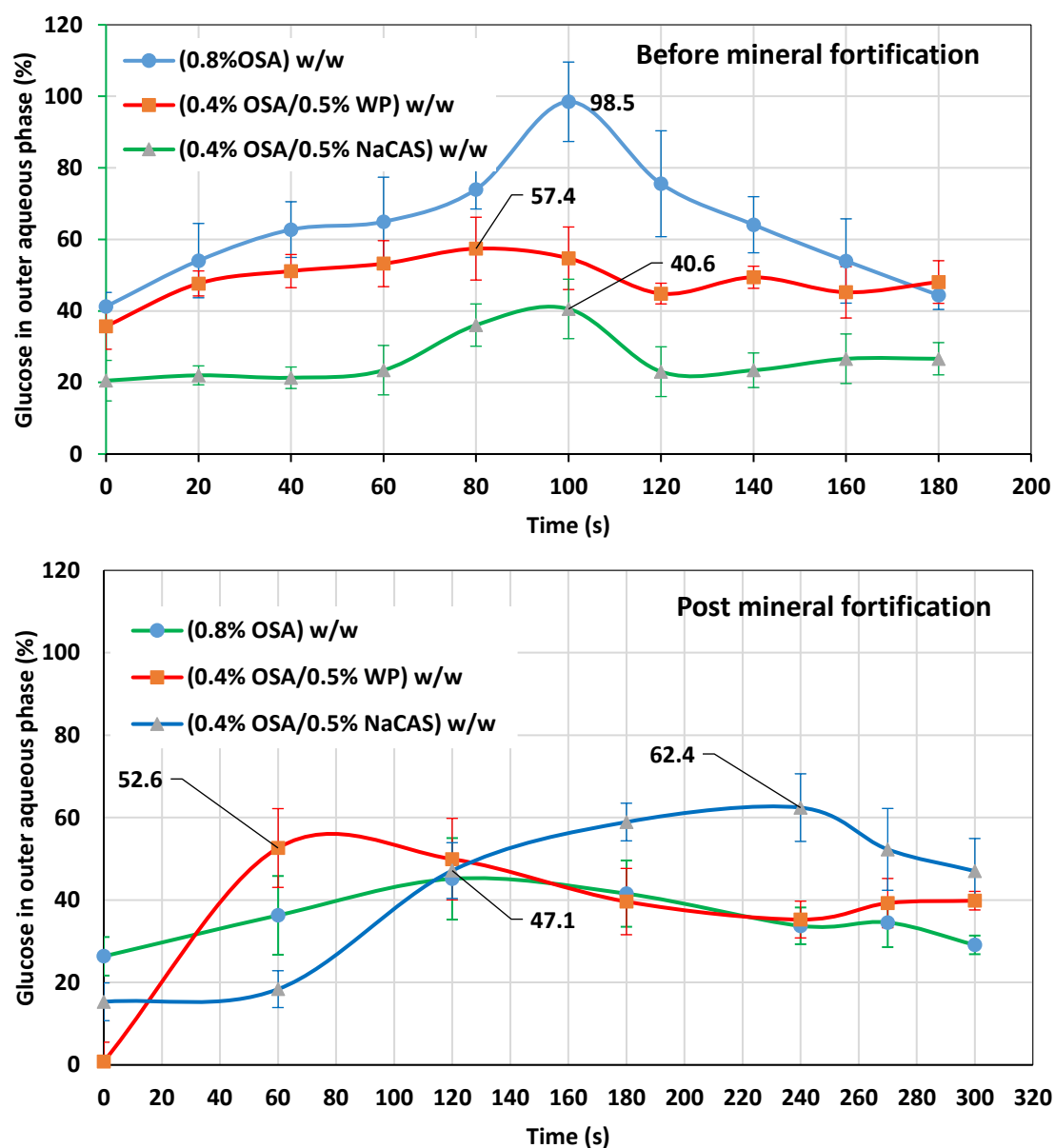


Figure 5-46. Encapsulated glucose release pre and post fortification with mineral salts of NaCl, CaCl₂, KCl and MgCl₂ in both aqueous phases of W₁/O/W₂ emulsions during *in-vitro* digestion by α -amylase enzyme. Measurements were performed using the UV based method. Three types of W₁/O/W₂ emulsion were tested; stabilised with hydrophobic emulsifier (0.57% PGPR) w/w in the internal interface and either 0.8% OSA, 0.4% OSA/0.5% WP or 0.4% OSA/0.5% NaCAS w/w in the external interface. Data are presented as means of three replicates (n=3) $\pm$ standard deviation.

The levels of glucose release detected in the outer aqueous phase after mineral fortification were lower than that measured before fortification in

emulsions stabilised with OSA/protein mixed emulsifier. These results indicate to more stability was happened in presence of minerals which led to a lower release. This agreed with Márquez et al. (2010), who found more stability of the primary W_1/O post mineral fortification and with Bonnet et al. (2009) who attributed stability of $W_1/O/W_2$ to entrapping $MgCl_2$ in the core of the emulsion; Hino et al. (2001) attributed that to the NaCl. However, maximum releasing time in the OSA/WP stabilised emulsions was the same pre and post mineral salt inclusion with about 5% reduction in the glucose level detected after adding the salts.

The control emulsion, which was stabilised with 0.8% OSA showed the highest release as expected, compared to the emulsion samples that were stabilised with the mixed emulsifier systems. The glucose release was decreased to nearly 40% after the mineral fortification, whereas the emulsion samples stabilised with mixed emulsifier kept the same level of release; this can be considered as an advantage of the starch/protein mixed emulsifier systems. In addition, the time of maximum release in the control emulsion was delayed from 100 s before the fortification to 220 s after the fortification, while the sample emulsions kept the same time with only a slight delay in the OSA/NaCAS stabilised emulsion. Resistant starch showed a greater absorption of calcium, magnesium and iron compared to digestible starch (Morais et al., 1996, Schulz et al., 1993). Therefore in the case of using OSA modified starch the mineral could be influence on the hydrolysis of the starch leading to some delay in the sugar release. The initial and maximum levels of the entrapped glucose release in the OSA/NaCAS stabilised emulsion after the mineral fortification were higher than that measured before fortification. The reason for this higher release in these emulsions can be due the bonds

between caseinate divalent cations leading to the reduction of the stability of these emulsions, which accelerate the release of sugar. The droplet size of the inner aqueous phase is the main factor in determining the rates of release from $W_1/O/W_2$ hydrocarbon emulsions (Omotosho et al., 1986). $MgCl_2$ entrapped in the inner phase of $W_1/O/W_2$ emulsions stabilised with PGPR and NaCAS was investigated by Bonnet et al. (2010), who reported that increasing inner globule fraction led to more $MgCl_2$ encapsulation at a constant temperature due to the relative reduction in the outer phase volume and the interfacial magnesium binding by NaCAS over time. The presence of calcium enhances α -amylase enzyme thermo stability (Konsula and Liakopoulou-Kyriakides, 2004). It can be concluded that fortification with mineral salts in both aqueous phases of the OSA/NaCAS stabilised emulsion can facilitate and increase the glucose release, whereas fortification slightly reduces glucose release in OSA/WP stabilised emulsions.

Chapter 6: Conclusions and Recommendations for Future Work

6. Conclusions and Recommendations for Future Work

6.1. Conclusions

- Oil-in-water emulsions with 20% w/w oil dispersed phase and stable against coalescence for a 14-day storage period at ambient temperature were produced with a small concentration of OSA starch, namely 0.8% w/w. This low concentration of OSA starch fitted with the research aim of reducing the starch concentration in emulsion-based sugar reduced foods. However, these emulsions were not stable when 0.1M sodium phosphate buffer at pH 7.0 was used as the aqueous phase instead of water. The reason for this behaviour was due to the ability of the buffer to screen the charge on the OSA starch molecule, or because of an interaction between starch and phosphate ions (HPO_4)⁻² in the buffer.
- The two milk proteins WP or NaCAS were used to stabilise O/W emulsions with a range of concentrations from 0.5 to 1.0% w/w, and it did not matter whether the aqueous phase was water or 0.1M sodium phosphate buffer at pH 7.0.
- WP and NaCAS were used as co-emulsifiers to OSA starch in which to assess the emulsifying ability of both OSA/WP and OSA/NaCAS mixtures. Solutions of either OSA/WP or OSA/NaCAS highly reduced the interfacial tension at the oil/water interface.
- In light of the interfacial tension data (oil purification see section 2.3.1), O/W emulsions with 20% w/w oil dispersed phase were successfully stabilised with these mixed emulsifiers systems. Emulsions stabilised with OSA/WP or OSA/NaCAS mixed emulsifiers showed no statistically significant differences ($p > 0.05$) in the mean droplet size ($d_{4,3}$)

during a two-week period of storage at ambient temperature, and when either water or 0.1 M sodium phosphate buffer at pH 7.0 were used as aqueous phase.

- The Zeta potential for the emulsions stabilised with either OSA/WP or OSA/NaCAS was (-39.04 ± 2.56) mV and (-57.35 ± 3.92) mV, respectively, when the aqueous phase was water, and it was (-36.64 ± 2.27) mV and -64.39 ± 0.68 mV, respectively in case of using buffer as aqueous phase. So, these emulsions exhibited a highly negatively charge at the interface ensuring repulsion between droplets preventing the emulsion from coalescence instability.
- Confocal Laser Scanning Microscopy (CLSM) was used to investigate the emulsions' microstructure, which confirmed that both starch and protein were indeed at the interface of the O/W emulsion droplets. The micrographs of the OSA/WP emulsions suggested the formation of a layer-by-layer interface. However, in the micrographs of the OSA/NaCAS emulsions some areas of the oil droplets surface seemed to be have been covered by protein and other areas covered by starch.
- The interfacial composition was inferred from the continuous phase analysis pre and post emulsification. In emulsions stabilised with OSA/WP, 0.26% of the starch used and 0.12% of the protein were adsorbed at the droplets interface. In OSA/NaCAS stabilised emulsions 0.25% of the starch used and 0.14% of the protein used were adsorbed in the droplets interface. Therefore, O/W emulsions can be stabilised at a lower emulsifying starch content if combined with WP or Na-CAS as a co-emulsifier.
- The O/W emulsions stabilised with the OSA/WP mixed emulsifier system in the presence of 0.57% w/w PGPR led to a slight increase in the mean droplet

size. This increase was not statistically significant for water or buffer as aqueous phase. Adding the same amount of PGPR to these emulsions when they were stabilised with the OSA/NaCAS mixed emulsifier led to a decrease in the mean droplet size, however, this was also non-significant.

- The O/W emulsions stabilised with either of the mixed emulsifier systems were developed into sucrose-rich $W_1/O/W_2$ emulsions by adding an internal aqueous phase into the droplets of these emulsions. This addition required the formulation of a stable W/O emulsion as primary stage of the double emulsion.
- The sucrose-rich $W_1/O/W_2$ emulsions, which were stable against coalescence, contained 7.66% sucrose w/w in total, in which 4.29% was encapsulated in the emulsion droplets whereas 3.37% was in outer aqueous phase. A dark appearance of the oil droplets and the formation of droplet-in-droplet microstructure were clearly recognisable in the microscopic evidences.
- No appreciable difference in the morphology of these emulsions or a visible increase in droplet size within the emulsion were seen between day 1 and 14 of the storage period at ambient temperature. This outcome was confirmed by the volume fraction distribution and the mean droplet size measurements, where no significant differences in the mean droplet diameter were found between the time points of measurements for each emulsion ($p > 0.05$). There was no sign of flocculation during the storage period. The pH values of these emulsions at day 1 and 14 were nearly the same.
- Using WP or NaCAS protein as co-emulsifiers with OSA starch providing more stability to the $W_1/O/W_2$ emulsions compared to the use of each protein alone.

• The encapsulation efficiency (EE) of $W_1/O/W_2$ emulsions was evaluated by (i) a liquid chromatography mass spectrometry (LCMS) and (ii) an ultraviolet (UV) absorption based method. The EE measured using the LCMS based method and the UV based method was between 70.6 – 78.6 % in emulsions stabilised with starch/protein mixed emulsifier systems at day 1. However, at day 14 the EE efficiency acquired from the UV based method was higher by about a third compared to that acquired using the LCMS based method, which indicates to sucrose diffusion from the internal to the external aqueous phase. These differences in the EE could be because of the difference in the sugar type or an occasional accident happened in the samples preparation for the analysis. The LCMS method was not really applicable because it indicated to high EE at day one and low EE at day, so at least the sucrose was temporally encapsulated.

• The presence of the protein did not inhibit microstructure breakdown of $W_1/O/W_2$ as initiated by starch hydrolysis when α -amylase enzyme was added to $W_1/O/W_2$ *in-vitro* emulsions after one day of formulation. A peak in behaviour occurred in the release curves of all $W_1/O/W_2$ emulsions, in which the level of sucrose decreased after reaching the maximum level. It could be a result of coalescence between oil droplets as they increase in size, as the microstructure after digestion of the emulsions showed. Re-emulsified some of the external aqueous phase into the big oil droplets during the procedure of digestion led to lost sugar. These large droplets were discarded after centrifuging to separate the external aqueous phase for analysis. The release of either glucose or sucrose in OSA/WP stabilised emulsions was greater than in those stabilised with the OSA/NaCAS mixed emulsifier

system. In all emulsions more than 18.3% of the encapsulated sucrose and 20% of the encapsulated glucose were released from the inner to the outer aqueous phase.

- Emulsions were fortified with the typical levels of mineral salts in two types of milkshakes; thick chocolate milk shakes and thick vanilla milk shakes. Adding these mineral salts to $W_1/O/W_2$ emulsions stabilised with OSA/WP did not affect the stability against coalescence ($P>0.5$) during 28 day of storage at ambient temperature, whereas adding $CaCl_2$ and $MgCl_2$ to OSA/NaCAS stabilised emulsions caused instability. The reason for this instability is because caseinate strongly binds divalent cations like Ca^{2+} and Mg^{2+} due to the presence of phosphoryl groups.
- Complex emulsion interfaces for sugar reduction in emulsions-based food were designed. The level of sugar or overall carbohydrate content that can be reduced depends on the type of food the emulsion will be applied in. A comparable level of starch reduction was achieved because a stable $W_1/O/W_2$ emulsion was produced with only 0.8% w/w OSA starch as emulsifier and then this percentage was reduced up to 50% by replacing half the starch content by milk proteins.

6.2. Future work

- The structure of the interfaces and the interaction between the OSA/WP and OSA/NaCAS would be investigated to optimise the interfacial composition to validate the hypothesis of this research in terms of the interfacial composition results. In addition, evaluation of the enzymatic induced breakdown of OSA/WP and OSA/Na-CAS designed interfaces, and to apply the stabilisation concept $W_1/O/W_2$ emulsions.

- Finding a natural and label-friendly lipophilic emulsifier instead of PGPR still is a practical challenge. Ammonium phosphatide (AMP) and sucrose ester O-170 have been used as alternative emulsifiers to the PGPR. Therefore, as a future work, it will be worth formulating $W_1/O/W_2$ emulsions with these emulsifiers and studying the effect on encapsulation of sucrose, the enzymatic breakdown of the emulsion and the release of sucrose.
- To look at another hydrocolloid and interactions that is purely there to prevent creaming.
- Any future work that supports the present data would be suggested e.g. droplet measurements after digestion could give information on how the breakdown and the size of droplets be affected.
- The colour of the emulsions can be measured before and after digestion using Colour QUES machine could provide additional data on sucrose release; the colour of oil droplets should be less darkness after the release.
- The release in $W_1/O/W_2$ stabilised with starch/protein mixed emulsifier systems would be investigated with using saliva α -amylase instead of porcine pancreas α -amylase enzyme to identify whether the release will be improved or not because it has been reported that the former led to better results than the latter for the release of sodium chlorite.
- A sensory study to evaluate the digestion of $W_1/O/W_2$ emulsions and the release of sucrose in the presence saliva amylase would be designed as future work to identify whether these emulsions will deliver a sweet taste in the mouth cavity to be available to the taste receptor or not.
- Looking into the effect of thermal treatments that mimicked those used in the pasteurization processes would be suggested as future work to identify whether the microstructures of $W_1/O/W_2$ emulsions will be stable against this

process or not, especially emulsion that stabilised with OSA/NaCAS, because it is known that NaCAS and PGPR resist several of thermal treatments.

- Adding divalent mineral salts to the emulsions stabilised with OSA/WP do not affect their stability, whereas adding these salts to the emulsions stabilised with OSA alone or OSA/NaCAS led to instability. So, a future research would be suggested in which these salts will be added only to inner aqueous phase to know whether these unstable emulsions would be stable or not.
- Beverage clouds have been prepared with freshly made $W_1/O/W_2$ emulsions, a research question can be asked on how these designs of $W_1/O/W_2$ will be used in beverage industry for sugar reduction, so a future work is need to answer this question.

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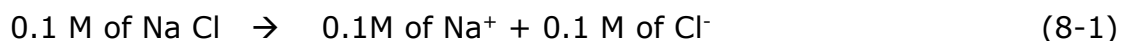
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Appendices

Calculation of the ionic strength of (0.1M) Na Cl and (0.1 M) Mg Cl₂

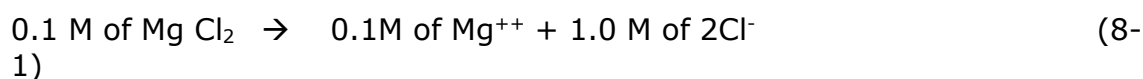
Ionic strength (I) of (0.1M) Na Cl:



$$I = 1/2 \sum C_i z_i^2 = 1/2 [(\text{molality of ion sodium} * \text{its charge}) + (\text{molality of ion chloride} * \text{its charge})]$$

$$= 1/2 [(0.1 * (+1)^2) + (0.1 * (-1)^2)] = 1/2 [0.1 + 0.1] = 1/2 [0.2] = 0.1\text{M}$$

Ionic strength (I) of (0.1M) Mg Cl₂:



$$I = 1/2 \sum C_i z_i^2 = 1/2 [(\text{molality of ion magnesium} * \text{its charge}) + (\text{molality of ion chloride} * \text{its charge})]$$

$$= 1/2 [(0.1 * (+2)^2) + ((0.1*2) * (-1)^2)] = 1/2 [0.4 + 0.2] = 1/2 [0.6] = 0.3\text{M}$$

(Ellis and Morrison, 1982)

Table 8-24. Data used for drawing the standard curve used for proteins determination in emulsions serum.

BSA stocks conc. (µg/mL)	Absorbance
0	0
25	0.016
125	0.145
250	0.303
500	0.610
750	0.784
1000	1.146
1500	1.565
2000	2.110

Table 8-25. Incremental amounts taken from two calibration solutions, concentration of sugar in each amount “from experiment calculation - section 2.4.1.1” and area under curve (AUC) against each concentration measured using a LCMS. Calibration solutions prepared on day 1 of EE measurements base on LCMS method.

Calibration solution No.	Amount of millilitres taken from calibration solution	Content of sucrose in the external phase (g)	AUC
1	2.00	0.9500	27908900
	1.50	0.7125	23843518
	1.00	0.4750	19287376
	0.50	0.2375	12759233
	0.25	0.1200	7070694
2	2.00	0.9500	25384698
	1.50	0.7125	21650388
	1.00	0.4750	16224503
	0.50	0.2375	11599513
	0.25	0.1200	67182170

Table 8-26. Incremental amounts taken from two calibration solutions, concentration of sugar in each amount “from experiment calculation - section 2.4.1.1” and area under curve (AUC) against each concentration measured using a LCMS. Calibration solutions prepared on day 14 of encapsulation measurements base on LCMS method.

Calibration solution No.	Amount of millilitres taken from calibration solution	Content of sucrose now release to external phase (g)	AUC
1	2.00	0.9500	24001620
	1.50	0.7125	20990794
	1.00	0.4750	18375604
	0.50	0.2375	11901702
	0.25	0.1200	6814616
2	2.00	0.9500	24251620
	1.50	0.7125	20700656
	1.00	0.4750	17509904
	0.50	0.2375	11144344
	0.25	0.1200	6814617

Table 8-27. The absorbance measurements for seven concentrations of a glucose calibration solution using the UV based method at wavelength 510nm at 20°C. These concentrations were prepared following the procedure in section 2.4.2.1 and used to draw a calibration curve for the EE measurements at day 1 by the UV based method.

Glucose conc. µg/mL	Absorbance	STDEV
0	0.000587	0.000115
10000	0.100767	0.000133
20000	0.206507	0.000081
30000	0.30659	0.000075
40000	0.447607	0.001509
60000	0.622257	0.000148
100000	1.080143	0.000301

Table 8-28. The absorbance measurements for sex concentrations of a glucose calibration solution using the UV based method at wavelength 510nm at 20°C. These concentrations were prepared following the procedure in section 2.4.2.1 and used to draw a calibration curve for the EE measurements at day 1 by the UV based method.

Glucose conc. µg/mL	Absorbance	STDEV
0	0.00059	0.000115
10000	0.19758	0.000151
20000	0.40828	0.000193
40000	0.60261	0.00021
60000	0.80915	0.00039
100000	1.00737	0.000488

Table 8-29. Absorbance measurements for six concentrations of glucose calibration solutions performed at wavelength 510nm and 20 °C using the UV based method see section 2.4.2. This data was processed using Microsoft Excel software to draw a calibration curve for glucose quantification in the UV based method.

Glucose conc. µg/mL	Absorbance	STDEV
0	0.00059	0.00012
200	0.19988	0.00007
400	0.40331	0.00001
600	0.61256	0.00013
800	0.81560	0.00008
1000	1.01791	0.00025

OSA starch

Day 1

Table 8-30. Statistical differences between OSA concentrations at day 1 using ANOVA test

ANOVA					
sizes					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	71.051	7	10.150	16.584	.000
Within Groups	9.793	16	.612		
Total	80.844	23			

Table 8-31. Statistical differences between OSA concentrations at day 1 using multiple comparisons test

Multiple Comparisons						
Dependent Variable: sizes						
Tukey HSD						
(I) concentration	(J) concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.40	.80	3.47300*	.63877	.001	1.2615	5.6845
	1.20	3.03333*	.63877	.004	.8218	5.2449
	1.60	3.15767*	.63877	.003	.9461	5.3692
	2.00	3.41867*	.63877	.001	1.2071	5.6302
	2.40	2.95733*	.63877	.005	.7458	5.1689
	2.80	5.43933*	.63877	.000	3.2278	7.6509

	3.20	6.15567*	.63877	.000	3.9441	8.3672
.80	.40	-3.47300*	.63877	.001	-5.6845	-1.2615
	1.20	-.43967	.63877	.996	-2.6512	1.7719
	1.60	-.31533	.63877	1.000	-2.5269	1.8962
	2.00	-.05433	.63877	1.000	-2.2659	2.1572
	2.40	-.51567	.63877	.990	-2.7272	1.6959
	2.80	1.96633	.63877	.101	-.2452	4.1779
	3.20	2.68267*	.63877	.012	.4711	4.8942
1.20	.40	-3.03333*	.63877	.004	-5.2449	-.8218
	.80	.43967	.63877	.996	-1.7719	2.6512
	1.60	.12433	.63877	1.000	-2.0872	2.3359
	2.00	.38533	.63877	.998	-1.8262	2.5969
	2.40	-.07600	.63877	1.000	-2.2875	2.1355
	2.80	2.40600*	.63877	.028	.1945	4.6175
	3.20	3.12233*	.63877	.003	.9108	5.3339
1.60	.40	-3.15767*	.63877	.003	-5.3692	-.9461
	.80	.31533	.63877	1.000	-1.8962	2.5269
	1.20	-.12433	.63877	1.000	-2.3359	2.0872
	2.00	.26100	.63877	1.000	-1.9505	2.4725
	2.40	-.20033	.63877	1.000	-2.4119	2.0112
	2.80	2.28167*	.63877	.041	.0701	4.4932
	3.20	2.99800*	.63877	.005	.7865	5.2095
2.00	.40	-3.41867*	.63877	.001	-5.6302	-1.2071
	.80	.05433	.63877	1.000	-2.1572	2.2659
	1.20	-.38533	.63877	.998	-2.5969	1.8262
	1.60	-.26100	.63877	1.000	-2.4725	1.9505
	2.40	-.46133	.63877	.995	-2.6729	1.7502
	2.80	2.02067	.63877	.087	-.1909	4.2322
	3.20	2.73700*	.63877	.010	.5255	4.9485
2.40	.40	-2.95733*	.63877	.005	-5.1689	-.7458
	.80	.51567	.63877	.990	-1.6959	2.7272
	1.20	.07600	.63877	1.000	-2.1355	2.2875
	1.60	.20033	.63877	1.000	-2.0112	2.4119
	2.00	.46133	.63877	.995	-1.7502	2.6729
	2.80	2.48200*	.63877	.022	.2705	4.6935
	3.20	3.19833*	.63877	.003	.9868	5.4099
2.80	.40	-5.43933*	.63877	.000	-7.6509	-3.2278
	.80	-1.96633	.63877	.101	-4.1779	.2452
	1.20	-2.40600*	.63877	.028	-4.6175	-.1945
	1.60	-2.28167*	.63877	.041	-4.4932	-.0701
	2.00	-2.02067	.63877	.087	-4.2322	.1909
	2.40	-2.48200*	.63877	.022	-4.6935	-.2705
	3.20	.71633	.63877	.942	-1.4952	2.9279
3.20	.40	-6.15567*	.63877	.000	-8.3672	-3.9441
	.80	-2.68267*	.63877	.012	-4.8942	-.4711
	1.20	-3.12233*	.63877	.003	-5.3339	-.9108
	1.60	-2.99800*	.63877	.005	-5.2095	-.7865
	2.00	-2.73700*	.63877	.010	-4.9485	-.5255
	2.40	-3.19833*	.63877	.003	-5.4099	-.9868
	2.80	-.71633	.63877	.942	-2.9279	1.4952

*. The mean difference is significant at the 0.05 level.

OSA day 7

Table 8-32. Statistical differences between OSA concentrations at day 7 using ANOVA test

ANOVA					
sizes					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	89.086	7	12.727	21.595	.000
Within Groups	9.429	16	.589		
Total	98.515	23			

Table 8-33. Statistical differences between OSA concentrations at day 7 using multiple comparisons test

Multiple Comparisons						
Dependent Variable: sizes						
Tukey HSD						
(I) concentration	(J) concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.40	.80	4.27667*	.62681	.000	2.1066	6.4468
	1.20	3.62667*	.62681	.001	1.4566	5.7968
	1.60	4.07561*	.62681	.000	1.9055	6.2457
	2.00	3.42533*	.62681	.001	1.2552	5.5954
	2.40	5.37467*	.62681	.000	3.2046	7.5448
	2.80	5.93233*	.62681	.000	3.7622	8.1024
	3.20	6.79333*	.62681	.000	4.6232	8.9634
.80	.40	-4.27667*	.62681	.000	-6.4468	-2.1066
	1.20	-.65000	.62681	.961	-2.8201	1.5201
	1.60	-.20105	.62681	1.000	-2.3712	1.9691
	2.00	-.85133	.62681	.863	-3.0214	1.3188
	2.40	1.09800	.62681	.657	-1.0721	3.2681
	2.80	1.65567	.62681	.211	-.5144	3.8258
	3.20	2.51667*	.62681	.017	.3466	4.6868
1.20	.40	-3.62667*	.62681	.001	-5.7968	-1.4566
	.80	.65000	.62681	.961	-1.5201	2.8201
	1.60	.44895	.62681	.995	-1.7212	2.6191
	2.00	-.20133	.62681	1.000	-2.3714	1.9688
	2.40	1.74800	.62681	.166	-.4221	3.9181
	2.80	2.30567*	.62681	.033	.1356	4.4758
	3.20	3.16667*	.62681	.002	.9966	5.3368
1.60	.40	-4.07561*	.62681	.000	-6.2457	-1.9055
	.80	.20105	.62681	1.000	-1.9691	2.3712
	1.20	-.44895	.62681	.995	-2.6191	1.7212
	2.00	-.65028	.62681	.961	-2.8204	1.5198
	2.40	1.29905	.62681	.469	-.8711	3.4692
	2.80	1.85672	.62681	.124	-.3134	4.0268
	3.20	2.71772*	.62681	.009	.5476	4.8878
2.00	.40	-3.42533*	.62681	.001	-5.5954	-1.2552
	.80	.85133	.62681	.863	-1.3188	3.0214
	1.20	.20133	.62681	1.000	-1.9688	2.3714
	1.60	.65028	.62681	.961	-1.5198	2.8204
	2.40	1.94933	.62681	.095	-.2208	4.1194
	2.80	2.50700*	.62681	.018	.3369	4.6771
	3.20	3.36800*	.62681	.001	1.1979	5.5381
2.40	.40	-5.37467*	.62681	.000	-7.5448	-3.2046
	.80	-1.09800	.62681	.657	-3.2681	1.0721
	1.20	-1.74800	.62681	.166	-3.9181	.4221

	1.60	-1.29905	.62681	.469	-3.4692	.8711
	2.00	-1.94933	.62681	.095	-4.1194	.2208
	2.80	.55767	.62681	.983	-1.6124	2.7278
	3.20	1.41867	.62681	.368	-.7514	3.5888
2.80	.40	-5.93233*	.62681	.000	-8.1024	-3.7622
	.80	-1.65567	.62681	.211	-3.8258	.5144
	1.20	-2.30567*	.62681	.033	-4.4758	-.1356
	1.60	-1.85672	.62681	.124	-4.0268	.3134
	2.00	-2.50700*	.62681	.018	-4.6771	-.3369
	2.40	-.55767	.62681	.983	-2.7278	1.6124
	3.20	.86100	.62681	.856	-1.3091	3.0311
3.20	.40	-6.79333*	.62681	.000	-8.9634	-4.6232
	.80	-2.51667*	.62681	.017	-4.6868	-.3466
	1.20	-3.16667*	.62681	.002	-5.3368	-.9966
	1.60	-2.71772*	.62681	.009	-4.8878	-.5476
	2.00	-3.36800*	.62681	.001	-5.5381	-1.1979
	2.40	-1.41867	.62681	.368	-3.5888	.7514
	2.80	-.86100	.62681	.856	-3.0311	1.3091
*. The mean difference is significant at the 0.05 level.						

OSA day 14

Table 8-34. Statistical differences between concentrations at day 14 using ANOVA test

ANOVA					
sizes					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	140.475	7	20.068	19.416	.000
Within Groups	16.537	16	1.034		
Total	157.013	23			

Table 8-35. Statistical differences between OSA concentrations at day 14 using multiple comparisons test

Multiple Comparisons						
Dependent Variable: sizes						
Tukey HSD						
(I) concentration	(J) concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.40	.80	5.89367*	.83010	.000	3.0197	8.7676
	1.20	5.97233*	.83010	.000	3.0984	8.8463
	1.60	6.54133*	.83010	.000	3.6674	9.4153
	2.00	5.27467*	.83010	.000	2.4007	8.1486
	2.40	7.00100*	.83010	.000	4.1271	9.8749
	2.80	7.35500*	.83010	.000	4.4811	10.228
	3.20	8.71133*	.83010	.000	5.8374	11.585
.80	.40	-5.89367*	.83010	.000	-8.7676	-3.0197
	1.20	.07867	.83010	1.000	-2.7953	2.9526
	1.60	.64767	.83010	.992	-2.2263	3.5216
	2.00	-.61900	.83010	.994	-3.4929	2.2549
	2.40	1.10733	.83010	.873	-1.7666	3.9813
	2.80	1.46133	.83010	.652	-1.4126	4.3353
	3.20	2.81767	.83010	.057	-.0563	5.6916
1.20	.40	-5.97233*	.83010	.000	-8.8463	-3.0984
	.80	-.07867	.83010	1.000	-2.9526	2.7953
	1.60	.56900	.83010	.996	-2.3049	3.4429

	2.00	-.69767	.83010	.988	-3.5716	2.1763
	2.40	1.02867	.83010	.908	-1.8453	3.9026
	2.80	1.38267	.83010	.707	-1.4913	4.2566
	3.20	2.73900	.83010	.068	-.1349	5.6129
1.60	.40	-6.54133*	.83010	.000	-9.4153	-3.6674
	.80	-.64767	.83010	.992	-3.5216	2.2263
	1.20	-.56900	.83010	.996	-3.4429	2.3049
	2.00	-1.26667	.83010	.784	-4.1406	1.6073
	2.40	.45967	.83010	.999	-2.4143	3.3336
	2.80	.81367	.83010	.971	-2.0603	3.6876
	3.20	2.17000	.83010	.220	-.7039	5.0439
2.00	.40	-5.27467*	.83010	.000	-8.1486	-2.4007
	.80	.61900	.83010	.994	-2.2549	3.4929
	1.20	.69767	.83010	.988	-2.1763	3.5716
	1.60	1.26667	.83010	.784	-1.6073	4.1406
	2.40	1.72633	.83010	.465	-1.1476	4.6003
	2.80	2.08033	.83010	.260	-.7936	4.9543
	3.20	3.43667*	.83010	.014	.5627	6.3106
2.40	.40	-7.00100*	.83010	.000	-9.8749	-4.1271
	.80	-1.10733	.83010	.873	-3.9813	1.7666
	1.20	-1.02867	.83010	.908	-3.9026	1.8453
	1.60	-.45967	.83010	.999	-3.3336	2.4143
	2.00	-1.72633	.83010	.465	-4.6003	1.1476
	2.80	.35400	.83010	1.000	-2.5199	3.2279
	3.20	1.71033	.83010	.476	-1.1636	4.5843
2.80	.40	-7.35500*	.83010	.000	-10.228	-4.4811
	.80	-1.46133	.83010	.652	-4.3353	1.4126
	1.20	-1.38267	.83010	.707	-4.2566	1.4913
	1.60	-.81367	.83010	.971	-3.6876	2.0603
	2.00	-2.08033	.83010	.260	-4.9543	.7936
	2.40	-.35400	.83010	1.000	-3.2279	2.5199
	3.20	1.35633	.83010	.725	-1.5176	4.2303
3.20	.40	-8.71133*	.83010	.000	-11.585	-5.8374
	.80	-2.81767	.83010	.057	-5.6916	.0563
	1.20	-2.73900	.83010	.068	-5.6129	.1349
	1.60	-2.17000	.83010	.220	-5.0439	.7039
	2.00	-3.43667*	.83010	.014	-6.3106	-.5627
	2.40	-1.71033	.83010	.476	-4.5843	1.1636
	2.80	-1.35633	.83010	.725	-4.2303	1.5176

*. The mean difference is significant at the 0.05 level.

Whey protein

Day 1

Table 8-36. Statistical differences between WP concentrations at day 14 using ANOVA test

ANOVA					
Particle, whey protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	87.122	5	17.424	4.470	.016
Within Groups	46.774	12	3.898		
Total	133.896	17			

Table 8-37. Statistical differences between WP concentrations at day 1 using multiple comparisons test

Multiple Comparisons						
Dependent Variable: particle, whey protein						
Tukey HSD						
(I) Samples	(J) Samples	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.50	.60	-2.36667	1.61201	.689	-7.7813	3.0479
	.70	-.23333	1.61201	1.000	-5.6479	5.1813
	.80	3.08000	1.61201	.441	-2.3346	8.4946
	.90	1.85333	1.61201	.851	-3.5613	7.2679
	1.00	4.19000	1.61201	.171	-1.2246	9.6046
.60	.50	2.36667	1.61201	.689	-3.0479	7.7813
	.70	2.13333	1.61201	.768	-3.2813	7.5479
	.80	5.44667*	1.61201	.048	.0321	10.8613
	.90	4.22000	1.61201	.166	-1.1946	9.6346
	1.00	6.55667*	1.61201	.015	1.1421	11.9713
.70	.50	.23333	1.61201	1.000	-5.1813	5.6479
	.60	-2.13333	1.61201	.768	-7.5479	3.2813
	.80	3.31333	1.61201	.369	-2.1013	8.7279
	.90	2.08667	1.61201	.783	-3.3279	7.5013
	1.00	4.42333	1.61201	.136	-.9913	9.8379
.80	.50	-3.08000	1.61201	.441	-8.4946	2.3346
	.60	-5.44667*	1.61201	.048	-10.8613	-.0321
	.70	-3.31333	1.61201	.369	-8.7279	2.1013
	.90	-1.22667	1.61201	.969	-6.6413	4.1879
	1.00	1.11000	1.61201	.980	-4.3046	6.5246
.90	.50	-1.85333	1.61201	.851	-7.2679	3.5613
	.60	-4.22000	1.61201	.166	-9.6346	1.1946
	.70	-2.08667	1.61201	.783	-7.5013	3.3279
	.80	1.22667	1.61201	.969	-4.1879	6.6413
	1.00	2.33667	1.61201	.699	-3.0779	7.7513
1.00	.50	-4.19000	1.61201	.171	-9.6046	1.2246
	.60	-6.55667*	1.61201	.015	-11.9713	-1.1421
	.70	-4.42333	1.61201	.136	-9.8379	.9913
	.80	-1.11000	1.61201	.980	-6.5246	4.3046
	.90	-2.33667	1.61201	.699	-7.7513	3.0779
*. The mean difference is significant at the 0.05 level.						

WP day 7

Table 8-38. Statistical differences between WP concentrations at day 7 using ANOVA test

ANOVA					
Particle. Whey protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	88.986	5	17.797	8.784	.001
Within Groups	24.312	12	2.026		
Total	113.297	17			

Table 8-39. Statistical differences between concentrations at day 7 using multiple comparisons test

Multiple Comparisons						
Dependent Variable: particle, wp						
Tukey HSD						
(I) Samples	(J) Samples	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.50	.60	.22333	1.16218	1.000	-3.6803	4.1270
	.70	-.73000	1.16218	.987	-4.6337	3.1737
	.80	1.22667	1.16218	.890	-2.6770	5.1303
	.90	4.31000*	1.16218	.028	.4063	8.2137
	1.00	5.11333*	1.16218	.009	1.2097	9.0170
.60	.50	-.22333	1.16218	1.000	-4.1270	3.6803
	.70	-.95333	1.16218	.958	-4.8570	2.9503
	.80	1.00333	1.16218	.948	-2.9003	4.9070
	.90	4.08667*	1.16218	.038	.1830	7.9903
	1.00	4.89000*	1.16218	.012	.9863	8.7937
.70	.50	.73000	1.16218	.987	-3.1737	4.6337
	.60	.95333	1.16218	.958	-2.9503	4.8570
	.80	1.95667	1.16218	.565	-1.9470	5.8603
	.90	5.04000*	1.16218	.010	1.1363	8.9437
	1.00	5.84333*	1.16218	.003	1.9397	9.7470
.80	.50	-1.22667	1.16218	.890	-5.1303	2.6770
	.60	-1.00333	1.16218	.948	-4.9070	2.9003
	.70	-1.95667	1.16218	.565	-5.8603	1.9470
	.90	3.08333	1.16218	.157	-.8203	6.9870
	1.00	3.88667	1.16218	.051	-.0170	7.7903
.90	.50	-4.31000*	1.16218	.028	-8.2137	-.4063
	.60	-4.08667*	1.16218	.038	-7.9903	-.1830
	.70	-5.04000*	1.16218	.010	-8.9437	-1.1363
	.80	-3.08333	1.16218	.157	-6.9870	.8203
	1.00	.80333	1.16218	.980	-3.1003	4.7070
1.00	.50	-5.11333*	1.16218	.009	-9.0170	-1.2097
	.60	-4.89000*	1.16218	.012	-8.7937	-.9863
	.70	-5.84333*	1.16218	.003	-9.7470	-1.9397
	.80	-3.88667	1.16218	.051	-7.7903	.0170
	.90	-.80333	1.16218	.980	-4.7070	3.1003

*. The mean difference is significant at the 0.05 level.

WP day 14

Table 8-40. Statistical differences between WP concentrations at day 14 using ANOVA test

ANOVA					
Particle, whey protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	126.523	5	25.305	14.676	.000
Within Groups	20.690	12	1.724		
Total	147.213	17			

Table 8-41. Statistical differences between concentrations at day 14 using multiple comparisons test.

Multiple Comparisons						
Dependent Variable: particle, whey protein						
Tukey HSD						
(I) Samples	(J) Samples	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.50	.60	2.95000	1.07212	.135	-.6512	6.5512
	.70	2.69000	1.07212	.196	-.9112	6.2912
	.80	5.31000*	1.07212	.003	1.7088	8.9112
	.90	6.64000*	1.07212	.001	3.0388	10.2412
	1.00	7.87667*	1.07212	.000	4.2755	11.4778
.60	.50	-2.95000	1.07212	.135	-6.5512	.6512
	.70	-.26000	1.07212	1.000	-3.8612	3.3412
	.80	2.36000	1.07212	.304	-1.2412	5.9612
	.90	3.69000*	1.07212	.044	.0888	7.2912
	1.00	4.92667*	1.07212	.006	1.3255	8.5278
.70	.50	-2.69000	1.07212	.196	-6.2912	.9112
	.60	.26000	1.07212	1.000	-3.3412	3.8612
	.80	2.62000	1.07212	.216	-.9812	6.2212
	.90	3.95000*	1.07212	.029	.3488	7.5512
	1.00	5.18667*	1.07212	.004	1.5855	8.7878
.80	.50	-5.31000*	1.07212	.003	-8.9112	-1.7088
	.60	-2.36000	1.07212	.304	-5.9612	1.2412
	.70	-2.62000	1.07212	.216	-6.2212	.9812
	.90	1.33000	1.07212	.810	-2.2712	4.9312
	1.00	2.56667	1.07212	.232	-1.0345	6.1678
.90	.50	-6.64000*	1.07212	.001	-10.2412	-3.0388
	.60	-3.69000*	1.07212	.044	-7.2912	-.0888
	.70	-3.95000*	1.07212	.029	-7.5512	-.3488
	.80	-1.33000	1.07212	.810	-4.9312	2.2712
	1.00	1.23667	1.07212	.850	-2.3645	4.8378
1.00	.50	-7.87667*	1.07212	.000	-11.4778	-4.2755
	.60	-4.92667*	1.07212	.006	-8.5278	-1.3255
	.70	-5.18667*	1.07212	.004	-8.7878	-1.5855
	.80	-2.56667	1.07212	.232	-6.1678	1.0345
	.90	-1.23667	1.07212	.850	-4.8378	2.3645
*. The mean difference is significant at the 0.05 level.						

Statistical differences between days in each concentration alone

Table 8-42. Statistical differences between days in the concentration 0.5% w/w of WP using ANOVA test.

ANOVA					
Whey. Protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.958	2	8.479	2.635	.151
Within Groups	19.310	6	3.218		
Total	36.267	8			

Table 8-43. Statistical differences between days in the concentration 0.6% w/w of WP using ANOVA test.

ANOVA					
Whey. Protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.497	2	8.749	1.827	.240
Within Groups	28.725	6	4.788		
Total	46.222	8			

Table 8-44. Statistical differences between days in the concentration 0.7% w/w of WP using ANOVA test.

ANOVA					
Whey. Protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.185	2	.093	.018	.982
Within Groups	30.786	6	5.131		
Total	30.971	8			

Table 8-45. Statistical differences between days in the concentration 0.8% w/w of WP using ANOVA test.

ANOVA					
Whey. Protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.082	2	1.041	.869	.460
Within Groups	8.384	7	1.198		
Total	10.465	9			

Table 8-46. Statistical differences between days in the concentration 0.9% w/w of WP using ANOVA test.

ANOVA					
Whey. Protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.563	2	7.282	18.617	.003
Within Groups	2.347	6	.391		
Total	16.910	8			

Table 8-47. Statistical differences between days in the concentration 0.9% w/w of WP using multiple comparisons test.

Multiple Comparisons						
Dependent Variable: Whey Protein						
Tukey HSD						
(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	7.00	3.01333*	.51064	.003	1.4466	4.5801
	14.00	2.19333*	.51064	.012	.6266	3.7601
7.00	1.00	-3.01333*	.51064	.003	-4.5801	-1.4466
	14.00	-.82000	.51064	.314	-2.3868	.7468
14.00	1.00	-2.19333*	.51064	.012	-3.7601	-.6266
	7.00	.82000	.51064	.314	-.7468	2.3868

*. The mean difference is significant at the 0.05 level.

Table 8-48. Statistical differences between days in the concentration 1.0% w/w of WP using ANOVA test.

ANOVA					
Whey. Protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.299	2	.150	.837	.478
Within Groups	1.072	6	.179		
Total	1.371	8			

Sodium Caseinate (NaCAS)

Statistical differences between concentrations in each day alone

Table 8-49. Statistical differences between NaCAS concentrations at day 1 using ANOVA test.

ANOVA					
Particle, sodium caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23.709	5	4.742	.953	.483
Within Groups	59.724	12	4.977		
Total	83.433	17			

Table 8-50. Statistical differences between NaCAS concentrations at day 7 using ANOVA test

ANOVA					
Particle, sodium caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28.696	5	5.739	1.679	.214
Within Groups	41.014	12	3.418		
Total	69.711	17			

Table 8-51. Statistical differences between NaCAS concentrations at day 14 using ANOVA test.

ANOVA					
Particle, sodium caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	29.572	5	5.914	1.757	.196
Within Groups	40.397	12	3.366		
Total	69.969	17			

Statistical differences between days in each concentration alone

Table 8-52. Statistical differences between days in the concentration 0.5% w/w of NaCAS using ANOVA test.

ANOVA					
Sodium. Caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.347	2	.174	.065	.937
Within Groups	15.929	6	2.655		
Total	16.276	8			

Table 8-53. Statistical differences between days in the concentration 0.6 % w/w of NaCAS using ANOVA test.

ANOVA					
Sodium. Caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.552	2	3.276	.324	.735
Within Groups	60.613	6	10.102		
Total	67.165	8			

Table 8-54. Statistical differences between days in the concentration 0.7 % w/w of NaCAS using ANOVA test.

ANOVA					
Sodium. Caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.960	2	.980	1.012	.418
Within Groups	5.811	6	.969		
Total	7.771	8			

Table 8-55. Statistical differences between days in the concentration 0.8 % w/w of NaCAS using ANOVA test.

ANOVA					
Sodium. Caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.704	2	.352	.441	.662
Within Groups	4.786	6	.798		
Total	5.490	8			

Table 8-56. Statistical differences between days in the concentration 0.9 % w/w of NaCAS using ANOVA test.

ANOVA					
Sodium. Caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.404	2	.702	.152	.862
Within Groups	27.784	6	4.631		
Total	29.189	8			

Table 8-57. Statistical differences between days in the concentration 1.0 % w/w of NaCAS using ANOVA test.

ANOVA					
Sodium. Caseinate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	2	.000	.000	1.000
Within Groups	34.567	6	5.761		
Total	34.567	8			

Statistical differences between days per and post adding PGPR to oil phase of O/W emulsions

Table 8-58. Statistical differences between days before adding PGPR to O/W emulsions stabilised with OSA/WP/water.

ANOVA					
particle					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.827	2	3.413	1.551	.286
Within Groups	13.203	6	2.200		
Total	20.030	8			

Table 8-59. Statistical differences between days after adding PGPR to O/W emulsions stabilised with OSA/WP/PGPR/water.

ANOVA					
particle					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.886	2	.943	1.684	.263
Within Groups	3.359	6	.560		
Total	5.245	8			

Table 8-60. Statistical differences between days before adding PGPR to O/W emulsions stabilised with OSA/WP/buffer.

ANOVA					
particle					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.680	2	.840	5.566	.043
Within Groups	.905	6	.151		
Total	2.585	8			

Table 8-61. Statistical differences between days before adding PGPR to O/W emulsions stabilised with OSA/WP/buffer using multiple comparisons test.

Multiple Comparisons						
Dependent Variable: particle						
Tukey HSD						
(I) day	(J) day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	7.00	-1.03067*	.31715	.040	-2.0038	-.0576
	14.00	-.72293	.31715	.135	-1.6960	.2502
7.00	1.00	1.03067*	.31715	.040	.0576	2.0038
	14.00	.30773	.31715	.620	-.6654	1.2808
14.00	1.00	.72293	.31715	.135	-.2502	1.6960
	7.00	-.30773	.31715	.620	-1.2808	.6654

*. The mean difference is significant at the 0.05 level.

Table 8-62. Statistical differences between days after adding PGPR to O/W emulsions stabilised with OSA/WP/PGPR buffer.

ANOVA					
particle					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	49.709	2	24.854	3.968	.080
Within Groups	37.582	6	6.264		
Total	87.291	8			

Table 8-63. Statistical differences between days before adding PGPR to O/W emulsions stabilised with OSA/NaCAS/water.

ANOVA					
droplet					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.796	2	7.398	21.349	.002
Within Groups	2.079	6	.347		
Total	16.875	8			

Table 8-64. Statistical differences between days before adding PGPR to O/W emulsions stabilised with OSA/NaCAS/water using multiple comparisons test.

Multiple Comparisons						
Dependent Variable: droplet						
Tukey HSD						
(I) days	(J) days	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	7.00	-2.39770*	.48064	.006	-3.8724	-.9230
	14.00	-2.95567*	.48064	.002	-4.4304	-1.4809
7.00	1.00	2.39770*	.48064	.006	.9230	3.8724
	14.00	-.55797	.48064	.516	-2.0327	.9168
14.00	1.00	2.95567*	.48064	.002	1.4809	4.4304
	7.00	.55797	.48064	.516	-.9168	2.0327

*, The mean difference is significant at the 0.05 level.

Table 8-65. Statistical differences between days after adding PGPR to O/W emulsions stabilised with OSA/NaCAS/PGPR/water.

ANOVA					
droplet					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.162	2	.081	1.011	.419
Within Groups	.482	6	.080		
Total	.644	8			

Table 8-66. Statistical differences between days before adding PGPR to O/W emulsions stabilised with OSA/NaCAS/buffer.

ANOVA					
droplet					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.925	2	2.962	1.004	.420
Within Groups	17.695	6	2.949		
Total	23.620	8			

Table 8-67. Statistical differences between days after adding PGPR to O/W emulsions stabilised with OSA/NaCAS/PGPR/buffer.

ANOVA					
droplet					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.252	2	.126	1.253	.351
Within Groups	.604	6	.101		
Total	.856	8			

Statistical differences in EE between multiple emotions in LCMS based method

Day 1

Table 8-68. Statistical differences in EE at day 1 between multiple emotions in LCMS based method, emulsions stabilised with either OSA, WP, OSA/WP, NaCAS or OSA/NaCAS.

ANOVA					
EE					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	98.863	4	24.716	.853	.505
Within Groups	724.417	25	28.977		
Total	823.280	29			

Day 14

Table 8-69. Statistical differences in EE at day 14 between multiple emotions in LCMS based method, emulsions stabilised with either OSA, WP, OSA/WP, NaCAS or OSA/NaCAS.

ANOVA					
EE					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	85.019	4	21.255	1.845	.152
Within Groups	288.020	25	11.521		
Total	373.039	29			

Statistical differences in EE between multiple emotions in UV based method

Day 1

Table 8-70. Statistical differences in EE at day 1 between multiple emotions in UV based method, emulsions stabilised with either OSA, WP, OSA/WP, NaCAS or OSA/NaCAS.

ANOVA					
EE					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	96.404	4	24.101	6.156	.009
Within Groups	39.149	10	3.915		
Total	135.553	14			

Table 8-71. Statistical differences in EE at day 1 between multiple emotions in UV based method, emulsions stabilised with either OSA, WP, OSA/WP, NaCAS or OSA/NaCAS using multiple comparisons test.

Multiple Comparisons						
Dependent Variable: EE						
Tukey HSD						
(I) type	(J) type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	4.60096	1.61552	.099	-.7159	9.9178
	3.00	6.13229*	1.61552	.023	.8155	11.4491
	4.00	4.17769	1.61552	.147	-1.1391	9.4945
	5.00	7.51784*	1.61552	.006	2.2010	12.8347
2.00	1.00	-4.60096	1.61552	.099	-9.9178	.7159
	3.00	1.53133	1.61552	.872	-3.7855	6.8481
	4.00	-.42327	1.61552	.999	-5.7401	4.8935
	5.00	2.91688	1.61552	.421	-2.3999	8.2337
3.00	1.00	-6.13229*	1.61552	.023	-11.4491	-.8155
	2.00	-1.53133	1.61552	.872	-6.8481	3.7855
	4.00	-1.95460	1.61552	.746	-7.2714	3.3622
	5.00	1.38555	1.61552	.906	-3.9313	6.7024
4.00	1.00	-4.17769	1.61552	.147	-9.4945	1.1391
	2.00	.42327	1.61552	.999	-4.8935	5.7401
	3.00	1.95460	1.61552	.746	-3.3622	7.2714
	5.00	3.34015	1.61552	.304	-1.9767	8.6570
5.00	1.00	-7.51784*	1.61552	.006	-12.8347	-2.2010
	2.00	-2.91688	1.61552	.421	-8.2337	2.3999
	3.00	-1.38555	1.61552	.906	-6.7024	3.9313
	4.00	-3.34015	1.61552	.304	-8.6570	1.9767
*. The mean difference is significant at the 0.05 level.						

Day 14

Table 8-72. Statistical differences in EE at day 14 between multiple emotions in UV based method, emulsions stabilised with either OSA, WP, OSA/WP, NaCAS or OSA/NaCAS.

ANOVA					
EE					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	196.516	4	49.129	1.731	.219
Within Groups	283.855	10	28.385		
Total	480.370	14			

Adding of Minerals to W₁/O/W₂ emulsions

OSA stabilised emulsions

Table 8-73. Statistical differences between days after adding NaCl.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.121	2	1.060	.177	.842
Within Groups	35.939	6	5.990		
Total	38.059	8			

Table 8-74. Statistical differences between days after adding CaCl₂.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	523.637	2	261.819	32.926	.001
Within Groups	47.711	6	7.952		
Total	571.348	8			

Table 8-75. Statistical differences between days after adding CaCl₂ using multiple comparisons test.

Multiple Comparisons						
Dependent Variable: size						
Tukey HSD						
(I) days	(J) days	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	14.00	-1.97667	2.30243	.684	-9.0411	5.0878
	28.00	-17.07833*	2.30243	.001	-24.1428	-10.0139
14.00	1.00	1.97667	2.30243	.684	-5.0878	9.0411
	28.00	-15.10167*	2.30243	.001	-22.1661	-8.0372
28.00	1.00	17.07833*	2.30243	.001	10.0139	24.1428
	14.00	15.10167*	2.30243	.001	8.0372	22.1661

*. The mean difference is significant at the 0.05 level.

Table 8-76. Statistical differences between days after adding KCl.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.450	2	.225	.054	.948
Within Groups	25.114	6	4.186		
Total	25.564	8			

Table 8-77. Statistical differences between days after adding MgCl₂.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.723	2	19.862	6.556	.031
Within Groups	18.176	6	3.029		
Total	57.899	8			

Table 8-78. Statistical differences between days after adding MgCl₂ using multiple comparisons test.

Multiple Comparisons						
Dependent Variable: size						
Tukey HSD						
(I) days	(J) days	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	14.00	-5.10000*	1.42111	.027	-9.4604	-.7396
	28.00	-3.14500	1.42111	.147	-7.5054	1.2154
14.00	1.00	5.10000*	1.42111	.027	.7396	9.4604
	28.00	1.95500	1.42111	.410	-2.4054	6.3154
28.00	1.00	3.14500	1.42111	.147	-1.2154	7.5054
	14.00	-1.95500	1.42111	.410	-6.3154	2.4054

*. The mean difference is significant at the 0.05 level.

OSA/WP stabilised emulsions

Table 8-79. Statistical differences between days after adding NaCl to OSA/WP stabilised emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.754	2	1.877	.270	.772
Within Groups	41.710	6	6.952		
Total	45.464	8			

Table 8-80. Statistical differences between days after adding CaCl₂ to OSA/WP stabilised emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.648	2	.824	.503	.628
Within Groups	9.832	6	1.639		
Total	11.480	8			

Table 8-81. Statistical differences between days after adding KCl to OSA/WP stabilised emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	29.792	2	14.896	.825	.482
Within Groups	108.328	6	18.055		
Total	138.120	8			

Table 8-82. Statistical differences between days after adding MgCl₂ to OSA/WP stabilised emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.375	2	2.688	.483	.639
Within Groups	33.408	6	5.568		
Total	38.783	8			

OSA/NaCAS stabilised emulsions

Table 8-83. Statistical differences between days after adding NaCl to OSA/NaCAS stabilised emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21.972	2	10.986	.576	.590
Within Groups	114.427	6	19.071		
Total	136.399	8			

Table 8-84. Statistical differences between days after adding CaCl_2 to OSA/NaCAS stabilised emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1730.316	2	865.158	7.073	.026
Within Groups	733.935	6	122.322		
Total	2464.251	8			

Table 8-85. Statistical differences between days after adding CaCl_2 to OSA/NaCAS stabilised emulsions using multiple comparisons test.

Multiple Comparisons						
Dependent Variable: size						
Tukey HSD						
(I) days	(J) days	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	14.00	-6.13333	9.03041	.784	-33.8411	21.5744
	28.00	25.86333	9.03041	.064	-1.8444	53.5711
14.00	1.00	6.13333	9.03041	.784	-21.5744	33.8411
	28.00	31.99667*	9.03041	.028	4.2889	59.7044
28.00	1.00	-25.86333	9.03041	.064	-53.5711	1.8444
	14.00	-31.99667*	9.03041	.028	-59.7044	-4.2889

*. The mean difference is significant at the 0.05 level.

Table 8-86. Statistical differences between days after adding KCl to OSA/NaCAS stabilised emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	123.315	2	61.657	1.455	.305
Within Groups	254.182	6	42.364		
Total	377.497	8			

Table 8-87. Statistical differences between days after adding to MgCl_2 OSA/NaCAS stabilised emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3064.401	2	1532.201	.350	.718
Within Groups	26267.602	6	4377.934		
Total	29332.003	8			

Adding of a group of mineral to $W_1/O/W_2$ glucose-rich emulsions

Table 8-88. Statistical differences between days after adding NaCl, CaCl_2 , KCl and MgCl_2 to OSA stabilised $W_1/O/W_2$ emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.007	4	2.252	.774	.567
Within Groups	29.097	10	2.910		
Total	38.103	14			

Table 8-89. Statistical differences between days after adding NaCl, CaCl₂, KCl and MgCl₂ to OSA/WP stabilised W₁/O/W₂ emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	55.765	4	13.941	1.882	.190
Within Groups	74.095	10	7.410		
Total	129.861	14			

Between day in OSA/NaCAS stabilised emulsions

Table 8-90. Statistical differences between days after adding NaCl, CaCl₂, KCl and MgCl₂ to OSA/NaCAS stabilised W₁/O/W₂ emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	178.134	4	44.533	1.751	.215
Within Groups	254.343	10	25.434		
Total	432.477	14			

Adding of a group of mineral to W₁/O/W₂ sucrose-rich emulsions

Table 8-91. Statistical differences between days after adding NaCl, CaCl₂, KCl and MgCl₂ to OSA stabilised W₁/O/W₂ sucrose-rich emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	108.295	4	27.074	10.022	.002
Within Groups	27.013	10	2.701		
Total	135.308	14			

Table 8-92. Statistical differences between days after adding NaCl, CaCl₂, KCl and MgCl₂ to OSA stabilised W₁/O/W₂ sucrose-rich emulsions using multiple comparisons.

Multiple Comparisons						
Dependent Variable: size						
Tukey HSD						
(I) days	(J) days	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	3.00	-.44000	1.34197	.997	-4.8565	3.9765
	7.00	1.08000	1.34197	.923	-3.3365	5.4965
	14.00	.23667	1.34197	1.000	-4.1799	4.6532
	28.00	-6.38333*	1.34197	.005	-10.7999	-1.9668
3.00	1.00	.44000	1.34197	.997	-3.9765	4.8565
	7.00	1.52000	1.34197	.787	-2.8965	5.9365
	14.00	.67667	1.34197	.985	-3.7399	5.0932
	28.00	-5.94333*	1.34197	.009	-10.3599	-1.5268
7.00	1.00	-1.08000	1.34197	.923	-5.4965	3.3365
	3.00	-1.52000	1.34197	.787	-5.9365	2.8965
	14.00	-.84333	1.34197	.967	-5.2599	3.5732
	28.00	-7.46333*	1.34197	.002	-11.8799	-3.0468
14.00	1.00	-.23667	1.34197	1.000	-4.6532	4.1799
	3.00	-.67667	1.34197	.985	-5.0932	3.7399

	7.00	.84333	1.34197	.967	-3.5732	5.2599
	28.00	-6.62000*	1.34197	.004	-11.0365	-2.2035
28.00	1.00	6.38333*	1.34197	.005	1.9668	10.7999
	3.00	5.94333*	1.34197	.009	1.5268	10.3599
	7.00	7.46333*	1.34197	.002	3.0468	11.8799
	14.00	6.62000*	1.34197	.004	2.2035	11.0365

*. The mean difference is significant at the 0.05 level.

Table 8-93. Statistical differences between days after adding NaCl, CaCl₂, KCl and MgCl₂ to OSA/WP stabilised W₁/O/W₂ sucrose-rich emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	84.263	4	21.066	2.380	.121
Within Groups	88.510	10	8.851		
Total	172.773	14			

Table 8-94. Statistical differences between days after adding NaCl, CaCl₂, KCl and MgCl₂ to OSA/NaCAS stabilised W₁/O/W₂ sucrose-rich emulsions.

ANOVA					
size					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1521.260	4	380.315	4.529	.024
Within Groups	839.782	10	83.978		
Total	2361.042	14			

Table 8-95. Statistical differences between days after adding NaCl, CaCl₂, KCl and MgCl₂ to OSA/NaCAS stabilised W₁/O/W₂ sucrose-rich emulsions using multiple comparisons.

Multiple Comparisons						
Dependent Variable: size						
Tukey HSD						
(I) days	(J) days	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	3.00	.59667	7.48234	1.000	-24.0283	25.2217
	7.00	-.50667	7.48234	1.000	-25.1317	24.1183
	14.00	-19.74667	7.48234	.136	-44.3717	4.8783
	28.00	-21.23667	7.48234	.100	-45.8617	3.3883
3.00	1.00	-.59667	7.48234	1.000	-25.2217	24.0283
	7.00	-1.10333	7.48234	1.000	-25.7283	23.5217
	14.00	-20.34333	7.48234	.120	-44.9683	4.2817
	28.00	-21.83333	7.48234	.089	-46.4583	2.7917
7.00	1.00	.50667	7.48234	1.000	-24.1183	25.1317
	3.00	1.10333	7.48234	1.000	-23.5217	25.7283
	14.00	-19.24000	7.48234	.150	-43.8650	5.3850
	28.00	-20.73000	7.48234	.111	-45.3550	3.8950
14.00	1.00	19.74667	7.48234	.136	-4.8783	44.3717
	3.00	20.34333	7.48234	.120	-4.2817	44.9683
	7.00	19.24000	7.48234	.150	-5.3850	43.8650
	28.00	-1.49000	7.48234	1.000	-26.1150	23.1350
28.00	1.00	21.23667	7.48234	.100	-3.3883	45.8617
	3.00	21.83333	7.48234	.089	-2.7917	46.4583
	7.00	20.73000	7.48234	.111	-3.8950	45.3550
	14.00	1.49000	7.48234	1.000	-23.1350	26.1150