FACULTY OF BIOSCIENCE ENGINEERING

IMPROVED EMULSIFYING AND HEAT STABILIZING PROPERTIES OF WHEY PROTEIN CONCENTRATE BY DRY HEAT INDUCED GLYCATION

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List of Abbreviations

α-La	α-Lactalbumin
β-Lg	β-Lactoglobulin
µm	Micrometer
a_w	Water activity
BSA	Bovine Serum Albumin
DG	Degree of glycation
EGCG	Epigallocathecin gallate
EMR	Early Maillard Reaction
GA	Gum arabic
HMF	Hydroxymethylfurfural
HWP	Hydrolysed whey protein
IEP	Isoelectric point
Igs	Immunoglobulins
KCl	Potassium chloride
LF	Lactoferrin
LMP	Low Methoxyl Pectin
MW	Molecular weight
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NMR	Nuclear magnetic resonance
o/w	Oil in water
OPA	Ortho-phthalaldehyde
rpm	Rotation per minute
w/v	Weight per volume
w/w	Weight per weight

ABSTRACT

The background of the research is to evaluate the properties of whey protein concentrate with the presence of simple sugar (naturally present lactose) conjugated via Maillard reaction during dry heat incubation. In this study, whey protein concentrate (WPC) was conjugated with naturally present lactose to improve the heat stabilizing and emulsifying properties in the WPC conjugates. This experiment was conducted using a dry heat method at a defined temperature (80 °C). Two conjugation variables were observed: the relative humidity during incubation and preconditioning pH prior to lyophilisation. Concerning the relative humidity (RH), a RH of 64%, 74% and 79 was used. Meanwhile, the preconditioning pH was set to obtain a pH of 4, 6, 8, and 10. After the dry heat conjugation, the WPC conjugates were used as emulsifier in 10% O/W emulsion. For checking its stability in the heat treatment, the emulsions were further heated at 80 °C for 20 min. During this period, the analyses conducted were the particle size, viscosity, and creaming stability both before and after the heating test.

The average volume weighted diameter ($D_{4,3}$) of the emulsions stabilized by the conjugates obtained at all RH values was around 0.6 μm. After heating, emulsion stabilized by WPC-conjugates became unstable (increased in the particle size and viscosity), except for those incubated for 6 hours (at RH of 74% and 79%) and 8 hours (at RH of 64%). RH of 74% was then further used in the next evaluation in term of the effect of preconditioning pH prior to lyophilisation. Based on the analysis conducted, for WPC conjugates preconditioned at pH 4, we did not find a suitable incubation time to produce heat stable emulsions. However, a minimum incubation time of 4 hours and 2 hours was found in order to make stable emulsions for preconditioned WPC conjugates at pH 6 and 8, respectively. This conclusion was drawn based on the results of particle size and viscosity analysis, as they could maintain stability upon the heat treatment. From the conjugates characterisation, it was found that indeed the higher the preconditioned pH, the more pronounced the Maillard reaction occurred, as demonstrated from the degree of conjugation and browning color formation (visually). Finally, from our conducted experiments, it was found that, indeed, conjugation between whey protein concentrate and naturally present lactose could improve the heat stabilizing

properties of o/w emulsions. However, no sufficient evidence was found that the emulsifying properties were also improved with the conjugation of WPC and naturally present lactose.

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

1.1.1. Overview

Oil in water (o/w) emulsions are abundantly present in many foods, such as mayonnaise, milk, or creamers. Bearing in mind that emulsions are thermodynamically unstable, it is important to stabilize the emulsions. Their unstable behaviour is due to the fact that oil and water do not coexist harmoniously because of the surface energy of the oil-water interface. With the absence of surfactants, emulsions attempt to reduce the interfacial area by coalescence of the oil droplets (Friberg, Larsson, & Sjoblom, 2004). In order to prevent the emulsion destabilization, a stabilizer such as an emulsifier can be used to improve the kinetic stability in the emulsions (McClements, 2016).

Sodium caseinate is one of the common emulsifiers that has been used widely in food products. Nevertheless, whey proteins (a by-product from cheese- and casein-production) which offer high nutritional content and good functional properties, possess a potential to be used as an emulsifier. These past years, the functionality of whey proteins has been widely studied. Whey proteins are found to stabilize o/w emulsions by forming interfacial films between hydrophobic and hydrophilic groups which facilitate good surface activity (Haines, 2005; Wang, 2013). However, although the utilization of whey proteins seem promising, the biggest drawback is their heat labile characteristic as whey proteins lose solubility upon heating which leads to heat-induced aggregation of the proteins (Roefs & Kruif, 1994). It is crucial as in food production, heat treatment is one of the most important parameters as it is directly related to safety.

Dry heat conjugation, a method which optimizes the Maillard reaction of whey proteins with sugar, has been studied as an alternative to improve the heat stability of whey proteins. One attempt that had been conducted was conjugating whey proteins with polysaccharides. A study by Setiowati, Saeedi, Wijaya, & Van der Meeren (2017), showed that using whey protein isolate (WPI) demonstrated a good emulsion capacity via dry heat

treatment in the presence of Low Methoxyl Pectin (LMP). However, most of the protein conjugation with polysaccharides took hours to days to obtain the desired functionality which in fact was not industrially feasible. Moreover, economically speaking, WPI is relatively expensive as it contains a high protein content (around 90%)

Lactose is a simple sugar that is known to react faster with protein during Maillard reaction, resulting in less incubation time to carry out good heat stabilizing properties of the whey protein conjugates. Several researches have been conducted to study the glycation between whey proteins and lactose (Liu & Zhong, 2014; Morgan, Nouzille, Baechler, Vuataz, & Raemy, 2005; Schong & Famelart, 2019). Whereas glycation of whey proteins with lactose in those studies could improve the properties of the proteins in terms of emulsifying and heat stabilizing properties, the preparation methods were complicated, requiring the addition of external lactose. In order to ensure molecular mixing, solutions have to be mixed and subsequently lyophilised.

In this study, whey protein concentrate (WPC) was conjugated with its naturally present lactose through dry heat induced glycation. The use of naturally present lactose has a future perspective in the production of clean label products. Nevertheless, it is essential to note that the condition of dry heating such as temperature and water activity are among the most important parameters influencing the Maillard reaction, as well as the pH and the type of sugars (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016; Fenaille, Morgan, Parisod, Tabet, & Guy, 2003). Those factors, therefore, should be monitored carefully so that advanced Maillard reaction will not take place. Further on, the effect of relative humidity (RH) and preconditioning pH on the WPC conjugation was evaluated. With this study, the formation of glycoprotein complexes between WPC and naturally present lactose was observed to see whether there is an improvement in the heat stabilizing and the emulsifying activity of WPC conjugates in o/w emulsions. To that end, a characterisation of the emulsions, as well as of the WPC conjugates was conducted.

1.1.2. Research objective

The objective of this research was to produce heat stable WPC conjugates through dry heat induced glycation with its naturally present lactose. The effect of incubation conditions,

particularly the effect of relative humidity (RH) and preconditioning pH in the conjugation of WPC was evaluated. The emulsifying and heat stabilizing properties of the conjugates were observed by applying the WPC conjugates in an o/w emulsion system followed by a heating test of the emulsions.

1.2. Whey proteins

Whey protein, which once was considered as a by-product from the cheese and casein manufacturing, is highly utilized for many functions due to its functionality. There were approximately 20% of the milk proteins in whey protein composition (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001). Whey proteins are also versatile; they foam well in an aqueous solution due to the small amount of fat contained in the proteins (De Wit, 1998). Whey protein remains soluble from pH 2.0 to 10.0 and stabilizes emulsions by forming interfacial films between hydrophobic and hydrophilic components (Burrington, 2005; Haines, 2005). They can interact with gels and edible films, and create network association (Foegeding, Davis, Doucet, & McGuffey, 2002). Whey proteins are also temperature sensitive; they unfold and aggregate upon heating and are able to bind large amounts of water depending on the pH, thermal conditions, and ionic strength (Hudson, Daubert, & Foegeding, 2000).

1.2.1. Whey protein composition

Whey protein consists of several proteins, such as β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La), the heavy- and light-chain immunoglobulins (Igs), bovine serum albumin (BSA), lactoferrin (LF), lactoperoxidase and glycomacropeptide (De Wit, 1998). β-Lactoglobulin (β-Lg) is the most abundant component in the whey proteins, and is responsible for the solubility, foaming, gelation, emulsification and flavour-binding properties (Jiménez-Castaño, Villamiel, Martín-Álvarez, Olano, & López-Fandiño, 2005). Its native conformation is sensitive towards heat and pH. At temperatures below 25°C and pH values above 7.0, the protein forms octamers (Pessen, Purcell, and Farrell, 1985). β-Lactoglobulin has a molecular weight of approximately 18.3 kDa. At room temperature and at its physiological pH, β-Lactoglobulin exists mainly as a dimer, in which the monomers are noncovalently linked, but

it dissociates into monomers at elevated temperature (Hoffman & Van Mil, 1999). This dimer, however, is predominantly important in the heat-induced aggregation mechanism (Cairoli, Iametti, & Bonomi, 1994; Iametti, De Gregori, Vecchio, & Bonomi, 1996). It also has a high solubility at low pH, which makes it useful in acidic beverages (Smithers, et al., 1996). Previous studies demonstrated that the β-Lg fraction produced by selective isoelectric fractionation, showed total solubility and clarity within the pH range from 3.0 to 8.0 (Pearce, 1987).

Additionally, β-Lactoglobulin has binding and gelling properties and forms heat induced gels. The gel strength formed was, however, found to be pH-dependent (Solak & Akin, 2012). Heating of β-Lactoglobulin at 80°C and neutral pH (6-7) for 20 minutes was found to make 80% of the protein to denature (Law & Leaver, 2000). Further on, β-Lg is pH-sensitive; it has an IEP around 4.0 to 5.2 (Bryant & McClements, 1998; Kováčová, Synytsya, & Štětina, 2009).

α-Lactalbumin accounts for approximately 25% of the total whey protein and is one of the main proteins present in human and bovine milk (Solak & Akin, 2012). α-LA has a small molecular weight (14200 Da) and has a IEP of 4-5 (Permyakov & Berliner, 2000). It possesses a single strong Ca^{2+} binding site (Permyakov, et al., 1981). The presence of Ca^{2+} attributes to the heat stability of α-LA as the high energy requirement needed to break the bonding (Haque, et al., 2013). Moreover, in total, the structure of α-LA is stabilized by four disulfide bridges (6-120, 61-77, 73-91, and 28-111) (Permyakov & Berliner, 2000).

Immunoglobulins (Igs) contain approximately 10-15% of the total whey proteins. There are several classes of antibodies, *i.e.* IgA, IgD, IgE, IgG and IgM. Bovine serum albumin (BSA) makes up around 10-15% of the total composition of whey protein (Solak & Akin, 2012). It consists of 17 intramolecular disulphide bonds and one free sulfhydryl group (Eigel, et al., 1984). The IEP of BSA is around 4.8-5.1 (Bryant & McClements, 1998).

1.2.2. Whey protein concentrate

There are two basic types of whey, namely sweet whey and acid whey. Sweet whey is derived from the manufacture of rennet-produced cheeses. Acid whey is produced from the manufacture of acid-produced cheeses. The composition of whey products varies according

to the milk source, type of cheese, the methods of production, purification and concentration, and manufacturing process (Solak & Akin, 2012).

There are many forms of whey products such as whey powder, hydrolysed whey protein (HWP), whey protein concentrates (WPC), whey protein isolates (WPI), reduced-lactose whey and demineralized whey. WPC is whey with a protein content ranging from 34% to 85%, where WPI contains at least 90% of protein on dry weight basis (Solak & Akin, 2012). Ultrafiltration technology and spray drying are usually used in the manufacturing of WPC (Morgan et al., 2005; Morr & Ha, 1991). Whey protein concentrates also contain 3.3 – 7.4% of total lipid and as a result from ultrafiltration have a lactose content ranging from 51% to 5% (Morgan et al., 2005; Morr & Ha, 1991). There are several types of whey protein concentrates based on its protein content, e.g. WPC-80 which has 80% of protein and WPC-34 which has 34% of protein mass. WPC-34 has fewer purification steps in its manufacturing and therefore contains big particulates such as milk fat globule membrane material (Liu & Zhong, 2014).

1.3. Emulsifying activity of whey proteins

Due to their amphiphilic nature and their ability to form cohesive viscoelastic films at o/w interfaces, proteins are preferred as emulsifiers (Damodaran, 1997). It is noted that several factors may influence the emulsifying activity of proteins, such as surface hydrophobicity (Voutsinas, Cheung, & Nakai, 1983), protein flexibility, electrostatic interactions and steric effects (Phillips, Kinsella, Whitehead, 1994), ionic strength (Leman & Kinsella, 1989), and protein concentration (Yamauchi, Shimizu, & Kamiya, 1980). Among all the proteins in food which are available, whey proteins also demonstrate a potential to be an emulsifier. As whey proteins are globular proteins with a great surface hydrophobicity and many S-S bonds, their emulsifying capacity is enhanced by partial unfolding and moderate heating which result in gelation (Zayas, 1997). It has high solubility over a wide range of pH values, and is able to readily adsorb at the interface, reduce the interfacial tension at the oil in water interface, and thus prevent destabilization of the emulsion by creating an interfacial membrane around the oil droplets (Kinsella & Whitehead, 1989).

According to Zayas (1997), the hydrophobicity influences the emulsifying capacity of proteins as it affects the protein solubility in water. It means that with a larger number of

hydrophobic amino acids, proteins would interact more with the oil surface. Upon emulsification, the soluble proteins diffuse and become concentrated at the interface. This diffusion is influenced by many factors such as protein concentration, molecular size, temperature, pH, solubility and ionic strength, and also differs for each kind of protein; whey proteins diffuse rapidly to the interface due to their small molecules and molecular complexes. As there is a rapid diffusion to the surface, interfacial film formation takes place rapidly to prevent coalescence against oil droplets.

1.4. Heat treatments

Heat treatments, such as preheating, pasteurization, and sterilization, influence the structure and properties of whey proteins, either reversibly or irreversibly. Structure and solubility of whey proteins are interrelated and affected by commonly used heat treatments. The relation varies with the nature of the protein and the composition of the protein solution. Due to the heating, whey proteins become denatured. This is the main issue in the dairy industry as it may lead to other problems, e.g. flavour defects due to the release of small sulfur-containing compounds such as hydrogen sulfide and methanethiol and aggregates formation which causes fouling of heat exchangers (Wijayanti, Bansal, & Deeth, 2014).

1.4.1. Influence of heat treatment in whey proteins

Heating affects the functionality of protein since it may cause denaturation and increase the apparent viscosity in some proteins and even gelation, where the protein in the aqueous phase interacts strongly with the adsorbed whey protein of the emulsion droplet surfaces (Friberg et al., 2004). It was found that amino acids are responsible for the protein interactions during heating, *e.g.* β-LG is not heat resistant due to the presence of two disulphide bridges and one thiol group per monomer which makes it reactive and causes conformational changes, while α-LA is the most heat resistant which is partially due to its secondary structure which does not have a free –SH group (Calvo, Leaver, 1993). As β-LG is the most abundant component in the whey proteins, it is often assumed to be the main driver of the aggregation in whey proteins.

The change of structure in proteins is related to the temperature in the heat treatments. Mulvihill and Donovan (1987) explained that the aggregation in whey proteins

consists of two main stages. The first stage is unfolding of the initial folded structure of globular β-LG and this stage will be followed by the formation of protein complexes as a consequence of the accumulation of the unfolded molecules. At room temperature, β-LG presents in an equilibrium between its dimeric and monomeric forms. However, at temperatures above 30 °C and pH values between 6 and 9, the dimer dissociates mainly into monomers. The reversible change is taken place when mild heat treatment is applied (in the temperature range up to 60 °C), and this change is driven by hydrophobic bonding where no loss in solubility for β-LG is supposed to take place (deWit & Klarenbeek, 1984).

Upon heating above 65 °C, there will be a conformational change, accompanied by an exposure of highly reactive nucleophilic groups in the hydrophobic groups of amino acids and thus, the total surface activity will be enhanced which is usually demonstrated when moderate heating is applied, noting that too much heating may lead to aggregation that would reduce the concentration of effective protein molecules as well as make this structural change become irreversible and reduce its solubility (deWit & Klarenbeek, 1984; Kinsella & Whitehead, 1989; Zayas, 1997). There are two kinds of aggregates during the irreversible aggregation, i.e. small (via -SH group oxidation and/or –SH/S–S interchange) and large aggregates (via non-specific interaction without -SH groups occupied) (Mulvihill & Donovan, 1987). Additionally, at a more severe temperature ranging in between 100 to 150°C, irreversible changes take place such as Maillard reaction and cysteine breakdown (deWit & Klarenbeek, 1984).

The model from Mulvihill & Donovan is not the only explanation on how whey protein aggregates are formed during heating. Steventon et al. (1991) proposed an aggregation model for WPC heated at 85°C for 5 min. They suggested three important stages: reversible unfolding, initiation of aggregation, and propagation of aggregation. At first, the monomers create dimers and react with denatured monomers. During the propagation stage, those will form larger aggregates and lead to gelation of whey proteins. Those are the result from the reactions between similar whey proteins, *i.e.* β-LG/β-LG or BSA/BSA or between different whey proteins, *i.e.* β-Lg/α-La or β-Lg/BSA or α-La/BSA.

To conclude, there is no clear agreement as to the extent of the interactions during denaturation and aggregation of whey proteins, as there are many models postulated, but

every model has its own circumstances. Nevertheless, it can be assumed that β-LG is highly associated with the occurrence of whey protein aggregation during heat treatment.

1.4.2. Influence of heat treatment in whey protein stabilized emulsions

According to Demetriades, Coupland, & McClements (1997), factors such as ionic strength and pH as well as the processing and storage conditions (*e.g.* heating, cooling, and mechanical agitation) contribute to the physicochemical properties of food emulsions stabilized by whey proteins. In their study, they observed the physicochemical characteristics of whey protein stabilized emulsions respectively at pH 3, 5, and 7. Based on the results, the emulsions were highly flocculated when subjected to heating at a temperature of 65 °C and higher when there was no salt presence. As explained previously, the flocculation was caused by the exposure of hydrophobic groups of protein adsorbed to the oil-water interface, which had been proven by Dalgleish (1996) through differential scanning calorimetry analysis as β-LG and α-LA unfolded at that particular temperature.

Due to heat treatment, the unfolding of the proteins would lead to enhanced interactions between proteins through hydrophobic and thiol-disulphide interchanges (Dickinson & Matsumura, 1991; McClements, et al., 1993; Monahan, et al., 1993). There can be either the interaction between molecules adsorbed to the same droplet (intradroplet) or between those adsorbed to different droplets (interdroplet). It is assumed that intermolecular interaction takes place at the high molecular density in the adsorbed layer (Dickinson & Matsumura, 1991). Intramolecular interactions give rise to viscoelasticity of the surface layer (Dickinson & Matsumura, 1991), while intermolecular interactions lead to an increased tendency to flocculate (McClements et al., 1993). Dickinson (1991) further explained that in the formation of intermolecular interactions, the intramolecular disulphide linkages remain intact immediately after adsorption, which means that the intermolecular disulphide bond formation takes place slowly.

Nevertheless, it was found that the extent of droplet aggregation was reduced when emulsions were heated to higher temperature, possibly due to the competition between interdroplet and intradroplet protein-protein interaction (Demetriades, Coupland, & McClements, 1997). Additionally, it may also due to the partial unfolding of the protein molecules at the surface when subjected to temperature around 65-80°C, making the

arrangement of nonpolar amino acids not all directed towards the oil phase (Dalgleish, 1996). This leads to a droplet surface that becomes more hydrophobic, and thus, there is a higher possibility of droplet aggregation. Similarly, when subjected to higher temperatures, the proteins are fully unfolded and the nonpolar groups directed to the aqueous phase, making the droplets become less vulnerable towards aggregation as they have a lower surface hydrophobicity (Dalgleish, 1996).

1.4.3. Factors influencing heat stability of whey proteins

It can be generally assumed that the stability of whey proteins depends on the Van der Waals, electrostatic and hydrophobic interactions (Demetriades, Coupland, & McClements, 1997). To improve the characteristics of whey proteins, factors such as pH, heat treatment and protein concentration have been widely studied and applied.

1.4.3.1. pH

The stability of proteins depends on the electrostatic interactions. The electrostatic interactions between similarly charged droplets are repulsive, and if this interaction is dominant, the protein will not form aggregates. However, when Van der Waals attraction dominates, proteins are vulnerable to flocculation. This electrostatic attraction between protein-stabilized emulsion droplets is particularly sensitive to pH and ionic strength (Demetriades, Coupland, & Mcclements, 1997). By changing the pH, this is related to an increase in the electrostatic forces of repulsion between the stabilizing membranes formed around the fat globules by the whey proteins and by some favourable protein denaturation and thus, the conformation of the protein molecules and the net charge of the adsorbed proteins will be affected (Fachin & Viotto, 2005; Phillips, Kinsella, Whitehead, 1994).

The influence of pH is reflected in the rheological properties of the emulsions, *i.e.* viscosity. Demetriades et al. (1997) showed that whey protein stabilized emulsions retained a low viscosity at pH-values far away from its isoelectric point. If the pH is conditioned around its IEP, the viscosity increases. Around its IEP, the net charge is minimised, and as a result, the repulsion between the fat globules is weakened, leading to poor stability of the emulsions (Yamauchi et al., 1980). Another study reported that the stability of whey protein emulsions enhanced when the pH was increased from 5 to 7, most likely due to an increase in repulsion

by the electrostatic charges of the proteins (Yamauchi et al., 1980). When the emulsions are flocculated, their rheology will exhibit a strong shear thinning behaviour, as the viscosity decreases with increasing shear rate (Demetriades, Coupland, & Mcclements, 1997). Some studies also concluded that an increasing pH will lead to greater conversion of native to aggregated proteins (Hoffman & Van Mil, 1999; Hoffmann & Van Mil, 1997; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007) and this happens due to the greater exposure of reactive thiol groups (Hoffman & Van Mil, 1999; Hoffmann & Van Mil, 1997).

1.4.3.2. Temperature and heating time

The extent of aggregation can be controlled by adjusting the heating time and temperature. As hydrophobicity contributes to the extent of aggregation, it was demonstrated that by increasing heating time, the surface hydrophobicity decreased (Zuniga, Tolkach, Kulozik, & Aguilera, 2010). Previous studies showed that the aggregate formation increased up to 10 minutes, when heating whey proteins at 85 °C and neutral pH, while at 70 °C the aggregates formed increased up to 8 hours as they had slower denaturation and diffusion (Durand, Gimel, & Nicolai, 2002; Hoffmann & Van Mil, 1997; McSwiney, Singh, & Campanella, 1994; Zuniga et al., 2010). Other study indicated that the size distribution of whey protein stabilized emulsions was not affected by heating at a temperature of 70 °C. Nevertheless, heating at a higher temperature rose the droplet size; this effect decreased with increasing heating temperature (Monohan, McClements, & German, 1996). They proposed that this is due to the predominance of intermolecular interactions which aggregate the emulsion at a temperature of 75-80°C, but at a higher temperature, intramolecular interactions take place. A study from Sliwinski et al. (2003) concluded that heating at 75 °C produced a maximum sauter mean diameter (d_{32}), which is the average volume-surface diameter, after about 45 minutes of heating. Meanwhile, when heated at 90°C the maximum was reached after 6-8 minutes of heating. Therefore, it can be assumed that an increase of the average molecular weight can be observed sooner, when heating β-LG at higher temperature (Le Bon, Nicolai, & Durand, 1999).

In the temperature range of 65-80°C, due to partial unfolding at the oil-water interface, the surface would have a relatively high hydrophobicity as not all hydrophobic chains are oriented towards the oil phase, making it vulnerable to aggregation. On the other hand, when

heated to a higher temperature, intradroplet protein-protein interactions are enhanced due to the complete unfolding, and thus the droplet surface would have a lower hydrophobicity (Dalgleish, 1996).

Additionally, another consequence that may happen from the heating time is the amount of adsorbed proteins which will be further elaborated in the next section. The aggregates were found to be more compact with more prolonged heating (Sliwinski et al., 2003). Thus, finding an appropriate time-temperature combination is essential to have a stable emulsion.

1.4.3.3. Protein concentration

The conversion rate of β-Lactoglobulin increases with initial protein concentration, similarly to the average aggregate size at neutral pH (Ryan, Zhong, & Foegeding, 2013). Iametti et al. (1996) postulated that the increased protein concentration leads to the formation of multimeric species in β-Lactoglobulin from 3.8 to 16 mg/mL. Consequently, heating at a higher initial protein concentration would reflect fewer dimers and trimers as their study showed that the average size of aggregates at 65 °C for various times (0.67, 4, 6.75, 24 and 48 hours) increased with increasing concentrations of β-Lactoglobulin from 10 to 50 mg/mL (Hoffmann & Van Mil, 1997). In addition, the adsorbed amount of protein was studied for whey protein-stabilised oil-in-water emulsions as a function of heating temperature and heating time. An increase in the heating temperature resulted in an increase of the adsorbed amount of protein and a concomitant decrease in the amount of whey protein in the aqueous phase (Sliwinski et al., 2003). On the other hand, it means that at a lower temperature, more proteins will be available for aggregation due to its higher availability for adsorption.

1.5. Conjugation of whey protein and simple sugar

1.5.1. General description

Bearing in mind that heating may cause a change in whey protein characteristics, which may be reflected in the emulsifying properties such as particle size and viscosity, we can conclude that in order to produce a stable emulsion, we expect there is no significant change on these parameters after heating. One of the methods to achieve this is by enzymatic

hydrolysis. Through hydrolysis, whey proteins will have an improved heat stability. A study by Gauthier & Pouliot (2003) revealed that adding whey protein hydrolysate in acidic beverages demonstrated its stability to sterilization treatment.

Ultrasonication, microencapsulation and microparticulation are other ways to enhance the stability of whey proteins. The use of ultrasonication has been widely studied and this method has been patented by Ashokkumar et al. (2009). Microencapsulation can also be applied, as the thermal pretreatment irreversibly denatures the native whey proteins through hydrophobic and/or -SH/S-S intermolecular reactions. Hence, stable emulsions can be achieved. As the particles are in the nano-sized region, the formation of aggregates is also limited (Zhang & Zhong, 2010). Microparticulation can be applied as well to form denatured whey proteins in small spherical particles (1 to 10 μm in diameter) (Ryan et al., 2013). This is conducted by physically shearing a WPC solution during heating, causing denaturation. As the shearing is applied, it prevents the protein from forming a gel network and instead, creates small protein aggregates (Spiegel, 1999).

Besides all of the methods mentioned above, chemical modification can also be conducted to obtain heat stable whey proteins. Chemical modifications will have impacts, particularly on the structural changes at the secondary, tertiary, and quaternary level of proteins as well as adjust their hydrophobicity-hydrophilicity balance (Srinivasan Damodaran, 2005). Of these modifications, Maillard reaction with a reducing sugar has been reported (Damodaran, 1996). Conjugation with carbohydrates has been found to improve the emulsification ability of the proteins. The presence of naturally present lactose in whey protein or addition of sugars enables them to be involved in various modifications, such as early Maillard Reaction (EMR) where sugars will react with free amino groups in the protein, or physical modification, i.e. lactose crystallisation (Morgan et al., 2005).

As this research will be focusing on the Maillard reaction, only this part will be further elaborated. Controlling the Maillard reaction is crucial and a way to control it is by monitoring the dry heating. Too extensive Maillard reaction could lead to too much structural modifications of the whey proteins, *e.g.* aggregation and brown colour formation (Schong & Famelart, 2017). Therefore, factors such as temperature, RH and pH should be monitored and this will be elucidated further in the next section.

1.5.2. Maillard reaction

1.5.2.1. Principle of Maillard reaction

When subjected to thermal processing, a covalent linkage between the amino groups of protein and reducing-end carbonyl groups of (poly)saccharides, at controlled relative humidity and temperature conditions is a common reaction which we further call as Maillard reaction (Srinivasan Damodaran, 2005; Jaeger, Janositz, & Knorr, 2010; G. Liu, Wang, Hu, Cai, & Qin, 2019). As consequences, the formation of brown color and flavour compounds is inevitable, as well as undesired effects such as destruction of the amino acids and the production of anti-nutritive compounds (Jaeger et al., 2010).

1.5.2.2. Stages in Maillard reaction

In the presence of sugars, dry heating of whey proteins will lead to complex reactions which take place in multiple ways. The Maillard reaction has been summarised by Hodge (1953) as can be seen in **Figure 1.1.**

It is reported that the early steps of the Maillard reaction can improve the functional properties. At the beginning, a reducing sugar such as lactose, condenses with a compound possessing a free amino group, like the ε-group of lysine or the α-amino group of terminal amino acids, to form an N-substituted glycosylamine. Afterwards, this glycosylamine undergoes the Amadori rearrangement to form ketosamines, known as early glycation products (Schong & Famelart, 2017). However, excessive Maillard reaction will lead to advanced Maillard reaction. This will result in unfavoured consequences, such as significant changes in protein structure as well as protein polymerisation (Schong & Famelart, 2017). The advanced stage of the Maillard reaction starts with the degradation of Amadori products. In neutral and acidic conditions, the formation of furfural and hydroxymethylfurfural (HMF) is usually involved. Meanwhile, at pH values above 7, the degradation of the Amadori compound involves mainly 2,3 enolization, with reductones being formed, such as 4-hydroxy-5-methyl-2 ,3-dihydrofuran-3-one, and a variety of fission products, including acetal, pyruvaldehyde and diacetyl (de Oliveira et al., 2016).

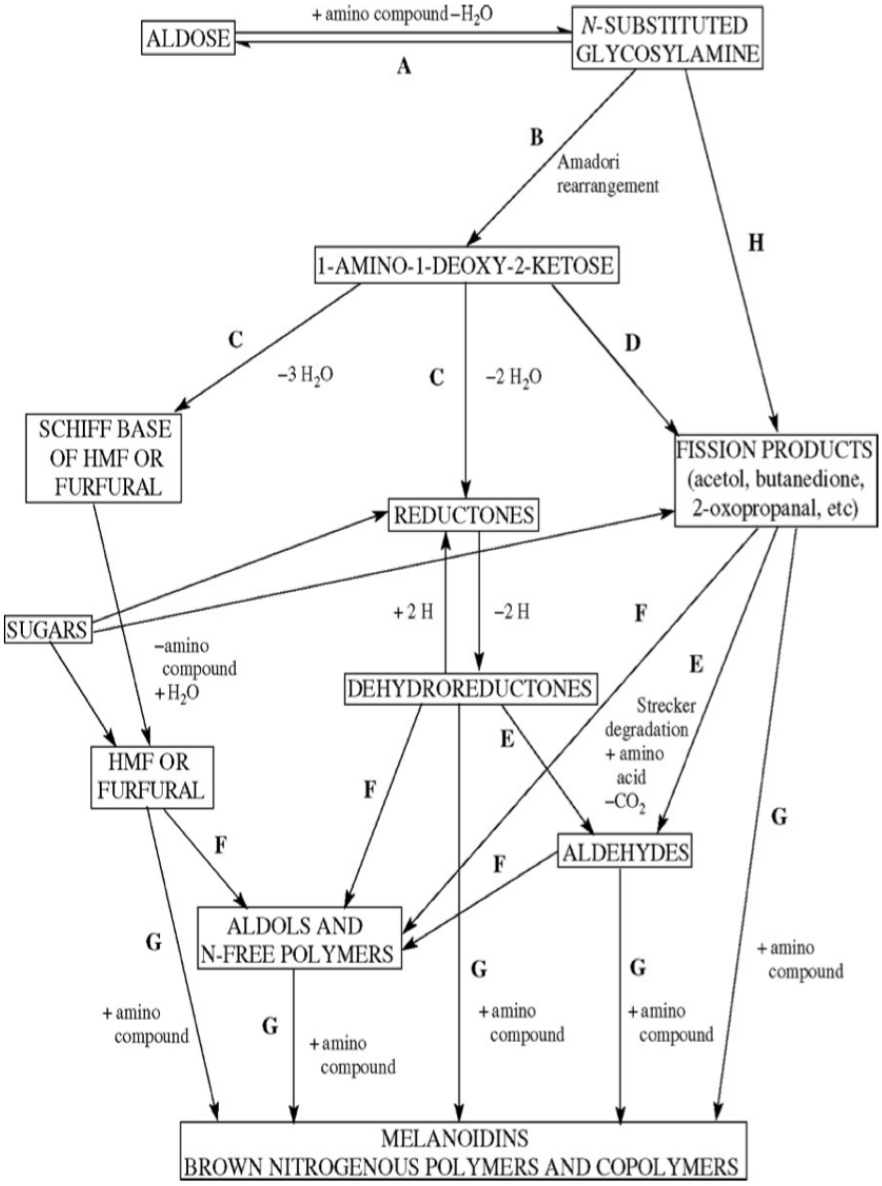


Figure 1.1. The Maillard reaction pathways as explained by Hodge (1953). In the initial stage, the products are colorless, without absorption in the UV region (280 nm) and two kinds of reactions can be taken place. These two are sugar amine condensation and Amadori rearrangement. In the intermediate stage, the products are colorless, with absorption in the UV region (280 nm). Reaction C: sugar dehydration. Reaction D: sugar fragmentation. Reaction E: amino acid degradation (Strecker degradation). Final stage: products are highly colored. Reaction F: Aldol condensation. Reaction G: Aldehyde-amine condensation and formation of heterocyclic nitrogen compounds

1.5.2.3. Factors influencing Maillard reaction

Several factors are influencing the degree of Maillard reaction, including protein and sugar type, pH, and reaction temperature (de Oliveira et al., 2016).

The type of sugar affects the degree of glycation (DG). Based on the type of sugar attached to the protein, it will provide different steric hindrances as well (Liu et al., 2019; G. Liu & Zhong, 2013) and thus minimize the effect of protein aggregation. It was also found that the smaller the sugar molecule is, the higher is its reactivity, which is most likely due to a higher access of the aldehyde group to the amino acids. Additionally, the higher the number of bound sugars, the lower the number of residual lysine residues in the WPI and the lighter the yellowness of the suspension (Schong & Famelart, 2017).

During Maillard reaction, the pH of the system affects the DG. The pH affects the surface charge of the protein; it will induce protein aggregation when it is close to the isoelectric point of the protein (Xi et al., 2020). Furthermore, the reactivity between the sugars and the nucleophilic amino groups is higher in alkaline environment (Martins, Jongen, & Boekel, 2001). This is because under alkaline conditions, the amino group is deprotonated (Chen, Ma, et al., 2019; Martins et al., 2001).

The temperature influences the reactivity during the Maillard reaction. Along with an increase in temperature, the more pronounced introduction of reactive amino groups to carbonyl groups via further unfolding of the protein structure occurs, leading to an increased reactivity of the carbonyl groups (Sedaghat Doost, Nikbakht Nasrabadi, Wu, A'yun, & Van der Meeren, 2019).

The RH affects the reaction rate between amino groups and sugars during the conjugation, as this is related to the water activity (a_w) of the food system. As Maillard reaction will induce browning color formation, it is also generally assumed that the maximum browning rate reaction occurs at intermediate moisture content, also considering that a high content of water inhibits the reaction (Ames, 1990; Labuza & Baisier, 1992). Pan & Melton (2007) observed the nonenzymatic browning of lactose and caseinate at different RH, ranging from 29 to 95%. They found that the degradation of the Amadori product, lactulosyl lysine, increased with increased of RH.

1.5.3. Studies on conjugation of whey proteins and simple sugars

Formation of color and flavour compounds are important characteristics in the Maillard reaction (Wang, 2013). This reaction leads to the conjugation/cross-linking between amino acids and sugars, also referred to as glycation, which can improve the functional properties of proteins (Xi et al., 2020). Conjugation of proteins can be done with the presence of high molecular weight components. Single sugar groups may alter the size, conformation and physical characteristics of proteins, such as the solubility, acid heat stability, and colloidal stability (Kinsella & Whitehead, 1989; Liu et al., 2019). Different reducing sugars varying in molecular weight and reducing power have a different effect on the Maillard reaction rate. The conjugation reaction between a polysaccharide and a protein is much slower than when mono-, di- or oligosaccharides are used (Wang, 2013). This enables better control of the Maillard reaction. Therefore, polysaccharides are widely used in the conjugation of proteins.

Contrary to the polysaccharides, utilizing simple sugars such as glucose and lactose would be more difficult to control as they will react quicker and therefore a shorter incubation time is needed to produce stable conjugates. Previous research (Liu & Zhong, 2013) demonstrated that reducing saccharides with smaller molecular weight were glycated at a large number of sites on each protein molecule. Particularly in that research, when evaluating glucose, lactose and maltodextrin, the estimation of the number of molecules of each saccharide attached to whey proteins was in the order: glucose > lactose > maltodextrin. It was postulated that a larger quantity of saccharides with a smaller molecular weight (MW) will make the aldehyde groups of reducing saccharides to amino groups during glycation (Liu & Zhong, 2013). Thus, a higher reactivity can be expected in reducing sugars with smaller MW (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007). The higher reactivity could be a drawback when it is not monitored carefully. Along with a higher reactivity, the glycation will be more pronounced, resulting in the formation of advanced Maillard reaction products. Consequently, modified charge characteristics of the whey proteins are obtained (Liu & Zhong, 2013).

Nevertheless, conjugating whey proteins with simple sugars (particularly lactose) might have a potential as lactose is naturally present in whey proteins. This means that lactose is readily available for the reaction, and thus no addition of sugars is needed. This can be

beneficial in a future approach, such as producing clean label whey protein conjugates. Several studies have been conducted to study the effect of conjugation between whey proteins and lactose (Liu et al., 2019; Liu & Zhong, 2013, 2014; Morgan et al., 2005; Schong & Famelart, 2019) and indeed, the conjugation between lactose and whey proteins demonstrated a potential in maintaining its stability and therefore could improve the functional properties of the protein.

1.6. Lactose

Lactose is the main component in milk and whey, composing about 5% in total (70-80% on dry basis) (Zárate & López-Leiva, 1990). Lactose is a disaccharide composed of glucose and galactose.

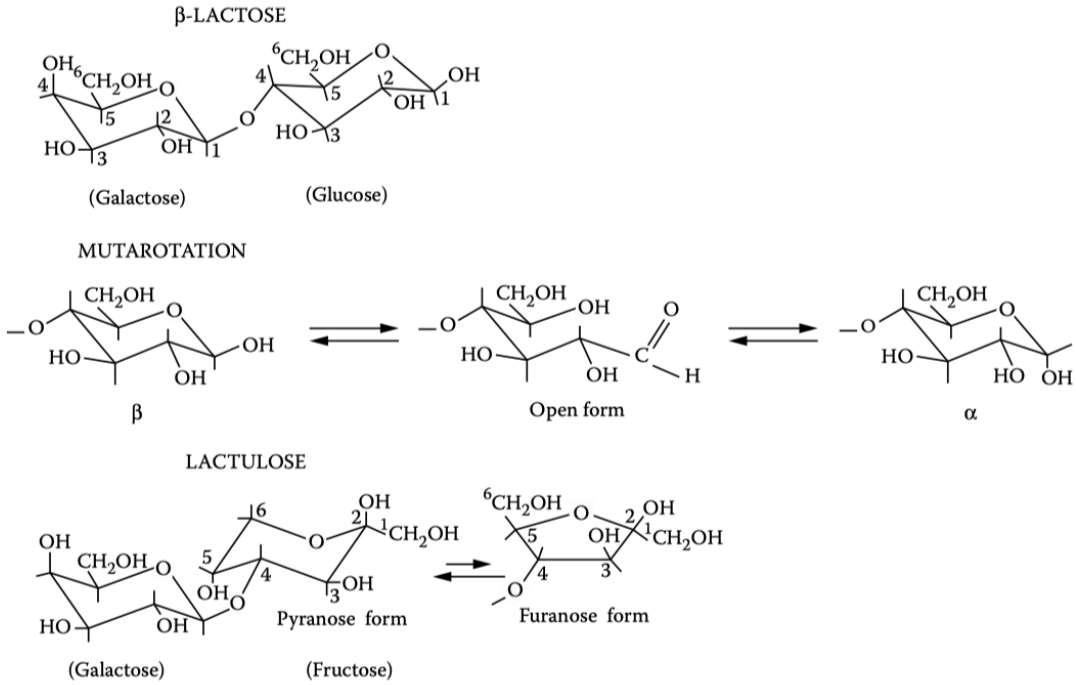


Figure 1. 2. Chemical structure of β-lactose and lactulose, and the mutarotation of the glucose moiety of lactose; adopted from Walstra et al. (2006).

Referring to Walstra et al. (2006), the aldehyde from galactose is connected to the C-4 group of glucose via a β-1,4-glycosidic linkage. These two sugar moieties occur predominantly in the pyranose ring form, and its chemical reactions include the hemiacetal linkage between C^1 and C^5 of the glucose moiety, the glycosidic linkage, the hydroxyl groups and the -C-C- bonds. Lactose also acts as reducing sugar, due to the presence of open-chain form which

contains an aldehyde group. Through this open chain form, in lactose solution, conversion of α- to β-lactose and vice versa may take place. This reaction is called mutarotation. Also due to the presence of the aldehyde group, lactose has a high reactivity. It leads to several reactions when milk is heated. For instance, lactose can isomerize into lactulose which then is used as an indicator of heat treatment intensity in milk. Caramelization and Maillard reaction may also be conducted upon heating. Maillard reaction occurs in the presence of amino groups, particularly the ε-amino group of lysine residues in proteins, which results in the formation of flavour compounds as well as enhanced brown color.

1.7. Hypothesis

The hypothesis was that the dry heat conjugation of WPC will improve the emulsifying and heat stabilizing capacity of WPC in o/w emulsions. RH and preconditioning pH would have impact on the rate on the WPC-lactose conjugation. A higher RH and preconditioned pH were expected to shorten the incubation time needed to produce WPC conjugates with good heat stabilizing capacity.

Chapter 2. Materials and Methods

2.1. Materials

Whey protein concentrate (WPC) was obtained from the commercial market (Royal Green® Organic Whey Protein, Frenchtop Natural Care Product BV, AL Hoorn, The Netherlands) containing 80% of protein and 12% lactose. Commercial sunflower oil was used to prepare the emulsion.

Imidazole buffer (pH 6.55±0.02) as diluting material contained 20 mM imidazole ($C_3H_4N_2$; Fisher scientific, ≥99% purity), 30 mM NaCl (VWR, ≥99% purity), and 1.5 mM sodium azide (NaN_3; Sigma Aldrich, ≥99% purity). Hydrochloric acid 1 N (Sigma Aldrich, 37% purity) and sodium hydroxide 2 N was used to arrange the pH of the buffer. To adjust the relative humidity at 64%, 74% and 79%, saturated salt solutions of $NaNO_3$, NaCl and KCl were used, respectively (Greenspan, 1977).

2.2. Methods

2.2.1. Conjugate preparation

For the preliminary analysis, ten gram of WPC was dry incubated in an oven at 80 ^oC and RH of 64% and 79% for up to 8 hours. After the dry heat treatment, the WPC-conjugated powder was placed in a closed plastic tube container. The results were then compared with those incubated at RH of 74%.

For the preparation of the WPC conjugates with the desired pH value (4, 6, 8, and 10), the WPC was diluted in distilled water (12% w/v) and stirred until completely dissolved. It was then stored at refrigerator temperature overnight and adjusted to the desired pH using HCl 1 N for the acid conditions, and NaOH 2 N for the base conditions. Subsequently, the WPC solution with the adjusted pH value was freeze-dried using a freeze dryer (Alpha 1-2 LD plus, Christ) until it became a powder. The powders were then incubated in an oven at 80 ^oC at RH of 74% during 1, 2, 3, 4, 5 and 6 hour. The WPC-conjugated powder was then placed in a closed tube container to be further analyzed.

2.2.2. Emulsion preparation

Oil in water emulsions were made by first diluting 0.5% of whey protein (equal to 0.625% WPC) in imidazole buffer of pH 6.55. The imidazole buffer contained 30 mM imidazole ($C_3H_4N_2$; Fisher scientific, ≥99% purity), 30 mM NaCl (VWR, ≥99% purity), and 1.5 mM sodium azide (NaN_3; Sigma Aldrich, ≥99% purity). Sodium azide was added to prevent microbial growth. The whey protein solutions were stored overnight in a refrigerator to fully hydrate the solution. After that, sunflower oil was added to produce 10% (w/w) O/W emulsions. The mixture was then prehomogenized using an Ultra Turrax TV45 (IKA) at 24000 rpm for 2.5 min followed by microfluidization at 4 bar of driving air pressure (*i.e.* 560 bar of liquid pressure) at 30^{o}C for 2 minutes (equal with 119-122 knocks) in a Microfluidizer M110S.

2.2.3. Heat Coagulation Analysis

The evaluation of heat stability was performed by heating the oil in water emulsions at 80 ^{o}C for 20 min in a water bath. Upon heating, the three-dimensional structure of the protein molecules will be modified, making that the internal hydrophobic and SH-groups become exposed (Shimada & Cheftel, 1989). The samples before and after heating were evaluated further as described below.

2.2.4. Emulsion characterization

2.2.4.1. Particle size measurement

Particle size is one of the parameters that can be used to further observe the stability of emulsions towards heating. A Mastersizer 3000 (Malvern Instrument Ltd, Malvern, UK) was employed to determine the particle size distribution of the unheated and heated emulsions. Using laser diffraction, this instrument measures the particle size distribution ranging from 0.01 – 3500 μm. Laser diffraction generates results based on its volume distribution; thus the result would be on a volume basis. As volume basis was used in this equipment, the value of D4,3 or the volume mean, was further used to evaluate the particle size distribution of the unheated and heated WPC-conjugated emulsions. For the conducted analyses, the refractive and absorbance index was set at 1.47 and 0.01, respectively. The sample was added dropwise

into the dispersion unit (Malvern Hydro MV) to obtain 10 to 20% of obscuration. During the measurement, the stirrer speed was set at 1500 rpm.

2.2.4.2. Viscosity measurement

The rheological properties of the emulsions were checked using an LV-DVII+pro (Brookfield) viscometer equipped with a small sample adapter in combination with a spindle SC4-18. The spindle was operated to obtain a minimum torque value of 10%. Thus, a shear rate of 30 to 100 s^{-1} was applied for viscous samples and 200 to 250 s^{-1} for the less viscous samples. During the measurement, 8 ml of each emulsion was filled into the small sample holder and analyzed at room temperature (20°C).

The data were fitted to a power law equation (Equation 1) where τ represents the shear stress (in Pa), γ the shear rate (s^{-1}), K the consistency coefficient (Pa.s), and n the flow behavior index.

$$\tau = K \cdot \gamma^n \qquad \text{(Equation 1)}$$

The consistency coefficient of emulsions behaving as Newtonian (n = 1) was determined from the average value of the viscosities at different shear rates.

2.2.4.3. Creaming stability analysis

The creaming stability of the emulsions was evaluated using a LUMIsizer (LUM GmbH, Germany). The prepared emulsion was filled into a rectangular polycarbonate cell, and then put into the equipment with a centrifugation speed of 3500 rpm. The analysis was conducted at 25°C. Each sample was recorded in two cycles, respectively for 10 s and 30 s, so that in total, two hours of centrifugation was conducted. Front tracking data analysis was applied to the raw data by setting the threshold value at 15% transmission. The range of the analyzed position was 112 to 127 mm for all samples. Using this method, a curve which shows the position of the interface between serum and cream phase during centrifugation was obtained at 1700 g centrifugal force.

2.2.4. Conjugate characterization

2.2.4.1. Degree of conjugation analysis

During the oven incubation, Maillard reaction occurs spontaneously between available amino groups from the protein with the reducing sugars. Further on during the Maillard reaction, the protein will be consumed and the available amino groups decrease. To observe the degree of conjugation, a free amino group analysis was performed using the O-phthalaldehyde (OPA) method.

First, the OPA reagent shall be prepared first. For each 50 mL of OPA reagent, 40 mg of OPA was dissolved in 1 mL of ethanol and 2.5 mL of 20% SDS solution was prepared. Separately, 100 µL of 2-merchaptoethanol to 24.9 mL of 0.1 sodium tetra borate buffer solution was also prepared. These two solutions were then combined, and deionized water was added until reached 50 mL of volume.

Each sample was prepared by diluting 156.25 mg WPC to the 50 mL of deionized water. Using those prepared samples, only 200 µL of the sample solution (equal with 0.5 mg of protein) was added in a tube. 4 mL of OPA reagent was added afterwards and the tube was stirred briefly and incubated for 4 minutes at room temperature. The samples were at last measured using a spectrophotometer at 340 nm.

In this method, the degree of conjugation was determined using this equation:

$$DG\ (\%) = \left[\left(\frac{A_0 - A_1}{A_0}\right)\right] \times 100$$

where A_0 = absorbance value before glycation

A_1 = absorbance value after glycation

2.2.4.2. Solubility analysis

The solubility analysis was performed using a simplified Lowry method, adapted from Schacterle & Pollack (1973). The solubility analysis was conducted by dissolving the WPC-conjugate powder in imidazole buffer (concentration 1% w/v). The solution was then stirred with a speed of 10 rpm for an hour. 5 ml of the sample was taken into a 10 ml reaction tube to be heated at 80 ^{o}C for 20 minutes in a water bath. After being heated, the sample was placed into cold water to stop the reaction. Two ml of both the unheated and heated samples were transferred to a centrifugation tube and centrifuged at 1300 rpm for 10 minutes. The supernatant was separated from the serum to be further proceeded to Lowry Analysis.

For the Lowry analysis procedure, samples were diluted 40 times using imidazole buffer and to each of the samples 1 mL of alkalic Cu-reagent was added. The tubes were put on a vortex and left undisturbed for 10 minutes. The phenol reagent was added and the mixture was turned upside down two times, to be then finally put in a water bath at 55 ^{o}C during 5 minutes. The solubility was then measured by using a spectrophotometer at 650 nm against a blank solution where imidazole buffer acted as a blank.

2.2.4.3. pH

For the sample preparation, the sample powder was dissolved in distilled water (2.5 mg/ml) and mixed using a magnetic stirrer bar. The pH of the solutions was measured by immersing the electrodes of a Hanna H 4222 pH meter into the solutions.

2.2.5. Statistical analysis

Statistical analysis was carried out using the SPSS program. A paired t-test was carried out at 95% significance level to compare the droplet size and viscosity before and after heating.

Chapter 3. Results and Discussion

The effect of the relative humidity and preconditioning pH towards the emulsifying and heat stabilizing characteristic of WPC conjugates will be further elucidated in this section. The parameters observed, including the emulsion and conjugates characterization, will be taken into account to observe its emulsion stability in relation with RH and preconditioning pH, as well as to conclude the minimum incubation time to produce conjugates with good heat stabilizing properties. The emulsion characterization can be explained by interpreting the results from particle size analysis, viscosity, and accelerated creaming. Taken together, the stability of o/w emulsions stabilized by WPC conjugates achieved through dry heat conjugation can be further elaborated.

The knowledge of the particle size distribution gives information on the efficiency of the emulsification process (Friberg et al., 2004). The efficiency of the emulsification process can be seen by comparing the particle size distribution before and after heat treatment. For heat stable emulsions, the particle size should be maintained even after heating. This is because of the natural characteristics of whey proteins which consist of a three-dimensional structure and can be modified through heating. Denaturation might happen when the internal hydrophobic and -SH groups are exposed upon heating, where they both finally have bonding interactions (Shimada & Cheftel, 1989). In severe conditions, the denaturation may lead to aggregation which is indicated by an increased particle size.

Droplet aggregation in whey protein stabilized emulsions also affects the viscosity of the emulsion (Sliwinski et al., 2003). An increase in viscosity indicates that there is a change in the protein structure, such as unfolding of the polypeptide chain, disruption of hydrophobic interactions and aggregation by covalent and non-covalent bonding (Drapala, Auty, Mulvihill, & O'Mahony, 2016). Furthermore, an increase in the viscosity after the heat treatment means that the emulsion is heat unstable. Previous studies have shown that the viscosity is affected by many factors, but the main factor is the particle size in the protein solutions (Dissanayake, Liyanaarachchi, & Vasiljevic, 2012).

Therefore, by observing both parameters we can evaluate whether the emulsion is stable or not by observing the size of the spherical oil droplets based on the volume-weighed

mean diameter ($D_{4,3}$) and viscosity. In order to demonstrate a stable emulsion, the $D_{4,3}$ value of o/w emulsions after heating should be similar in comparison with the droplet size before heating.

With regard to the long-term stability, creaming analysis can be one of the tests to observe the stability of the emulsions during storage. Creaming is related to the density difference between the oil and aqueous phase, and changes in the density of the oil phase may cause changes during the storage of an emulsion (McClements, 2016). Creaming occurs when the droplets have a lower density than the surrounding medium, and thus tend to move upward (McClements, 2016). Creaming is related to the particle size: larger particles will tend to cream faster and therefore move upward more rapidly. As larger particles move quickly, they will collide with those which are smaller in size (Dukhin & Sjoblom, 1996), leading to aggregation. This will in turn result in a faster creaming rate.

3.1. The effect of Relative Humidity (RH) on the emulsifying and heat stabilizing capacity of the conjugated WPC

The relative humidity (RH) is one of the parameters that influence the Maillard reaction during dry heat conjugation. RH affects the reaction rate between amino groups and sugars during the conjugation, as this is related to the water activity (a_w) of the food system. As Maillard reaction will induce browning color formation, it is also generally assumed that the maximum browning rate reaction occurs at intermediate moisture content, also considering that a high content of water inhibits the reaction (Ames, 1990; Labuza & Baisier, 1992). Consequently, an increase in the RH particularly from 50 to 80%, is thought to improve the amount of reacted amino groups (Doost et al., 2019). As the dry heat incubation temperature was chosen to be at 80 ^{o}C, the variation of RH chosen were 64%, 74% and 79%. This particular RH range was decided as it seems that the range of 60-85% is needed for the maximum conjugation (Pan & Melton, 2007). It needs to be noted that the results of RH 74% used here were obtained from a former researcher and used as the comparison with the current study.

Both the non-incubated and incubated WPC were able to produce emulsions with a volume-weighted average particle diameter in the range of 0.6 to 0.8 μm. After the heating test at 80 ^{o}C for 20 min, the particle size of the emulsion stabilized by the non-incubated WPC

increased. Nevertheless, it is demonstrated in **Table 3.1** that at all RH values considered a stable particle size of the emulsions was obtained by using the conjugates with a minimum of 4 hour of incubation.

The viscosity analysis results, as can be seen in **Table 3.2**, demonstrated similar results in regard to heat stable emulsions, where the consistency coefficient for 74% and 79% RH, respectively, indicated stable emulsions after a minimum incubation time of 4 hours. The viscosity of the emulsions is directly related with the particle size. As previously explained, the larger droplet sizes upon heating are due to aggregate formation, which also results in an increased viscosity.

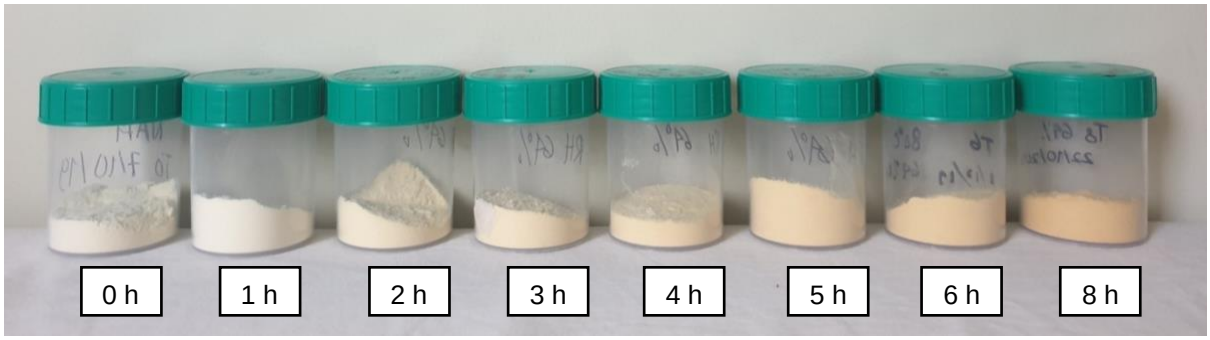


Figure 3.1. WPC conjugates conditioned at 64% RH (from left to right: incubation time of 0, 1, 2, 3, 4, 5, 6, and 8 hours).

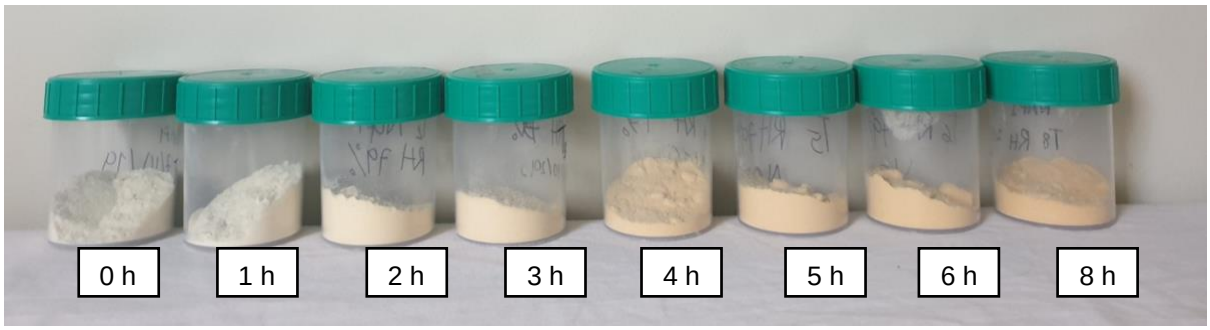


Figure 3.2. WPC conjugates conditioned at 79% RH (from left to right: incubation time of 0, 1, 2, 3, 4, 5, 6, and 8 hours).

Based on the results from the evaluation of the effect of RH on the emulsifying and heat stabilizing properties of the WPC conjugates in o/w emulsions, it was found that indeed dry heat conjugation could improve the heat stabilizing capacity in the WPC conjugates. This can be seen as in **Table 3.1.** and **Table 3.2.**, the native WPC could not demonstrate heat stable emulsions. Meanwhile, those which were pretreated with dry heat conjugation showed better heat stabilizing

properties. In conclusion, the minimum incubation time needed for WPC conjugates at 64% RH to produce heat stable conjugates was 6 hours, whereas for 74% and 79 % RH, 4 hours of incubation time required.

Through the Maillard reaction, the introduction of sugar molecules provides strong steric interactions for protein which results in the inhibition of droplet flocculation (Liu et al., 2019). This will enhance the thermal stability of the protein, and therefore a stabilized emulsion can be achieved. The improved performance of the emulsions through conjugation with a reducing sugar via the Maillard reaction has been proven in several studies (Liu et al., 2019; Liu & Zhong, 2013). It was indicated that WPI-lactose and epigallocatechin gallate (EGCG) could produce a thermally stable Pickering emulsion-based delivery system for curcumin (Liu et al., 2019) with incubation time of 24 hours at 79% RH and a temperature of 70 °C.

Table 3.1 Volume-weighted average particle diameter ($d_{4,3}$, expressed in μm) of 10% O/W emulsions stabilized by the non-incubated WPC (0 hour) and incubated WPC for 2 to 8 hours at 80 °C at different relative humidity conditions (64%, 74%, and 79%), before and after heating the emulsions at 80 °C for 20 min.

Incubation time (h)	**Before heating**			**After heating**		
	RH 64%	RH 74%	RH 79%	RH 64%	RH 74%	RH 79%
0	0.6 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	38.9 ± 2.6	21 ± 1.8	32.1 ± 0.0
2	0.6 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	5.5 ± 0.4	1.7 ± 0.3	4.1 ± 0.0
4	0.6 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	1.0 ± 0.2	0.6 ± 0.0
6	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.1	0.6 ± 0.0
8	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.0

Table 3.2. Consistency coefficient (expressed in mPa.s) of 10% O/W emulsions stabilized by the non-incubated WPC (0 hour) and incubated WPC for 2 to 8 hours at 80 °C at different relative humidity conditions (64%, 74%, and 79%), before and after heating the emulsions at 80 °C for 20 min.

Incubation time (h)	**Before heating**			**After heating**		
	RH 64%	RH 74%	RH 79%	RH 64%	RH 74%	RH 79%
0	1.7 ± 0.0	1.7 ± 0.0	1.9 ± 0.0	95 ± 24	275 ± 25	437 ± 51
2	1.7 ± 0.0	1.6 ± 0.0	1.9 ± 0.0	6.0 ± 0.4	2.0 ± 0.0	2.9 ± 0.0
4	1.7 ± 0.0	1.6 ± 0.0	1.9 ± 0.0	1.6 ± 0.0	1.8 ± 0.0	1.9 ± 0.0

6	1.8 ± 0.0	1.5 ± 0.0	2.0 ± 0.0	1.6 ± 0.0	1.7 ± 0.0	1.9 ± 0.0
8	1.7 ± 0.0	1.4 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	1.7 ± 0.0	1.9 ± 0.0

As previously mentioned, it is expected that an increase in the RH will enhance the amount of reacted amino groups (Sedaghat Doost et al., 2019), and this will lead to higher interaction with sugars during the Maillard reaction. This is further explained by Martinez-Alvarenga et al. (2014), where they observed that there was an increase in the glycation extent of whey protein isolate (WPI) and maltodextrin conjugates when increasing the RH from 50 to 80%. This has a relation to the water activity, as the reaction rate increases gradually with increasing a_w from 0.3 to 0.8. However, for a_w values below 0.3 and above 0.8, there will be a decrease in the reaction rate which leads to a higher percentage of blocked amino groups in whey proteins. Pan & Melton (2007) observed non-enzymatic browning of lactose and caseinate during dry heating at RH ranging from 29% to 95% and noted that the maximum reaction rates occurred at intermediate RHs. In the current study, the WPC-lactose conjugation times required to produce a stable emulsion within the RH range used (nearly 60% to 80%) were comparable. This result was in line with Malec, Pereyra Gonzales, Naranjo, & Vigo (2002) and Pan & Melton (2007) who indicated that conjugation usually takes place at a temperature in the range between 40 and 80 ^{o}C and a RH in the range between 60-85% for the maximum reaction rate. A visual illustration of the extent of browning reaction for RH 64% and 79% can be seen in **Figure 3.1.** and **3.2,** respectively. The relative humidity of 74% was then chosen for all further experiments, so that the current results will be comparable with the previously reported ones (A'yun et al., 2020; Sedaghat Doost et al., 2020; Setiowati, Vermeir, Martins, De Meulenaer, & Van der Meeren, 2016) which also used a RH of 74%.

3.2. Effect of preconditioning pH on the emulsion characteristics

Whey proteins are usually obtained from whey with different pH values, and the pH has been well known to influence the type and kinetics of chemical reactions during preparation and processing such as dry heating (Povey et al., 2009). The reactivity between amino groups and sugars during Maillard reaction is affected by the pH value. At higher pH value, the amino group is in the unprotonated form and is more reactive with the open chain form in the reducing sugar (Martins et al., 2001). Meanwhile, at lower pH value, more

protonated amino groups are present in the equilibrium. Consequently, they will become less reactive with the sugar (Martins et al., 2001). With the preconditioning pH, we would like to observe the influence of pH on the rate of the Maillard reaction in the conjugation. In our study, the preconditioning pH value was set to achieve WPC solutions at a pH of 4, 6, 8 and 10 prior to lyophilisation. The obtained conjugates from the WPC incubation were then applied in o/w emulsions. However, the conjugates preconditioned at pH 10 could not be dissolved in the imidazole buffer. Thus, the following sections only explain the effect of pH values of 4, 6 and 8 on the heat stability and emulsifying properties of WPC conjugates in o/w emulsions. To be sure that the preconditioning pH did not affect the final emulsion pH, and hence that the buffer used in emulsion preparation was sufficiently strong, the pH of all emulsions was measured. **Table 3.3** indicates that the pH of all emulsions was situated within a rather narrow range from pH 6.5 to pH 6.7 for WPC preconditioned at pH 6 and 8 in o/w emulsions, meanwhile for WPC preconditioned at pH 4 in o/w emulsions had lower pH. Hence, referring from the pH value of emulsions for WPC preconditioned at pH 6 and 8 in o/w emulsions, differences in emulsion stability are due to differences in conjugate characteristics, rather than in emulsion pH.

Table 3. 3. pH analysis of WPC conjugates in o/w emulsions preconditioned at pH 4, 6, and 8

Incubation time (hour)	pH 4	pH 6	pH 8
0	5.88±0.00	6.56±0.02	6.71±0.00
1	-	6.60±0.02	6.70±0.04
2	-	6.57±0.03	6.66±0.00
3	5.71±0.00	6.61±0.00	6.62±0,05
4	-	6.53 ±0.04	6.60±0.05
5	5.62±0.00	6.53±0.04	6.55±0.00
6	-	6.53±0,00	-

Remarks: (-) Data not available due to limitation during COVID-19 pandemy.

3.2.1. Particle size

Fig. 3.4., **Fig. 3.6.**, and **Fig. 3.8.** represent the result of the particle size distribution measurement of emulsions stabilized by the WPC conjugates preconditioned at pH 4, 6, and 8. Based on the average particle size at pH 4 (**Figure 3.3.**) and the distribution of the particle size in volume percentages at pH 4 (**Figure 3.4.**), they demonstrated significantly larger

emulsion droplet sizes after heat treatment, regardless of the incubation time, which means that the emulsions underwent severe heat aggregation. At pH 4, the protein denaturation was accelerated. This finding was in line with other research, where at pH 4.6, the solubility of the WPI proteins was also decreased during heating at 100 °C (Gulzar, Bouhallab, Jeantet, Schuck, & Croguennec, 2011) and thus reflected in a larger aggregate size. Paired t-test also revealed that the particle size of WPC conjugates for both heated and unheated, for preconditioned pH at 6 and 8 at the same incubation time was not significantly different ($p > 0.05$), while for preconditioned at pH 4 was indeed significantly different ($p < 0.05$).

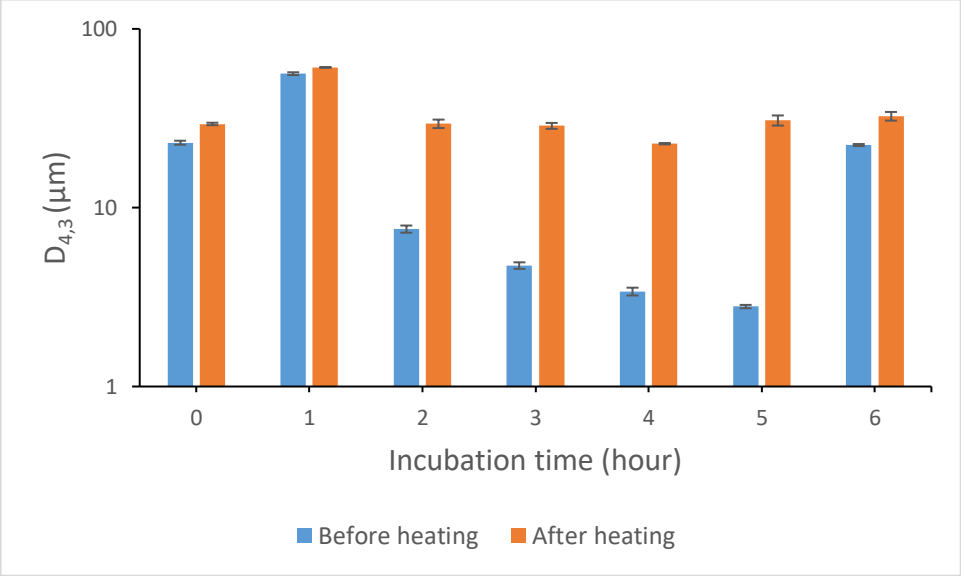


Figure 3.3. Volume-weighted average oil droplet size ($D_{4,3}$) of 10% O/W emulsions stabilized by dry heat incubated WPC, preconditioned at pH 4 obtained after different incubation times (at 80 °C, and RH 74%), before and after heating the emulsions at 80°C for 20 min.

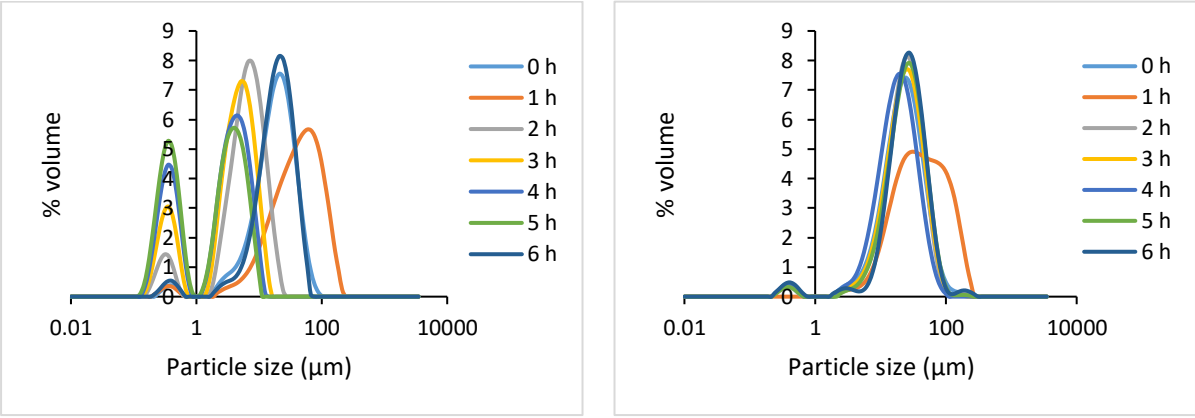


Figure 3.4. Particle size distributions of 10% o/w emulsions stabilized by dry heat incubated WPC (at 80 °C, RH 74%) preconditioned at pH 4 obtained after different incubation times, before (left) and after (right) heating the emulsions at 80°C for 20 min.

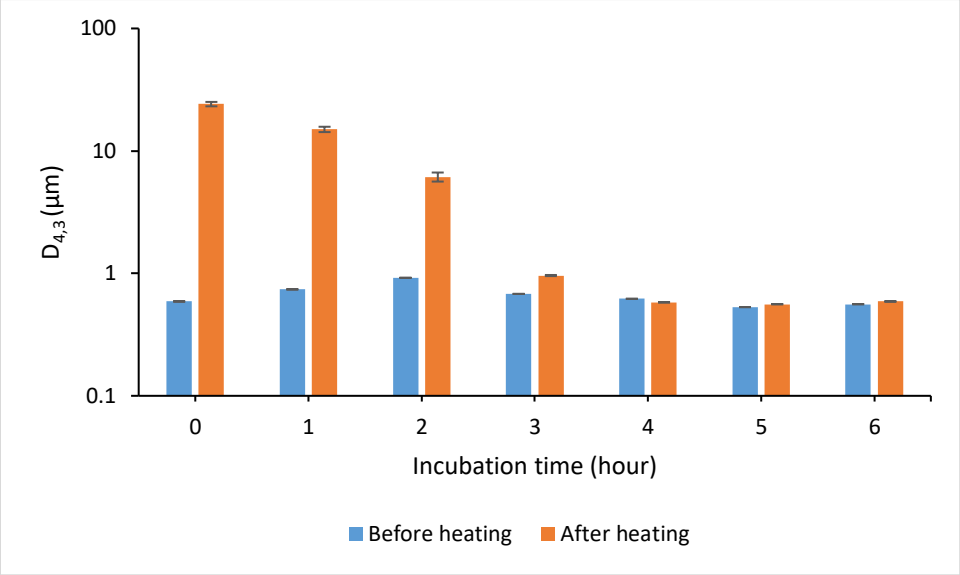


Figure 3. 5. Volume-weighted average oil droplet size ($D_{4,3}$) of 10% O/W emulsions stabilized by dry heat incubated WPC, preconditioned at pH 6 obtained after different incubation times (at 80 °C, and RH 74%), before and after heating the emulsions at 80°C for 20 min.

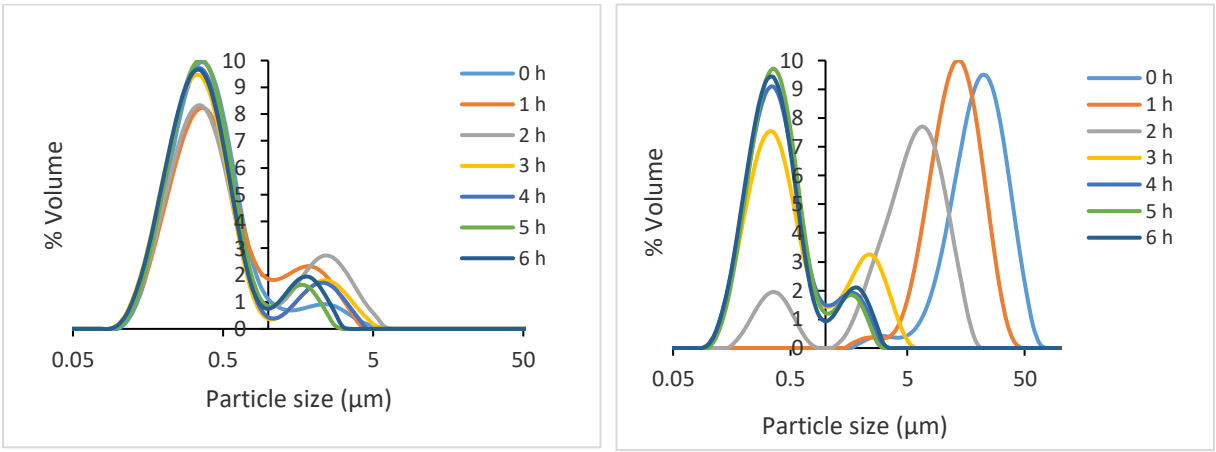


Figure 3.6. Particle size distributions of 10% O/W emulsions stabilized by dry heat incubated WPC (at 80 °C, RH 74%) preconditioned at pH 6 obtained after different incubation times, before (left) and after (right) heating the emulsions at 80°C for 20 min.

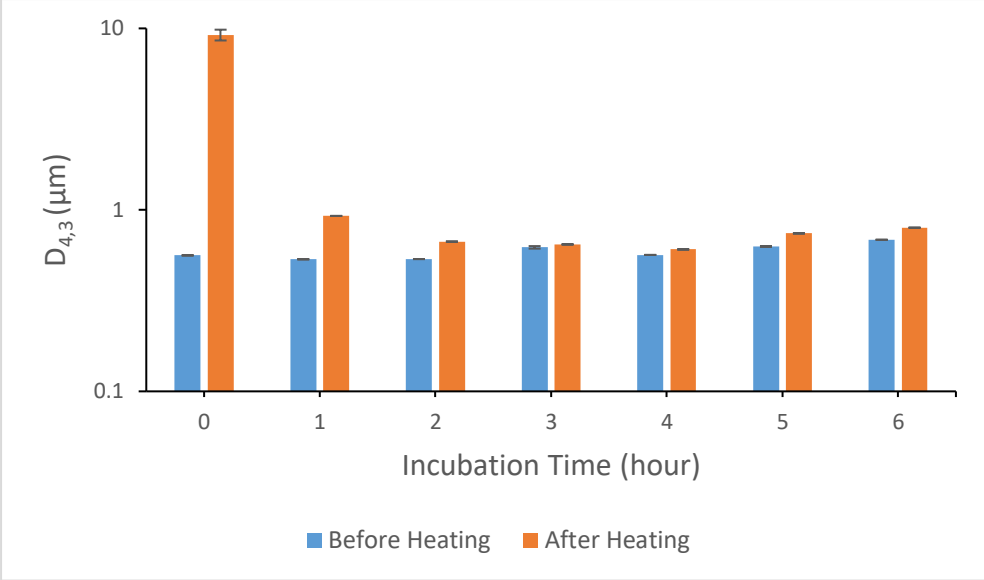


Figure 3.7. Volume-weighted average oil droplet size ($D_{4,3}$) of the O/W emulsions stabilized by dry heat incubated WPC, preconditioned at pH 8, obtained after different incubation times, before and after heating the emulsions at 80°C for 20 min.

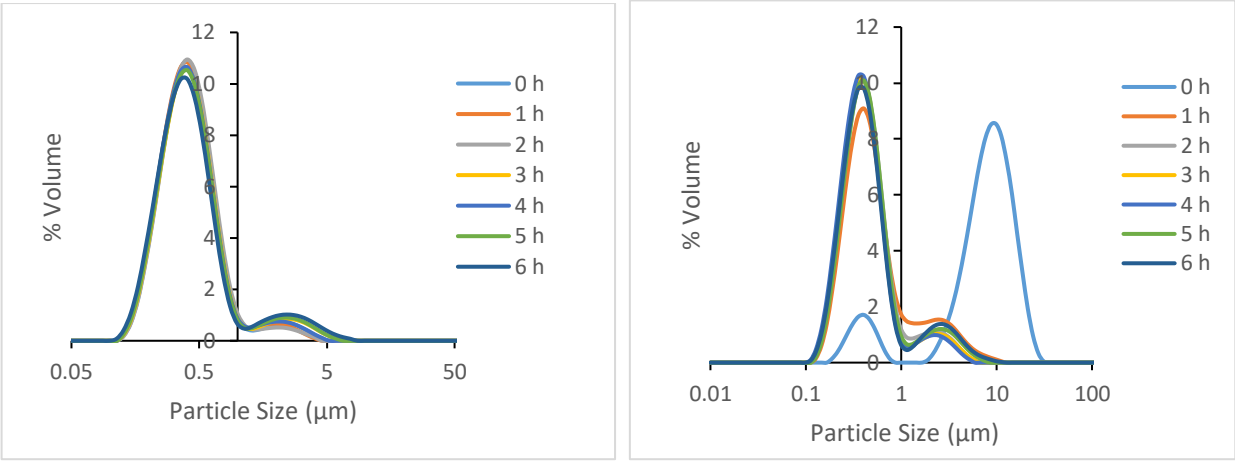


Figure 3.8. Particle size distributions of 10% O/W emulsions stabilized by dry heat incubated WPC (at 80 °C, RH 74%) preconditioned at pH 8 obtained after different incubation times, before (left) and after (right) heating the emulsions at 80°C for 20 min.

The isoelectric point of whey proteins is around 5.0 (Liu & Zhong, 2014). It means that around its IEP, the droplets have zero net charge, thus resulting in a weak electrostatic repulsion. Hence, the proteins unfold and aggregate through a combination of hydrophobic and disulphide bonds, and create irreversible aggregates (Jones & McClements, 2011). This explains the relatively large particle size distribution in o/w emulsions stabilised by WPC

conjugates preconditioned at pH 4, as demonstrated in **Figure 3.3**. **Figure 3.4.** also demonstrated the particle size distributions for emulsions containing WPC conjugates preconditioned at pH 4, as a function of incubation time. The dominant particle size distribution is in the range of 1 – 100 µm. Accordingly, to stabilize the emulsions, the electrostatic repulsion as well as steric hindrance should be dominant over hydrophobic and Van der Waals interaction for the emulsions not to be aggregated (Demetriades, Coupland, & Mcclements, 1997). This means that at a pH around its IEP, there will be irreversible protein aggregation as the steric and electrostatic hindrance is more limited. This will result in unstable emulsions.

Consequently, using WPC conjugates preconditioned at pH 4, the emulsion was already denatured, and demonstrated irreversible aggregates. This was most likely due to the buffering system used (imidazole buffer at pH 6.55), which could not stabilize the pH. Additionally, pH analyses of the o/w emulsions stabilized by the preconditioned WPC conjugates at pH 4, 6 and 8 were also conducted as can be seen in **Table 3.3.** For emulsions with conjugates preconditioned pH 4, the pH of the emulsions was decreasing. The pH of emulsions with WPC conjugates preconditioned at pH 4 was 5.88, 5.71, and 5.62 for an incubation time of 0, 3 and 5 hours, respectively. This indicated that the buffer used could not resist the addition of protons which took place during the preconditioning of the WPC. Nevertheless, considering that the pH of emulsions for WPC conjugates preconditioned at pH 4 was still considerably far enough from IEP of whey proteins, the pH of the emulsions could not be the only explanation why the emulsions formed large droplet size and were unstable upon the heat treatment. Besides pH of the system, the destabilisation mechanisms could occur because of several factors, such as the nature and concentration of emulsifier or stabiliser, ionic strength, temperature, homogenisation parameters, and interaction of dispersed with continuous phase (McClements, 2016; Sjöblom, 2006). During the emulsification of emulsions preconditioned at pH 4, there was a possibility that the homogenisation using microfluidizer was not conducted optimally considering that the microfluidizer was not at its best performance (intensive leakage at the microfluidizer during the period of emulsification at WPC preconditioned at pH 4). Nevertheless, a repetition could not be done due to the limitation during pandemy. Hence, it is important to note that a further

investigation for emulsions preconditioned at pH 4 is needed, as well as compare these results to native WPC stabilised emulsions at the same pH values where the WPC conjugates solution is titrated to the desired pH before emulsification.

In case of WPC conjugates preconditioned at pH 6 and 8, the electrostatic repulsion is greater as the pH value is further from the IEP. For the emulsion stabilized by preconditioned conjugates at pH 6 and 8, the imidazole buffer used could maintain its pH value. The pH range for emulsions containing WPC preconditioned at pH 6 was around 6.50 to 6.62, whilst for pH 8 it was around 6.53 to 6.73. Consequently, preconditioned WPC conjugates at pH 6 and 8 resulted in stable emulsions before the heating test, which resulted in smaller particle sizes as can be seen in **Fig. 3.5** and **Fig. 3.7** at the non-heated emulsions where they exhibited a small average droplet size. Clearer demonstrations can also be seen in **Fig. 3.6.** and **3.8**. where the particle size distributions can be maintained in the small size range with sufficient incubation time: the distribution size peak curve is inclined to the left side.

The conjugation of the WPC with the naturally present lactose improved the heat stabilizing properties of WPC in the o/w emulsions as shown by the stable particle size of the emulsions (**Figure 3.5.** and **3.7.**). During conjugation, the proteins and carbohydrates would be covalently linked through Maillard reaction, where with sufficient incubation time (4 hours and 2 hours for pH 6 and 8, respectively) the average droplet size remained stable after heating, as demonstrated in **Fig. 3.5.** and **Fig. 3.7**. The figures also showed that for pH 6 and 8, respectively, the droplet sizes of emulsions stabilized by the non-incubated WPC after heating were significantly higher than the conjugated ones. Before heating, the average oil droplet size was in the range of 0.5 to 0.7 µm for each pH value. Referring from the results, the droplet size remained stable after the dry heat treatment for 4 hours and 2 hours for the pH 6 and 8, respectively, while for pH 4 no heat-stable emulsions could be obtained. We can conclude that in our experiment, preconditioning of the conjugates at alkaline pH was favourable in making heat stable emulsions with less incubation time (2 hours). Hence, a more detailed study within the alkaline pH range (*e.g.* comparing pH values of 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5) would be desirable in future research. A first rapid screening can be done based on the solubility of the obtained conjugates in the imidazole buffer: good emulsification properties can only be obtained, provided that the protein conjugates are soluble.

3.2.2. Viscosity of emulsions

Based on our findings, the emulsions stabilized by the conjugated WPC preconditioned at pH 4 showed severe aggregation even before the heating test. The emulsion viscosity increased and exhibited a gel-like structure after being heated. This happens because protein aggregation is favoured near the isoelectric point of the proteins. Around its IEP, the net charge will be zero (Golovanov, Hautbergue, Wilson, & Lian, 2004). After the heating test, the emulsion stabilized by the non-incubated WPC preconditioned at pH 6 (**Fig. 3.9.**) and pH 8 (**Fig. 3.10.**), showed a non-Newtonian behaviour (shear thinning) where the viscosity was strongly shear rate dependent. Meanwhile, the emulsions stabilized by the conjugated WPC behaved as Newtonian fluids with an average viscosity of about 1 mPa.s.

In the presence of conjugates, at pH 6, the result demonstrated a heat stable emulsion for those conjugates incubated for 4 hours and more, while at pH 8, the minimum incubation time needed to produce a stable emulsion was 2 hours. These outputs are in agreement with the finding of the particle size distribution measurements. The stable emulsions obtained at a longer incubation time indicate that the conjugation link between the proteins and the naturally present lactose during Maillard reaction is effective at both pH 6 and 8.

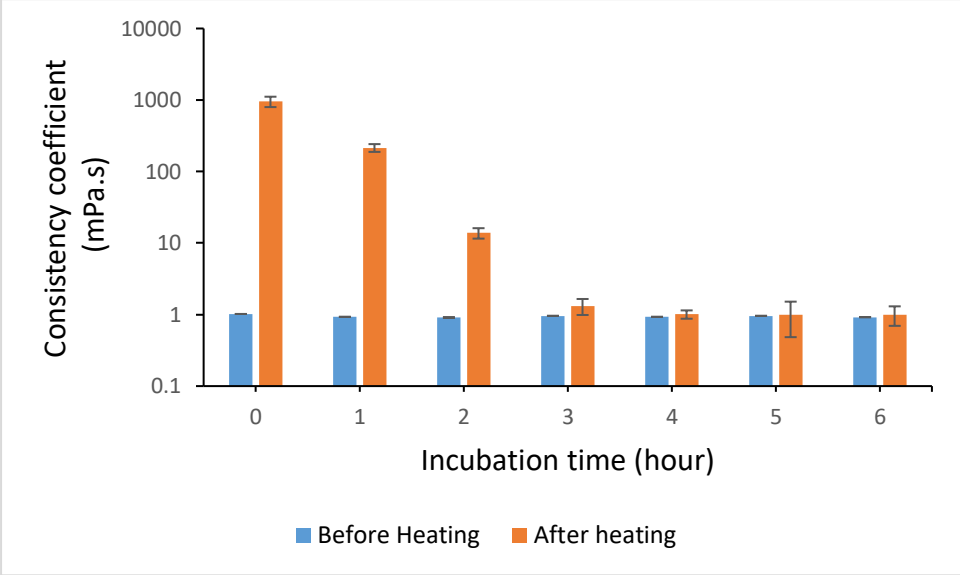


Figure 3.9. Consistency coefficient (in mPa.s) of the o/w emulsions stabilized by dry heat incubated WPC, preconditioned at pH 6, before and after heating of the emulsions at 80 °C for 20 minutes.

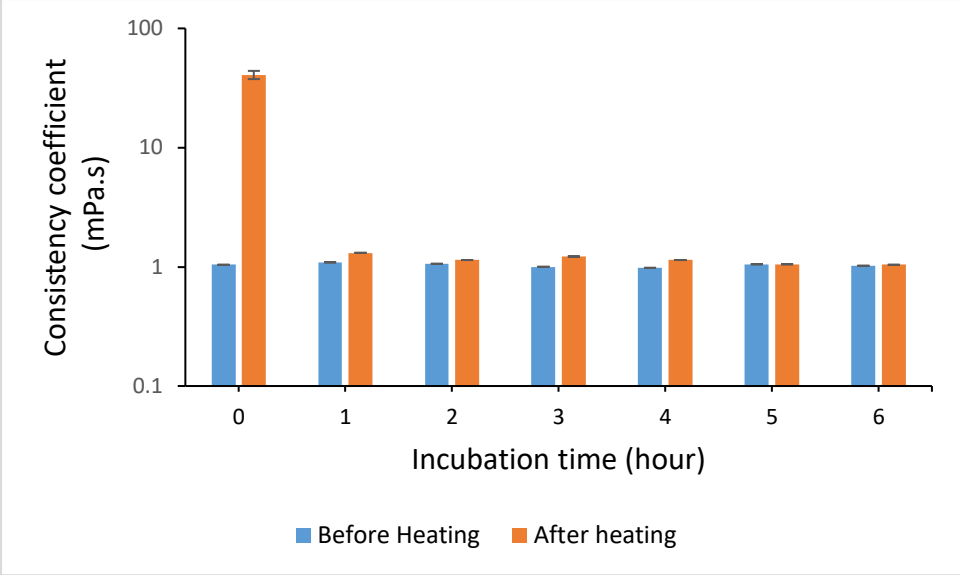


Figure 3.10. Consistency coefficient (in mPa.s) of the o/w emulsions stabilized by dry heat incubated WPC, preconditioned at pH 8, before and after heating the emulsions at 80 °C for 20 minutes.

Referring to the results of particle size distribution and viscosity analysis, it can be concluded that the preconditioning pH of WPC conjugates could shorten the incubation time needed to produce stable emulsions. Based on our findings, increasing the pH to a more alkaline environment is favourable to shorten the incubation time. This is due to the fact that in an alkaline environment, the amino groups are in the deprotonated form, making it more reactive to conjugate with sugars present (Martins et al., 2001). As the amino groups become more nucleophilic, Maillard reaction takes place quickly and thus, a shorter incubation time is feasible to produce stable emulsions. The extent of Maillard reaction will, however, be further explained in **Section 3.3.2**. Nevertheless, it is also important to note that in our findings, the preconditioning pH did not improve the emulsifying properties of the emulsions. As can be seen in the **Figure 3.3**., **3.5**., **3.7.** as well as **3.9** and **3.10.** respectively for each native (non-incubated) emulsion, we did not find any evidence that by preconditioning the WPC conjugates only, a stable particle size and viscosity could be maintained after the heat treatment. Dry heat treatment was always needed to enable the formation of a heat stable WPC.

3.2.3. Creaming stability of the emulsions

The stability of the emulsions against creaming was assessed using analytical centrifugation. The heated samples were evaluated to know whether the emulsion stability was affected by heating. Emulsions containing WPC that was not dry heat incubated were not evaluated as they already became aggregated after the heating test. Meanwhile, most of the emulsions at pH 4 were severely aggregated. That is why only those containing WPC preconditioned at pH 6 and 8 were measured during the analysis.

The rate of creaming is influenced by the droplet size as well as the density differences between the oil and water phases (McClements, 2016). Creaming rate can be analysed using the Lumisizer, an analytical centrifuge which measures the extinction of transmitted light across the sample. Upon centrifugation, accelerated migration of the particles take place and the graph (**Figure 3.11**) represents the transmission profile. The regions of well dispersed droplets (cream phase) scatter and absorb the light, hence the low transmission. Meanwhile, the region of clear dispersions (aqueous phase) do not scatter and absorb the light well, resulting in high transmission.

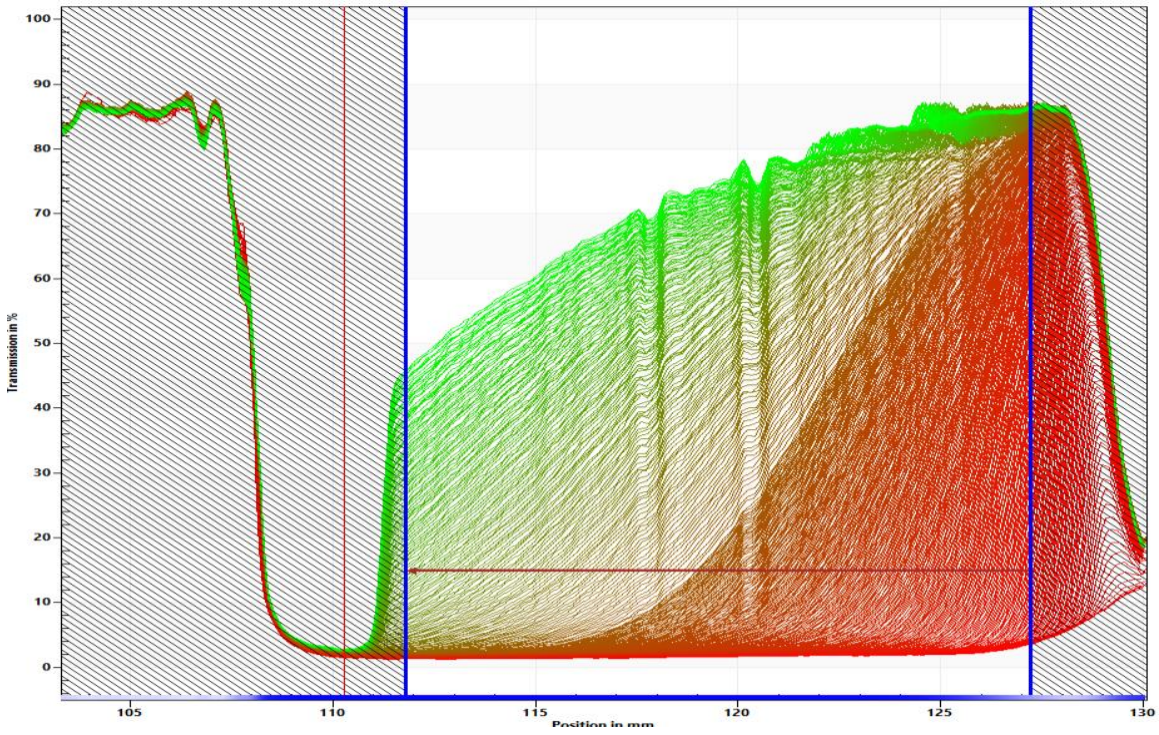


Figure 3.11. A typical centrifugation profile of emulsion stabilized by the WPC conjugates preconditioned at pH 6 in O/W emulsion (before heating test) with incubation time of 6 hours.

Front tracking data analysis was applied to the raw data by setting the trigger value at 15% transmission. Thus, creaming velocity rate can be obtained, respectively for emulsions preconditioned at pH 6 and 8. The results can be seen in **Table 3.4.**

Table 3. 4. Creaming velocity (in mm/day) (mean ± standard deviation) at 1700 g of 10% o/w emulsions stabilized by dry heat incubated WPC before and after heating the emulsions at 80 ^{o}C for 20 min, as a function of the preconditioning pH value.

Incubation time (h)	Creaming rate (mm)			
	pH 6		pH 8	
	before heating	after heating	before heating	after heating
0	344.8±4.1	-	-	-
1	350.4±4.1	-	-	-
2	361.6±4.1	1,130.6±4.7	323.4±4.4	279.4±4.6
3	334.5±4.1	480.2±4.0	254.7±4.7	263.6±4.6
4	341.9±4.1	406.5±4.0	280.4±4.5	262.8±4.6
5	343.9±4.1	364.5±4.1	281.9±4.6	290.9±4.6
6	338.7±4.1	347.2±4.1	272.2±4.6	277.8±4.6

Remarks: (-) Data was not available as the experiments were not conducted for that particular sample.

Table 3.4. indicated the creaming rate of emulsions stabilized by the preconditioned WPC conjugates at pH 6 and 8 obtained by an accelerated creaming test at a centrifugation of 1700 g. As a longer incubation time resulted the more pronounced conjugation which lead to the emulsions able to maintain their droplet size, it is expected that the creaming velocity should be lower as an indicator of stable emulsions.

Based on **Table 3.4**, the susceptibility to creaming can be observed. It can be seen that emulsions could maintain their stability against creaming, after sufficient incubation time was applied. For instance, at preconditioned pH 6, WPC conjugates in o/w emulsions could maintain its creaming velocity for incubation time of 5 and 6 hours. Meanwhile, at preconditioned pH 8, WPC conjugates in o/w emulsions exhibited stability for incubation time of 3, 4, 5 and 6 hours. The result of creaming analysis was in accordance with the result of particle size distribution and viscosity analysis of both pH condition. Droplet flocculation would lead to change in rheological properties, where increase in consistency and extensive shear thinning can be observed (Demetriades, Coupland, & Mcclements, 1997). This

flocculation would also cause an increase in tendency of droplets to cream, most likely due to the effective size of the particles increased (Demetriades, Coupland, & Mcclements, 1997).

Also, as mentioned previously, the droplet size is reflected in the creaming velocity, where small particle sizes will result in a small creaming velocity. In fact, creaming will not take place in emulsions when the diameter of the oil droplet is very small (typically submicron), making the creaming rate of the particles roughly equal to their Brownian motion (Srinivasan Damodaran, 2005). On the other hand, it can be noticed when the droplets size were considerably big, it did not demonstrate a good stability against creaming. This can be seen for example at WPC preconditioned at pH 6 with incubation time of 2 hours where the creaming velocity was much higher compared to the unheated ones.

3.3. Protein Characterization

3.3.1. Degree of conjugation

Maillard reaction is a spontaneous and natural reaction via heating, which consists of a condensation of a reducing sugar with the ε-amino group of lysine residues of proteins and through the formation of a Schiff base and the Amadori rearrangement, producing so-called Amadori products (Friedman, 1996; Ledl & Schleicher, 1990). Through this reaction, the conjugation of the sugar to the protein could happen and improve the functional properties of the protein. The degree of conjugation/glycation is influenced by several factors, such as the protein and sugar type, pH, and incubation temperature (de Oliveira et al., 2016). Free amino groups are reacted in the early stage of the Maillard reaction (Liu & Zhong, 2014). Therefore, in this study, the ortho-phthaldialdehyde (OPA) analysis method was conducted to know the quantity of available amino groups as the loss of available amino groups can be used to calculate the degree of glycation.

Table 3.5. Degree of conjugation (%) of the conjugated WPC preconditioned at pH 6 and 8 (RH 74%, 80 ^{o}C) as a function of incubation time.

Incubation time (hour)	pH 6	pH 8
1	2.9 ± 0.7	22.4
2	7.0 ± 5.2	18.3
3	12.1 ± 1.2	21.7

4	8.7 ± 1.1	16.9
5	1.4 ± 1.5	30.2
6	14.3 ± 4.6	22.9

Remarks: From the results demonstrated in **Table 3.5.**, we can see that there were some points for WPC conjugates preconditioned at pH 6 with a large standard deviation. This *standard deviation for preconditioned WPC at pH 6 was derived from two repetitions. For pH 8, due to the limitations during the COVID-19 pandemy, a second repetition could not be conducted.*

Presumably, the OPA method is highly dependent on the reaction time between the OPA reagent and the sample. In this case, as the results did not show a clear trend as a function of incubation time, the method seemed not to be fully optimised yet. This issue could not be solved due to the laboratory-work limitations during the COVID-19 pandemy.

The OPA analysis was only conducted for emulsions preconditioned at pH 6 and 8, referring to the previous results from the emulsion characterisation that only those two preconditioned pH value whose resulted an improved performance in term of the heat stabilizing properties. Thus, the OPA analysis was prioritised for evaluating the degree of conjugation for WPC conjugates preconditioned at pH 6 and 8. In general, the OPA method depends on the absorbance reading whereby a decreased absorbance reading at a wavelength of 340 nm (A_{340nm}) demonstrates the formation of glycoconjugates due to the Maillard reaction. The formation of conjugates reflects the loss of free amino acids. In previous research using the OPA assay, it was shown that the greatest loss of free amino groups was found in heated samples (Lillard, Clare, & Daubert, 2009). Additionally, through dry heat methods, the degree of glycation for whey protein isolate – gum arabic (WPI-GA) was found to be significantly higher for those mixtures incubated for a longer time (Chen, et al., 2019) which was plausible, considering that a longer incubation time meant that the Maillard reaction took place to a larger extent.

Previous research investigated the effect of pH on the degree of glycation (DG) of sugars using the OPA method, and showed that the DG value increased rapidly at pH 2-6 and decreased slightly at pH 8-9, similarly for all kinds of sugars (glucose, lactose, and dextran) (Xi et al., 2020). The study indicated that as the pH increased from 6 to 10, the protein unfolded and thus the DG increased. However, at pH values higher than pH 8, the free sulfhydryl content

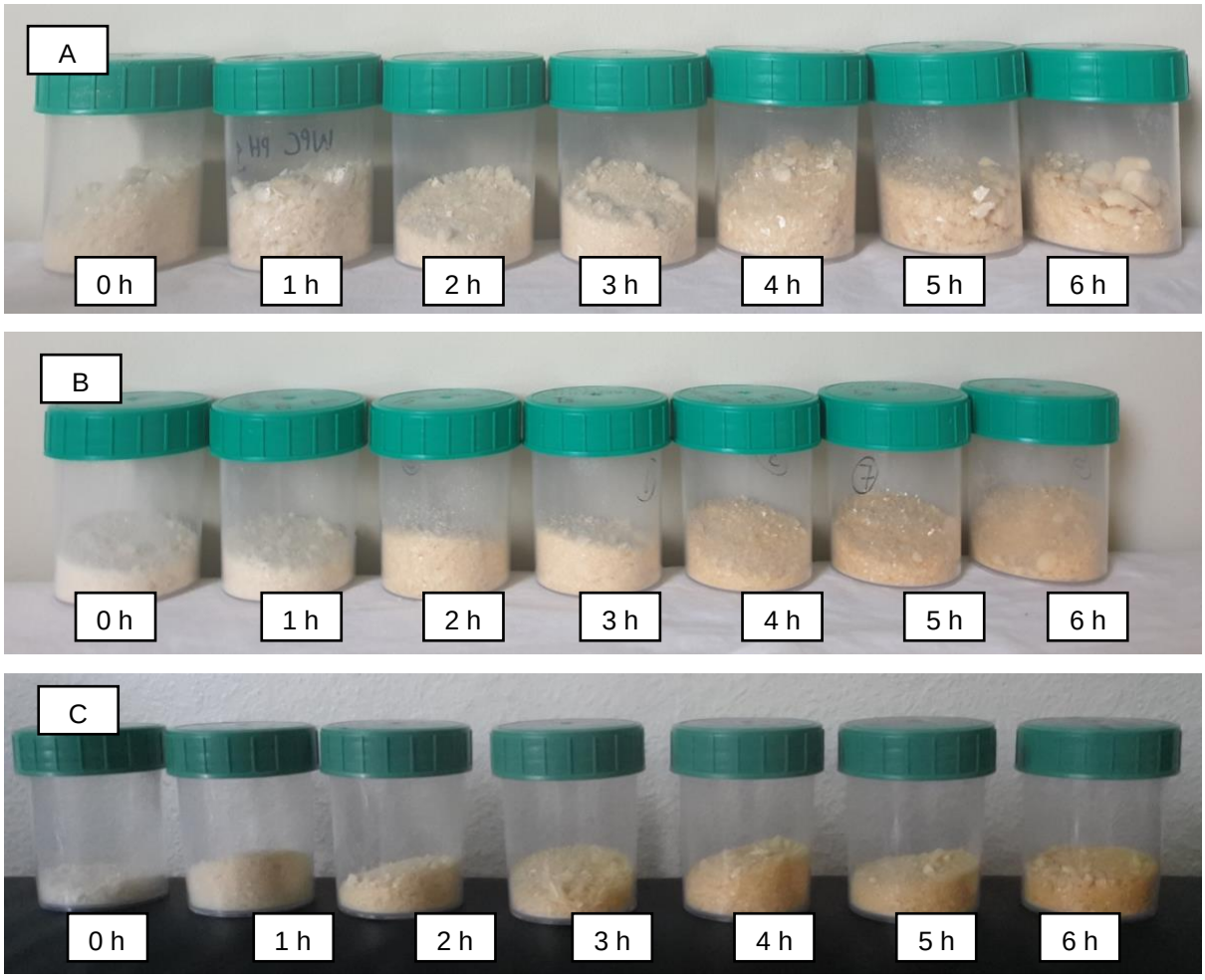
in the WPC-sugar conjugates was reduced, and disulfide bonds were formed, resulting in the aggregation between the protein molecules. As a further consequence, the DG decreased.

Referring to the other results, such as the particle size distribution and viscosity which demonstrated a better emulsion stability in the presence of conjugates preconditioned at pH 8, we can conclude that indeed an alkaline environment is favourable for the formation of WPC-Lactose conjugates, until some extent. The latter conclusion is based on our results at a preconditioning pH of 10, where the conjugated WPC could not be dissolved anymore, similar to the results from Xi et al. (2020). In an alkaline environment, the initial stage of glycation is enhanced by deprotonating the amino groups, and thus increases the reactivity with the carbonyl group of the reducing saccharide (Chen, Ma, et al., 2019). Therefore, the degree of glycosylation at pH 8 is supposed to be higher in comparison to pH 6 as an alkaline environment is more favourable for the glycosylation reaction. It is important to be noted that an increased pH value does not always mean an increase of DG: a decrease of the DG may take place concomitant with an increasing pH, e.g. at pH 9-10 in the previous research from Xi et al. (2020) due to the changes in the free sulfhydryl content, making it more vulnerable to aggregation upon heating which will further be reflected in its increase of the particle size.

In addition, NMR diffusometry measurements have been conducted in our group (under publication) on dry heat conjugated WPC. NMR diffusometry is a direct method to calculate the degree of conjugation by measuring the diffusion behaviour of lactose as a function of the conjugation with proteins. Hereby, unbound lactose is diffusing rapidly, whereas protein-bound lactose diffuses much more slowly. The result indicated that around 17% of conjugated amino groups were sufficient to produce WPC conjugates with good heat stabilizing capacity. Meanwhile, referring to the OPA analysis results at pH 6, where it showed heat stable emulsions with an incubation time of 4 hours, the required percentage of bound amino groups was around 7.6 – 9.8% to have heat stable emulsions. This means that the percentage of primary amino groups in the WPC which are no longer free due to the complex formation via Maillard reaction was around these values. Nevertheless, from the calculations based on these two measurements, we can conclude that a minimum number of conjugated amino group is required to produce conjugates with desirable heat stabilizing capacity.

3.3.2. Browning color development

Dry heating leads to the browning of powders and the formation of advanced glycation end-products upon the Maillard reaction. In the Maillard reaction, the initial pH or the presence of a buffer has a significant role as the basic amino groups could vanish (DeMan, 1999). Following the loss of amino groups, the pH decreases and this explains why the pH of a solution of the conjugates decreases linearly with the incubation time. The longer the incubation time, the more the Maillard reaction took place. Additionally in alkaline conditions, Schiff bases form easily and thus enhance the Maillard reaction (Liu, Yang, Jin, Hsu, & Chen, 2008).



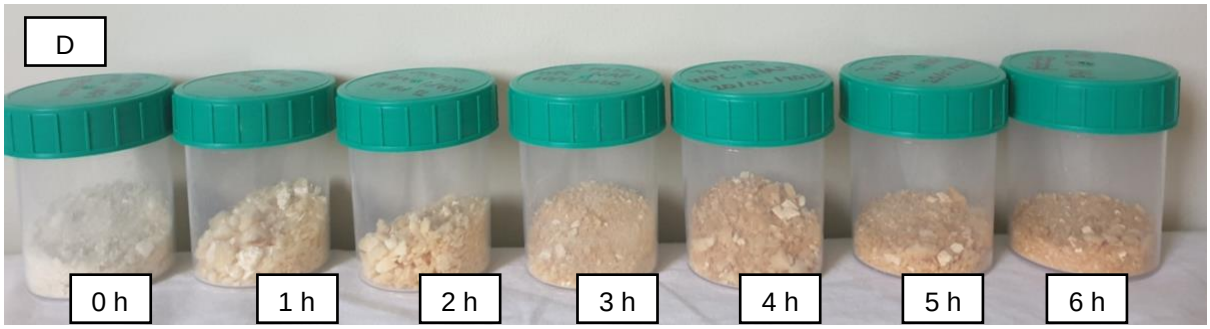


Figure 3.12. WPC conjugates preconditioned at (A) pH 4 (B) pH 6 (C) pH 8 and (D) pH 10 (incubation time: 0, 1, 2, 3, 4, 5 and 6 hours, from left to right).

The browning color development analysis was supposed to be conducted by using the L*a*b* Hunter method. The color development is then expressed as ΔE. Nevertheless, due to the limitations during the Covid-19 crisis, the experiment had to be cancelled. Nevertheless, it is expected that for WPC conjugates at pH 8, the brown color development is more distinctive in comparison to those at pH 4 and 6. From **Figure 3.12 (A to D)**, it can be visually seen that the WPC powder color tends to become darker along with the incubation time. Theoretically, it would result to a lower L* value and an increase in a* and b* value. In this context, Gómez-Narváez, Contreras-Calderón, & Pérez-Martínez (2019) discovered a correlation between the L* value and the amount of available lysine. Higher a* and b* values, on the other hand, were correlated with increased concentrations of furosine, hydroxymethylfurfural, and coloured compounds.

The expected result should be in accordance with a previous study from Schong & Famelart (2019) where they observed the effect of addition of lactose on the production of whey protein microparticles at pH 9.5 via dry heat treatment. From their results, a more intensive Maillard reaction was exhibited maximally after 3 hours of dry heating at pH 9.5. After that, no more color change was observed. They postulated based on their browning and carboxymethyllysine content analysis, that due to the pH adjustment, the reactivity between the sugar and the nucleophilic amino groups is more pronounced in alkaline conditions. Additionally, Schong & Famelart (2019) also suspected that an increasing content in lactose could also rise the production of volatile Maillard intermediates, such as ammonia, diacetyl, formic or acetic acid. More intense crosslinking of proteins and a lower ability to swell in the aqueous phase are also expected at the high alkaline pH (Schong & Famelart, 2019). Thus, for

WPC conjugates preconditioned at pH 10, the sample became insoluble. Meanwhile, in acidic conditions, the protonation of the amino groups could alter the system's reactivity and lower the browning reaction.

3.3.3. Protein solubility

A protein solubility increase generally coincides with a decrease of the droplet size (McClements, 2016); protein solubility is hence a determining factor to create an emulsion (Kinsella & Whitehead, 1989). Due to the heat treatment, a loss of solubility, structural unfolding and heat induced aggregation are some of the consequences of the induced changes in the protein structure (Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005). Heating increases the adsorbed amount of protein and causes a concomitant decrease of the amount of whey protein in the aqueous phase (Sliwinski et al., 2003). Therefore, a solubility analysis of the WPC conjugates was supposed to be conducted to evaluate the protein stability against heat. If the conjugates could show a good solubility, it means that they also have potential to produce a heat stable emulsion. However, this analysis had to be cancelled due to the limitations during the Covid-19 pandemy.

The solubility of whey proteins can be altered by temperature and pH changes. At pH values above the isoelectric point, the proteins have a net negative charge, while below it they would have a net positive charge (Demetriades, Coupland, & Mcclements, 1997). This results in an electrostatic repulsive force at pH values away from the isoelectric point, meaning that the protein is supposed to demonstrate stability at pH values far from its IEP. Meanwhile, at temperatures up to 70 °C, ß-Lactoglobulin shows irreversible denaturation (de Wit & Klarenbeek, 1984), and this will cause a decrease of the protein solubility. The conjugation pf protein with sugar was shown to be able to maintain the solubility of the protein upon heating, similar to the results from Setiowati et al. (2016) where the solubility in the non-conjugated WPI was lower compared to WPI conjugated with pectin.

Pelegrine & Gasparetto (2005) evaluated the whey protein solubility in the pH range of 4.5 – 7.8, and discovered that a pH value of 4.5 demonstrated the minimum solubility. This is because the protein-protein interactions increase as the electrostatic repulsive forces are reduced and therefore, less water interacts with the protein molecules. It is then hypothesized

that the solubility of WPC preconditioned at pH 4 will the lowest among the studied samples (i.e. compared to pH 6 and 8). Similarly, those preconditioned at pH 8 will have a higher solubility, considering the excess of similar charges which result in a larger solubility (Pelegrine & Gasparetto, 2005). It was indeed seen from the results of Pelegrine & Gasparetto (2005) that the solubility at pH 7.8 was the largest. Nevertheless, as explained previously, we analysed the pH of the emulsions using WPC conjugates preconditioned at pH 4, 6, and 8. For the emulsions containing conjugates preconditioned at pH 6 and 8, the pH results were comparable. Thus, it can be said that electrostatic effects most likely do not contribute that much in regard to emulsion stability, when comparing between WPC preconditioned at pH 6 or 8.

Conjugation should have a protective effect against the decrease of the solubility due to heating. This result was demonstrated in previous research from Chevalier et al. (2001) where ß-Lactoglobulin was glycated with different sugars (arabinose, galactose, glucose, lactose, rhamnose and ribose) at 60 °C. From this study, it was proven that the conjugates between proteins and sugars have a relatively high thermal stability in comparison to the native protein. In addition, due to the major conformational modification due to the glycation, a decrease in solubility is observed (Chevalier et al., 2001). It is also important to note that prolonged incubation might result in a decreased of solubility due to the formation of high molecular weight AGEs.

3.3.4. pH analysis

Table 3.6. shows the pH of aqueous dispersions of the WPC-conjugates as a function of the incubation time. After the dry heat incubation, it was found that, in general, the pH demonstrated a decreasing trend as the incubation time got longer. Particularly for WPC conjugates preconditioned at pH 6, the pH of the solution was higher than the previously adjusted pH value regardless of the incubation time and conjugated or non-conjugated conditions.

Table 3.6. pH analysis of a aqueous dispersions containing 2.5 mg/mL of WPC conjugates preconditioned at pH 6 or 8

Incubation time (hour)	pH of WPC solution	
	pH 6	pH 8

0	7.02	8.24
1	6.87	7.90
2	6.77	7.88
3	6.64	7.75
4	6.57	7.56
5	6.58	7.16
6	6.56	7.47

The Maillard reaction can significantly affect the pH of a protein solution as the basic amino groups could vanish (DeMan, 1999). Following the loss of amino groups, the pH would decrease. This explained the behaviour seen in Table 3.6, where the pH decreased linearly with the incubation time: the longer was the incubation time, the more pronounced the Maillard reaction took place.

3.4. Overall comparison

The conditioning RH in order to produce protein conjugates may vary depending on the circumstances. Nonetheless, several studies have been conducted to observe the emulsifying capacity and heat stabilizing properties of whey proteins when conjugated with a particular sugar at a particular RH. A study conducted by Liu et al. (2019) experimented WPI/WPI-Lactose with and without the presence of EGCG. Those mixtures were incubated at 70 ^{o}C, 79% RH, and pH 7.0 for 24 hours. The results showed that in the presence of lactose, a better thermal stability was obtained as there was no significant difference in the particle size before and after heating of the emulsions. Additionally, the presence of EGCG revealed that the WPI-Lactose/EGCG particles had an excellent thermal stability. Another study examined the glycation of WPI and saccharides (lactose or maltodextrin) at 80 ^{o}C, and 80% RH for 2 hours, and also showed improvements with regard to its heat stabilizing properties (Liu & Zhong, 2013). At lower RH, a study investigated the conjugation of whey proteins with glucose-6-phosphate at 50 ^{o}C, and 65% RH for 1 – 3 days: in general, with a longer incubation time, they observed more pronounced Maillard reaction (Aoki, Fukumoto, Kimura, Kato, & Matsuda, 1994).

Referring to the literature described above, in our study, we conducted an experiment to check the influence of the variation of RH on the emulsifying activity and heat stabilizing

properties of WPC conjugated with naturally present lactose ranging from 64% to 79% RH. From the results, we could conclude that a RH of 74% as well as 79% could produce heat stable emulsions with 4 hours incubation time, which is shorter than the incubation time used in most studies. Furthermore, we tried to evaluate the influence of the preconditioning pH on the rate of Maillard reaction. A previous study conducted by Liu & Zhong (2014) evaluated WPC conjugated with lactose at 130 ^{o}C for 20 and 30 minutes and 60 ^{o}C for 24 and 48 hours. Those two groups had a comparable heat stability and degree of glycation. Other research studied the conjugation of WPC with lactose and dextran at pH 3.5, and from the results, it was demonstrated that the functionality of WPC was enhanced, especially with regard to its emulsifying attributes (Lillard et al., 2009). Meanwhile, based on our results, it was found that a preconditioning pH in the alkaline environment (i.e. preconditioning at pH 8), could improve the heat stabilizing properties of WPC conjugates in o/w emulsions with a shorter incubation time (2 hours). It is indeed known that both the RH and pH are crucial factors in the production of WPC conjugates with improved heat stabilizing as well as emulsifying properties. Thus, further research is encouraged to evaluate the extent and impact of Maillard reaction towards protein properties.

Chapter 4. Conclusion and Future Perspectives

Overall, the results indicated that the conjugation of WPC with naturally present lactose by dry heat treatment could improve the functional properties of the protein, particularly the heat stabilizing property. The present work revealed that WPC conjugates obtained through the dry heating method at a RH of 74% and 79% showed a good performance in o/w emulsions. Nevertheless, the difference between both condition was limited. Therefore, a RH of 74% at 80 °C was chosen as the method to conduct the dry heat induced glycation in the WPC. Stable emulsions could be produced upon incubation at a RH of 74% with a minimum incubation time of 4 hours.

Additionally, the effect of pH was also examined during this study. The results revealed that by increasing the pH until some extent, a faster Maillard reaction could be induced, as reflected by a shorter incubation time required to produce WPC with improved functionality. However, it is important to note that no improvement in the emulsifying activities was shown by conjugation of WPC with its naturally present lactose. With regard to the heat stabilizing properties, in comparison between pH 6 and 8, WPC conjugates preconditioned at pH 8 demonstrated better results referring to the particle size and viscosity analysis after heat treatment. The incubation time also had an influence on their properties: for pH 6 and 8, the minimum incubation time needed to have stable emulsions was 4 and 2 hours, resp. Additionally, these two emulsions, stabilised by WPC preconditioned at pH 6 and 8 showed stability against creaming and a higher degree of glycosylation for pH 8.

In future research, an optimization of the OPA analysis method should be conducted, or another analysis should be used to measure the degree of glycosylation, which is essential to further learn about the glycosylation in the WPC caused by the Maillard reaction. The planned analyses that could not be conducted, such as the browning color development, as well as solubility and zeta potential analysis, should also be performed to gather more evidence regarding its performance maintaining the stability. More analyses to learn about the structural changes such as sulfhydryl analysis, SDS-page, and circular dichroism can also be applied in future research. As denaturation is supposed to increase with a longer incubation time in dry heating, conducting similar experiments with a wider range of

incubation times is also suggested to see until what extent the WPC conjugates could maintain their stability. Moreover, evaluating the conjugates' emulsifying and heat stabilizing properties in emulsions at various pH values and salt concentrations can be one step further to have a more in-depth knowledge to see its industrial feasibility, e.g. in acid beverages production.

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