Re-dispersible Dry Emulsions stabilised with Protein-Polysaccharide Conjugates

By

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Abstract

Recently the need for more functional food products has opened new fields of research on the subject. Dry emulsions present an opportunity not only for the production of functional ingredients but also for the reduction of industrial costs. However drying emulsions comes with limitations fixed by both the composition of the emulsified product and the drying process used. In the case of reconstitution by rehydration it is important that the emulsified product keep the same textural properties to be a viable commercial product.

The work presented in this thesis investigates the use of promising emulsifiers for the drying and rehydration of emulsions. Maillard conjugates also called glycoconjugates have been used for the production of oil-in-water emulsions. Because of the thicker membrane they form at the oil/water interface, Maillard conjugates are potential candidates for the stabilisation of emulsions destined to be dried. The key challenge when drying complex structures rests in their preservation. The strength and the nature of the interface are two of the parameters that need to be taken into account when attempting to dry an emulsion.

Due to the constant relationship between product formulation and drying process this study proposes a combination of formulation development and process engineering.

To carry out this project conjugates were produced, analysed and tested in model emulsions. Conjugate stabilised oil-in-water emulsions with identical formulation (20% w/w sunflower oil, 80% w/w water) were produced and analysed in different scenarii. Subsequently their ability to be dried and rehydrated was assessed using freeze-drying and spray-drying.

The results showed that the conjugates particle size was highly dependent on the pH of the conjugation reaction. Smaller particle size could be produced without affecting the yield of reaction.

The enhanced stability of the systems to external stresses was confirmed. However experiments using a charged polysaccharide showed that at pH close to pK_a the stability of the system was challenged.

All emulsions showed good stability to drying at different emulsifier concentrations. The textural components (viscosity and friction coefficient) were well preserved.

The adjustment of the formulation by addition of additives significantly improved the stability of the systems to freeze-drying and spray drying.

Overall this study filled some gap of knowledge in the use of Maillard conjugates in oil-in-water emulsions. The study of the stability to external stresses such as salt addition or pH modification contributed to the understanding of the functional properties of these conjugates.

Drying and rehydration experiments provided a better understanding of dry emulsions behaviour.

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Nomenclature

°C	Celsius degrees
µm	Micrometer
σ	Surface tension
Δσ	Surface tension variation
ζ-potential	Zeta potential
a_w	Water activity
C_a	Adsorbed concentration
$D_{3,2}$	Surface mean diamater
$D_{4,3}$	Volume mean diameter
g	Gram
g/mL	Density
MC	Moisture content
mg	Milligram
mg/mL	Milligram per millilitre
mL	millilitre
mL/min	Feeding Rate
mM	Millimolar
mm/s	Ball speed
mN/m	Interfacial tension/surface tension millinewton per meter
mV	Millivolt
nm	Nanometer
Pa.s	Pascal second
rpm	Revolution per minute

s	Second
s^{-1}	Shear rate
T_g	Glass transition Temperature
w/w	Mass concentration
$CaCl_2$	Calcium chloride
CN-De	Sodium caseinate-dextrin conjugate
DLS	Dynamic light scatering
DSC	Differential scanning calorimetry
FD	Freeze-drying
FT	Freeze-thawing
H^+	Proton
HCl	Hydrogen chloride
IFT	Interfacial tension
N_2	Liquid nitrogen
NaCN	Sodium caseinate
NaCl	Sodium chloride
NaN_3	Sodium azide
NaOH	Sodium hydroxide
pI	Isoelectric point
RH	Room humidity
RT	Room temperature
SBP	Sugar beet pectin
SD	Spray drying
SEM	Scanning electron microscopy
SFT	Surface tension

WPI	Whey protein isolate
WPI-La	Whey protein isolate-lactose conjugate
WPI-SBP	Whey protein isolate-sugar beet pectin conjugate

Chapter 1

Introduction

1.1 Introduction

Emulsions are the mixture of oil and water, one phase dispersed as droplets in the other. Ingredients such as surfactants, particles and hydrocolloids are used to stabilise these dispersions.

Protein provide excellent surface-active properties and electrostatic stabilisation through droplet repulsion (Crenwelge, Dill et al. 1974, Haque, Timilsena et al. 2016). Polysaccharides on the other hand, are typically used as texturizing agents that enhance the stability by increasing the viscosity thus reducing droplet collisions.

Polysaccharides and proteins have been associated and used to produce emulsified products with enhanced stability (freeze-thaw stability (O'Regan and Mulvihill 2010), heat stability (O'Regan and Mulvihill 2010), resistance to change of pH (Zeeb, Gibis et al. 2012) and ionic strength). These complexes can be formed through enzymatic reaction (Zeeb, Gibis et al. 2012), electrostatic interaction (Guzey and McClements 2006, Turgeon, Schmitt et al. 2007, Zinoviadou, Scholten et al. 2012) and heat induced covalent link (the Maillard reaction) (Akhtar and Dickinson 2007, O'Regan and Mulvihill 2009, Evans, Ratcliffe et al. 2013).
The enhanced stability provided by such systems is mainly attributed to the bulkier interfacial membrane they form at the oil/water interface.

The naturally occurring Maillard reaction (or conjugation) is the reaction responsible for the browning of meat, bread or pastry through cooking and baking. The browning occurs when a covalent link is formed between the amino groups of the proteins and the reducing sugar of the polysaccharides. The conjugation takes place at high temperature and under controlled humidity conditions.

Several couples of conjugated proteins and polysaccharides have been studied so far to stabilise oil-in-water emulsions; whey protein and maltodextrin (Drapala, Auty et al. , Akhtar and Dickinson 2007), sodium caseinate and maltodextrin (O'Regan and Mulvihill 2009), egg white protein and pectin (Al-Hakkak and Al-Hakkak 2010), β-lactoglobulin and dextran (Wooster and Augustin 2006) and many others (Akhtar and Dickinson 2003, Neirynck, Van der Meeren et al. 2004, Perrechil, Santana et al. 2014).

In most of these studies dry heating; a mix of proteins and polysaccharides powders heated under controlled humidity for several hours, was chosen over wet heating; a solution of the two ingredients is heated for several hours to several weeks, to produce the conjugates because of its rapidity. Experiments revealed that the effectiveness of the conjugation depended on the nature of the couple, the ratio of each component, the incubation time (Al-Hakkak and Al-Hakkak 2010, Perrechil, Santana et al. 2014) and also the molecular weight of the polysaccharide (O'Regan and Mulvihill 2009).

Because of the thicker membrane they form at the interface, Maillard conjugates are interesting candidates for the stabilisation of emulsions destined to be dried. Drying is commonly use in the food industry to increase the shelf life of emulsified products but also to reduce the storage and transport costs. The key challenge when drying complex structures is the retention their structures. The strength and the nature of the interface are two of the parameters that need to be taken into account when attempting to dry emulsions. Maillard conjugates have potential to be used for this purpose.

On the other hand, few studies address the question of the reconstitution of emulsions after drying (Christensen, Pedersen et al. 2001, Dollo, Le Corre et al. 2003, Whitby, Scarborough et al. 2017).

1.2 Research Objectives

The aim of this study was to assess the ability of Maillard conjugates to stabilised re-dispersible dry emulsions. This work addresses the reconstitution of the emulsions after rehydration focusing on the microstructure and textural properties. The relationship between emulsions composition and rehydration outcome is explored. Additionally, a contribution to the existing knowledge on the preparation of conjugates and their functional properties oil-in-water emulsions was given in two introductory chapters.

The research objectives can be listed as follow:

- Provide additional information on the functional properties of Maillard conjugates in oi-in-water emulsions.
- Assess the ability of the conjugates to stabilised emulsion destined to be dried using high and low temperature processes.
- Establish a relationship between the nature of the emulsifier and the dry emulsions behaviour before, during and after rehydration.
- Optimise emulsion formulations and processing parameters to favour the reconstitution after drying and rehydration.

1.3 Thesis Layout

This thesis is organised into six chapters;

Chapter 2 Literature Review and Theoretical Background gives an overview of the existing knowledge. Similar studies are critically review in order to highlight the knowledge gaps the present thesis intends to fill.

Chapter 3 Conjugates Preparation and Emulsifying Properties in Oil-in-Water Emulsions presents an investigation of the effects of different protein/polysaccharide ratios on the conjugation efficiency of the Maillard reaction. The effect of the pH of conjugation on conjugates made with a charged polysaccharide is also studied. Lastly the effects of conjugation on the interfacial tension, on the thickness of the interfacial film and on the coverage of the emulsifier are investigated. The intention of this work was to set the best conditions for the production of the conjugates.

Chapter 4 Influence of External Stress on the Stability of Oil-in-Water Emulsions Stabilised with Maillard Conjugates displays the results from experiments studying the effects of external stresses on the emulsions formulated in Chapter 3. The effects on the conjugates emulsifying properties and on the emulsion stability of addition of salt and pH changes are studied. Emulsion stability to high and low temperature treatments is investigated. Heat treatment and freeze-thaw stability test were performed. Results from these experiments were used to assess the ability of the conjugates to stabilised emulsions to be dried using both freeze-drying and spray-drying. Additionally, the addition of salt and changes of pH were used to evaluate the suitability for food applications.

Chapter 5 Drying and Rehydration of Oil-in-Water Emulsions using Different Drying Processes is the first chapter treating of drying and rehydration of emulsions using spray-drying and freeze-drying. In addition to drying and rehydration experiments dry emulsions are studied and their storage stability investigated.

Chapter 6 Influence of the Processing Parameters and Emulsion Formulations on Dry Powder Characteristics and Reconstitution Ability After Rehydration is the last chapter of this study. It presents the effects of different process parameters on the reconstitution of the emulsions. Emulsions prepared in Chapter 4 are also dried and rehydrated. Additives are added in an attempt to favour the reconstitution after rehydration.

Chapter 7 General Conclusions and Future Recommendations summarises the findings of this study and suggests complementary and future experiments.

1.4 Posters and presentations

Oral presentations:

Manecka, G.-M., Mills, T, Norton, I.T., Formulation of Re-dispersible Freeze-Dried Emulsions. *Centre for Innovative Manufacturing in Food Conference*, Loughborough, 2016.

Poster presentations:

Manecka, G.-M., Mills, T., Norton, I.T., Formulation of Re-dispersible Freeze-Dried Emulsions. *Centre for Innovative Manufacturing in Food Conference*, Loughborough, 2016.

Manecka, G.-M. Mills, T., Norton, I.T., Maillard Conjugates as Emulsifiers of Re-dispersible Freeze-Dried Emulsions, *Centre for Innovative Manufacturing in Food Conference*, Birmingham, 2017.

Manecka, G.-M., Mills, T., Norton, I.T., Maillard Conjugates as Emulsifiers of Re-dispersible Dry Emulsions in Food, *Oxford Females in Engineering, Science and Technology Conference*, Oxford, 2018.

Chapter 2

Literature Review
and
Theoretical Background

2.1 Introduction

This chapter aims to give an overview of the current knowledge on re-dispersible dry emulsions, in terms of emulsifiers used, drying processes, characterisation methods and applications.

For this purpose, it is important to introduce emulsion systems, the different types of emulsifiers and the functional properties they offer. The theoretical background on emulsion systems is presented and the literature discussed in order to identify what scientific gaps the work presented in this thesis is filling.

The theories in emulsion stabilisation using surfactants, Pickering particles and proteins-polysaccharides complexes are presented. Although this thesis focusses on the use of protein and polysaccharide conjugates to produce emulsions, the functional properties of other systems are examined and discussed in order to understand the advantages and disadvantages they present. The emphasis is made on the resistance to environmental changes as the emulsifier used for this research has demonstrated enhanced stability to ionic strength and pH changes.

Dry emulsions and re-dispersible dry emulsions are then presented. The different drying processes and up to date studies will be critically summarised. This part will give an understanding of the direction of the present work.

2.2 Emulsions

When two immiscible liquids are mixed together, the two phases separate in two layers. These layers are the most thermodynamically stable state for the mixture; the free energy of the system is at its lowest point.

2.2.1 Emulsion Formation

"Emulsion" is the term used to describe the mixture of two immiscible liquids one being dispersed as fine droplets in the other, commonly water and oil. To fight the thermodynamic instability that pushes the system back into separation, an emulsifier is needed. The emulsifier reduces the interfacial tension between the oil and aqueous phases enabling their proximity. Depending on the emulsifier two types of emulsions can be produced; oil-in-water emulsions (o/w) consist of oil droplets (dispersed phase) dispersed in water (continuous phase) or water-in-oil (w/o), water droplets dispersed in oil (Figure 2-1).

Double or multiple emulsions are more complex structures that can be produced. They are the result of an already formed emulsion phase dispersed into a continuous phase (for example oil-in-water-in-oil) (Figure 2-1).

For all systems, the nature of the continuous phase will greatly depend on the emulsifier used.

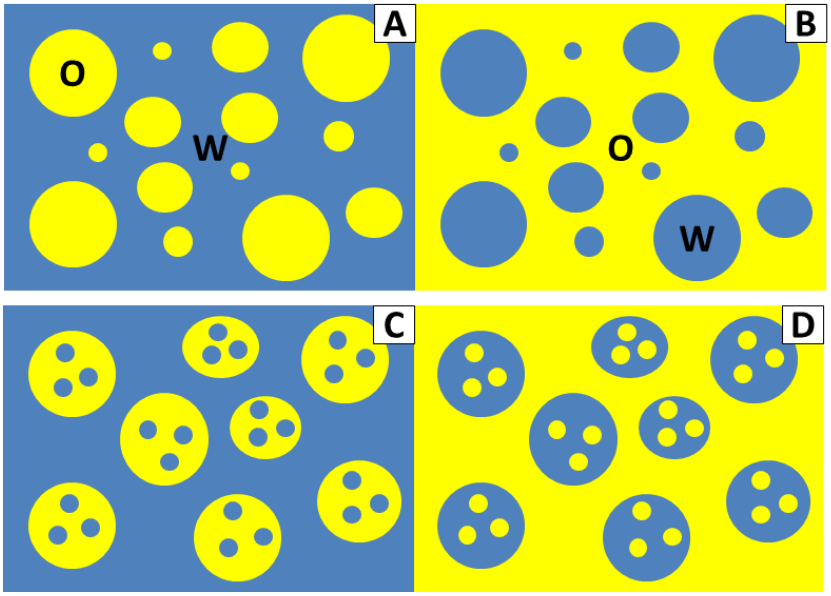


Figure 2-1: Schematic representation of (A) oil-in-water emulsion, (B) water-in-oil emulsion, (C) water-in-oil-water emulsion and (D) oil-in water-in oil emulsion

Less commonly encountered are water-in-water and oil-in-oil emulsions. They respectively correspond to the mixture of two incompatible macromolecule solutions and two immiscible organic solvents or oils with different polarities.

To form the emulsion phase, energy is input to the system. Homogenisation or emulsification is carried out using devices that break down the two phases into droplets of varying sizes. High speed, high pressure, sonication or membranes are ways to produce emulsions.

Emulsions can be classified into three categories based on the size of their droplets and structural aspects;

- Macroemulsions have a droplet diameter greater than 0.1µm and are kinetically stable and thermodynamically unstable

- Microemulsions have a droplet diameter below 100-200 nm, generally comprised between 5 and 50 nm. They are thermodynamically stable.
- Nanoemulsions have a droplets diameter below 100 nm, generally in the 20 to 200 nm range. They are thermodynamically unstable and kinetically stable.

Despite sharing the same size range, microemulsions are not to be confused with nanoemulsions. They differ mainly by their thermodynamic stability and structural aspects.(McClements 2012, Singh, Meher et al. 2017)

2.2.2 Stabilisation and mode of destabilisation

The stability of an emulsion is complex to predict since it depends on several factors such as the droplets size, the viscosity of the system or the speed of homogenisation. However two different types of stabilities characterise emulsions; the kinetic stability and the thermodynamic stability. Most emulsions are kinetically stable, meaning that the phase separation occurs at such a slow pace that they appear stable over long periods of time. On the other hand, only microemulsions are thermodynamically stable, meaning that the free energy of the emulsion state is lower than the free energy of the two separated phases (Tadros 2013).

As mentioned previously emulsions that are unstable tend to break down or show signs of destabilisation. Different modes of destabilisation have been identified (Figure 2-2);

- Creaming and sedimentation are visually noticeable by the formation of a layer of droplets at the top (creaming) or at the bottom (sedimentation) of the emulsion. These processes are not the result of a breaking down of the emulsions; two phases separate one being richer in droplets than the other. The displacement towards the top or towards

the bottom depends on the density of the dispersed phase and the viscosity of the continuous phase. Gravitational and centrifugal forces are the main driver of this phenomenon (Tadros 2013).

The Stokes law can be used as a tool to estimate the creaming or setting rate. Its equation takes into consideration the radius of the dispersed droplets, the viscosity of the bulk and the difference of density between the two phases.

$$\nu = \frac{2r^2(\rho - \rho_0)g}{9\eta}$$

Equation 2-1

Where ν is the creaming or setting rate, r the droplet radius, ρ the droplet density and ρ_0 the density of the dispersion medium, η the viscosity of the dispersion medium and g the acceleration of gravity.

- "Flocculation" is the term used to describe the aggregation of droplets in the medium. It occurs when repulsive forces that drive droplets away from each other are weaker than the Van Der Waals attraction forces.
- Ostwald ripening is a thermodynamically driven process that happens when the droplet size distribution is wide. The system displays a big size difference between the largest and the smallest droplets. Large droplets are energetically favoured. Over time, the size of the smallest droplets decreases increasing their solubility. The molecules of the smaller droplets eventually start diffusing in the bulk and contribute to the growth of the larger droplets (Tadros 2013). The stability of the droplets increases as their size increases due to a decrease of the Laplace pressure of the system.

  Laplace pressure is given by the Young-Laplace equation:

$$\Delta P = \gamma \frac{2}{R}$$

Equation 2-2

Where ΔP is the difference of pressure inside and outside the droplet, R the radius of curvature, γ the interfacial tension between the two phases.

- Coalescence designates the merging of at least two neighbouring droplets resulting in the formation of a larger droplet in the emulsified system. This type of instability ultimately leads to phase separation of the two liquids. This phenomenon is the consequence of collisions and disruption of the interfacial membrane surrounding the droplets. Coalescence depends on factors such as the turbulence of the medium, the concentration of droplets but also the colloidal interactions and physicochemical properties of the emulsifier (Tadros 2013).

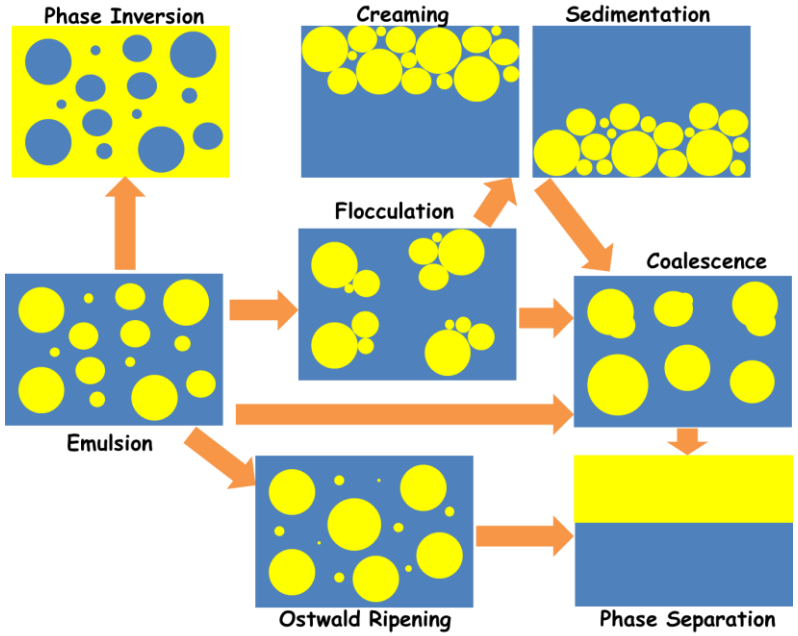


Figure 2-2 : Schematic representation of destabilisation mechanisms in emulsions.

- Phase inversion is the phenomenon describing the change of orientation of the emulsions from oil-in-water to water-in-oil or vice versa. This happens when the composition or the temperature of the emulsion changes.

2.2.3 Stability Testing

Experimentally the long term stability of an emulsion can be tested using different methods; freeze-thaw cycling; the emulsions are frozen below -10˚C and melted several times, storage at elevated temperatures for several weeks or month, temperature cycling; the temperature is changed from 4˚C to 45˚C over a 48 hours period for a month, refrigeration, light exposure or centrifugation at 3000 to 4000 rpm for ~30 minutes.

Temperature changes are generally used to assess the stability of emulsions. However techniques consisting of decreasing the temperature for several cycles also induced other phenomena unrelated to creaming that can influence the stability of the emulsion (e.g. change of pH) (van den Berg and Rose 1959, Banin and Anderson 1974, Ghosh, Cramp et al. 2006).

Centrifugation induces creaming (or sedimentation) and to an extent increases the probability of occurrence of other destabilisation processes such as flocculation and coalescence.

2.3 Surfactants

2.3.1 Structure and properties

Surfactants have been used to stabilise emulsions for decades (Campanella, Dorward et al. 1995, Al-Sabagh 2002, Yang, Leser et al. 2013). They are amphiphilic molecules consisting of a hydrophilic “head” and a hydrophobic “tail”. When added to a liquid or a mixture of liquids, they migrate at the liquid/gas interface or the liquid/liquid interface and lower the free energy between the two phases; the surface tension at the liquid/gas interface, the interfacial tension at the liquid/liquid interface.

Depending on the nature of their “head” surfactants are classified as anionic, cationic, non-ionic or amphoteric (or Zwitterionic).

Their “tail” consists of aliphatic, linear, branched or aromatic hydrocarbon chains.

In some cases, surfactants can have complex structures. Multi-tailed surfactants and dimeric surfactants (several “heads” linked with complex designs) exist. The latter are often less efficient in lowering the surface and interfacial tension than their conventional equivalents (Zana 1997).

In solvents, surfactants are characterised by their critical micelle concentration. It is the concentration above which surfactants start to form aggregates called “micelles” in solution. This is an important value to identify since above this point further addition of surfactant doesn’t affect the surface tension. This concentration is influenced by external factors (e.g. the temperature of the system) (Mohajeri and Noudeh 2012, Sidim and Acar 2013).

The orientation of the micelle depends on the nature of the medium; the most energetically favoured micelle forms (Figure 2-3).

In polar solvents, the hydrophobic tails meet exposing the hydrophilic heads in the medium.

In non-polar solvents, the outer layer of the micelle consisting of the hydrophobic tails.

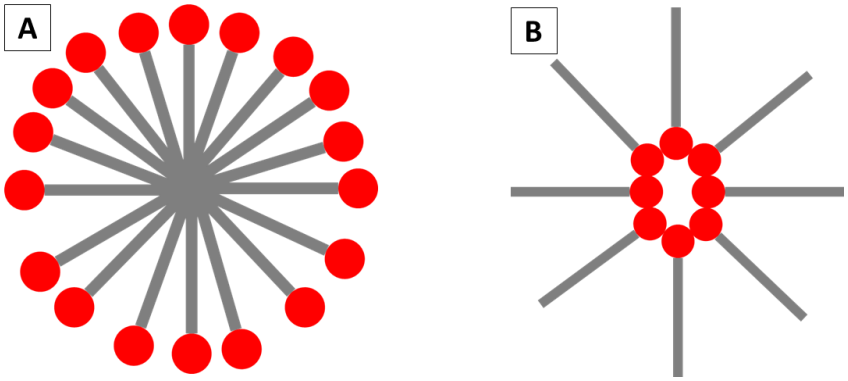


Figure 2-3 : Schematic representation of (A) a micelle and a (B) inverse/reverse micelle

2.3.2 Emulsion stabilisation

During the emulsification process, the interfacial area between the oil and water phase is extended. The surfactants place themselves at the interface areas created and droplets are formed. In systems containing surfactants the energy needed to produce small droplets is reduced (Tadros 2013).

Non-ionic surfactants are the most effective at stabilising emulsions. They provide a steric repulsion that keeps droplets away from each other. On the other hand, the stabilisation by ionic

surfactants is enhanced by electrostatic repulsion between droplets. However such emulsions are sensitive to the introduction of electrolytes in the medium.

To predict the orientation of an emulsion, the Bancroft rule states that the solubility of the surfactant in one or the other phase governs the orientation of the emulsion (i.e. oil-in-water or water-in-oil). The phase in which the surfactant is the most soluble will be the continuous phase of the emulsion. Consequently, hydrophobic surfactants tend to form water-in-oil emulsions while hydrophilic surfactants tend to form oil-in-water emulsions (Tadros 2006).

The HLB (Hydrophilic Lipophilic Balance) was introduced in 1949 and extended in 1954 by Griffin to help in the preparation of the desired emulsion (Griffin 1949). It is based on the calculation of the relative percentage of hydrophilic and hydrophobic groups in the surface active molecule. Griffin developed a series of simple equations to calculate the HLB of given surfactants. Later Davies upgraded this method by taking into account the charged groups present in the molecules (Davies 1957, Hutchinson 1961).

Based on this number, the surfactants can be classified as shown in Table 2-1. Different HLB values brackets can be found in the literature for the same properties.

Table 2-1 : HLB values and corresponding surfactant application (Griffin 1949)

HLB value	Properties
1-3	Anti-foaming agent
3-6	W/O emulsifier
7-9	Wetting agent
8-18	O/W emulsifier
13-15	Detergent
15-18	Solubiliser

In terms of industrial applications, surfactants are widely used in different sectors where emulsions are present. From food (Campanella, Dorward et al. 1995); to cosmetics (Lukic, Pantelic et al. 2016); pharmaceuticals (Tadros 2005); and detergents formulations; surfactants can be used in a variety of emulsions.

2.4 Pickering emulsions

2.4.1 Pickering particles

Pickering particles and Pickering emulsions were first discovered by Ramsden in the early 20th century (Ramsden 1903). They were studied in depth by Pickering who gave his name to these new systems (Pickering 1907). They observed that particles can adsorb at the interface of immiscible liquids and stabilise their mixture. Pickering emulsions are indeed stabilised by densely packed particles that form a rigid layer at the interface. The particle size varies generally from 100 nm to 5 μm. The emulsions display an enhanced kinetic stability in comparison with emulsions stabilised with surfactants. The rigidity of their interface makes Pickering emulsions systems of interest for a variety of applications. Such an interface can resist harsh processing conditions and environmental changes (Marefati, Rayner et al. 2013, Whitby, Scarborough et al. 2017, Zhu, Zhang et al. 2017, Mikulcová, Bordes et al. 2018).

Natural organic and inorganic particles or synthetic particles can be used to produce Pickering emulsions for example for food applications; silica, clays, starch and egg yolk granules (Ashby and Binks 2000, Frelichowska, Bolzinger et al. 2009, Marefati, Rayner et al. 2013, Rayner, Marku et al. 2014).

The nature of the particle greatly influences the emulsions formation and stability.

2.4.2 Emulsion stabilisation

Emulsions are formed when the particles adsorb at the oil/water interface. The adsorption of the Pickering particles is essentially irreversible. This irreversibility is due to the free energy of detachment. The high desorption energy makes their presence at the interface energetically favourable. The densely packed particles at the interface prevent coalescence, Ostwald ripening and to some extent potentially enhance the oxidation stability of the oil (in the case of oil-in-water emulsions). The packing at the interface can follow a disorganised pattern. Particles form single or multiple ordered or disorganised layers at the oil/water interface.

In addition to providing an increased stability, Pickering particles enable the production of emulsions with larger droplets. However, because they adsorb slowly at the interface in comparison with surfactants, forming droplets can be continuously broken down during the emulsification process. Higher concentrations of particles are needed to stabilise Pickering emulsions in comparison with surfactant concentrations needed for the production of surfactant stabilised emulsions.

The droplet size is controlled by adjusting the concentration of particles and emulsification parameters.

Unlike surfactants, the particles do not have any effect on the interfacial tension. Electrostatic and steric repulsion (volume exclusion) ensure the overall stability of the systems. The orientation of emulsions is predicted by measuring the contact angle Θ of the particles. This calculation can be tricky since contact angle measurements on bulk powder are approximations.

Lipophilic particles have a contact angle $\Theta > 90^{\circ}$. When introduced in a mixture of oil and water they will adsorb at the interface and be mostly wetted by the oil. The particle laden

interfacial layer will curve around the water and a water droplet will form through homogenisation. A water-in-oil emulsion is produced.

Hydrophilic particles have a contact angle $\Theta < 90^{\circ}$. When introduced in a mixture of oil and water they will adsorb at the interface and are mostly wetted by the water. Through homogenisation oil droplets will form. An oil-in-water emulsion is produced.

Nevertheless, the particles need to have both a hydrophilic and lipophilic component. Being wetted by only one of the two phases would result in the formation of a dispersion of particles in this particular phase.

At $\Theta = 90^{\circ}$, oil-in-water and water-in-oil emulsions can potentially be produced. The orientation will depend on the nature of the particle used and on the proportions of the two phases. Inversion will be possible by increasing the proportion of one phase or the other.

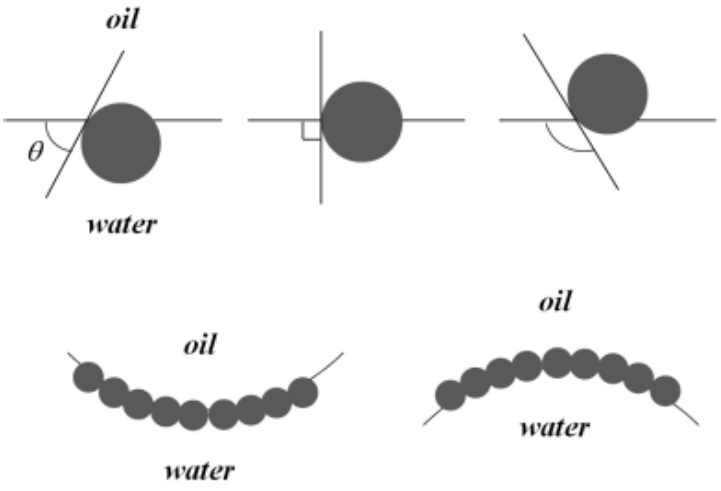


Figure 2-4: Schematic representation of Pickering particles positioning at the oil/water interface based on their contact angle(Aveyard, Binks et al. 2003).

2.5 Hydrocolloids

This section presents the different types of proteins (caseins and whey proteins) and polysaccharides (dextrins and pectins) used for emulsion formulation. The role of the different hydrocolloids is succinctly presented. The focus is made on the structures and properties of the proteins and the polysaccharides used in this study.

The functional properties of emulsions stabilised with proteins, polysaccharides and their association will also be discussed.

2.5.1 Proteins

2.5.1.1 Structure and properties

Proteins are biological macromolecules made of chains of amino acids. They can be of different origins, vegetal (e.g. pea protein, rice protein) or animal (e.g. egg white protein, casein, whey protein). Essential actors of the metabolism, they take part in cell signalling, immune response and cell adhesion. However not all amino acids are synthesised by animals and need to be obtained from food, making proteins an important part of dietary needs.

Their structure can be described on four different levels (Figure 2-5):

- The primary structure refers to the linear succession of amino acids

- The secondary structure is the arrangement of the chains in alpha helix or β-sheets via hydrogen bonds.

- The tertiary and quaternary structures are three dimensional architectures of polypeptide chains.

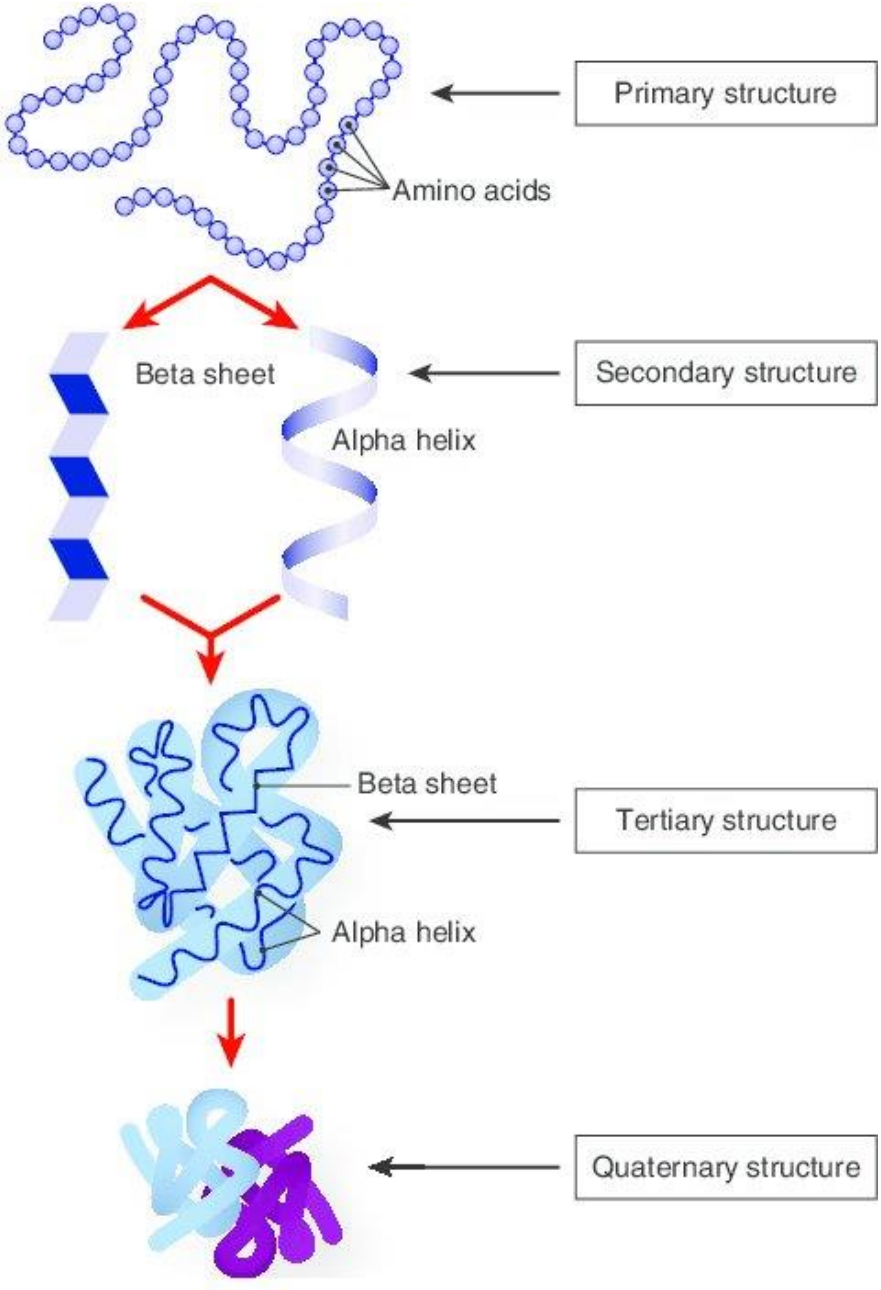


Figure 2-5: Schematic representation of the different structural level of protein chains (Haque, Timilsena et al. 2016).

Proteins properties can change with their environment. Temperature and pH can alter the structure and physicochemical behaviour of the proteins.

Depending on the pH of the medium they are in, proteins can be positively or negatively charged. They are characterised by their isoelectric point (pI), the pH at which the protein has no apparent charge. When in solution at pH = pI, the protein precipitates due to aggregation triggered by the electrostatic attraction of opposite charges.

An increase of temperature generally leads to the denaturation of the protein. The heat induces a change of structure (or conformation) that can be reversible or irreversible (e.g. when cooking egg white, the white coloration is the result of the denaturation of the egg proteins). The denaturation is the result of the disruption of intramolecular interactions (i.e. hydrogen bonds). Potential denaturation at high temperature is important to consider when proteins are used for emulsion formulation.

Milk proteins have been extensively studied and used to produce food emulsions (Dickinson 1997, Dickinson and Golding 1997). Caseins and whey proteins are the two components of milk proteins, caseins represent 80% of milk protein and whey protein the 20% remaining (Madureira, Pereira et al. 2007).

Caseins are extracted from skimmed milk using different industrial processes. They are made of four different type of proteins with different physicochemical properties; α-casein (αs1 and αs2), β-casein, κ-casein and γ-casein (Modler 1985);

- α-caseins (αs1 and αs2) are found in milk of ruminant but absent in human milk. They represent ~38% of all the caseins in cow milk.
- β-caseins are soluble in the serum at low temperature (below 5˚C).
- κ-caseins stabilise the caseins micelles.

- γ-caseins are the result of enzymatic cleavage of β-caseins.

In milk, these different caseins interact with calcium phosphate ions and form complex aggregates (micelles) that also contain other minor ions and enzymes. Casein micelles are insoluble unlike caseinate salts that are highly soluble in water. Caseinate salts (sodium, potassium or calcium caseinate) are obtained through neutralisation of casein solutions using a base containing the salt followed by drying (Bylund 2003, Sarode, Sawale et al. 2016).

Experimentally the isoelectric point of caseins has been measured between 4.4 and 4.8 (Modler 1985, O'Kennedy 2011).

At their pI they coagulate and precipitate in solution. At acidic pH, below their pI, they have the ability to associate and form gels. At pH above their pI, they are soluble in water and generally result in clear solution depending on their concentration. The interactions between casein micelles are strongly influenced by the pH, the ionic strength and the temperature of the medium.

Whey proteins are also known as serum proteins since they are the proteins fraction remaining in skimmed milk after the extraction of casein micelles. The term "whey proteins" refers to a number of different proteins found in the serum. The main ones are β-lactoglobulins, α-lactalbumins, serum albumins and the immunoglobulins (Deeth and Bansal 2019);

- β-lactoglobulins are the most abundant whey proteins in milk. They represent 50% of the whey proteins and 10 to 12% of all the milk proteins(O'mahony and Fox 2013). They have a highly globular structure that changes conformation with increasing temperature. At high temperatures they denature forming supramolecular structures. Reversible denaturation of β-lactoglobulins occurs at temperature below 60˚C, while

above 60˚C those structural changes are irreversible (Prabakaran and Damodaran 1997, Bhattacharjee, Saha et al. 2005).

- α-lactalbumin is the most heat stable. It is the second whey protein in number (20% of the whey proteins and ~3.5% of all the milk proteins).
- Serum albumin represents 8% of whey proteins (~1.5% of milk proteins)
- Immunoglobulins are the antibodies found in milk of mammals (Hanson and Söderström 1981, Telemo and Hanson 1996, Marnila and Korhonen 2011).

Aside from these main proteins, lactoferrin, osteopontin, glycomacropeptide are proteins found in a lesser amount in the serum.

Experimentally the pI of whey proteins samples has been measured between 4.4 and 5.2 (Bystrický, Malovíková et al. 1990, Bystrický, Malovíková et al. 1991). However, the most reported pI is ~5.2, the pI of β-lactoglobulins, the major protein in whey proteins (Farrell Jr, Jimenez-Flores et al. 2004, Boland 2011).

As mentioned previously, unlike caseins, whey proteins are sensitive to temperature changes. In their studies, *Dissanayake et Al*. (Dissanayake, Ramchandran et al. 2013, Dissanayake, Ramchandran et al. 2013) demonstrated that changing the temperature and the pH of whey proteins solutions at the same time affected their heat-induce denaturation. At acidic pH higher temperatures are needed to induce the denaturation of the proteins.

Proteins present on their molecular chain disparities of polarity, thus they can be used in food products not only as texture modifiers when modifying the processing conditions but also to stabilise emulsions.

2.5.1.2 Emulsion stabilisation

Proteins are widely used as emulsifiers of food emulsions (Dickinson 1997, McCarthy, Kennedy et al. 2016, Tang 2017). They adsorb at the oil/water interface and have the ability to lower the interfacial tension like classic surfactants. The ability of proteins to stabilise oil/water interfaces rest on the difference of polarity in their structure. It is not accurate to state that they have clearly separated hydrophobic and hydrophilic components, the disparities in their structure (the presence of hydrophilic groups and hydrophobic chains on the amino acids) creates a difference of polarity that gives them amphiphilic properties. The hydrophilic and hydrophobic groups are spread all over their primary structure without following a design like surfactants. In their globular conformation, the surface presents random hydrophobic and hydrophilic patches. Therefore, they are not as good as surfactants at lowering the interfacial tension and tend to diffuse at a slower rate at the interface. At the oil/water interface the hydrophobic patches protrude in the oil, while hydrophilic moieties remain in water. However, depending on the pH, charged amino acids would preferentially stay in one phase rather than the other (i.e. charged amino acid would tend to orientate themselves towards the water phase). This change of conformation can potentially affect the dispersed droplets in o/w emulsions.

Nevertheless, they form thicker interfacial membranes, sometimes described as interfacial film, making the resulting emulsions more kinetically stable. The interfacial film forms a gel-like structure through protein-protein non-covalent interactions making them irreversibly adsorbed at the interface. This highly viscoelastic film also acts as physical barrier preventing coalescence. Flocculation, on the other hand is avoided thanks to the charges present in the proteins at the surfaces of the droplets; at pH below or above the isoelectric point of the protein, droplets bearing the same charges undergo electrostatic repulsion enhancing the stability.

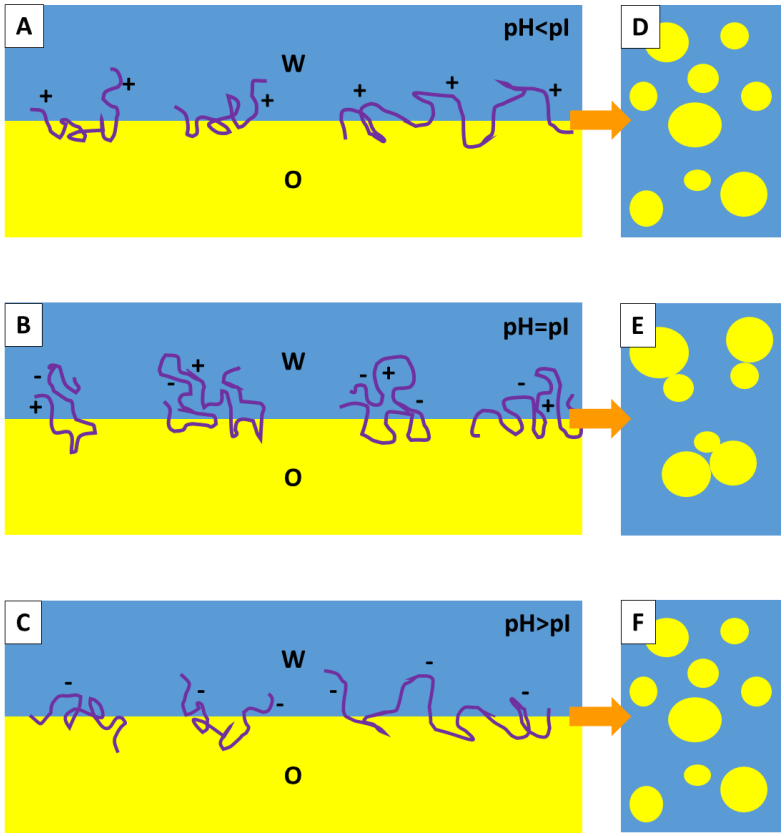


Figure 2-6: Schematic representation of an oil/water interface in protein stabilised emulsions at pH<pI (A), pH=pI (B) and pH>pI (C), and the macroscopic effects in oil-in-water emulsions (D,E,F).

Zeta potential measurements can be used to predict flocculation (and coalescence). Zeta potential gives an idea of the charges surrounding particles in suspension or dispersion, in the case of emulsions, droplets. When the net charges surrounding the droplet are same, repulsion occurs. However, if the ionic strength of the medium changes, the zeta potential also changes. The reduction of the charges reduces the repulsion between droplets enabling their proximity. As explained by the DLVO theory, a change of ionic strength changes the energy barrier (i.e. the droplets can get closer). As the droplets get closer, they enter the range of effect of the Van Der Waals attractive forces leading to flocculation (and coalescence if the interfacial membrane is disrupted).

Contrary to surfactants and their HLB, it is impossible to predict the surface activity of proteins. Even with similar primary structures, two proteins can exhibit different abilities to lower the surface and interfacial tension. This is due to the unpredictable rearrangement of their hydrophilic and hydrophobic moieties when folded into their tertiary or quaternary structures. The main factor that affects proteins surface activity is their ability to undergo conformational changes when put in a different medium and the speed at which it happens. Furthermore the solubility of the protein is a key parameter for the emulsification (Crenwelge, Dill et al. 1974, Kinsella, Melachouris et al. 1976, Voutsinas, Cheung et al. 1983); if a protein shows low solubility the emulsion formed will display early signs of instability (De Wit 1989).

The mechanism of stabilisation of food emulsions by proteins presents another challenge. Indeed, proteins used to form food emulsions are often a mixture of several proteins, whey proteins and caseins are a good example. Thus, the surface activity of these type proteins depends on the proportion of each protein.

2.5.2 Polysaccharides

2.5.2.1 Structure and properties

Polysaccharides are biopolymers consisting of chains of monosaccharides. Like proteins, polysaccharides can be of vegetal (e.g. cellulose, starch, amylopectin, etc…) or animal origin (e.g. chitin, glycogen, etc…). The presence of hydroxyl groups in their molecular structures make them highly hydrophilic (highly water soluble) thus used in the food industry mainly as additives. In solution, polysaccharides increase the viscosity and have the ability to form gels, making them molecules of interest for texturizing purposes.

The high hydrophilicity and the lack of molecular chain flexibility of polysaccharides prevent them from having surface active properties. In rare cases, some polysaccharides can have an effect on the interfacial tension if they bear hydrophobic groups or proteinaceous moieties (Dickinson, Galazka et al. 1991, Funami, Zhang et al. 2007, Sabahelkhier, Yagoub et al. 2008). However, their efficacy to lower the interfacial tension is significantly lower than proteins.

The next section will detail the structure and properties of the main polysaccharides used in this study.

"Dextrins" is a generic term used to describe low molecular weight products of the incomplete hydrolysis of starch (BeMiller 2003). They are characterised by their dextrose equivalent (DE), a number ranging from 0 to 100 used to quantify the reducing power of sugars. The reducing power of glucose is 100% (DE = 100) while that of starch is 0. The dextrose equivalent of dextrins is typically comprised between 1 and 15.

These polysaccharides can be extracted from corn, wheat, potatoes or rice. They are polymers of D-glucose linked through α-(1→4) or α-(1→6) glycosidic bonds. The result is a mixture of linear and branched polymer chains. Dextrins are fully soluble in water and result in solutions with low viscosity in comparison with starch solutions.

Although they are edible products, their use in food products is anecdotal. They are mainly used for the production of paper adhesives (Kennedy and Fischer 1984, Liu, Wang et al. 2011, Mathias, Grédiac et al. 2016).

Sugar beet pectin (SBP) is extracted from a by-product of the sugar refining industry, the sugar beet pulp. Pectin, in general, can be extracted from apple or citrus peels, it is an anionic polysaccharide made of chains of D-galacturonic acid, sometimes L-rhamnose, D-arabinose, D-galactose and other monosaccahrides (Vincken, Schols et al. 2003). Sugar beet pectin is

mostly made of (1→4) partly methyl esterified α-D-galacturonic acid chains at times interrupted with α-(1→2)-L-rhamnopyranosyl units and side chains of galactose and arabinose (Figure 2-6) (Maxwell, Belshaw et al. 2012). Beside this polysaccharide chain, SBP contains in its structure a high amount of proteinaceous material, making it a surface active polysaccharide with excellent emulsifying properties (Funami, Zhang et al. 2007, Ma, Yu et al. 2013). In their study *Zhang et Al.* (Funami, Zhang et al. 2007) highlighted the implication of the proteinaceous moieties in the increased surface activity of SBP.

Because of the presence of proteinaceous moieties and the composition of its chain, SBP does not display gelling properties unlike other the types of pectins (Rejaii and Salehi 2016).

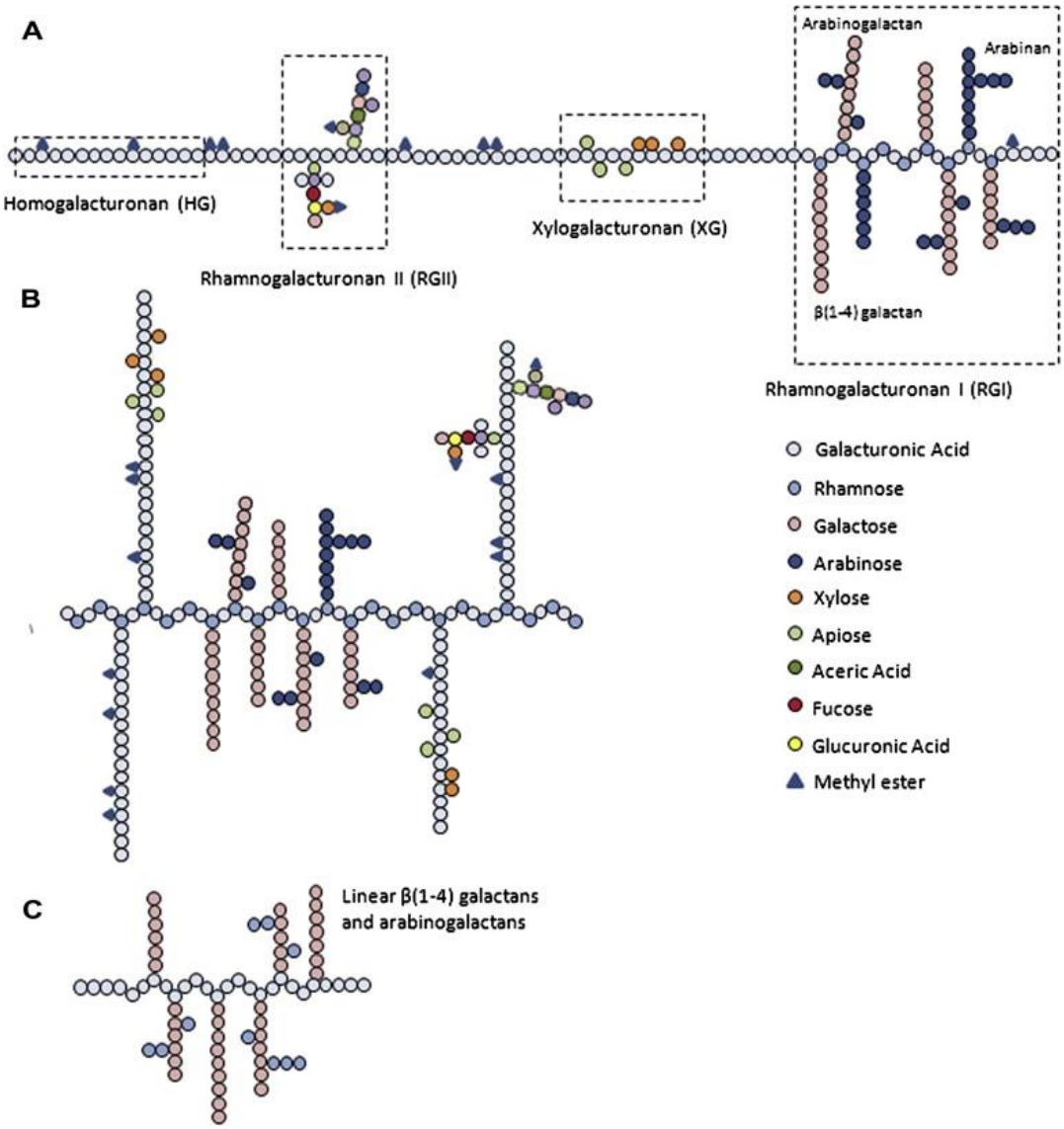


Figure 2-7 : Schematic representation of Pectin structure (Maxwell, Belshaw et al. 2012)

Because of the presence of D-galacturonic acid on their chains, pectins are negatively charged polymers at pH above their pKa (~3 (Cape, Cook et al. 1974)). This characteristic is key for electrostatic stabilisation when used as emulsifiers. Electrostatic repulsion between droplets enhance the stability of emulsions produced using pectin.

2.5.2.2 Emulsion stabilisation

Polysaccharides have two roles in emulsion stabilisation; texturizer (the most common) or emulsifier. As texturizers, they increase the viscosity of the continuous phase and by extension improve the stability of the emulsion, since the stability of an emulsion can be directly linked to its viscosity (Derkach 2009). When the viscosity of an emulsion increases, the droplet movements in the bulk are limited, the probability of droplets interactions decreases at the same time. With a lower probability of droplet collisions, destabilisation mechanisms such as coalescence and flocculation are limited or inhibited.

A few polysaccharides can be classified as “surface active polysaccharides” and therefore have the ability to adsorb at the oil/water interface and lower the interfacial tension and stabilise emulsions. The most popular is *Gum Arabic*.

Gum Arabic surface active properties are provided by proteinaceous moieties in its structure i.e. arabinogalactan, arabinogalactan-protein complex and glycoprotein.

Recently, there has been a growing interest in sugar beet pectin previously presented as it has exhibited a better ability to stabilise emulsions.

2.5.3 Proteins-polysaccharides complexes

Under specific environmental conditions, proteins and polysaccharides can interact and form supramolecular structures of interest. Indeed, depending on the pH, ionic strength or temperature, electrostatic or covalent interactions are possible between proteins and polysaccharides forming structures with improved capacity to adsorb at the oil/water interface and lower the interfacial tension.

2.5.3.1 Electrostatic complexes

There are two identified routes to create electrostatic protein-polysaccharides complexes. The complex can be pre-formed in solution leading to formation of coacervates (insoluble complexes) and soluble complexes or proteins and polysaccharides can be added in order the form layers at the oil/water interface. In both cases, the key parameter for attractive electrostatic interactions to take place is the presence of opposite charges on both biopolymers. For this purpose, the ideal pH must be determined. Moreover, the protein/polysaccharide ratio, the ionic strength, the temperature, the biopolymers molecular weight and concentration as well as the charge density come into play (Schmitt and Turgeon 2011). Electrostatic interactions formed between the two polymers are generally reversible when modifying the environmental parameters (pH, ionic strength, etc…).

Proteins commonly form complexes with polysaccharides when they are charged positively (pH<pI) and the latter charged negatively (pH>pKa, most polysaccharides having a pKa≈3).

The only positively charged polysaccharide used to form electrostatic complexes with proteins is *Chitosan*.

The same process is used to produce emulsions stabilised with a coacervate or a soluble complex. Proteins and polysaccharides are both dispersed into water to create two separated solutions. At this point, the pH can be adjusted to a value where no interaction between the two biopolymers is possible or it can be adjusted at a value where interactions occur before mixing of the two solutions. After mixing, depending of the nature of the hydrocolloids use, coacervates or soluble complexes are formed. From these solutions emulsions can be produced by addition of oil.

Electrostatic proteins-polysaccharides soluble complexes and coacervates were the first type of supramolecular structures reported in the literature. Later another method for the complexation of protein-polysaccharides has been developed. The need for emulsions with increase resistance to environmental changes led to this innovation in interfacial engineering. Emulsions with layered interfaces through in-situ interactions between a protein and a polysaccharide were produced.

The layer by layer (LBL) technique consists in the deposition of alternated layers of oppositely charged proteins and polysaccharides (Guzey and McClements 2006, Dickinson 2008, Bouyer, Mekhloufi et al. 2012, Evans, Ratcliffe et al. 2013). Two possible ways to obtain layered complexes at the oil/water interface exist.

The first route is to produce a primary protein-stabilised emulsion; the pH is then adjusted to a value below the isoelectric point to increase the proportion of NH_3^+ at the surface of the oil droplets. At this pH the polysaccharide is introduced in the continuous phase and complexation occurs on top of the interfacial protein layer forming a second layer around the droplets. The charges surrounding the droplet are reversed allowing the process to be repeated by addition of a biopolymer of the opposite charge. If so, a multi-layered interface is created (Moreau, Kim et al. 2003, Ogawa, Decker et al. 2003, Guzey, Kim et al. 2004).

Alternatively, both proteins and polysaccharides can be introduced in solution before emulsification. In first instance, a protein solution is mixed with a polysaccharide solution at a pH where the two cannot interact (same charge). After addition of oil and shear stress, an emulsion is produced. The only surface active entity in the medium being the protein, the resulting emulsion is stabilised by the protein, the polysaccharide remaining free in the continuous phase. The next step consists of lowering the pH to enable the electrostatic attraction between protein and polysaccharide leading to the deposition of the polysaccharide on top of

the protein layer at the oil/ water interface. Experimentally, this technique showed limitations. Indeed, in their study *Surh et al.* showed that the free polysaccharide interfered with the emulsification process leading to early signs of instability compared with the same emulsion produced without any polysaccharide (Surh, Gu et al. 2005).

2.5.3.2 Covalent complexes

In the hope to effectively combine the surface active properties of proteins with the hydrophilic and texturizing properties of polysaccharides, while mimicking the amphiphilic structures of surfactants, the products of covalent bonding between the two biopolymers have been used to produce emulsions. The most widely reported method to create covalent complexes of protein-polysaccharide is the Maillard reaction. The Maillard reaction (or conjugation), named after the French chemist Louis Camille Maillard, is the reaction between the free amino groups of a proteins and the reducing sugars of carbohydrates happening at high temperature and under humid conditions. This naturally occurring reaction is the reaction responsible for the browning of meat, bread or pastry through cooking and baking. In 1912 the chemist described the reaction as a succession of non-enzymatic reactions. Later, *Hodge* divided this reaction into three consecutive stages;

- The first stage is the reaction between the free Ɛ-amino groups of the protein and the reducing carbonyl groups of the polysaccharide. The amino groups capable of engaging in the condensation reaction are found mainly on lysine and to a lesser extent, histidine or tryptophan. However, some studies reported a possible ability of arginine to take part into the reaction. This stage leads to the formation of a Schiff base that is then converted, due to the presence of water, into molecules called the *Amadori products* (or Heyns

product). This stage is spontaneous and can happen in stored food under mild conditions.

- The second step is the degradation of the Amadori products into a range of different rearranged structures that can lead to the cross linking of the protein. The nature of the by-products obtained at the stage depends highly on the pH of the medium.
- During the last step also referred to as the advanced stage of the Maillard reaction, a large number of by-products are produced. These products are undesirable since some of them are insoluble, lead to off flavours and smells or are potentially toxic.

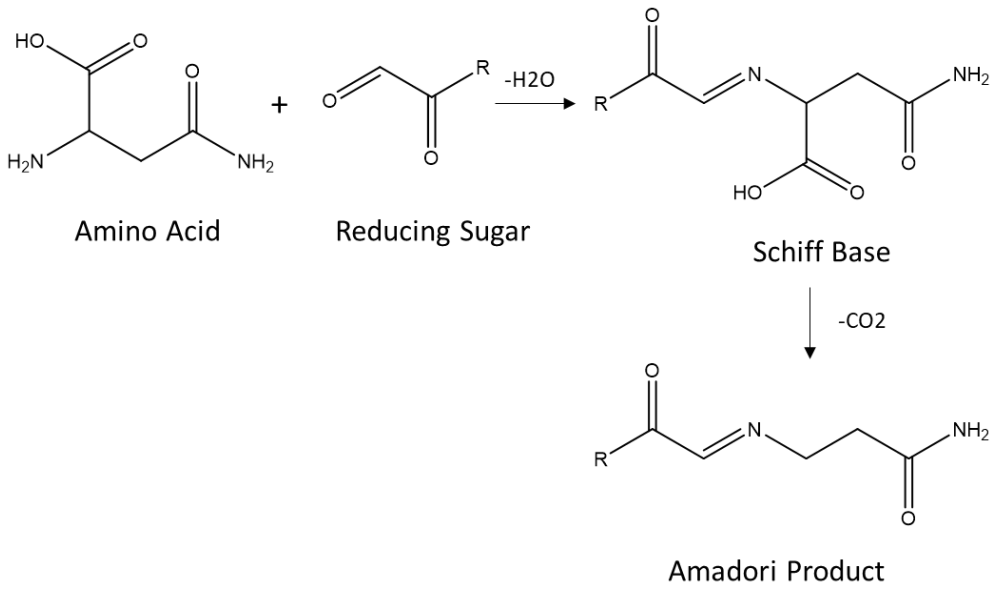


Figure 2-8: Reaction between the ε-amino group of an amino acid and a reducing sugar.

The product of interest in the stabilisation of emulsions is the *Amadori product* obtained at the early stage of the reaction.

Different methods have been proposed to studies the reaction and evaluate its efficiency. NMR, SDS-Page and most recently the OPA-Assay, are all methods that give an idea of the extent of the Maillard reaction.

NMR and the OPA-Assay are quantitative approaches that give an estimation of the efficiency of the reaction by monitoring the number of Ɛ-amino groups before and after conjugation. By opposition, the SDS-page analysis only gives a qualitative answer. This method shows the disappearance of the amino groups however extrapolation on the percentage of reaction is not possible. Other methods such as HPLC have also been used.

Given their structures, the covalent complexes obtained from the Maillard reaction, called Maillard conjugates, are believed to behave like surfactants at the oil/water interface. The hydrophobic moieties of the proteins protrude in the oil phase, while the hydrophilic carbohydrates part stays in the water phase. Emulsions produced using these conjugates have shown increased stability to external stress when compared with the associated protein alone. This increase of stability is mainly due to the thicker interfacial membrane they provide at the oil/water interface.

2.5.4 Functional properties: effects of external stresses

Emulsions stabilised with hydrocolloids have found applications in the food and pharmaceutical industry since the early 90's. For these applications it was crucial to study the effects of environmental conditions and external parameters such as change of pH, ionic strength or temperature. The first studies of emulsions stabilised with proteins focused mainly on the physico chemical properties of the latter. Then, the focus was shifted on the effect of pH and salts (ionic strength) on the stability of the emulsions. In their study, *Hunt et al.* (Hunt and Dalgleish 1996) investigated the effect of KCl on milk protein stabilised emulsions. They showed that the addition of salt induced flocculation for both proteins used, whether it was made before or after emulsification. The salt can also change the composition of the interface.

As the concentration of salt was increased in whey proteins stabilised emulsion, the proteins underwent conformational changes. Due to these changes, progressively α-lactalbumin replaced β-lactoglobulin at the oil/water interface. The combination of pH changes and salt addition was also considered in this study and others (Demetriades, Coupland et al. 2006). Increasing the ionic strength of an emulsion stabilised with whey protein showed to broaden the range of pH at which it was unstable.

On another note, the study of the effects of temperature changes on milk proteins stabilised emulsion, revealed that the increasing the temperature after emulsification induced instability. The presence of free unabsorbed proteins enhancing the instability mechanisms (Liang, Patel et al. 2013).

For food applications, the effects of pH, salt and temperature changes are of great interest. Naturally, when protein-polysaccharide complexes were introduced, the same attention was directed to the effects of these parameters. Emulsions stabilised by such complexes showed increase stability to salt addition and pH (Aoki, Decker et al. 2005, Salminen and Weiss 2014). However, the layer-by-layer technique exposed some limitation to these environmental changes. Indeed, studies showed that the emulsions with layered interfaces had a stability limited to a certain range of pH (Aoki, Decker et al. 2005, Zinoviadou, Scholten et al. 2012). As soon as the pH was adjusted to a value at which the charge of the outer layer changed, it provoked desorption from the interface.

When it comes to stability to temperatures changes, the layer-by-layer method showed increased stability to both freezing and heating in comparison with monolayered emulsions. Nevertheless, a resistance to freezing (tested through freeze-thaw cycling) was only observed with more than two layers of hydrocolloids at the interface. On the other hand, emulsions stabilised with preformed complexes showed no signs of enhanced freezing stability.

Resistance to heat shows the opposite result. Emulsions stabilised with preformed complexes display a better stability to heat than emulsions stabilised with layered hydrocolloids. In their studies using the layer-by-layer technique, *Suk Gu et al*. concluded that the instability to increased temperatures was the result of partial irreversible desorption of the outer layer of ι-carrageenan. The first layer made of β-lactoglobulin underwent conformational changes at high temperatures leading to desorption of the second layer.

The final step of the formation of preformed electrostatic complexes is often a heat treatment (Krzeminski, Prell et al. 2014, Oduse, Campbell et al. 2017), therefore, emulsions stabilised with these complexes demonstrate good heat stability at temperature greater than or equal to the temperature used during heat treatment.

Far less similar studies are available in the literature on Maillard conjugates. A few authors studied the effect of heat treatment and pH changes on emulsions stabilised with the glycoconjugates. Yet, studies investigating the freezing stability or influence of ionic strength are rare (Akhtar and Dickinson 2003, Xu, Yuan et al. 2010).

Maillard conjugates have shown an increased emulsifying capacity at pH close to the isoelectric point of the protein in comparison with the protein taken alone. This observation was attributed to the protection against precipitation provided by the polysaccharide linked to protein. Steric repulsion inhibited the meeting of the neighbouring conjugate particles before emulsification. After emulsification, the steric hindrance around the droplets kept the droplet out of the range of effect of electrostatic forces avoiding droplet flocculation.

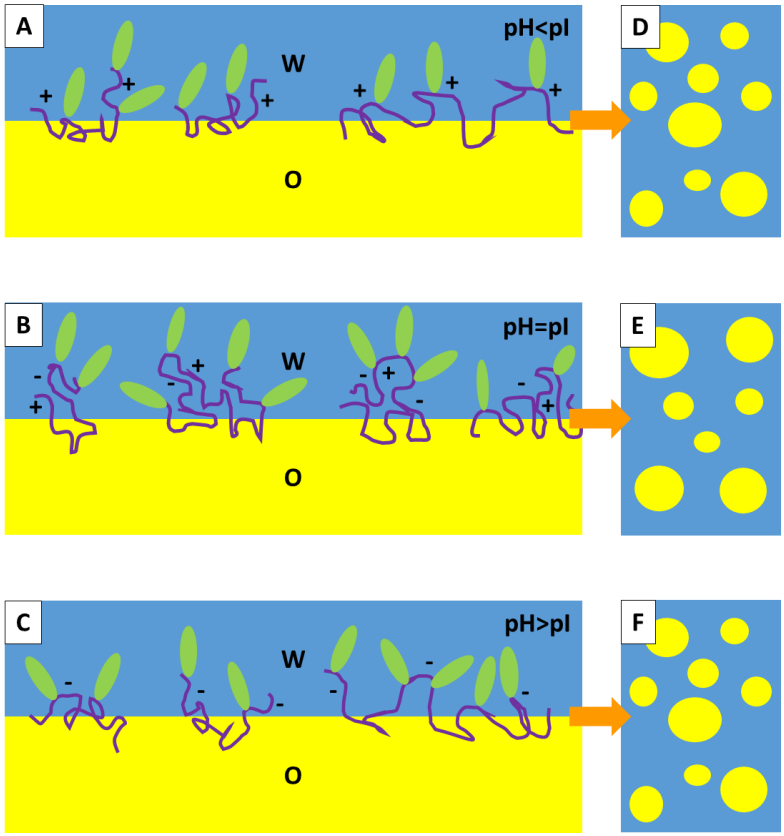


Figure 2-9: ***Schematic representation of an oil/water interface in conjugate stabilised emulsions at pH<pI (A), pH=pI (B) and pH>pI (C), and the macroscopic effects in oil-in-water emulsions (D,E,F).***

Unlike the extensive literature available on the effect of ionic strength on proteins stabilised emulsions, studies on the effect of ionic strength on conjugate stabilised emulsions are rare. In their studies, Setiowati et al., succinctly covered the subject, still their study was focusing on investigating the effects of pH and ionic strength changes on the heat stability.

Emulsion stabilised with the glycoconjugates show enhanced stability to heat. It has been demonstrated by several authors, using different proteins including whey proteins at temperature greater than their denaturation point.

The freeze stability of emulsions mainly rests on the thickness of the interfacial film and the percentage of unadsorbed materials in the continuous phase (O'Regan and Mulvihill 2010, Xu,

Yuan et al. 2010). In the case of multi layered emulsions, the freeze-thaw stability observed with tri-layered emulsions was attributed to the thickness of the interface (Aoki, Decker et al. 2005). Similarly, Maillard conjugates, in theory, provide a thicker interfacial membrane that has the ability to protect the droplets through the stress endured during ice formation in the continuous phase. The use of glycoconjugates as emulsifiers does not enable the retention of the structure after freeze-thawing like the multi-layered emulsions. Nevertheless, the experiments investigating the freezing stability of conjugates stabilised emulsions highlighted that the conjugates enhanced the stability of the emulsions in comparison with the associated protein alone without providing full recovery after thawing.

Overall electrostatic or covalent complexes have shown good potential to be used for specific applications. The current literature, however lacks information on the salt stability of Maillard conjugates, an important knowledge for the production of calcium fortified formulation for instance.

2.6 Dry Emulsions

In most applications emulsions are liquids or pastes. However, in food, cosmetic and pharmaceutical products they can be found as powders. In food, they are creamers, or can be found in instant soup where the fat is emulsified before being dried to be incorporated in the final mixture. By removing the water from the emulsions, the shelf life is increased i.e. the development of mould and the growth of bacteria is inhibited. It is also used as a way to prevent undesirable oil oxidation.

The key challenge faced when drying emulsions is the retention of the microstructure, indeed upon drying the structure and more specifically the interfacial membrane is subject to high stress sometimes leading to its disruption. The failure to maintain the microstructure through drying directly impacts the desired application. To protect the droplets, the interfacial membrane needs to show resistance to temperature changes.

In this section, freeze-drying and spray-drying, the processes used in this thesis, are presented in detail.

2.6.1 Freeze-drying

Freeze-drying is a low temperature, low pressure drying technique relying of the sublimation of ice in a frozen water containing sample. This is a process commonly used to improve the stability of pharmaceutical products i.e. viruses, vaccines, peptides, proteins or for the encapsulation lipophilic molecules i.e. nanoparticles, nanoemulsions. Its use in the food

industry is less reported. Due to its cost and slowness (several days or weeks are sometimes needed to complete drying) compared with other drying techniques, freeze-drying is generally reserved for high value products.

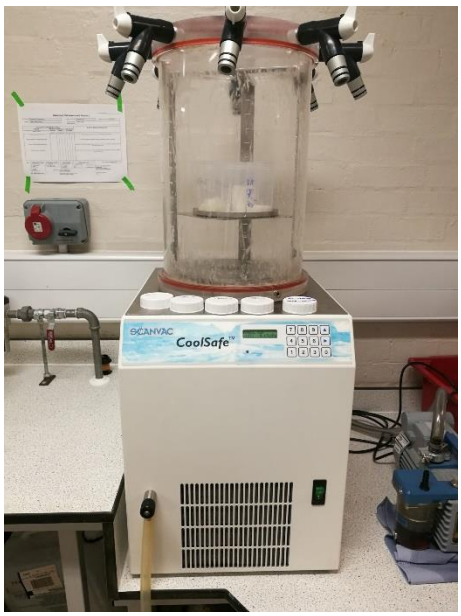


Figure 2-10: Photograph of a freeze-dryer Scanvac CoolSafe (University of Birmingham).

However, the lower temperatures are beneficial for the preservation of bioproducts. Indeed, most proteins and polysaccharides are degraded at high temperature or undergo structural changes affecting their properties (Voutsinas, Cheung et al. 1983, Prabakaran and Damodaran 1997, Dissanayake, Ramchandran et al. 2013, Dissanayake, Ramchandran et al. 2013, Liang, Patel et al. 2013, Wijayanti, Brodkorb et al. 2019).

The entire freeze-drying process from sample preparation to the obtainment of the dry resulting product can be divided into three stages; freezing, primary drying and secondary drying.

2.6.1.1 Freezing

The first step of freeze drying is the preparation of the sample for the process. The water containing sample is frozen at a temperature around -20˚C. For optimal freezing it is important to make sure that the freezing temperature is lower than the freezing point of the sample. Indeed, if the freezing temperature is close to the freezing point of the sample or higher, the unfrozen water inside the product remains in a state close to glass (high viscosity water due to the freezing temperatures). Upon drying, at low pressure, the glassy water boils resulting in undesirable structural changes in the final freeze-dried product.

The freezing mechanism of a liquid material can be divided in four successive stages: supercooling, nucleation, crystal growth and recrystallization.

Supercooling is the decrease of temperature until a liquid material reaches a metastable state before the formation of the first crystals. It is characterised by a glassy state mentioned previously. Phase diagrams only display three states of the matter; liquid, glass and solid. Yet, studies have shown that a portion of a material can stay somewhat liquid under the freezing temperature reported on its phase diagram (for water below 0˚C). At this stage, freezing can be triggered by bringing changes to the system (e.g. vibrations, addition of substances affecting the freezing point).

The rate of cooling affects the temperature at which the liquid stays in the supercooled state. In the case of water, at slow cooling rates, it remains liquid a few degrees below 0˚C. If the cooling happens quickly, the water stays in a glassy metastable state until it reaches temperature close to -42˚C or below if the parameters of the system are altered (Gallo and Stanley 2017).

Nucleation describes the formation of crystals when a product in a supercooled state is frozen below its melting point. The molecules of the liquid start to gather in clusters that progressively aggregate. Over time the growing aggregates form larger architectures orientated in a predefined manner giving the crystalline structure of the solid. Two types of nucleation have been identified; homogenous or heterogeneous nucleation. Homogenous nucleation happens spontaneously when no predefined nucleation site exists in the liquid. Typically ice formation in pure water takes place through homogenous nucleation. Heterogeneous nucleation defines the formation of crystals triggered by impurities or physical disturbance. The addition of salt, presence of dust, or any other solute provides a nucleation site where the nucleation process can start. In food products some ingredients can be used to control the nucleation process. Slowing down or accelerating the nucleation process can result in the formation of larger or smaller ice crystals or increase their number. Controlling the nucleation in a product destined to be freeze-dried can help controlling the porosity of the final product.

After nucleation the ***crystal growth*** happens. The nuclei formed during nucleation start growing to form crystals. The crystal growth will continue until the system approaches an equilibrium and then stop.

In the case of water solutions, the presence of solutes will alter the crystal growth. As they increase in size, the crystals push away the solutes in the remaining unfrozen water. At the end of the process, a part of the water stays unfrozen, resulting in an ice crystal phase and an unfrozen water phase concentrated in solutes. Locally this increase of concentration (the solutes can be salts, hydrocolloids, alcohol, etc…) induces changes of physico chemical properties of the water, i.e. pH, ionic strength or viscosity.

Similar to the nucleation, the crystal growth can be controlled by adjusting the cooling. Typically, slow cooling will lead to a slow formation of nuclei allowing the crystal to grow. The final product will contain a small amount of large crystals.

Fast cooling leads to rapid nucleation, a lot of nuclei form at the same time limiting the space available for crystal growth. The final product will contain a large amount of small crystals.

Once again, in a product destined to be freeze-dried, the ice structure will affect the structure of the final freeze-dried product.

Recrystallization happens in case of prolonged storage of the frozen sample. The shape, size and number of crystal changes over time due to physico chemical changes in the system. The crystals can aggregate and large crystals continue to grow at the expense of smaller crystals.

In emulsified products, the freezing step has been identified as a crucial step to allow the retention of the structure when the food is thawed after storage at low temperatures. During freezing several events can lead to instability of the thawed emulsion. While expanding, the forming ice can force the oil droplets together leading to the fusion of the interfacial membranes of neighbouring droplets causing irreversible droplets aggregation. The same phenomenon can lead to coalescence if the interfacial membrane is disrupted and at least two droplets merge together. Freezing also makes less water available to keep the emulsifier hydrated at the droplet surface, the emulsifier slowly desorbs from the surface and coalescence occurs. If the fat crystallises before the water freezes the fat crystals can protrude through the interfacial membrane causing its disruption, this can cause coalescence in the thawed emulsion (Degner, Chung et al. 2014).

2.6.1.2 Primary drying

Once a product has been frozen, the water is removed by sublimation; at low pressure, generally between 0.05 to 0.5 hPa, the ice transitions from solid to vapour. The sublimation progresses from the surface to the centre of the sample. The water vapour moves through the sample by diffusion or convection. At the centre, the vapour diffuses through the layers of already dried product to the surface. The size of the pore influences the rate of water diffusion. Smaller pores make it difficult for the vapour to navigate to the surface, while larger pores allow the water to be eliminated quickly. As mentioned in the previous section, the pore size is directly linked to ice crystal size which can be controlled by the freezing rate. The speed of sublimation is thus indirectly linked to the freezing rate. For this reason, the primary drying is the longest part of the freeze-drying process.

Another parameter influencing the diffusion of the vapour through the solid is the pressure. The pressure is the driving force of the water vapour diffusion. Lower pressure leads to faster sublimation.

Since the sublimation begins at the surface of the frozen product, the surface area is also a parameter that can tremendously affect the efficiency of the drying.

The last parameter to consider is the temperature. The temperature must be maintained at a temperature below the glass transition temperature T_g of the system. If the system starts to melt, and reaches a temperature called the “collapse temperature” (T_c), the structure is lost during the drying process.

2.6.1.3 Secondary drying

The secondary drying corresponds to desorption of the remaining water molecules from the surface of the product. This step is governed by the adsorption/desorption equilibrium of the system. At the end of this step the moisture content percentage is reduced to an optimal value for long term storage.

The surface area is also an important factor to consider for this, since desorption occurs mainly at the surface of the sample; the greater the area the lower moisture content in the resulting dry product.

2.6.2 Spray Drying

Spray-drying is currently the most used encapsulation technique in the food industry and it is also widely used for the encapsulation of actives in pharmaceutical and nutraceutical industries. The technique consists in exposing dispersed or dissolved materials to a flow of hot air. When entering in the hot air flow, the water evaporates converting the atomised droplets into fine dry particles. Owing to its speed (the water evaporation occurs within seconds) spray drying is a less costly process in comparison with freeze-drying. The evaporation step takes place at the surface of the droplets, the variation of temperature and partial pressure of water vapour makes it almost instantaneous (Schuck 2008). The spray drying process can be divided into three steps; atomisation, water evaporation and powder recovery.

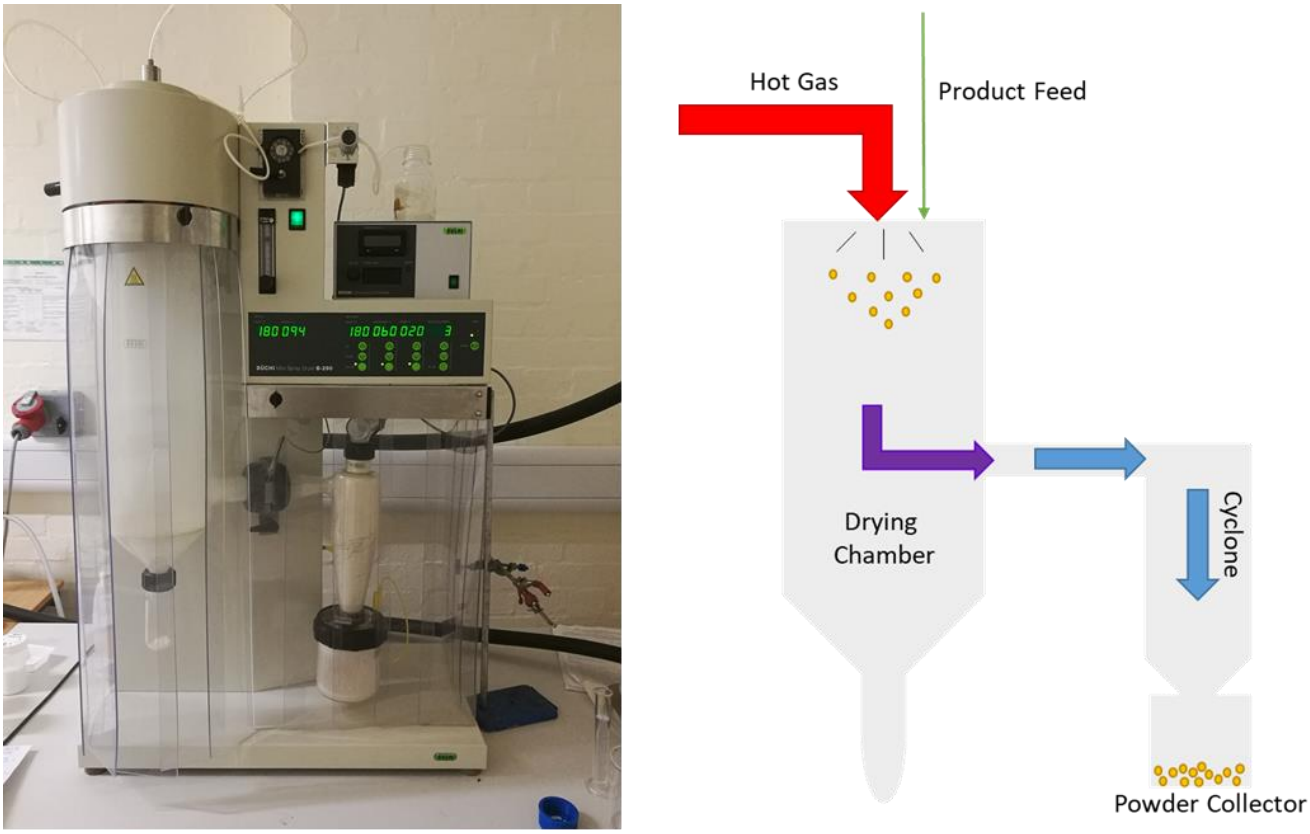


Figure 2-11: Photograph (left) of a Buchi Mini Spray DryerB-290 (University of Birmingham) and schematic representation (right) of the spray drying process.

2.6.2.1 Atomisation

The atomisation is the first step of the spray drying process. The feed solution is atomised into microdroplets. The main driving force for atomisation is pressure or centrifugal forces. The main purpose of this step is to create the highest surface area possible to enter in contact with the flow of hot air and consequently optimise the drying. The size of the droplets after atomisation not only depends on the surface tension and viscosity of the solution, but also on the pressure and velocity of the spray. Ultimately the size of the droplets will be a key parameter for the drying efficiency. Therefore the preparation of the feed solution is an important step indeed the viscosity and particle size will greatly impact the size of the atomise droplets (Fang and Bhandari 2012).

2.6.2.2 Water/solvent evaporation

The evaporation of the solvent occurs at the same time as the atomisation. As they are atomised the droplets enter in contact with a flow of hot air. Depending on the orientation of the air flow, the drying can be described as co-current drying or counter-current drying. In co-current, the direction of droplet atomisation and the flow of air are the same. In opposition, in case of counter-current drying, the direction of the droplet atomisation and hot air flow are opposite. For the drying of most food ingredients, co-current drying is used. Because the air is blown from the top of the equipment, although the inlet air temperature is typically between 150-200˚C (depending on the solution), the temperature of the dry powder stays relatively low (between 50-80˚C) (Fleming 1921).

As the heat travels from the hot air to surface of the droplet, the difference of partial pressure of water between the droplet and the air flow induces the evaporation. The water migrates from the core of the droplet to its surface, where it evaporates.

The drying kinetics are governed by three factors (Schuck 2008);

- The evaporation surface area directly linked to size of the particles.
- The difference of partial pressure of the water between the air and the droplet previously mentioned. An increase of the air humidity can slow down the drying process.
- The rate of migration of water from the centre of the droplet to its surface.

2.6.2.3 Powder recovery

Powders are separated based on their density in the drying chamber. Depending on the design of the equipment, the dense particles fall into the base of the drying chamber whilst the finer

particles travel to the cyclone and can be recovered at its end. The moisture content of the final powder is influenced by the difference of temperature between the inlet air and the outlet of the spray dryer. Studies using spray drying have shown that higher outlet temperatures (i.e. small difference between inlet and outlet temperatures) led to lower moisture content and water activity values.

To tailor the powder resulting from spray drying both external and processing parameters need to be adjusted. The solution needs to be prepared mindfully, with an adequate viscosity, particle size, composition (i.e. wall material, emulsifier in the case of emulsions, etc…). The parameters of the spray drying can as well help predict the final particle size distribution, the yield of drying and final moisture content of the dry product.

parameter / dependence	aspirator rate ↑	air humidity ↑	inlet temperature ↑	spray air flow ↑	feed rate ↑	solvent instead of water	concentration ↑
outlet temperature	↑↑ less heat losses based on total inlet of energy	↑ more energy stored in humidity	↑↑↑ direct proportion	↓ more cool air to be heated up	↓↓ more solvent to be evaporated	↑↑↑ less heat of energy of solvent	↑↑ less water to be evaporated
particle size	-	-	-	↓↓↓ more energy for fluid dispersion	(↑) more fluid to disperse	(↓) less surface tension	↑↑↑ more remaining product
final humidity of product	↑↑lower partial pressure of evaporated water	↑↑ higher partial pressure of drying air	↓↓ lower relative humidity in air	-	↑↑ more water leads to higher particel pressure	↓↓↓ no water in feed leads to very dry product	↓ less water evaporated, lower partial pressure
yield	↑↑ better separation rate in cyclone	(↓) more humidity can lead to sticking product	(↑) eventually dryer product prevent sticking	-	(↓↑) depends on application	↑↑ no hygroscopic behaviour leads to easier dying	↑ bigger particles lead to higher separation

Figure 2-12: Summary of spray drying parameters and their influence on the spray drying process and powder quality (Buchi)

2.6.3 Re-dispersible dry emulsions

The ability of a dry emulsion to be reconstituted after exposure to an aqueous solution presents potential applications in food and pharmaceutical products.

Generally, an emulsion is considered reconstituted if its structure (droplet distribution) is maintained through drying and rehydration. However, for specific applications such as in vivo drug delivery, the encapsulation activity has to be considered. Equally, for food applications the viscosity (rheology) and traction coefficient (tribology) to correlate to mouthfeel are parameters that should as well be restored after rehydration.

Rheology is the method used to study and characterise the flow of a material. Different properties can be studied (e.g viscoelastic properties with the measurement of the storage *G'* and loss moduli *G"* or the apparent viscosity η of a system). The shear viscosity or the shear stress are typically measured as function of the shear rate. Shear-thinning behaviour is the rheological behaviour most described in emulsions. The shear viscosity decreases as the shear rate increases. Higher shear viscosities are witnessed at low shear rates. However, NaCN stabilised emulsions are known for displaying Newtonian behaviour; the apparent viscosity is independent on the shear rate.

Information on the rheological behaviour of emulsions (shear-thinning or Newtonian) is key for certain application but also for the transport and distribution of emulsion based products.

In emulsions rheological behaviour and viscosity are greatly influenced by the nature of the oil/water interface but also by the composition of the bulk (Derkach 2009).

On the other hand, tribology can be used to correlate to mouth feel. Indeed, tribology is the science that studies friction, lubrication and wear. The instrument measures the friction of a ball

rolling onto the surface of a disc immerged into the fluid of interest (e.g. emulsions). More specifically the traction coefficient (sometimes called friction coefficient) is measured. The action of the rolling ball onto the surface of the disc is correlated to the movements of the tongue in the mouth. Nevertheless, the information giving by these measurements before and after drying and rehydration of emulsions is just indicative of the fluid friction. The authentic mouthfeel cannot be deducted from these results.

Considering the retention of the structure and the restoration of the physicochemical properties, the choice of the drying process together with the choice of a suitable emulsifier is a determining factor if the emulsion needs to be re-dispersed.

For the structure to be retained, the emulsifier must undergo minimal chemical and physical changes through drying; it has to form a strong enough barrier at the oil/water interface (mechanical barrier), but also resist the changes in temperature brought by the drying process.

For retention and restoration of the physicochemical properties, the emulsifier must be chemically stable explicitly, resist the changes of temperatures it undergoes during drying. Indeed, most proteins and polysaccharides undergo structural changes at high temperature. When using spray drying for instance, the plasticisation of hydrocolloids can directly affect the physicochemical properties of the dispersion they form in solution (Roos 2002, Drapala, Auty et al. 2017).

The dispersibility specifically of dry emulsions has rarely been thoroughly studied. Nevertheless, the dispersibility of spray-dried powders in aqueous medium started to be investigated in the late 50's, with the study of dried milk dispersibility in water (Bockian, Stewart et al. 1957).

From a physical point of view, dry emulsions when re-dispersed in water behave like particles. Therefore, to study redispersion of dry emulsions it is valid to apply the existing knowledge on powder dispersion.

A solid dispersion describes the distribution of soluble or partially soluble particles in a liquid medium (Kissa 1999). Similarly to emulsions, the continuous phase corresponds to medium in which the powder (the dispersed phase) is dispersed. In the case of emulsions, the powder is soluble since the outer surface of the particle is mainly composed of the emulsifier.

The action of dispersing a solid particle in a medium is characterised by the dispersibility; the ease with which the powder is dispersed in the medium (Kissa 1999). In the case of emulsions, *Klinkesorn et al.* measured the dispersibility of dry emulsions by monitoring the evolution of the droplet size of a dry emulsion being rehydrated (Klinkesorn, Sophanodora et al. 2006). The emulsion was considered fully rehydrated when the droplet size remained constant overtime.

The most important physical properties of the dry particles are their shape, size, density, rigidity, the surface area and the morphology of the surface (Kissa 1999).

Once a powder is poured onto the surface of a liquid its dispersion mechanism can be divided into four steps;

- Wetting
- Penetration
- Dispersion
- Dissolution

These steps can occur successively but most of the time they happen at the same time.

The ***wetting*** happens when the powder comes into contact with the liquid. Capillary forces drive the liquid through the particles. The interparticular porosity is a key parameter of this step. The wetting rate is governed by the affinity of the powder for the chosen liquid. Characteristically, if the solid-liquid interactions are stronger than the solid-solid interactions this step will happen spontaneously.

Under the action of gravity, the particles eventually penetrate the surface. The ***penetration*** is governed by the density of the particles; when the density of the particles is higher than the density of the liquid, the penetration is spontaneous.

Once the particles are past the surface barrier, they are free in the liquid medium and the ***dispersion*** occurs. At this step, the aggregates and agglomerates are broken down into dispersed individual particles.

The ***dissolution*** happens when the particles are soluble in the medium. Chemical interactions (hydrogen bonds, ionic interactions) occur between the particles and the medium.

Bringing this theory back to the dispersibility of dry emulsions, in addition to the physical properties mentioned previously as important factors, the wettability of the dry emulsions and solubility of the emulsifier are parameters that will affect the rehydration.

2.6.3.1 Systems, processes and current knowledge

The first publications reporting drying of oil-in-water emulsions were published as early as the beginning of the 70's. However, the drying and rehydration of oil-in-water emulsions started to be reported in the mid 90's (Fäldt and Bergenståhl 1995, Fäldt and Bergenståhl 1996). At this time emulsifiers used to produce those emulsions were generally surfactants or proteins often

associated with a hydrophilic wall material that would form a continuous matrix between the droplets in the bulk powder (Fäldt and Bergenståhl 1996). The most frequently reported drying technique was spray drying and still is to this day. In the majority of these studies the attention was focused on the encapsulation efficiency of the dry powders (Gejl-Hansen and Flink 1978, Fäldt and Bergenståhl 1996, Klinkesorn, Sophanodora et al. 2006, Li, Wang et al. 2015). As mentioned previously, the main application of dry emulsion is encapsulation in both the food and pharmaceutical industry. The authors focused on the lipid oxidation of the lipid phase, and in case of re-dispersion of the powder, on the lipid oxidation of the oil after rehydration. The storage stability was also a point of focus. Since the goal when drying emulsions is to enable longer storage period, naturally, the storage stability of the powder is a parameter to study. For this purpose, the powders are generally stored in different conditions (e.g. high temperatures, high humidity), and studied afterwards. A few studies investigated the effect of storage on the ability of an emulsion to be rehydrated. After storage and rehydration, the authors observed that the emulsions showed signs of aggregation or coalescence (Fäldt and Bergenståhl 1996).

Beside the use of surfactants and proteins, that in some cases showed a mediocre ability to effectively encapsulate the oil, the use of protein-polysaccharides complexes has been more and more reported since their introduction in food technology. The layer-by-layer technique described in a previous section has attracted a lot of interest for this specific application (Klinkesorn, Sophanodora et al. 2006, Serfert, Schröder et al. 2011, Serfert, Schröder et al. 2013). When using spray drying, the layered interfacial membrane displayed a better resistance to the process, and a better ability to encapsulate oils however wall materials were still needed (Klinkesorn, Sophanodora et al. 2006).

Lately, Maillard conjugates have been used to produce spray dried oil-filled powders and re-dispersible emulsions (Drapala, Auty et al. , Drapala, Auty et al. 2017, Toikkanen, Outinen et al. 2018). The conjugates displayed a better ability to encapsulated oil than protein or layered

hydrocolloid interfaces without the need for a wall material. In a recent study, the conjugation of whey protein hydrolysate to maltodextrin not only allowed to decrease the surface oil percentage of the spray dried powders but also resulted in reconstituted emulsions after rehydration, once again without any wall material (Drapala, Auty et al. 2017).

In the available literature it is easily noticeable that spray drying is the main drying technique used to produce oil filled powders. Freeze-drying, as mentioned previously, is a method usually reserved for high value products due to its cost and less damaging operating temperatures, for this reason its use for food applications is less common than for pharmaceutical applications. Naturally when investigating this process, the focus was on the encapsulation efficiency and the stability of the powders over time (i.e. oil oxidation) (Gejl-Hansen and Flink 1978, Heinzelmann and Franke 1999).

Freeze-drying being a multistep process, some studies also looked at the different steps of the process and their effect on the physical aspect, the encapsulation stability and the oxidation stability of the powders (Marefati, Rayner et al. 2013). Freezing can be a source of destabilisations of emulsified products; therefore, the influence of different freezing rates and the use of cryoprotectants were two parameters of interest to those investigating this process. In their study, *Heinzelmann et al.*, looked at the effect of freezing rate and the nature of the wall material on the oil oxidation stability. They observed that the freezing rate had an effect on the oxidation stability of the resulting powders (Heinzelmann, Franke et al. 2000).

More recently, *Iyer et Al.* reported that emulsions containing sucrose as cryoprotectant showed a limited droplet diameter increase after freeze-drying and rehydration in comparison with ones containing trehalose or a mixture of trehalose and mannitol (Iyer, Cayatte et al. 2017).

2.7 Conclusions and Remarks

Studies about dry emulsions produced through different drying processes are available in the literature however the same emulsifiers are often used. The protein-polysaccharide complexes have offered a new option that has been investigated. The layer-by-layer technique and the use of Maillard conjugates to stabilise oil-in-water emulsions to be dried, has attracted interest due to the thicker interfacial membrane they form at the oil/water interface. The increased stability of oil-in-water emulsions to external stresses was another indicator of the potential suitability for drying applications.

The studies demonstrated that when using Maillard conjugates, wall materials were not needed in the formulation. And oil-filled powders showed better encapsulation efficiency than when other emulsifiers were used. Despite the fact that these emulsions displayed an increased stability to ionic strength or pH modifications, the effects of these parameters on drying are still unknown. Additionally, there is a gap of knowledge on the effect of the addition of salts to oil-in-water emulsions stabilised with the glycoconjugates. The few studies available focused only on the combined effects of salt and pH changes.

Moreover, even fewer studies propose to establish a link between the drying process, the choice of emulsifier and the ability of an emulsion to be reconstituted after rehydration.

Thus, there are still areas in which knowledge is lacking for the study of both oil-in-water emulsions produced with Maillard conjugates and re-dispersible emulsions.

Chapter 3

Conjugates Formation and Emulsifying Properties in Oil-in-Water Emulsions

3.1 Introduction

As mentioned previously in the literature review, the study of the effects of external stresses on emulsions stabilised with Maillard conjugates need further investigation.

The aim of this chapter was on one hand to offer a comparison between the glycoconjugates and their associated proteins in term of emulsifying properties and resistance to external stresses (ionic strength and pH changes, and temperature changes). On the other hand, to add new data to the currently available literature on conjugate stabilised emulsions.

The effects of different protein/polysaccharide ratios and parameters such as the pH were firstly investigated. The protein/polysaccharide ratio and pH have been identified as major factors influencing the conjugation efficiency of the Maillard reaction (Ajandouz, Tchiakpe et al. 2001, Cerny and Briffod 2007, O'Regan and Mulvihill 2009, Arachchi, Kim et al. 2017). Additionally, in this study sugar beet pectin was used. Depending on the pH used for the preparation of the conjugates, sugar beet pectin would bear positive or negative charges leading to electrostatic interactions with the oppositely charged whey protein. The formation of electrostatic complexes prior to conjugation was thus expected. The potential for these complexes to affect the ability of the polysaccharide moieties to undergo conjugation was also assumed.

Since an increased interfacial thickness in conjugate stabilised emulsions is one of the main motives for the use of Maillard conjugates, the thickness of the conjugated layer was measured. The results from these measurements were compared with the results from the measurements of the thickness of protein layers.

In the context of the present study a significantly higher thickness would confirm the adequacy of the choice of conjugates for the stabilisation of emulsions mechanically resistant to drying.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Proteins

Casein sodium salt from bovine milk and egg white protein were purchased from Sigma Aldrich, UK. Whey protein isolate was ordered from Volac.

3.2.1.2 Polysaccharides

Dextrin from corn starch (DE ≤ 5.0. Mw = 7 kDa), maltodextrin (DE = 4.0-7.0, Mw = 3.6 kDa) and guar gum were purchased from Sigma Aldrich, UK. Sugar Beet Pectin (degree of esterification >50%, Mw = 45.3 kDa) was purchased from Herbstreith & Fox KG (named Betapec RU 301).

3.2.1.3 Other materials

The sunflower oil (Solesta) used for the production of oil-in-water emulsions was purchased from a local store.

The O-phtaldialdehyde and the polystyrene latex beads (100 nm) were purchased from Sigma Aldrich UK.

3.2.2 Methods

3.2.2.1 Preparation of the conjugates

All conjugates were prepared as follow; protein and polysaccharide were dissolved separately in deionized water at a concentration of about 10% (w/v) and stirred overnight. The two solutions were then mixed together under magnetic stirring for an hour. The mixture was then frozen at -22˚C for at least 24 hours and lyophilised for 4 days.

To carry out the Maillard reaction, the dried mixture was placed in a desiccator over a saturated solution of KBr to ensure a relative humidity of 79%, and heated at 60˚C for 96 hours. After which the conjugates were placed in a freezer at -22˚C for 24 hours to stop the reaction. The conjugate was then freeze dried for 24 hours before being used for experiments.

Table 3-1: List of protein and polysaccharides used for the preparation of conjugates

Protein	Polysaccharide
Egg Protein	Guar Gum
Sodium Caseinate	Maltodextrin
Whey Protein Isolate	Dextrin
	Dextran
	Sugar Beet Pectin

3.2.2.2 Characterisation of the conjugates

3.2.2.2.1 O-pthaldialdehyde assay

The conjugation efficiency was assessed by comparing the amount of free amino groups in conjugated samples with a protein sample, using the OPA assay *(Pan, Mu et al. 2006)*. A mixture of 80 mg of o-phtaldialdehyde (OPA) (dissolved in 2 mL of a 95% ethanol solution), 50 mL of borate buffer pH 9.5, 5 mL of a 20% sodium dodecyl sulphate solution and 0.2 mL

β-mercaptoethanol was prepared and adjusted to a final volume of 100 mL. Conjugates solutions were prepared with a 3 $mg.mL^{-1}$ protein concentration. The samples were made by adding 0.1 mL of conjugate solution to 2.70 mL of OPA solution, and allowed to incubate at room temperature for one minute.

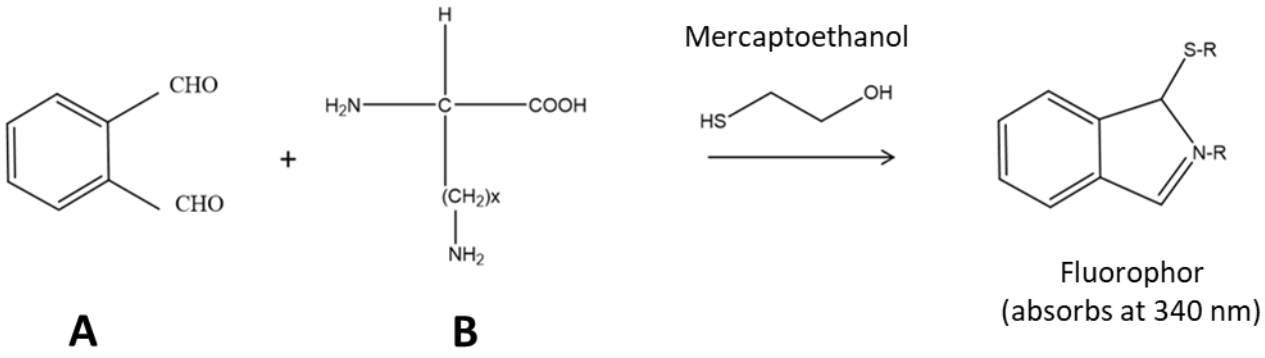


Figure 3-1: Reaction between o-phtaldialdehyde (a) and protein amino groups (b)

An absorbance scanning between 330 and 350 nm was then performed on the samples using a UV-Visible Spectrometer Orion Aquamate 8000 from Thermo Scientific. The conjugation efficiency was calculated with the absorbance at 340 nm using the following equation:

$$\%\ conjugation = \left(1 - \frac{\text{A after conjugation}}{\text{A before conjugation}}\right) \times\ 100 \qquad \textit{Equation 3-1}$$

Where A is the absorbance.

3.2.2.2.2 Conjugates particle size measurements

The conjugate particle size was measured using dynamic laser diffraction measured with a Mastersizer 2000 (Malvern Instruments UK). Drops of ~10% w/w conjugate solution were dispersed in the equipment. The measurements displayed represent an average of three distinct

measurements from three different solution samples. The error when mentioned corresponds to the standard deviation of these averages.

3.2.2.3 Emulsifying properties of the conjugates

3.2.2.3.1 Interfacial tension measurements

The interfacial tension (IFT) between sunflower oil and protein (or conjugate) solutions was determined at room temperature using the Wilhelmy plate method on a K100 Tensiometer from Krüss, Germany.

The protein (or conjugate) solutions were prepared with an increasing protein concentration, 0.03%, 0.08%, 0.17%, 0.25%, 0.33%, 0.83% w/w for NaCN and CN-De and 0.07%, 0.17%, 0.33%, 0.5%, 0.67%, 1.67% w/w for WPI and WPI-SBP. The sunflower oil was poured at the surface of the solution in the vessels using a pipette. Data points were taken at 1400 minutes, value at which interfacial tension equilibrium was achieved. Measurements were performed in triplicate on the same or different solutions. Error bars correspond to the standard deviation of the average of these measurements.

3.2.2.3.2 Interfacial membrane thickness measurements

The interfacial membrane thickness was measured using a technique inspired by the one described by *Wooster and Augustin* (Wooster and Augustin 2006). The conjugate interfacial thickness was measured by adsorbing the Maillard conjugate onto the surface of monodisperse polystyrene latex beads. The variation of hydrodynamic diameter was measured using dynamic light scattering (using a Zetasizer from Malvern Instruments UK). The value for the interfacial

thickness was taken as the different of hydrodynamic diameter between the naked beads and the conjugate coated ones. Samples for these experiments were prepared in buffer 20 mM Tris/HCl 30 mM NaCl containing 0.15% latex bead. The buffer solution was added to the desired protein/conjugate to avoid packing at the surface of the polystyrene beads (Wooster and Augustin 2006). All samples were prepared from 1% w/w conjugate solution divided in three separate samples for each data point.

3.2.2.3.3 Optical microscopy

The microstructure of the emulsions was observed using an optical microscope Leica DM 2500 LED equipped with a camera LEICA DFC 450 C. The images were collected with the software Leica Application Suite V 4.8. The 40x or 100x objective lenses were mainly used for the obtainment of the images featured in this study.

3.2.2.3.4 Surface coverage

Emulsion samples containing 2g of conjugate were centrifuged at 20000 rpm for 20 minutes at 20 °C using a centrifuge Sigma 3K30 centrifuge (Sigma Laborzentrifugen). The cream layer was discarded with a syringe. The emulsion was centrifuge until most of the dispersed phase was withdrawn. The continuous phase was then successively filtered with 0.45 μm and 0.32 μm filters to eliminate the remaining oil droplets. The unadsorbed mass of conjugate was determined by freeze-drying the samples.

The surface coverage Γ_s was calculated using the following equation:

$$\Gamma s = \frac{Ca}{S} = \frac{Ca \times D3,2}{6\Phi Ve}$$

Equation 3-2

Given in $mg.m^{-2}$

Where C_a is the concentration of adsorbed emulsifier, $D_{3,2}$ the surface mean diameter of the droplets, Φ a constant detailed below and V_e the volume of emulsion.

With $$\Phi = \frac{\Phi_m \rho 1}{\rho 1 \Phi_m + (1 - \Phi_m)\rho 2} \qquad \Phi m = \frac{mD}{mE}$$

Where ρ_1 is the density of water, ρ_2 the density of oil, m_D the mass of oil droplets and m_E the mass of emulsion.

3.2.2.3.5 Emulsion preparation

All emulsions were prepared following the same protocol. Conjugates, protein or a mixture of protein and polysaccharide were dissolved in 80 g of distilled water; 0.08 g, 0.2 g, 0.4 g, 0.6 g, 0.8 g and 2 g for the conjugates, 0.05 g, 0.13g, 0.27 g, 0.4 g, 0.5 g and 1.33 g for WPI and 0.03 g, 0.07 g, 0.13 g, 0.2 g, 0.27 g and 0.67 g for NaCN. The mixture was put under magnetic stirring for 1 hour after which 20 g of sunflower oil were added and the temperature increased to 50 ˚C. NaN_3 (0.01% w/w) was also added to prevent bacterial growth and increase long term stability. The mixture was stirred for another 30 minutes.

Pre-emulsions were made using a high shear mixer Silverson L5M (10 000 rpm for 15 s).

The final emulsions were produced by passing the pre-emulsions three times through a microfluidiser (Microfluidics model M-110S) at a pressure of 1250 bars.

The droplet size was measured using laser diffraction with a Malvern Mastersizer 2000.

3.2.2.3.6 Storage stability

Emulsions were placed in the oven at 40 °C for a week.

3.3 Results and Discussion

Several protein/polysaccharide couples were assessed and the conjugation efficiency measured. Sodium caseinate, whey protein isolate and egg white protein were chosen. While the polysaccharides tested were guar gum, maltodextrin, dextrin and dextran. Table 3-2 displays the results from conjugation efficiency measurements.

Table 3-2: Conjugation efficiencies of conjugates at different protein/polysaccharide ratios

Ratio (wt:wt)	Conjugation efficiency (%)
Sodium caseinate-guar gum	
2:1	36.86
1:1	23.04
1:2	7.06
1:4	0
Sodium caseinate-maltodextrin	
2:1	53
1:1	44.79
1:2	38.63
1:3	6.94
1:4	0
Sodium caseinate-dextrin	
1:2	88.37
Whey protein isolate-dextrin	
2:1	27
1:2	42.61
1:3	44.37
1:4	43.9
Whey protein isolate-maltodextrin	
2:1	2.3
1:4	6.34
Whey protein isolate-sugar beet pectin	
2:1	76.03
Whey protein isolate-dextran	
1:2	42.02
1:3	40.14
1:4	34.74
Egg white-guar gum	
2:1	12.73
1:1	24.85
1:2	0

The different proteins were chosen for their conformation in solution. Indeed, egg white protein and whey protein isolate are mainly globular in solution. Used for emulsion stabilisation they are likely to behave like particles. While sodium caseinate present a rod-like arrangement making its behaviour in emulsion stabilisation closer to the one of classic surfactants.

The choice behind the different polysaccharides rests in their properties, structures or both. Guar gum was chosen as it has gelling properties. The hope was to increase the viscosity of the emulsions prepared from conjugated guar gum while forming a gel like structure around the oil/water interface. Due to their branched or partially branched structure dextrin and dextran were chosen for similar reasons. Previous authors have shown that conjugated dextrin could lead to the production of gel. Using conjugated dextrin or dextran at the interface would lead to the formation of a gel like interfacial membrane increasing the mechanical resistance of the latter.

Sodium caseinate associated with dextrin and whey protein isolate associated with sugar beet pectin gave the highest conjugation efficiencies. Those conjugates were selected for further experiments.

3.3.1 Preparation and characterisation of the conjugates

3.3.1.2 Sodium caseinate-dextrin conjugate

The occurrence of the Maillard reaction was visually observed before any chemical characterisation. After dry heating the mixture of sodium caseinate (NaCN) and dextrin was of a brownish colour.

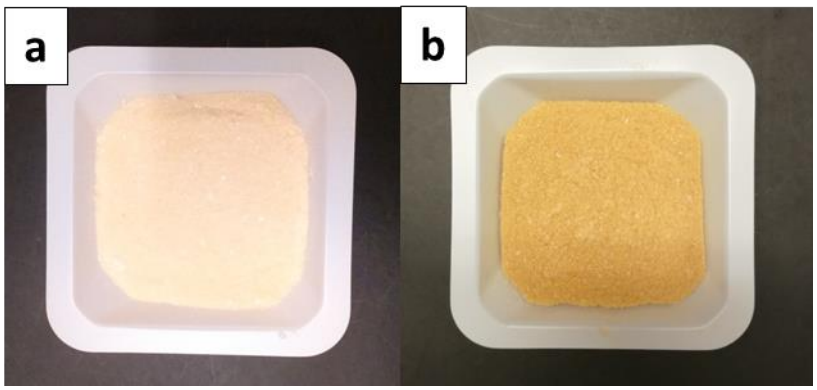


Figure 3-2: Photographs of NaCN/dextrin dry mixture before (a) and after (b) dry heating at 40°C for 4 days, ratio 1:2.

To quantify the extent of the Maillard reaction the OPA-assay was used. The amount of amino groups was measured before and after conjugation.

To find the weight ratio giving the highest efficiency, the conjugation efficiencies of different NaCN/dextrin ratios were measured (Table 3-1).

Table 3-3: Conjugation efficiencies of CN-De conjugates at diffrent sodium caseinate/dextrin ratios

NaCN:dextrin (w:w)	Conjugation efficiency (%)
2:1	66.79 ± 4.34
1:1	71.05 ± 1.92
1:2	88.37 ± 1.06
1:3	83.38 ± 3.09
1:4	83.79 ± 2.99

Results showed that at the highest protein content (weight ratio 2:1) the conjugation efficiency was the lowest (66.79 ± 4.34%). At a protein: polysaccharide weight ratio of 1:2 the highest conjugation efficiency was reached (88.07 ± 1.06%).

At high protein content the amount of dextrin was not enough to react with the majority of the free amino groups of the sodium caseinate, leading to the lowest conjugation efficiency.

For this reason, the proportion of dextrin was increased while the proportion of sodium caseinate was kept constant. Conjugation efficiencies for the ratios 1:3 and 1:4 was measured. Despite this increase the highest efficiency stayed for the ratio 1:2 (88.07 ± 1.06%). This is explained by the conditions of reaction. During dry heating the mixture is left stationary throughout the process. The increasing amount of dextrin increased the hindrance around the amino groups of NaCN thus limiting the reaction.

The NaCN/dextrin ratio 1:2 (w/w) was kept for further experiments. At this ratio, the conjugation efficiency was 88.07 ± 1.06%.

The solubility of the chosen conjugate was measured. The solubility of an emulsifier can be a good indicator of its emulsifying ability (De Wit 1989). Despite being able to form emulsions, proteins with lower solubility produce emulsions that tend to form droplet aggregation (flocculation) rapidly after homogenisation (Jiang, Sontag-Strohm et al. 2015).

The solubility measured for the sodium caseinate–dextrin conjugate (CN-De) was 67.46 ± 1.00%.

The particle size of the conjugate was measured after conjugation on 1 $g.mL^{-1}$ solutions. The CN-De conjugate displayed an average particle size ($D_{4,3}$) of 334.98 ± 6.19 µm. This value compared with the particle size of NaCN and dextrin. The two hydrocolloids when unconjugated displayed a smaller particle size, respectively 143.98 ± 4.58 µm and 17.1 ± 2.4 µm for NaCN and dextrin respectively.

3.3.1.3 Whey protein isolate-sugar beet pectin conjugate

After dry heating the mixture of WPI and SBP changed colour. The conjugated product was of a darker yellow in comparison with the dry mixture before dry heating.

Whey proteins and sugar beet pectin are two hydrocolloids that have often been used together to form electrostatic complexes (Salminen and Weiss 2014, Tamm, Härter et al. 2016, Faridi Esfanjani, Jafari et al. 2017, Wagoner and Foegeding 2017). Studies performed at the University of Birmingham on these complexes showed that the optimum level of electrostatic interactions between the two entities was achieved for a weight ratio whey protein/pectin of 2 to 1 at pH 4. For this study, the same ratio was chosen with the expectation to obtain the highest conjugation efficiency possible.

Unlike the mixture of NaCN and dextrin, mixing whey protein and sugar beet pectin offers a range of options of environmental pH. Indeed, both pectins and whey proteins are charged macromolecules depending on the pH. Sugar beet pectins bear in their polysaccharide chains carboxyl groups (c.f. Chapter 2 Figure 2-6) and whey proteins are characterised by their pI. In order to properly identify the best conjugation conditions, it was important to measure the conjugation efficiency at different pH. The pH of the mixture of hydrocolloids prior to dry heating is a factor greatly influential on the conjugation efficiency of the Maillard reaction (Steinhart 2005). Additionally, the effects of the formation of electrostatic complexes before dry heating on the efficiency of the conjugation reaction needed to be investigated. To do so, conjugation at different pH was studied.

Two separate solutions of sugar beet pectin and whey protein isolate were prepared and their pH adjusted to 7. At this pH both hydrocolloids were negatively charged. The two solution were then mixed, lyophilised and dry heated (at 40°C for 4 days) to produce a conjugate. Due to their negative charges at pH 7 no electrostatic complexation was possible upon mixing the solutions.

In a second test, two separate solutions of SBP and WPI were prepared and the pH kept unchanged. The native pHs of sugar beet pectin solutions and whey protein isolate solutions were respectively ~3.9 and ~6.8. After mixing the solutions the pH was measured at ~4.5. At

this pH, SBP was negatively charged while WPI chains bore a mixture of amine (NH_2) and ammonium (NH_3^+) groups if we refer to the pI of WPI (pI 5.2). Some electrostatic complexes could form upon mixing of the two solutions.

The last batch consisted of adjusting the pH of both protein and polysaccharide solutions to 7. After mixture of the two solutions the pH was lowered at 4. At this pH, WPI was positively charged (NH_3^+) while SBP was negatively charged (COO^-). This pH adjustment would induce the formation of electrostatic aggregates (complexes). Thus the effect of complexation of WPI with SBP on the conjugation efficiency could be studied.

The results of the conjugation efficiency measurements as a function of pH conditions of pre dry heating the mixture are displayed in Table 3-2.

Table 3-4: Conjugation efficiencies and mean particles diameters of WPI-SBP conjugates under different conjugation pH conditions

pH conditions	Conjugation efficiency (%)	Surface Mean Diameter $D_{3,2}$ (µm)	Volume mean Diameter $D_{4,3}$ (µm)
Unchanged	76.03 ± 0.57	72.17 ± 2.08	137.68 ± 2.27
Adjusted at pH 7	87.39 ± 0.59	171.91 ± 34.43	350.94 ± 53.15
From pH 7 to 4	13.93 ± 1.33	97.96 ± 3.83	166.1 ± 11.9

When a WPI solution was mixed with a SBP solution without any pH adjustments, the resulting solution had a pH of ~4.5. After conjugation through dry heating (40 °C for 4 days), the conjugation efficiency and particle size were measured (Table 3-3). According to these results, 76.03 ± 0.57 % of the amino groups had reacted with SBP's reducing sugars.

At a pH close to 4, WPI and SBP were oppositely charged. Meaning, at this pH, when mixed together, they spontaneously formed electrostatic aggregates of about 100 μm.

These results showed that not only the formation of electrostatic aggregates had no effect on the capacity of the hydrocolloids to engage in the conjugation reaction, but also that the particle size of the resulting conjugates could be controlled by pH adjustments. After preparation electrostatic complexes are often exposed to high temperatures for short period of time, as it has demonstrated positive effects on their emulsifying properties (Schmitt and Turgeon 2011).

In the production of electrostatic complexes of WPI and SBP, a way to form nano-sized complexes instead of larger aggregates is to mix the two hydrocolloids solution at neutral pH before inducing complexation by lowering the pH towards acidic values (~pH 4) (Wagoner and Foegeding 2017). In the present study this technique was used in order to investigate the ability of nano-sized protein/polysaccharide complexes to undergo the Maillard reaction in comparison with larger aggregates. The use of nano-sized conjugates instead of larger ones to stabilise emulsions could improve the packing at the oil/water interface providing a stronger interface beneficial for drying experiments.

Results showed that the formation of smaller complexes before dry heating inhibited the reaction. The conjugation efficiency when the pH was decreased from 7 to 4 was the lowest observed out of three conditions studied. However, the particle size of the conjugates was comparable with that of the conjugates produced at pH 4.

To avoid electrostatic interactions and the formation of complexes through electrostatic attraction upon mixing of the solutions, the pH of each solution was adjusted to 7 a value at which both polymers are negatively charged. After dry heating, the same experiments as previously were performed. In these pH conditions, the largest particles were produced along with the highest conjugation efficiency. The conjugation efficiency measured after reaction was

87.39 ± 0.59%. The size of the conjugated particles, ~ 350 μm was similar with conjugates particle sizes frequently reported. This result was expected since the Maillard conjugation is optimum at pH between 6 and 9 (Arachchi, Kim et al. 2017).

For further experiments, the pH was adjusted to 7 to prepare the solution that would be used to produce the conjugates.

The solubility of this conjugate was 91.12 ± 0.26%.

3.3.2 Emulsifying properties

3.3.2.1 Effects of conjugation on interfacial layer thickness

One of the reasons why Maillard conjugates have attracted interest in the past decades is the thicker interfacial layer they provide at the oil/water interface in emulsions they stabilise. The thickness of the conjugated layer can be calculated using Dynamic Light Scattering (DLS) (Wooster and Augustin 2006).

The conjugates were adsorbed onto the surface of 100 nm latex particles. The thickness of the conjugated layer was then estimated from the difference of diameter between the uncoated particles and the particles coated with conjugate. Results displayed in Figure 3-2 show the evolution of the thickness layer with an increasing protein concentration.

The measurements were performed on latex particles covered with the protein and the conjugates to note the increase due to conjugation. The interfacial thickness was taken where a plateau in the increase of the thickness layer was observed.

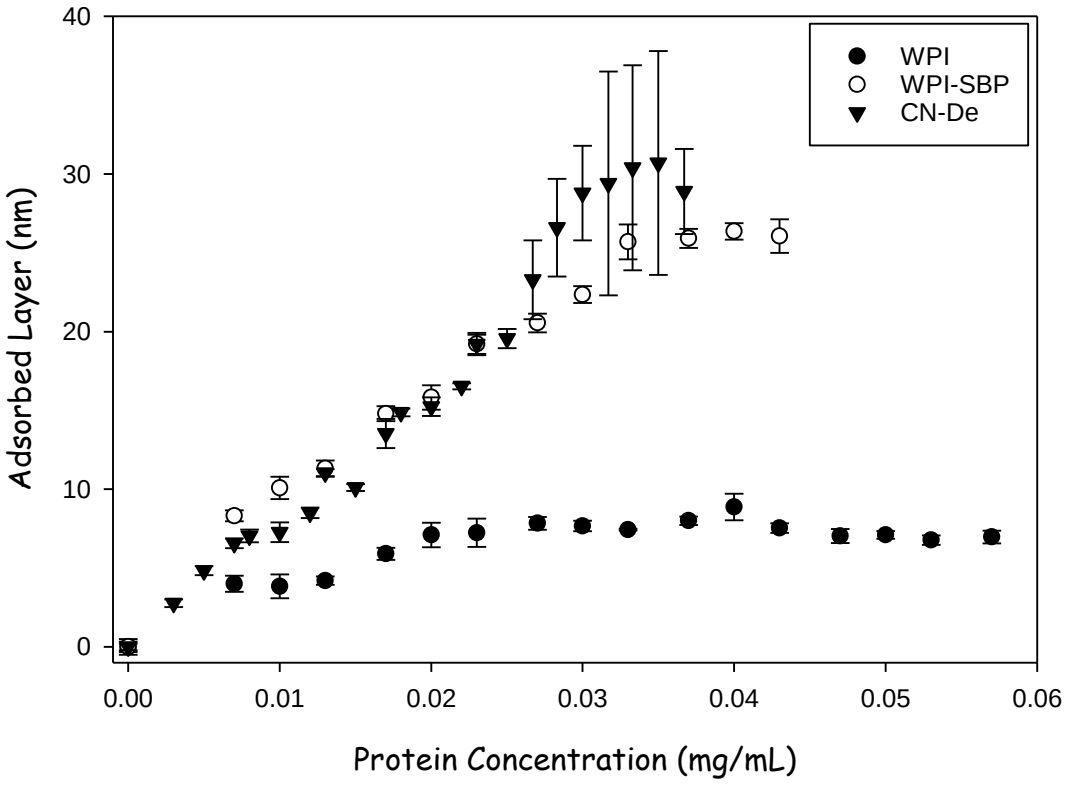


Figure 3-3: Adsorbed thickness layer of WPI-SBP, CN-De and WPI. Adsorbed layer thickness taken from the difference between the naked and covered polystyrene latex beads. Measurements were carried out at room temperature (22 °C), pH 7.5. All samples were measured on triplicate. Error bars represent standard deviation.

The experiments with NaCN presented an irregular pattern. The interfacial layer measured showed no coherent increase therefore the thickness of its interfacial layer considered for this study was that reported in the literature. In their experiments *Fang et Al.* measured an interfacial layer of 5-10 nm for NaCN (Fang and Dalgleish 1993).

Analysis of the data from these experiments showed that the adsorption of the conjugates resulted in a drastic increase of the layer thickness in comparison with particles covered by proteins; from ~7 nm for WPI against ~26 nm for WPI-SBP and from 5-10 nm for

NaCN against ~ 29-30 nm for CN-De. These values were consistent with interfacial film thicknesses measured with other conjugates (Wooster and Augustin 2006).

3.3.2.2 Effects of conjugation on interfacial tension

The interfacial tension (IFT) is a crucial parameter for the determination of emulsion stability. Surfactants due to their amphiphilic properties have the ability to lower the interfacial tension between water and oil. The effects of conjugation on the interfacial tension have been studied. The association of polysaccharides with proteins is believed to bestow amphiphilic properties to the final structure (Dickinson and Galazka 1991, Dickinson 1995). It is however not clear if the addition of the hydrophilic moieties (polysaccharides) further affects the interfacial tension. IFT was measured for WPI, CN, WPI-SBP and CN-De with increasing protein concentrations. Results are displayed in Figure 3-3.

Expectedly, for both the proteins and the conjugates, the IFT decreased as the protein concentration increased. However, the IFT was not affected by the conjugation. Indeed, the similar values were obtained for the protein alone and the glycoconjugate in both cases. This means that the difference of polarity induced by the presence of the polysaccharides was not significant enough to increase the ability to lower the interfacial tension of the protein. These results confirmed that the enhanced stability observed for emulsions stabilised with Maillard conjugates solely rests on the thicker interfacial membrane and the steric and electrostatic repulsion they provoke between the droplets.

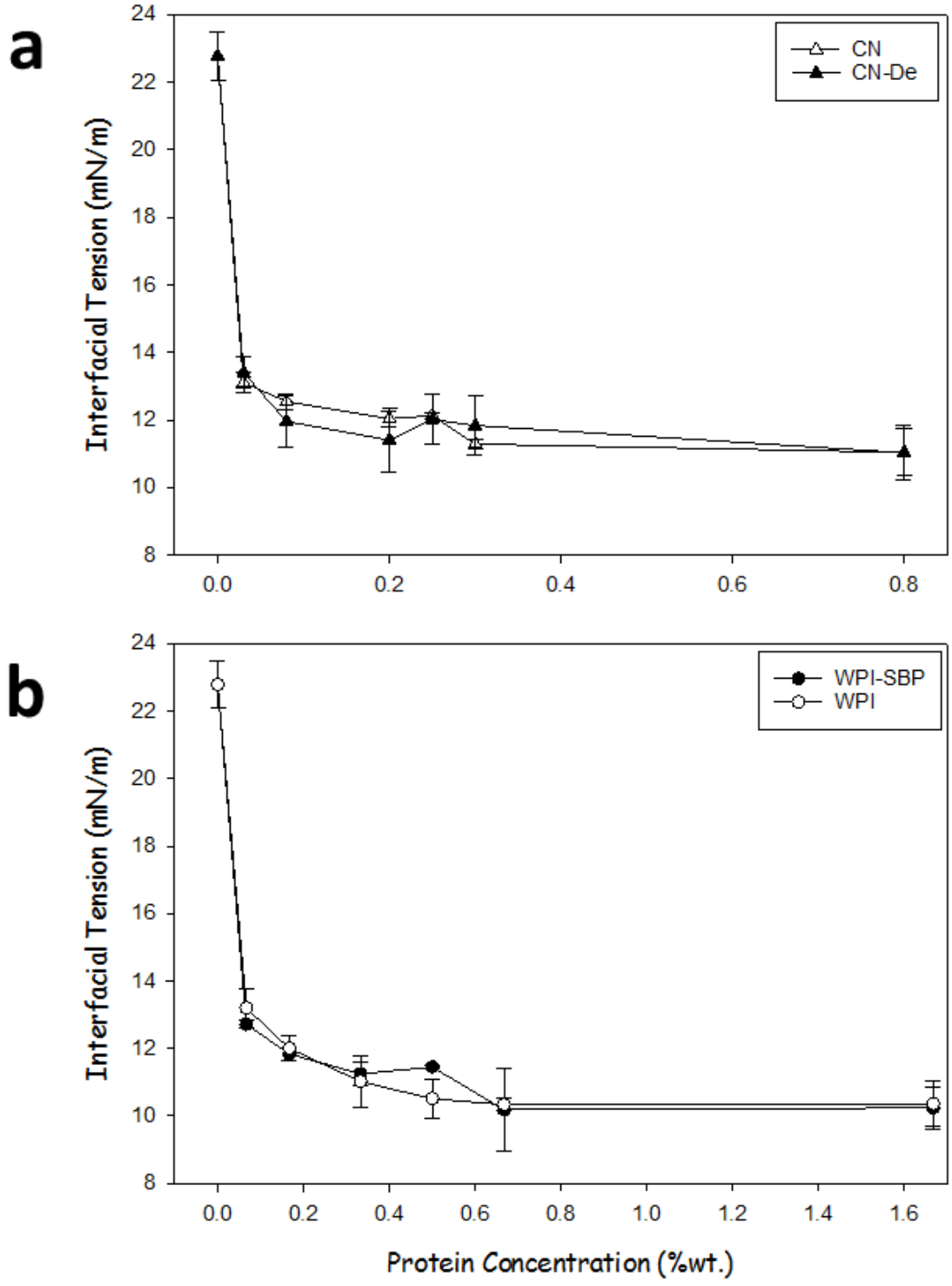


Figure 3-4: Equilibrium conjugate solution/sunflower oil IFT and protein solution/sunflower oil IFT as a function of protein concentration at room temperature (22 °C). Data point are averages of three measurements. Error bars represent standard deviation.

3.3.2.3 Oil-in-water emulsions

Oil-in-water emulsions were produced using CN-De and WPI-SBP. For this study the chosen ratio was 20% sunflower oil and 80% water.

In order to find the suitable amount of conjugate needed to stabilise the emulsions, several concentrations were tested. Concentrations ranging from 0.1% to 2.5% w/w conjugate (from ~0.03% to ~0.8% w/w NaCN or from ~0.07% to ~1.7% w/w WPI) were used to produce emulsions. The emulsions were all visually homogenous immediately after emulsification. After being stored at room temperature for 24 hours, the emulsions from 0.1% to 1% w/w conjugate had creamed under gravity for both conjugates used.

To induce creaming of the stable emulsions, centrifugation at moderate speed can be used. The stable emulsions, 2.5% w/w CN-De (~0.8% w/w. NaCN) and 2.5% w/w WPI-SBP (~1.7% w/w WPI), were centrifuged to test their creaming stability. After 30 minutes at 4000 rpm, the emulsions showed no signs of creaming. This concentration of conjugate was chosen as a standard for further experiments.

Emulsions stabilised with CN-De and WPI-SBP at 2.5% w/w were used to study the long term storage stability.

Two methods were used to evaluate the stability of the emulsions over time; monitoring the evolution of the droplet diameters over time and analysing droplet diameters and microstructures (optical microscope) after exposing the emulsions to increased temperature over an extended period of time (emulsions were kept in the oven at 40°C for a week).

The evolution of the surface and volume mean diameter of the emulsions was monitored over time. In-between measurements, the emulsions were kept in a fridge at 5˚C. Results are shown in Figure 3-5.

All emulsions appeared visually homogenous over the chosen period of time. However DLS measurements revealed that the oil droplet size of the CN-De stabilised emulsion increased over time. The volume mean diameter increased from 457 ± 59 nm on day 0 to 4.48 ± 1.66 µm after 17 weeks of storage.

The WPI-SBP stabilised emulsions showcased better storage stability. Indeed both the surface mean diameter and volume mean diameter showed minimal variation between day 0 and the week 6. Respectively from 158 ± 3 nm to 157 ± 2 nm for $D_{3,2}$ and from 361 ± 91 nm to 341 ± 1 nm for $D_{4,3}$ with little to no major fluctuations between these two measurements.

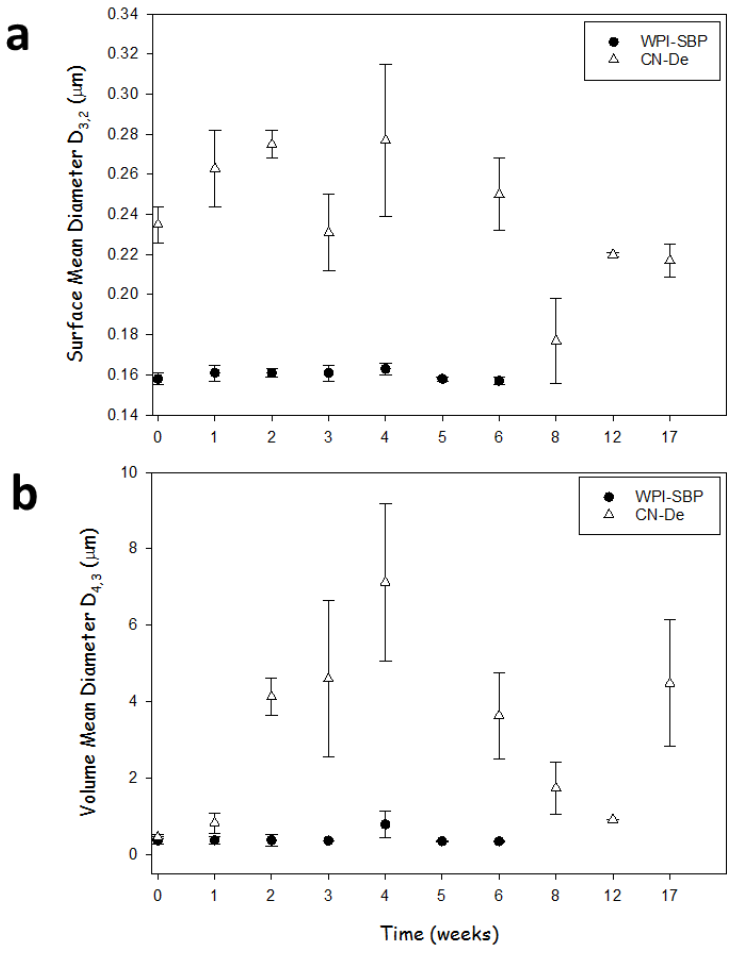


Figure 3-5: Droplet size evolution as function of time of 20% sunflower oil, 80% water oil-in-water emulsions stabilised with 2.5% w/w CN-De (pH 6.8) and 2.5% w/w WPI-SBP (pH 5.8). Surface mean diameter $D_{3,2}$ (a) and volume mean diameter $D_{4,3}$ (b).

The long term stability of emulsions can be assessed by increasing the storage temperature over a long period of time. After a week in an oven at 40 °C, the mean droplet diameter of the emulsions was measured. The same experiment was performed on emulsions stabilised with the equivalent protein concentration. For the case of CN-De an emulsion stabilised with a mixture of sodium caseinate and dextrin in the same proportion of the conjugate was produced to assess the effects of conjugation on the storage stability.

For all emulsions the droplet diameter displayed a multimodal distribution after storage at 40 °C for 7 days (Figure 3-6). There was no distinct enhancement of the stability when comparing NaCN stabilised emulsions and CN-De stabilised emulsions droplet size distributions. In both cases aggregates detected in the larger size range formed upon storage at moderately high temperature.

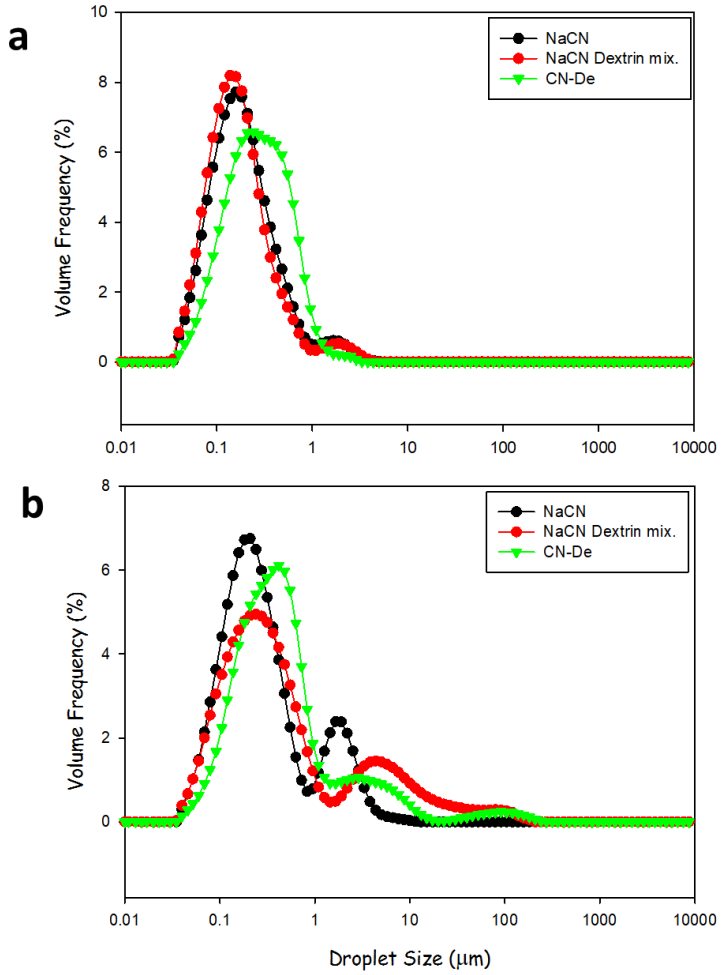


Figure 3-6: Droplet size distribution of oil-in-water emulsions (20% w/w sunflower oil, pH 6.8) stabilised with ~ 0.8% w/w NaCN, 2.5% w/w CN-De and NaCN dextrin mixture (~0.8% w/w NaCN and ~1.7% w/w dextrin) before (a) and after (b) storage for 7 days at 40˚C.

The WPI-SBP stabilised emulsions displayed the same surface and volume mean diameter values after storage (Figure 3-7). This occurrence was consistent with previous observations made for a longer storage period (O'Regan and Mulvihill 2009).

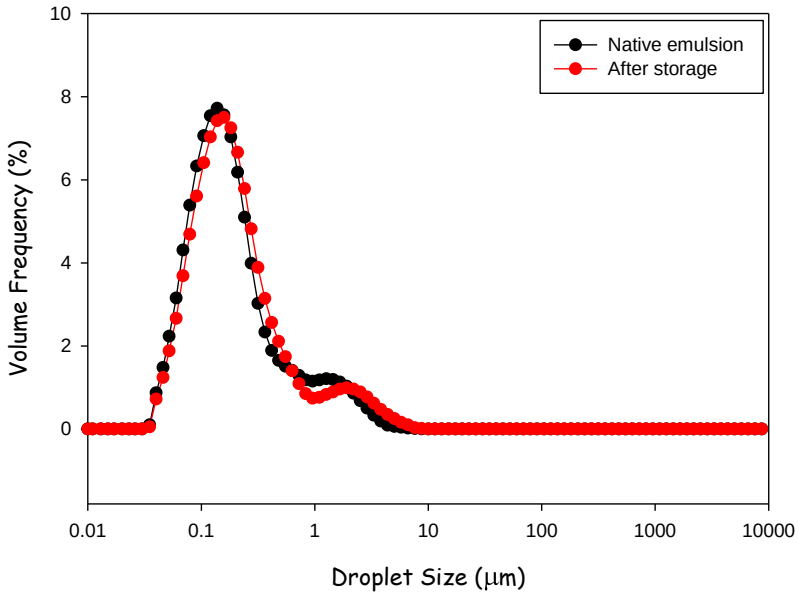


Figure 3-7: Droplet size distribution of oil-in-water emulsions (20% w/w sunflower oil, pH 5.8) stabilised with 2.5% w/w WPI-SBP before and after storage for 7 days at 40˚C.

The latter result was the most interesting since whey protein solution form aggregates at high temperatures (Ju, Hettiarachchy et al. 1999).

After being stored for a week at 40 ˚C, the WPI stabilised emulsions exhibited a gel-like aspect due to droplet aggregation (Dickinson and Chen 1999). The conjugation to SBP inhibited the formation of this structure in the WPI-SBP stabilised emulsions.

3.3.2.4 Surface coverage

The surface coverage or surface load corresponds to the amount of emulsifier at the oil/water interface of an emulsified system. In the case of proteins, it can be calculated using different methods (Dickinson and Hong 1994, Srinivasan, Singh et al. 1996). The principle of these methods consists in measuring the non-adsorbed emulsifier and deducting from it the mass of emulsifier per surface area unit at the interface. The conjugation can affect the surface coverage

of an emulsifier. The conjugation of polysaccharides to proteins increases the steric crowding at the oil/water interface. Consequently the amount of conjugated molecules that can potentially adsorb at the interface becomes limited (Wooster and Augustin 2006).

The hydrocolloid surface coverage for emulsions stabilised with CN-De at ~0.8% w/w NaCN (2.5% w/w conjugate) were measured and compared with the value of the surface coverage of NaCN only at the same concentration. The surface concentration of protein calculated for CN-De at the oil droplets surface was 0.75 ± 0.14 $mg.m^{-2}$. In literature at the same protein concentration the surface load reported for NaCN is ~0.7 $mg.m^{-2}$ (Srinivasan, Singh et al. 1996, Foegeding 2005). In their study, Wooster et Al. found that the conjugation to dextran greatly influenced the surface coverage of β-lactoglobulin. Indeed, in their study, the conjugation to dextran not only decreased the surface coverage of the protein, but this decrease was also influenced by the molecular weight of the sugar.

The conjugation to dextrin had no effect on the surface coverage of the emulsifier. The conclusions made by *Wooster and Augustin* (Wooster and Augustin 2006) were not corroborated by the present results. However, the difference of results can be explained by the molecular weight of the dextrin used. In their studies *Wooster and Augustin* (Wooster and Augustin 2006) compared the coverage of conjugates made of dextran with molecular weights ranging from 36 kDa to 440 kDa. The average molecular weight of the dextrin used in this experiment was about 7 kDa, that is to say five times smaller than the smallest dextran of their study. This showed that the coverage of Maillard conjugates decreases in comparison with the coverage of the protein alone only when larger polysaccharides are used.

3.4 Conclusion

Several protein/polysaccharide conjugates were produced to stabilise oil-in-water emulsions. The conjugates giving the highest conjugation efficiencies were selected for further studies. Experiments varying the ratio of protein and polysaccharide confirmed that the conjugation efficiency of the Maillard reaction was greatly affected by the hydrocolloids ratio (Xu, Wang et al. 2012). A higher mass proportion of polysaccharides seemed to lead to higher conjugation efficiencies (Table 3-2).

Experiments varying the pH of the hydrocolloid solution revealed that the use of a charged polysaccharide such as sugar beet pectin offered the possibility to control the size of the conjugate particles. At acidic pH smaller conjugates were produced.

The present study confirmed that the main source of enhanced stability of the conjugate stabilised emulsions is the thickness of their interface. Interfacial thickness measured showed interfacial films up to 5 times thicker than their protein counterpart.

Chapter 4

Influence of External Stress on The Stability of Oil-in-Water Emulsions Stabilised with Maillard Conjugates

4.1 Introduction

The influence of external changes on emulsions is important to consider. The pH or ionic strength of emulsified food products is often adjusted in order to suit a specific application. From one system to another salt concentration and pH can vary and strongly affect the stability of the emulsion phase.

The aim of this chapter was to understand how theses parameters affect the present emulsions allows to better grasp their suitability for food applications. Apart from physico chemical changes, this study covers the challenges of producing re-dispersible dry emulsions obtained through temperature dependent processes therefore the effects of temperatures changes are also important to consider.

In this section the emphasis is on the ability of emulsions to keep their appearance as it is important for food products to be visually appealing. Consequently, in most cases emulsions presenting signs of droplet aggregation and creaming were considered unsuitable for further examinations.

The same set of experiments carried out on the NaCN and CN-De stabilised emulsions were replicated for WPI and WPI-SBP stabilised emulsions.

The effects of salt and pH on this particular conjugate were expected to affect more severely the solubility of the conjugate. Indeed, sugar beet pectin is a charged polysaccharide depending on the pH. This characteristic increases the probability of screening by salts leading to aggregation of conjugate. Intermolecular interactions could also be expected at pH where opposite charges were present on the conjugate.

On the other hand, unlike sodium caseinate, whey proteins have shown physicochemical changes when exposed to increased temperatures (Bryant and McClements 1998, Ju, Hettiarachchy et al. 1999, Dissanayake, Ramchandran et al. 2013, Dissanayake, Ramchandran

et al. 2013). Conjugation to polysaccharides has been identified as a way to increase the heat stability of both whey protein solution and whey protein stabilised emulsions (Jiménez-Castaño, López-Fandiño et al. 2005, Jimenez-Castano, Villamiel et al. 2007). This property makes WPI-SBP conjugate potentially suitable emulsifier for spray-drying applications.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Proteins

Casein sodium salt from bovine milk and egg white protein were purchased from Sigma Aldrich, UK. Whey protein isolate was ordered from Volac.

4.2.1.2 Polysaccharides

Dextrin from corn starch (DE = ≤5. Mw = 7kDa) was purchased from Sigma Aldrich, UK. Sugar Beet Pectin (degree of esterification >50%, Mw = 45.3kDa) was purchased from Herbstreith & Fox KG (named Betapec RU 301).

4.2.1.3 Other materials

The sunflower oil (Solesta) used for the production of oil-in-water emulsions was purchased from a local store.

A pH 4.65 acetate buffer from Sigma Aldrich, UK was used for the production of emulsions.

The sodium chloride was purchased from Sherman Chemicals Ltd and the calcium chloride GBiosciences.

4.2.2 Methods

4.2.2.1 Emulsifying Properties

4.2.2.1.1 Zeta potential measurements

Zeta potential measurements were performed using a Zetasizer from Malvern Instruments UK.

For ζ-potential measurements as a function of pH a 1% w/w conjugate solution was prepared. The pH was lowered from 6.8 and 5.8, for CN-De solutions and WPI-SBP solutions respectively, to pH 2. Another solution at the same conjugate concentration was then prepared and the pH adjusted from native pH to 8. pH adjustments were performed by addition of concentrated hydrochloride acid or potassium hydroxide. Three separate samples were taken from the solution at each pH and their ζ-potential measured. The values displayed are an average of these data accompanied by the standard deviation.

For ζ-potential measurements as function of salt concentration a 1% w/w conjugate solution was prepared and separated in three to produce a solution at one given salt concentration. The mass of salt introduced was calculated in order to be in adequacy with the conjugate/salt proportion in emulsions; 0.04 g, 0.1 g, 0.2 g, 0.3g and 0.4 g of salt were introduced in 100 mL of 1% w/w conjugate solution.

The values displayed are an average of these data accompanied by the standard deviation.

4.2.2.1.2 Solubility of the conjugates as a function of pH

The solubility of the conjugate as a function of pH was measured using a method similar to one presented by *Mohanty, Mulvihill and Fox* (Mohanty, Mulvihill et al. 1988, O'Regan and Mulvihill 2009). Solutions containing 1% w/w protein or conjugate were prepared and the pH

adjusted at the desired value (from pH 2 to 8 with 0.5 increments) using concentrated HCl or NaOH. The solutions were stirred for 30 minutes and the pH checked and readjusted if needed. The solutions were centrifuged using a centrifuge Sigma from Scientific Lab Supplies Ltd at 3000 rpm for 20 minutes at room temperature (~22 °C). After centrifugation each supernatant was decanted. The pellets and supernatants of all samples were frozen at -22 °C for at least 24 hours and freeze dried until all water was removed. The solubility (S) was calculated using Equation 3-2 as the percentage of protein (or conjugate) in the supernatant in comparison with the total protein (or conjugate) in the initial dispersion. Samples were measured in triplicate. The error bars correspond to the standard error of the average of these three measurements.

$$S = \frac{supernatant\ protein}{total\ protein} \times 100$$

Equation 4-1

4.2.2.1.3 Surface tension measurements

The surface tension (SFT) of the protein (or conjugate) solutions was determined at room temperature using the Wilhelmy plate method on a K100 Tensiometer from Krüss, Germany.

The protein (or conjugate) solutions were prepared with an increasing protein concentration. Data points were taken at 200 seconds, value at which surface tension equilibrium was achieved. Measurements were performed in triplicate on the same or different solutions. Values are reported as average ± standard deviation.

4.2.2.1.4 Emulsion preparation

See Chapter 3.

Emulsions were prepared according to the method described in chapter 3. However for the preparation of emulsions at different pH some additional steps when undertaken.

For the preparation of emulsion at pH 2, 5.2 and 12, the pH of distilled water was adjusted to the desired pH using concentrated HCl and/or NaOH. A conjugate or protein solution at the desired pH was prepared and stirred for 30 minutes at room temperature. After 30 minutes the pH was checked and adjusted if needed.

The pH was checked after emulsification.

For the preparation of emulsions at pH 4.65. A conjugate or protein solution at the desired concentration was prepared in an acetate buffer pH 4.65. The rest of the preparation was carried out according to method previously described.

4.2.2.2 Texture Analysis

4.2.2.2.1 Shear Viscosity Measurements

The shear viscosity of the emulsions was measured at 25 ˚C, from 0.1 to 100 s^{-1} shear rate using a Kinexus rheometer equipped with a cylindrical double gap and cup geometry. The maximum sampling was set at 5 minutes and the integration time at 1 minute.

4.2.2.3 External stress stability

4.2.2.3.1 Heat stability

Emulsions previously prepared were placed in the oven at 95 ℃ for 5, 10, 15 and 20 minutes.

4.2.2.3.2 Freeze-thaw stability

Emulsions were frozen in a commercial freezer at -22 °C for at least 24 hours before being allowed to melt at room temperature.

4.3 Results and discussion

4.3.1 Effects of salt addition

Two different salts were used to study the effects of low to moderate concentration of salt on the stability of the emulsions. Sodium chloride (NaCl) and calcium chloride ($CaCl_2$) were added before and after emulsification.

Upon addition of salt, the charges born by the conjugates are screened promoting the formation of aggregates through electrostatic attraction (Ju, Hettiarachchy et al. 1999, Kastelic, Kalyuzhnyi et al. 2015, Xu, Liu et al. 2015). The screening of the charges was monitored by measuring the zeta-potential (ζ-potential) of conjugates solution as a function of salt concentration. Likewise, to better understand the effect of salt addition on the interfacial properties of the conjugates, the surface tension (SFT) of conjugates solutions was measured as function of the salt concentration.

SFT and IFT are linked by the Equation 4-2 (Girifalco and Good 1957, Fowkes 1964).

Where $\gamma(a,b)$ is the interfacial tension between *a* and *b*, $\sigma(a)$ is the surface tension of a, $\sigma(b)$ the surface tension of b and $F(a,b)$ a value representative of the interactions between *a* and *b*.

Based on this equation it is notable that an increase or decrease of the SFT (σ) of one of the components of the system will lead to the same variation for the IFT (γ). Therefore, SFT measurements can be used to extrapolate on the variation of IFT with an increasing salt concentration.

$$\gamma(a,b) = \sigma(a) + \sigma(b) - F(a,b)$$

Equation 4-2

Additionally, the effects of salt addition on the ζ-potential of NaCN and WPI solutions were studied for comparison purposes.

Results for NaCN and CN-De are displayed in Figure 4-1 and Table 4-1.

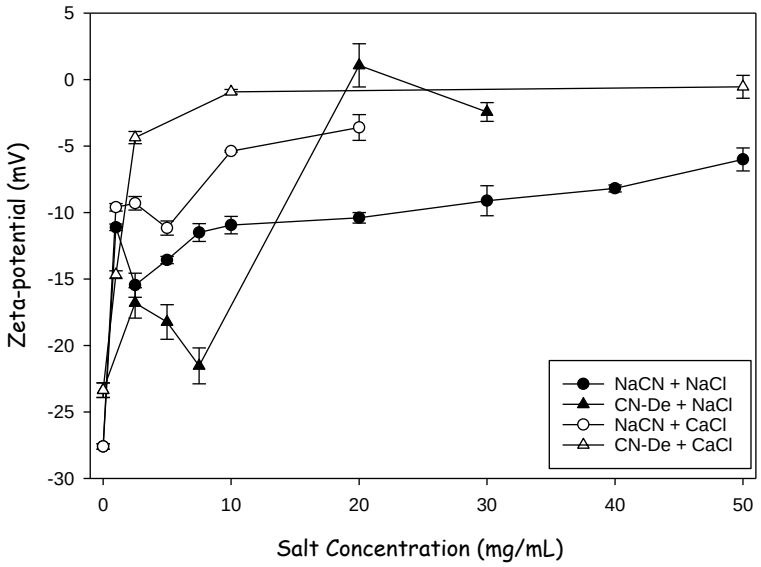


Figure 4-1: Zeta potential as a function of salt concentration for NaCN and CN-De at room temperature (22 °C), pH 6.8. Data point are averages of three measurements. Error bars represent standard deviation.

The addition of salt led to an increase of the ζ-potential for both the protein and the conjugate (Figure 4-1). Initial potentials were negative for both hydrocolloid solutions, respectively -28 and -23 mV for NaCN and CN-De. Indeed, the pH of the solutions (i.e. ~ 7) was higher than the pI of NaCN (i.e. 4.65) prior to salt addition. As predicted upon salt addition, the negative charges of the protein were progressively screened by Na^+ or Ca^{2+} ions. The increase of the ζ-potential was a consequence of this occurrence. Due to the bivalency of Ca^{2+}, the ζ-potential in the presence of $CaCl_2$ increased at a greater rate than with NaCl. However above 10 mg/mL of

NaCl the ζ-potential showed a sudden increase. The steric hindrance caused by dextrin could be an explanation for this sudden increase. The conjugation of dextrin to NaCN affected the rate of penetration of Na^+ to neutralise the negative charges. Above a certain concentration, further addition of salt resulted in an accumulation of free ions in solution.

The effects of the addition of salt on the surface tension of protein and conjugate solution were studied in order to assess the effect of salt on the emulsifying ability of both the different hydrocolloids. The SFT of conjugate solutions containing increasing concentrations of salt was measured and compared with the SFT of conjugates solution without salt. Table 4-1 displays the values for Δσ a value corresponding to the difference between the SFT of a conjugate solution and the SFT of a conjugate solution with added salt at the indicated concentration.

As the concentration of salt increased the variation of the surface tension noted Δσ was expected to progressively increase as well. Conjugates were expected to aggregate in the presence of salt decreasing their number at the air/water interface. Less conjugate at the air/water interface would result in an increasing SFT.

Table 4-1: Variation of equilibrium surface tension (Δσ) as function of salt concentration at room temperature (22°C), pH 6.8. All samples were measured in triplicate. Values are presented as mean ± standard deviation.

Salt Concentration (mg/mL)	**Δσ (mN/m)**			
	NaCN solution		**CN-De solution**	
	NaCl	**$CaCl_2$**	**NaCl**	**$CaCl_2$**
1	7.41 ± 0.62	11.12 ± 0.50	-3.73 ± 1.03	5.96 ± 0.23
2.5	12.64 ± 0.17	12.37 ± 0.05	5.34 ± 0.17	4.68 ± 1.01
5	12.6 ± 0.21	12.8 ± 0.03	-0.88 ± 1.33	6.84 ± 0.78
7.5	2.45 ± 0.95	12.52 ± 0.2	1.51 ± 0.26	0.46 ± 1.31
10	9.95 ± 0.12	12.59 ± 0.08	-5.06 ± 0.28	-4.36 ±0.23

The results from these experiments shown in Table 4-1 displayed a lack of consistency. The confirmation of logical pattern was impossible from these values. Although for NaCN the $\Delta\sigma$ increased at every concentration tested no trend was observed.

Analysis of the ζ-potential data (Figure 4-2) revealed that the conjugation to SBP led to lower values indicating a greater number of negative charges on the conjugate. This observation can be explained by the presence of negative charges on both the protein and the polysaccharide. The measured pH of a solution of WPI-SBP was ~6. At this pH both polymer were negatively charged.

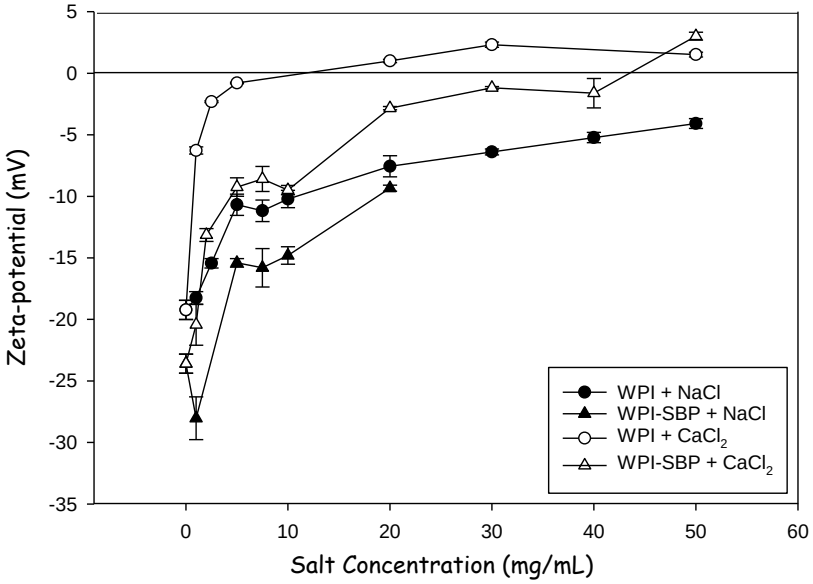


Figure 4-2: Zeta potential measurements at room temperature (22°C) of 1% w/w WPI (pH 6.8) and 1% w/w WPI-SBP solutions (pH 5.8) as function of concentration ranging from 0 to 50 mg.mL^{-1} (1, 2.5, 5, 7.5, 10, 20, 30, 40 and 50 mg.mL^{-1}) of NaCl or $CaCl_2$. All samples were measured in triplicate. Error bars represent standard deviation.

When comparing the effect of the two different salts, $CaCl_2$ decreased the ζ-potential of the solution at a greater rate than NaCl. As previously, the bivalency of Ca^{2+} was suggested as the main reason for this difference. The intensity of the screening of the negative charges born by the conjugate or the protein was higher in the presence of Ca^{2+} ions.

As with CN-De the addition of salt to WPI-SBP solutions led to random variations of the SFT (Table 4-2).

However, the effects on the SFT of WPI-SBP solutions were negligible in comparison with the values obtained for WPI solutions. With both NaCl and $CaCl_2$, the SFT varied from 0.16 to 1.65 $mN.m^{-1}$ in comparison with the SFT of WPI-SBP in distilled water.

Table 4-2: Variation of equilibrium surface tension (Δσ) as function of salt concentration at room temperature (22 °C), pH 6.8 (WPI) and pH 5.8 (WPI-SBP). All samples were measured in triplicate. Values are presented as mean ± standard deviation.

Salt Concentration (mg/mL)	**Δσ (mN/m)**			
	WPI solution		**WPI-SBP solution**	
	NaCl	**$CaCl_2$**	**NaCl**	**$CaCl_2$**
1	1.58 ± 0.36	1.84 ± 0.45	0.24 ± 0.56	0.65 ± 0.23
2.5	0.98 ± 0.44	-0.79 ± 0.24	n/a	1.65 ± 1.20
5	0.3 ± 0.3	1.31 ± 0.34	0.85 ± 0.20	-0.16 ± 0.01
7.5	1.01 ± 0.31	1.48 ± 0.27	0.15 ± 0.22	0.14 ± 0.11
10	1.01 ± 0.39	1.03 ± 0.08	0.26 ± 0.15	-0.39 ±0.05

These results coupled with the ζ-potential data (Figure 4-2) suggested that the addition of salt before preparation to WPI-SBP stabilised emulsions would have limited effect on the

emulsifying ability of the conjugate. After emulsification the previous results suggested that the conjugate coated droplets would resist droplet aggregation.

4.3.1.2 Effects of salt addition before emulsification

The experiments studying the effects of salt addition on the ζ-potential of conjugate and protein solutions suggested to the formation of aggregates through electrostatic attraction. The presence of opposite charges at the surfaces of droplets formed from these aggregates could later lead to early creaming in the emulsions.

Emulsions stabilised with NaCN and CN-De, with a protein concentration of ~ 0.8% w/w (conjugate protein/polysaccharide ratio 1:2) were produced. Increasing salt concentrations were introduced before emulsification.

Emulsions (20 % w/w oil) stabilised with NaCN (~0.8% w/w) in presence of $CaCl_2$ (1, 2.5, 5, 7.5 and 10 $mg.mL^{-1}$) creamed immediately after homogenisation (Figure 4-3). In solutions, calcium ions specifically bond with the different caseins fractions leading to formation of micron sized aggregates thus reducing the electrostatic repulsion between the protein fractions (Pitkowski, Nicolai et al. 2009). This phenomenon explained the droplets aggregation and creaming observed after emulsification of the NaCN/$CaCl_2$ solutions with sunflower oil.

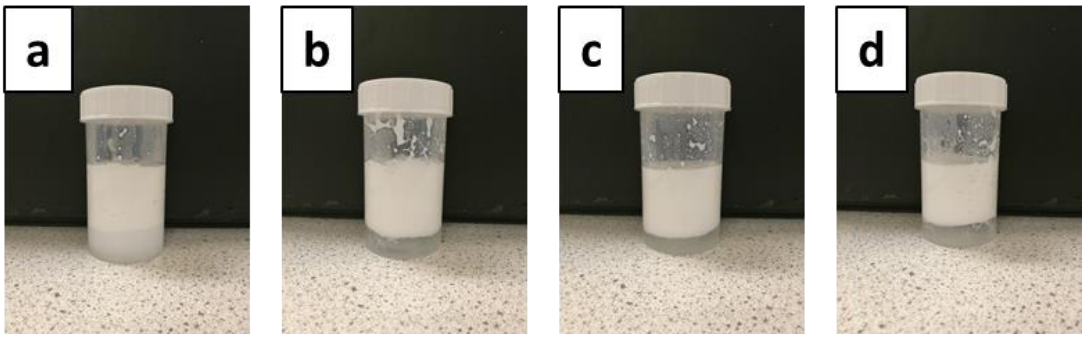


Figure 4-3: Photographs of pots containing ~0.8% w/w NaCN stabilised oil-in-water emulsions (20% w/w sunflower oil) with 1 mg.mL^{-1} (a), 2.5 mg.mL^{-1} (b), 5 mg.mL^{-1} (c) and 10 mg.mL^{-1} (d) $CaCl_2$ added before emulsification. Photographs were taken immediately after emulsification.

Emulsions (20% w/w sunflower oil) stabilised with CN-De (2.5% w/w conjugate) showed a better salt stability. All emulsions were visually homogenous after emulsification (with salt concentration ranging from 1 to 10 mg.mL^{-1}). However, the long-term stability was assessed using centrifugation testing. All emulsions had creamed after centrifugation.

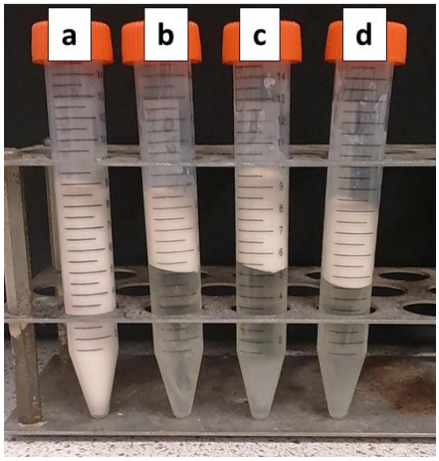


Figure 4-4: Photographs of centrifugation tubes containing 2.5% w/w CN-De stabilised oil-in-water emulsions (20% w/w sunflower oil) with 0 mg.mL^{-1} (a), 2.5 mg.mL^{-1} (b), 5 mg.mL^{-1} (c), and 10 mg.mL^{-1} (d) $CaCl_2$ added before emulsification. Photographs were taken immediately after centrifugation.

Nevertheless, the conjugate stabilised emulsions were visually homogenous immediately after emulsification. The increased salt stability displayed by the conjugate stabilised emulsions was still consistent with observations made by previous authors (Nakamura, Kato et al. 1992, Kato, Minaki et al. 1993, Akhtar and Dickinson 2003). Despite the screening of the negative charges of NaCN by the calcium ions, the dextrin provided enough steric repulsion between the oil droplets to improve the short-term stability.

The results for the addition of salt before emulsification highlighted that NaCN and CN-De both had poor emulsification properties in the presence of calcium chloride. However, the creaming rate seemed to be slow down by the conjugation as CN-De stabilised emulsions were visually homogenous immediately after homogenisation.

Emulsions stabilised with WPI and WPI-SBP with a protein concentration of ~1.7% w/w (conjugate protein/polysaccharide ratio 2:1) were produced. Increasing salt concentrations were introduced before emulsification.

Adding $CaCl_2$ (1, 2.5, 5, 7.5 and 10 $mg.mL^{-1}$) before emulsification induced the aggregation of the droplets in the WPI stabilised emulsions (20% w/w sunflower oil, ~1.7% WPI) upon homogenisation. The texture formed was visually comparable to a gel structure. The aggregation and gelation of WPI can be induced by the addition of calcium chloride (Bryant and McClements 1998, Kharlamova, Nicolai et al. 2017).

The state of the protein particles before emulsification plays an important role in the emulsifying properties of these ones (Sobhaninia, Nasirpour et al. 2017). The instant droplet aggregation was a direct consequence of the aggregated state of the particles (Delahaije 2014). Conjugation to a polysaccharide could potentially limit the aggregation process through two

mechanisms; by decreasing the percentage of charge screening due to the presence of polysaccharide around the protein; by increasing steric repulsion between the particles, making electrostatic attraction impossible to occur.

WPI-SBP conjugate stabilised emulsions (20% w/w sunflower oil, 2.5% w/w WPI-SBP) displayed a different stability to the addition of calcium chloride (from 1 to 10 mg.mL-1) before emulsification. All the emulsions were visually stable right after emulsification. The centrifugation stability was assessed. After 30 minutes at 4000 rpm, emulsions containing 7.5 and 10 mg.ml^{-1} a cream layer formed but was visually hard to distinguish (Figure 4-4).

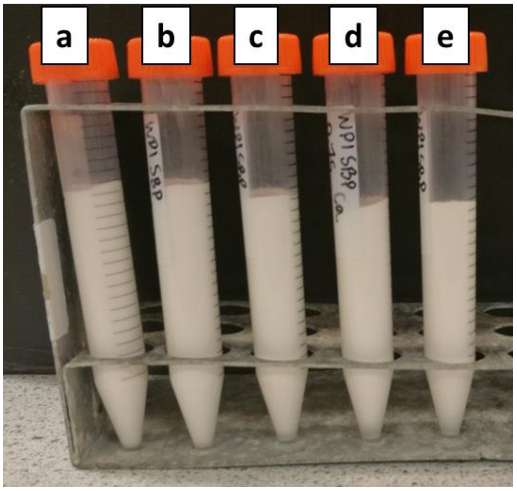


Figure 4-5: Photographs of centrifugation tubes containing 2.5% w/w WPI-SBP stabilised oil-in-water emulsions (20% w/w sunflower oil) with 1 mg.mL^{-1} (a), 2.5 mg.mL^{-1} (b), 5 mg.mL^{-1} (c), 7.5 mg.mL^{-1} (d) and 10 mg.mL^{-1} (e) $CaCl_2$ added before emulsification. Photographs were taken immediately after centrifugation.

The emulsions produced exhibited a visual increase of viscosity with an increasing salt concentration. The shear viscosity as a function of the shear rate of emulsions containing 0 to 10 mg.mL^{-1} $CaCl_2$ was thus measured (Figure 4-4 b). The droplet size was also measured and the microstructure observed as shown in Figure 4-3.

All emulsions exhibited a shear thinning behaviour unaffected by the presence of salt. The measurements confirmed that the addition of calcium chloride brought about an increase of the shear viscosity. Nevertheless this increase seemed to stop from 7.5 $mg.mL^{-1}$ $CaCl_2$. At 7.5 and 10 $mg.mL^{-1}$ salt, the two emulsions exhibited similar shear viscosities.

The observation of the microstructure of the emulsions coupled with the droplets size measurements (Figure 4-3 a) confirmed the presence of aggregates in the emulsions. The aggregates were responsible for the increase in viscosity. The emulsions stayed stable to centrifugation.

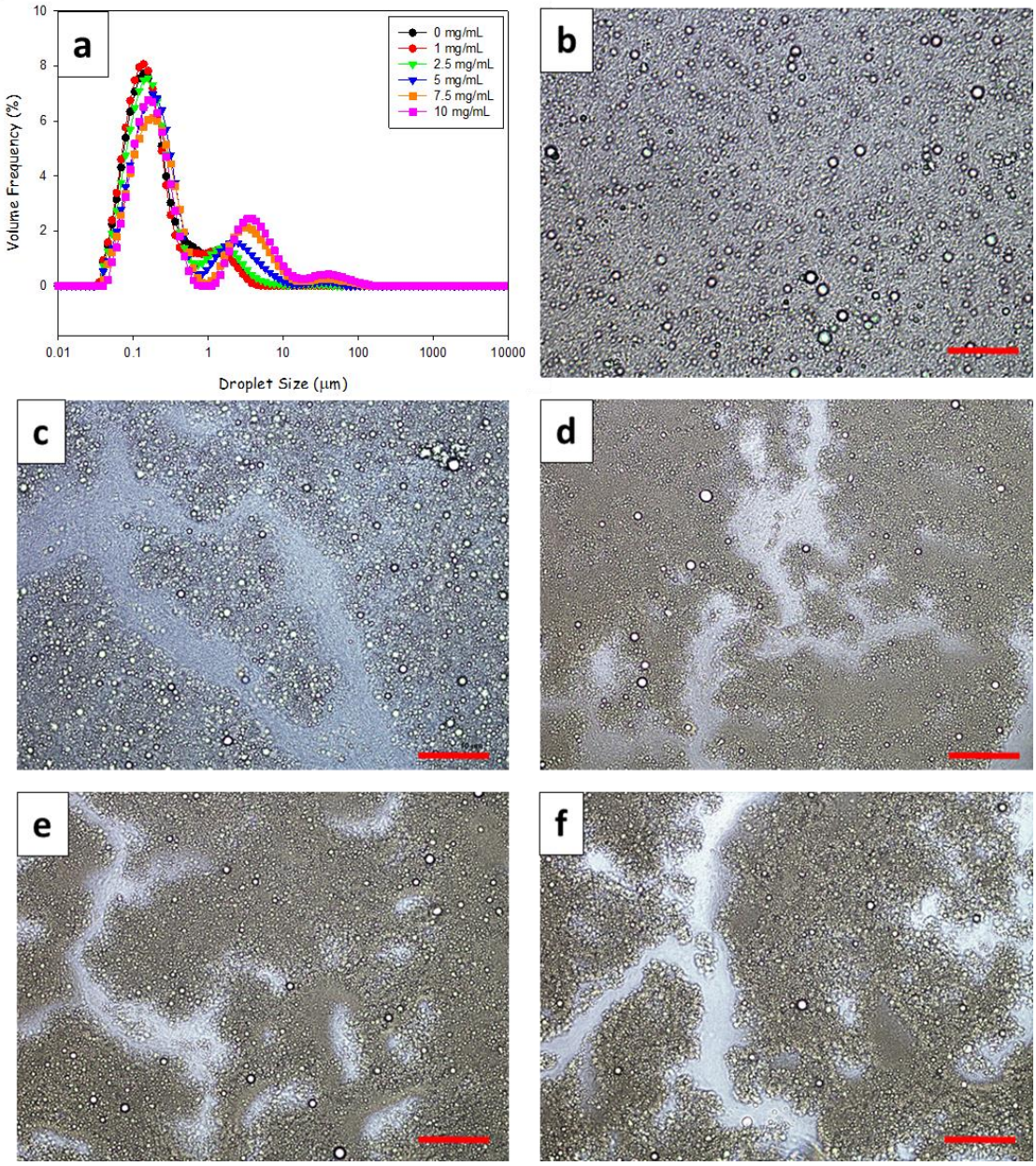


Figure 4-6: Droplet size distribution (a) and optical microscope images (b to f) of WPI-SBP stabilised oil-in-water emulsions (20% w/w sunflower oil) in the presence of increasing concentrations of $CaCl_2$. Microscope images were taken on emulsions stabilised with 2.5% w/w. WPI-SBP containing 1 $mg.mL^{-1}$ (b), 2.5 $mg.mL^{-1}$ (c), 5 $mg.mL^{-1}$ (d). 7.5 $mg.mL^{-1}$ (e) and 10 $mg.mL^{-1}$ (f) $CaCl_2$. Scale bars represent 50 µm.

The same experiments were performed using NaCl. The WPI stabilised emulsion were all visually stable after homogenisation. After centrifugation at moderate speed, emulsions from 5 $mg.mL^{-1}$ NaCl had creamed (Figure 4-6).

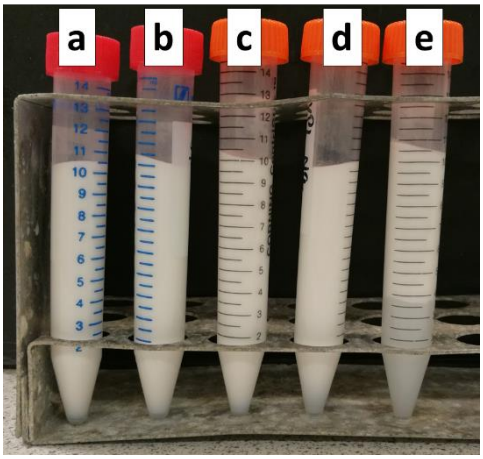


Figure 4-7: Photographs of centrifugation tubes containing ~1.7% w/w WPI stabilised oil-in-water emulsions (20% w/w sunflower oil) with 1 mg.mL^{-1} (a), 2.5 mg.mL^{-1} (b), 5 mg.mL^{-1} (c), 7.5 mg.mL^{-1} (d) and 10 mg.mL^{-1} (e) NaCl added before emulsification. Photographs were taken immediately after centrifugation.

Figure 4-7 shows that for WPI-SBP stabilised emulsions the shear viscosity was less affected by the addition of NaCl in comparison with $CaCl_2$. The viscosity of an emulsion is greatly influenced by droplets movements in the bulk (Pal 1996, Derkach 2009). Adding calcium chloride generated more interactions between the droplets than the addition of sodium chloride, once more due to the bivalency of the calcium ions.

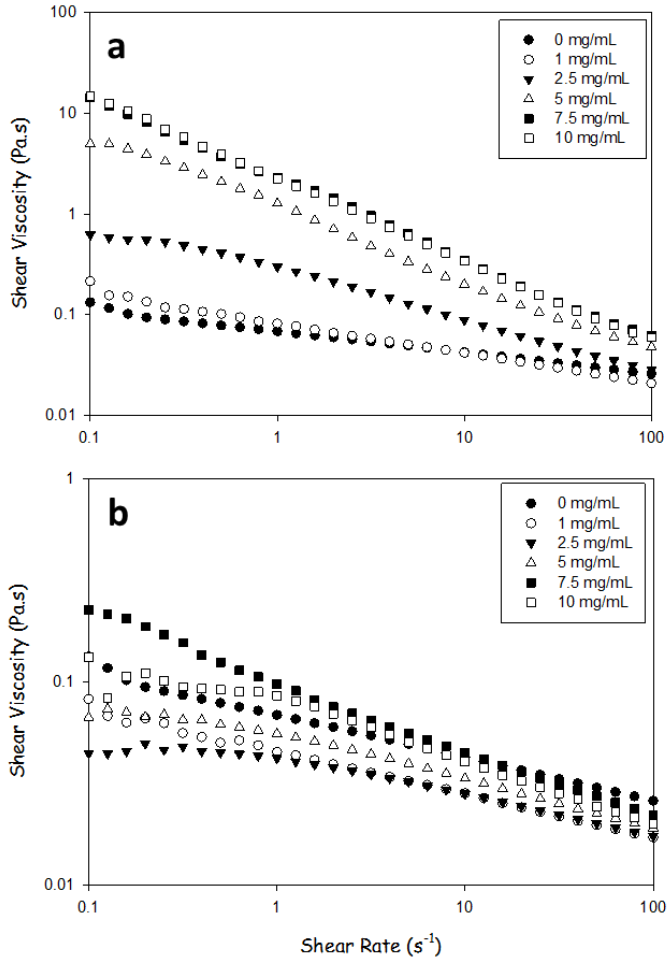


Figure 4-8: Effect of the addition of calcium chloride (a) and sodium chloride (b) before emulsification on the shear viscosity of oil-in-water emulsions stabilised with 2.5% w/w WPI-SBP (~1.7% w/w WPI, conjugate protein/polysaccharide ratio 2:1).

The droplet size measurements (Table 4-3) were consistent with this conclusion. As the salt concentration increased both the surface and volume mean diameter displayed a negligible increase.

Table 4-3: Droplet size parameters of 2.5% w/w WPI-SBP stabilised oil-in-water as a function of NaCl concentration.

Salt Concentration (mg/mL)	$D_{3,2}$ (µm)	$D_{4,3}$ (µm)	Span
0	0.144 ± 0.003	0.375 ± 0.007	4.968 ± 0.465
1	0.132 ± 0.001	0.312 ± 0.001	4.688 ± 0.009
2.5	0.133 ± 0.001	0.321 ± 0.002	4.921 ± 0.029
5	0.136 ± 0.001	0.342 ± 0.003	5.199 ± 0.032
7.5	0.137 ± 0.001	0.360 ± 0.005	5.511 ± 0.063
10	0.143 ± 0.001	0.395 ± 0.007	5.825 ± 0.108

4.3.1.2 Effects of salt addition after emulsion

Adding the salt before emulsification affects directly the emulsifying properties and structure of hydrocolloids emulsifiers (Delahaije 2014, Xu, Liu et al. 2015). When added after emulsification, the salt affects the microstructure of the emulsion itself. Consequently, differences of stability can be expected depending on whether the salt is adding before or after homogenisation.

The effects of the addition of NaCl and $CaCl_2$ (1, 2.5, 5, 7.5 and 10 mg.mL-1) after emulsification were studied on NaCN and CN-De stabilised emulsions (80% w/w water, 20 % w/w oil, ~0.8% w/w protein, conjugate protein/polysaccharide ratio 1:2).

All the emulsions formulated remained visually stable shortly after salt addition. Therefore, the stability to centrifugation was assessed.

Emulsions stabilised with NaCN with added $CaCl_2$ showed limited stability to centrifugation. After 30 minutes at 4000 rpm, only the emulsions containing 1 $mg.mL^{-1}$ (0.009 M) of calcium chloride were stable. Different results were obtained in the presence of NaCl. After

centrifugation only the emulsions containing high salt concentrations (7.5 and 10 mg.mL^{-1}) showed sign of creaming. This difference in results between the two salts can certainly be explained by the difference of ionic strength changes undergone by the emulsions. The same mass concentration of salt led to different ionic strength for the solution. Based on the following equation (Equation 3-2) used to calculate the ionic strength of a solution, it is also noticeable that its value is dependent on the oxidation number of the species. Multivalent ions like Ca^{2+} will bring greater ionic strength changes. According to the Schulze-Hardy rule stating that the greater the valence of the counter ion the faster the coagulation of charged hydrocolloids in solution this would lead to a higher rate of aggregation with Ca^{2+}.

$$\mathrm{I} = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2$$

Equation 4-3

Table 4-3 summarises the conversion from mass concentration to molar concentration and ionic strength at the different salt concentrations. For NaCl, 1 mg.mL^{-1} resulted in an ionic strength of ~0.02 M. Whilst for $CaCl_2$ at the same mass concentration, the resulting ionic strength was 0.027 M. This value rose with increasing salt concentration. At a salt concentration of 5 mg.mL^{-1} the difference of stability to centrifugation between sodium chloride and calcium chloride was apparent. At this concentration the gap of ionic strength values between the two salts significantly widened; 0,085 M for NaCl and 0.135 M for $CaCl_2$.

Table 4-4: Conversion of sodium chloride and calcium chloride from mass concentration to molar mass and ionic strength.

Salt Concentration (mg/mL)	NaCl		$CaCl_2$	
	Molar Concentration (mol/L)	Ionic Strength (mol/L)	Molar Concentration (mol/L)	Ionic Strength (mol/L)
1	0.017	0.017	0.009	0.027
2.5	0.043	0.043	0.023	0.069
5	0.085	0.085	0.045	0.135
7.5	0.128	0.128	0.068	0.204
10	0.171	0.171	0.09	0.27

The same experiments were carried out on CN-De stabilised emulsions (20% w/w sunflower oil, 2.5% w/w conjugate). Emulsions with added calcium chloride (1, 2.5, 5, 7.5 and 10 $mg.mL^{-1}$) were stable to centrifugation up to 5 $mg.mL^{-1}$ of salt. These results were compared with results from experiments on NaCN stabilised emulsions (20 % w/w sunflower oil, ~0.8% w/w protein). The latter emulsions had creamed upon centrifugation from 2.5 $mg.mL^{-1}$ $CaCl_2$. Emulsions stabilised with CN-De exhibited a better stability to moderate salt addition. Improved salt stability had already been reported by other authors when using glycoconjugates as emulsifiers (Nakamura, Kato et al. 1992, Kato, Minaki et al. 1993, Akhtar and Dickinson 2003).

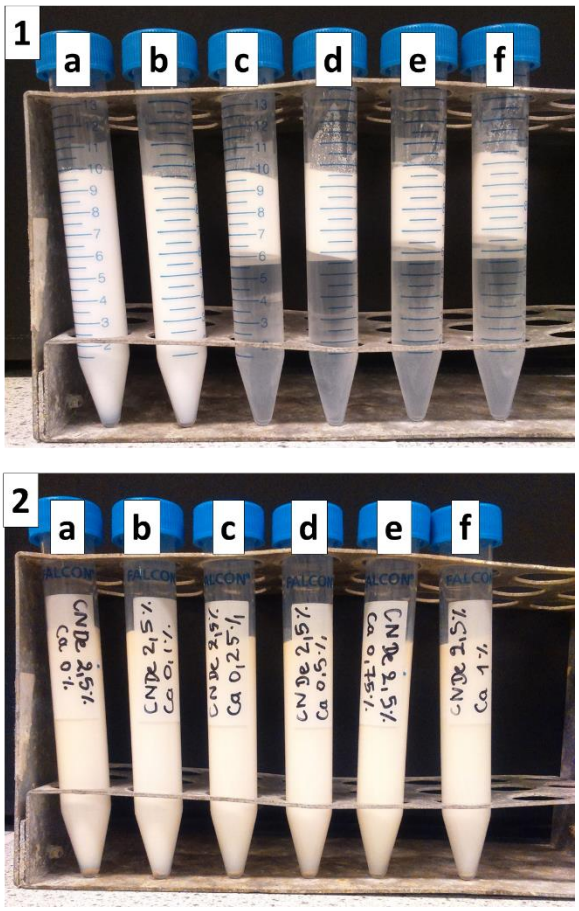


Figure 4-9: Photographs of centrifugation tubes containing ~0.8% w/w NaCN stabilised oil-in-water emulsions (20% w/w sunflower oil) (1) and 2.5% w/w CN-De stabilised oil-in-water emulsions (20% w/w sunflower oil) (2), with 0 mg.mL^{-1} (a), 1 mg.mL^{-1} (a), 2.5 mg.mL^{-1} (b), 5 mg.mL^{-1} (c), 7.5 mg.mL^{-1} (d) and 10 mg.mL^{-1} (e) NaCl added before emulsification. Photographs were taken immediately after centrifugation.

More drastic differences of results were obtained with calcium chloride. With NaCN as emulsifier only the emulsion containing 1 mg.mL^{-1} $CaCl_2$ was stable to centrifugation. With CN-De, after centrifugation only the emulsions containing high concentrations of salt (7.5 and 10 mg.mL^{-1}) had creamed, however the cream layer was hard to visually distinguish. Once again, this difference could be attributed to the steric repulsion provided by the dextrin moieties surrounding the oil droplets.

The observation of the microstructures of the emulsions (Figure 4-5) revealed signs of predictable flocculation over a longer period of time.

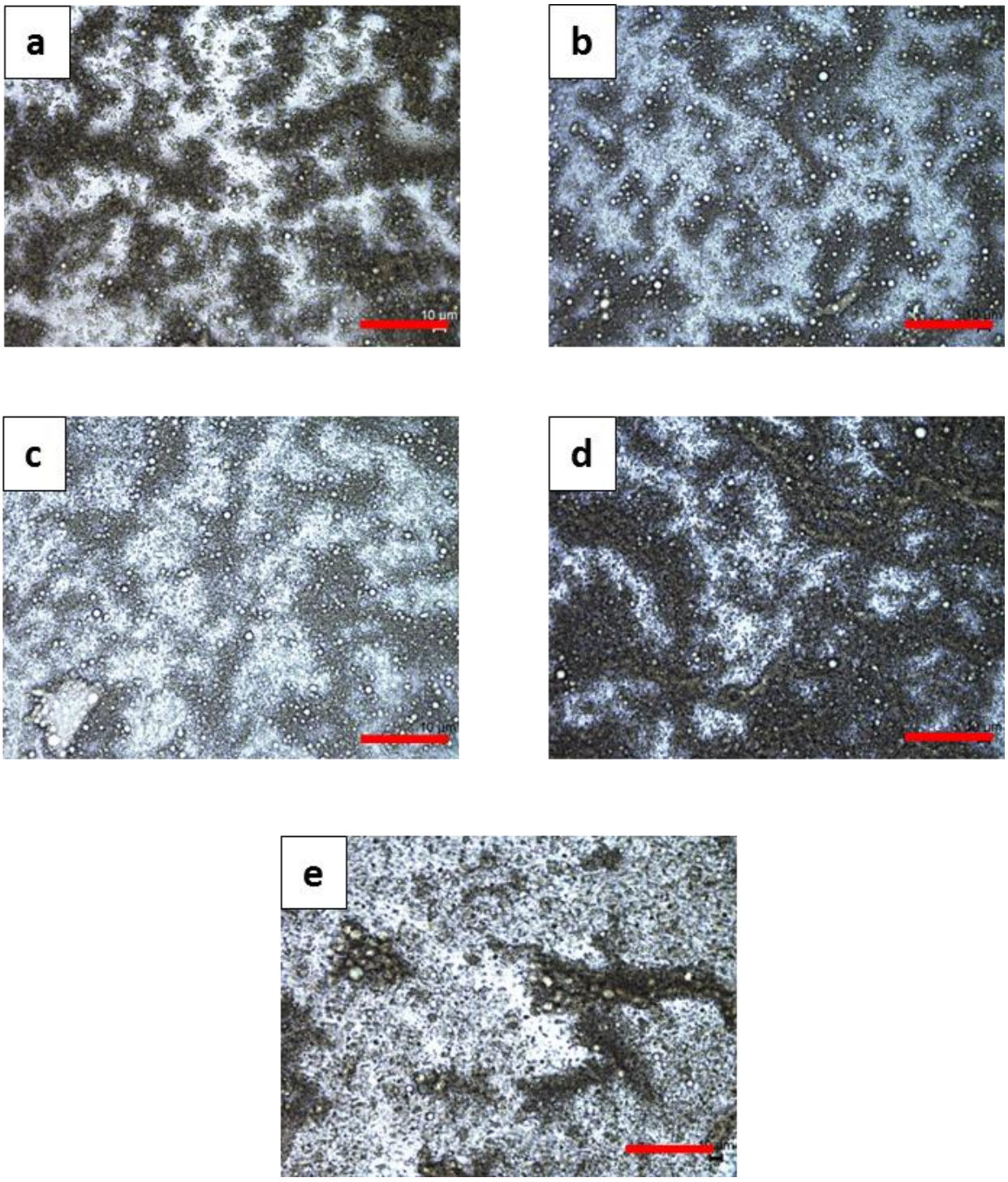


Figure 4-10:Optical microscope images of 2.5% w/w CN-De stabilised oil-in-water emulsions with 0.1% w/w (a), 0.25% w/w (b), 0.5% w/w (c), 0.75% w/w (d) and 1% w/w (e) added $CaCl_2$. Scale bars represent 100 µm.

Experiments on the addition of salt after emulsification of CN-De stabilised emulsions revealed that the screening and binding of the salt to protein moieties enable the formation of droplet aggregates.

The addition of NaCl to both WPI and WPI-SBP stabilised emulsions (~1.7% w/w WPI, 20% w/w sunflower oil) had no effect on the stability of the emulsions. Both series of emulsions appeared visually stable right after homogenisation and were stable to centrifugation at every salt concentration tested.

The droplet size and viscosity of the WPI-SBP stabilised emulsions of interest for this study were investigated after addition of NaCl (respectively Table 4-4 and Figure 4-6).

The addition of salt (up to 10 $mg.mL^{-1}$) had no effect on the droplet diameter of the emulsions (Table 4-4). The rheology data (Figure 4-6) revealed that the viscosity was also not affected meaning that the interaction between droplets remained the same after addition of salt.

As concluded for CN-De stabilised emulsions, the increased salt stability already observed by other researchers (Akhtar and Dickinson 2003) was due to the steric hindrance provided by SBP and preventing droplets encounter.

Table 4-5: DLS analysis data for 2.5% w/w WPI-SBP stabilised emulsions with increasing concentration of sodium chloride. Values are presented as mean ± standard deviation.

NaCl concentration (mg/mL)	D 3,2 (nm)	D 4,3 (nm)	Span
0	144 ± 3	375 ± 7	4.968 ± 0.465
1	138 ± 3	378 ± 23	5.699 ± 0.179
2.5	140 ± 5	406 ± 27	6.198 ± 0.201
5	135 ± 2	375 ± 27	5.855 ± 0.222
7.5	137 ± 4	401 ± 28	6.478 ± 0.359
10	138 ± 2	406 ± 13	6.437 ± 0.007

The difference in stability between the protein and the conjugate upon addition of $CaCl_2$ were intensified. After addition of the salt both emulsions stayed visually stable. Creaming was induced through centrifugation for all WPI stabilised emulsions with the formation of an obvious cream layer. On the other hand for emulsions stabilised with WPI-SBP the cream layer was hard to distinguish after centrifugation from 5mg.mL^{-1} $CaCl_2$. Emulsions containing low salt concentrations (i.e. 1 and 2.5 mg.mL^{-1}) were resistant to centrifugation.

These results were in agreement with previous observations. The conjugation of whey protein to the pectin helped enhancing the salt stability of the emulsions through steric repulsion. Figure 4-6 shows that the addition of salt after emulsification also led to an increase of the shear viscosity.

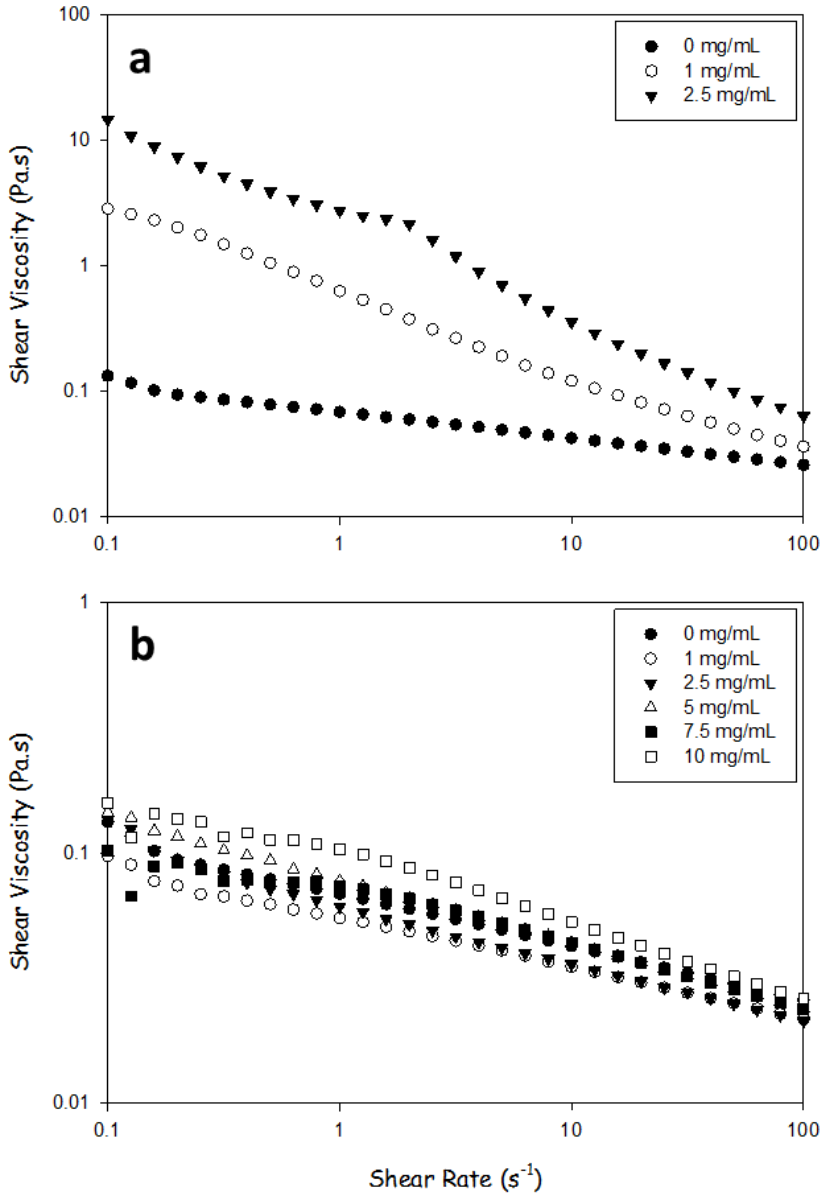


Figure 4-11: Effect of the addition of calcium chloride (a) and sodium chloride (b) after emulsification on the shear viscosity at room temperature (22°C) of oil-in-water emulsions 920% w/w sunflower oil) stabilised with 2.5% w/w WPI-SBP (~1.7% w/w WPI, conjugate protein/polysaccharide ratio 2:1, pH 5.8).

4.3.2 Effects of pH

The effect of pH on emulsifying properties of the conjugate was investigated. This study had for aim to compare the ability of the conjugates to stabilise emulsion under different pH conditions in comparison with the proteins. Several authors have demonstrated that the protein-polysaccharide conjugates displayed better emulsifying properties around the isoelectric point of their protein (O'Regan and Mulvihill 2009). Others showed that they also provided better emulsifying properties at acidic and alkaline pH (Wong, Day et al. 2011, Perrechil, Santana et al. 2014, Barbosa, Ushikubo et al. 2018).

Following these results, the emulsifying properties of CN-De were studied at pH 2, 4.65 (i.e. the isoelectric point of sodium caseinate) and at pH 12.

The pH range was not extended beyond pH 8 as no change in the trend was expected considering the charges present on the molecules at this pH.

Beforehand the solubility of CN-De and NaCN was measured from pH 2 to 8. The ζ-potential of CN-De was measured on that same range of pH.

Results from the solubility measurements gathered in Figure 4-7 corroborated observations made in previous studies (Chevalier, Chobert et al. 2001, O'Regan and Mulvihill 2009, Mulcahy, Park et al. 2016).

The solubility of the conjugate stayed constant over the range of pH while the solubility of NaCN progressively decreased. Starting at ~96%, it decreased as the pH approached the isoelectric point to finally reach ~20% at pH 4.5.

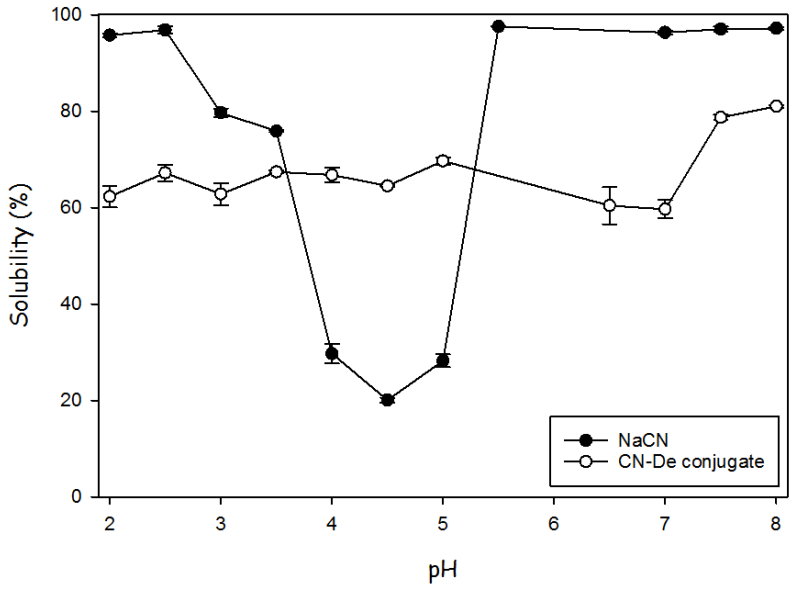


Figure 4-12: Solubility of sodium caseinate and CN-De as a function of pH. All samples were measured in triplicate. Error bars represent standard deviation

Zeta potential measurements (Figure 4-8) revealed that the values for ζ-potential of the conjugate as function of pH was similar with the ones for NaCN (Post, Arnold et al. 2012). The ζ-potential decreased with an increasing pH. At pH=pI, the zeta potential reached 0. Above this pH value, the zeta potential stayed negative.

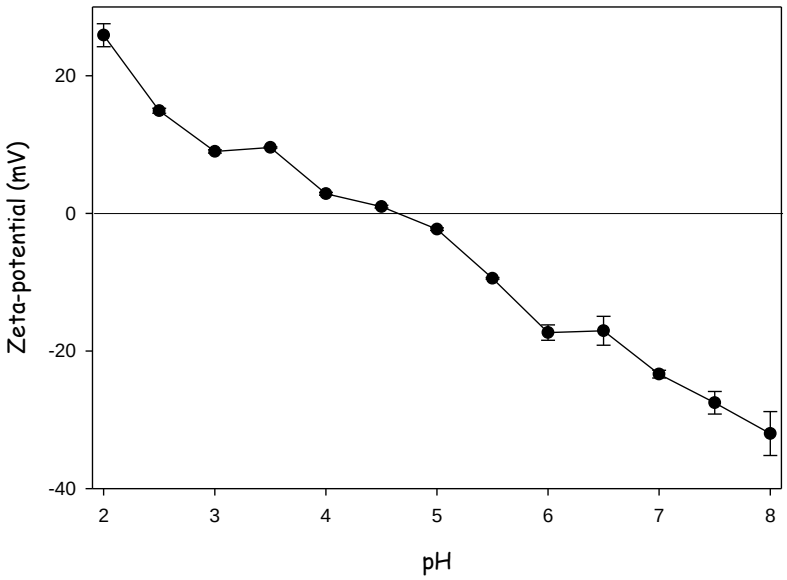


Figure 4-13: Zeta potential as function of pH for CN-De. Data points are averages of three measurements. Error bars represent standard deviation.

Unlike when using CN-De as emulsifier, the use of WPI-SBP presented two challenges. Both WPI and SBP are pH sensitive hydrocolloids. WPI has an isoelectric point reported in the region 4.5-5.2 (Bystrický, Malovíková et al. 1990, Bystrický, Malovíková et al. 1991) while SBP bears on its structure acid functions (galacturonic acid) and has a pKa of ~3 (Cape, Cook et al. 1974). Considering these values, the emulsifying properties of WPI-SBP were studied at pH 3, 5.2 and 12.

To assess the effect of the conjugation on the solubility it was relevant, in this case, to measure the solubility of sugar beet pectin on the same range of pH as the whey protein and the conjugate. Therefore, the solubility of WPI, SBP and WPI-SBP were measured from pH 2 to 8 (Figure 4-9). The ζ-potential of WPI-SBP was measured on the same range of pH (Figure 4-12). For the same reasons as mentioned for CN-De the solubility at pH 12 was disregarded.

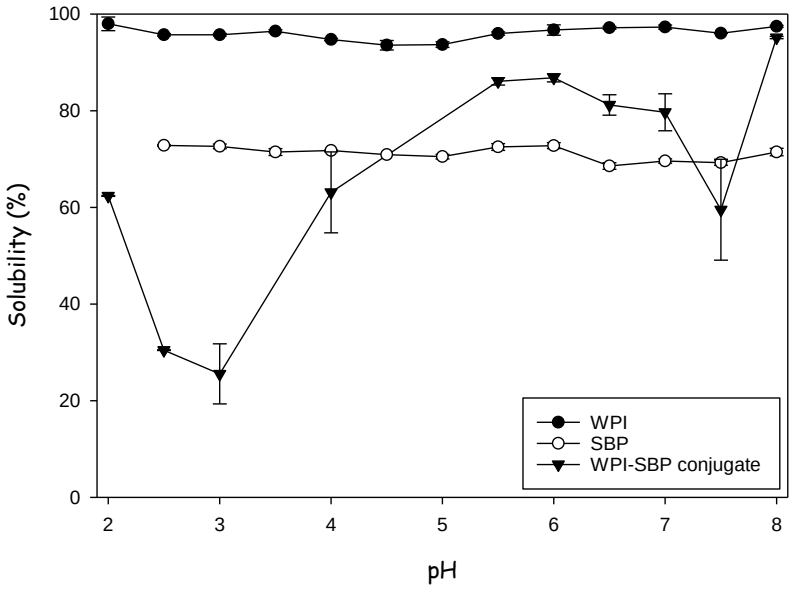


Figure 4-14: Solubility of whey protein, sugar beet pectin and WPI-SBP as a function of pH. All samples were measured in triplicate. Error bars represent standard deviation.

The solubility measurements results (Figure 4-9) differed from the one obtained from CN-De (Figure 4-7). Indeed, for WPI-SBP the solubility of the conjugate was lower than the one of the protein on the entire pH range expect for pH 8. The solubility of WPI was constant at ~96% over the range of pH while the solubility of SBP was constant at ~71%.

On the other hand, the solubility of WPI-SBP showed a drastic decrease from pH 5.5 reaching a minimum (25%) at pH 3.

The decrease of WPI-SBP solubility below pH 6 was a direct consequence of the coexistence of positive and negative charges in the conjugate over this range of pH. Figure 4-10 shows a schematic representation of the charges present on the conjugate depending on the environmental pH. Theoretically from pH ~6.3 to ~2, positive and negative charges coexist, born by WPI, SBP or both depending on the pH.

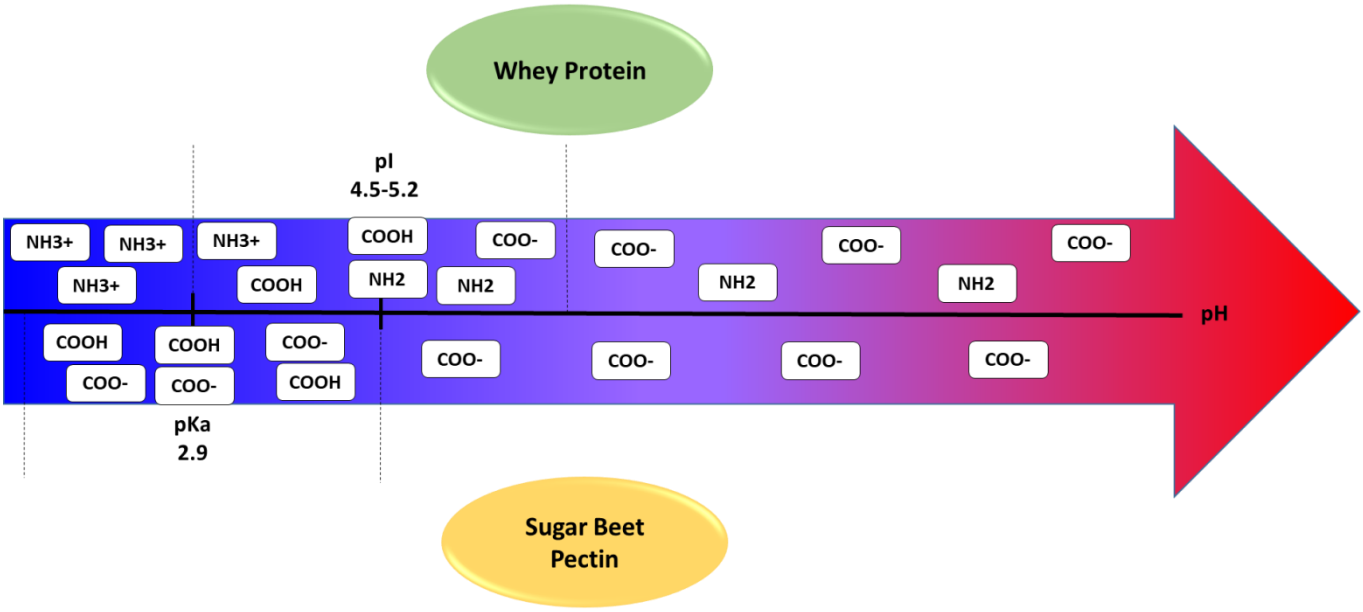


Figure 4-15: Schematic representation of the charges repartition on whey protein and sugar beet pectin as a function of pH.

Over this range of pH, interparticle attraction can lead to the formation of aggregates lowering the solubility of conjugate. These aggregates, considering their low solubility, seem similar to the coacervates described previously (c.f. Chapter 2).

The lowest solubility was measured at pH 3. The pKa of SBP has been reported at 2.9. Thus at pH 3, in theory, 50% of the acid functions of SBP were dissociated and the other half protonated.

Below pH 3, the amount of protonated acid functions slowly increased resulting in the breakdown of the previously formed aggregates hence the slow increase of the solubility below this value.

Zeta potential measurements (Figure 4-12) were consistent with the conclusion made from the solubility measurements.

Below pH 5.5 the ζ-potential displayed a drastic increase towards positive value. From -36 ± 2 mV at pH 5.5 to -8.9 ± 0.3 mV at pH 4. After pH 4 it slowly progressed to finally reach 1.5 ± 0.1 mV at pH 2.

At pH above 5.5 the ζ-potential was expected to stay negative and relatively consistent. The results obtained on this range of pH overall met the expectation. Nevertheless an inexplicable variation that could be the result of discrepancies in the samples.

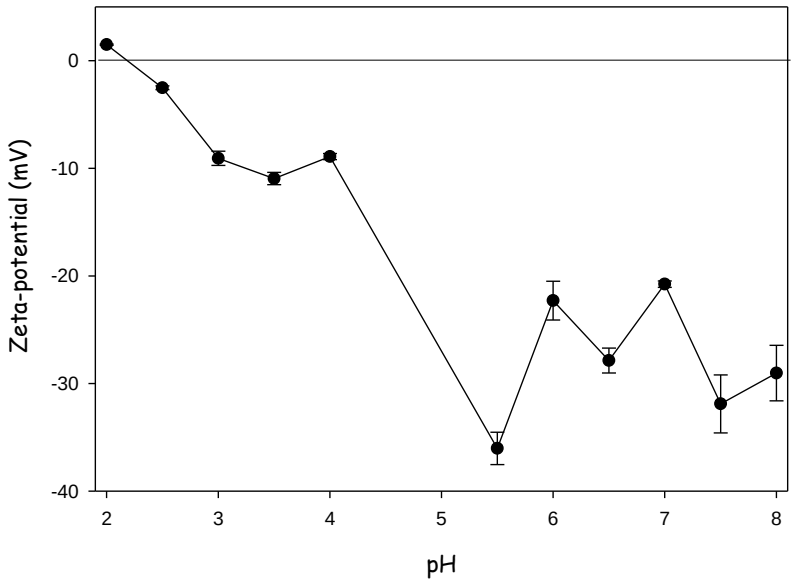


Figure 4-16: Zeta potential as function of pH for WPI-SBP. Data points are averages of three measurements. Error bars represent standard deviation.

The solubility experiments (Figure 4-9) revealed that from pH 5.5 the solubility of the conjugates decreased significantly. This decrease was attributed to the formation of insoluble aggregates (coacervates). Overlapping this data set with the decrease of the ζ-potential at the same pH supported this hypothesis.

4.3.2.1 Oil-in-water emulsions

Emulsions stabilised with 2.5% w/w CN-De (i.e. ~0.8% w/w NaCN) at the desired pH were produced and the pH checked after homogenisation. All emulsions were visually stable after emulsification. At pH=pI, the same emulsion was prepared using NaCN.

The protein stabilised emulsion displayed droplet aggregation during the homogenisation process. This result was consistent with protein precipitation at pH = pI. The precipitation of NaCN impacted the emulsifying properties of this latter.

In an attempt to induce creaming the conjugate stabilised emulsions were centrifuged. After centrifugation a clear cream layer formed in the emulsion at pH 4.65 (i.e. the isolectric point of sodium caseinate). Emulsions at pH 2 and 12 showed no visual signs of creaming or separation. The physical aspect of these emulsions was observed using an optical microscope. The microscope images along with the droplet size measurements are displayed in Figure 4-13.

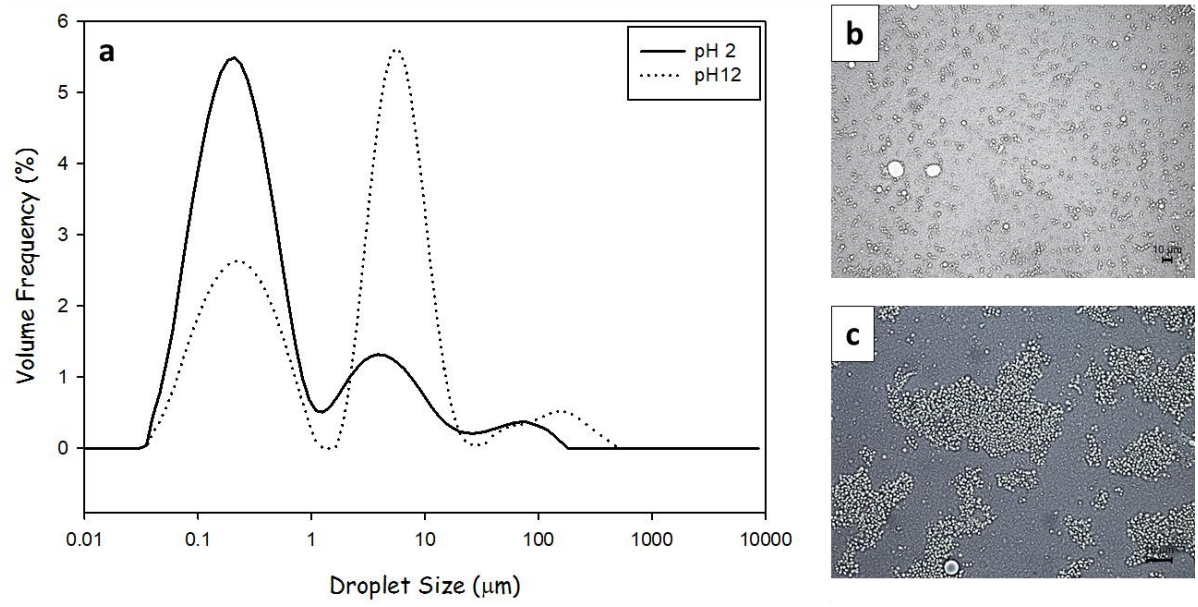


Figure 4-17: Droplet size distribution and optical microscope images of CN-De stabilised oil-in-water emulsions at pH 2 and 12. Microscope images were taken on an emulsion stabilised with 2.5% wt. CN-De at pH 2 (b) and 12 (c).

The emulsions showed signs of droplet aggregation both visually (Figure 4-13 b and c) and using DLS analysis (Figure 4-13 a).

The zeta potential measurements performed on the conjugates on the range of pH (Figure 4-8) suggested that at pH 2 and 12, the conjugates would enable emulsion stability through electrostatic repulsion. At pH 2, the oil-in-water emulsion displayed a ζ-potential of 20.2 ± 4.5 mV. At pH 12, it was -37.7 ± 1.4 mV (point not displayed in the figure). These values were consistent with the ζ- potentials measured on the conjugate solutions (Figure4-8).

However at acidic pH despite the protonation of the amino groups of NaCN to form NH_3^+ and induce electrostatic repulsion, carboxylic groups become protonated enabling the formation of hydrogen bonds between proteins chains (Goto, Calciano et al. 1990). The folding and unfolding mechanisms occurring at acidic pH in proteins when in solution generated droplet

aggregation in the emulsion. However, the mechanism leading to droplet aggregation at pH 12 remains unclear.

Emulsions stabilised with 2.5% w/w WPI-SBP (i.e. ~1.7% w/w WPI) at pH 2.9, 5.2 and 12 were produced. After homogenisation the pH was checked and adjusted if needed.

Out of all the emulsions produced only the emulsion prepared at pH 2.9 had creamed shortly after homogenisation. The solubility of the conjugate (25.57 %) at this pH was the lowest measured (Figure 4-9). Emulsifiers with high solubility show better emulsifying properties. Considering the low solubility of WPI-SBP at pH 3, creaming was expected in the emulsion.

The solubility of WPI-SBP was the lowest at this pH supposedly due to the formation of aggregates.

The ζ-potential also displayed higher value (-9.1 ± 0.7 mV) at this pH, meaning the presence of both negative and positive charges at the surfaces of the conjugates.

This value confirmed the formation of aggregates.

Emulsions at pH 5.2 and 12 were visually homogenous and stable to centrifugation. The microscope analyses revealed densely packed nano-sized droplets for both pH.

A WPI stabilised emulsion was produced at pH 5.2 as a control sample to confirm the enhanced emulsifying ability of WPI-SBP at pH=pI. The emulsion creamed right after homogenisation.

Despite the droplet aggregation observed at pH=pKa, the WPI-SBP conjugate still enabled the production of well dispersed emulsions at pH=pI.

4.3.3 Heat stability

Resistance of food emulsion to heat is a crucial parameter for different production processes. In this study investigating the resistance to heat of the emulsions was directly linked to the use of spray-drying as a means to later produce oil filled powders.

Proteins are known to be heat sensitive compounds (Dissanayake, Ramchandran et al. 2013, Dissanayake, Ramchandran et al. 2013, Wijayanti, Brodkorb et al. 2019). However casein defies this statement by displaying stability to moderately high temperatures (50 °C) (Raikos 2010).

Whey proteins undergo denaturation and aggregation when exposed to high temperatures (Wijayanti, Brodkorb et al. 2019). In emulsions this translates into droplets aggregation (Millqvist-Fureby, Elofsson et al. 2001, Raikos 2010).

Several studies have identified conjugation as a means to improve the heat stability of whey proteins (Jiménez-Castaño, López-Fandiño et al. 2005, Jimenez-Castano, Villamiel et al. 2007).

The heat stability of oil-in-water emulsions stabilised with either a protein (NaCN or WPI) or a conjugate (CN-De or WPI-SBP) was assessed by exposing the emulsions to a temperature above the denaturation temperature of whey proteins (i.e. 95 ˚C) for increasing time periods. The protein stabilised emulsions served as a control sample to highlight the effects of conjugation.

NaCN and CN-De stabilised emulsions displayed similar stability to thermal treatment (Figure 4-14 and Table 4-6). Minimal changes in the droplet size were observed with an increasing

temperature for both emulsifiers. Nevertheless, both the surface and volume mean diameters of emulsions stabilised with NaCN showed a greater increase than CN-De stabilised emulsions.

Respectively from 162 ± 4 nm to 190 ± 3 nm and from 364 ± 19 nm to 2.593 ± 1.272 μm for NaCN stabilised emulsions. In opposition with a surface mean diameter varying from 213 ± 9 nm to 202 ± 12 nm and a volume mean dimeter increasing from 372 ± 59 nm to 1.745 ± 0.956 μm for CN-De stabilised emulsions.

These results suggested that exposing the NaCN stabilised emulsions and CN-De stabilised emulsions to high temperature for longer period time or to higher temperatures would have revealed the difference of heat stability between the two systems.

These experiments taken alone failed to confirm the increased stability to heat of the CN-De stabilised emulsions.

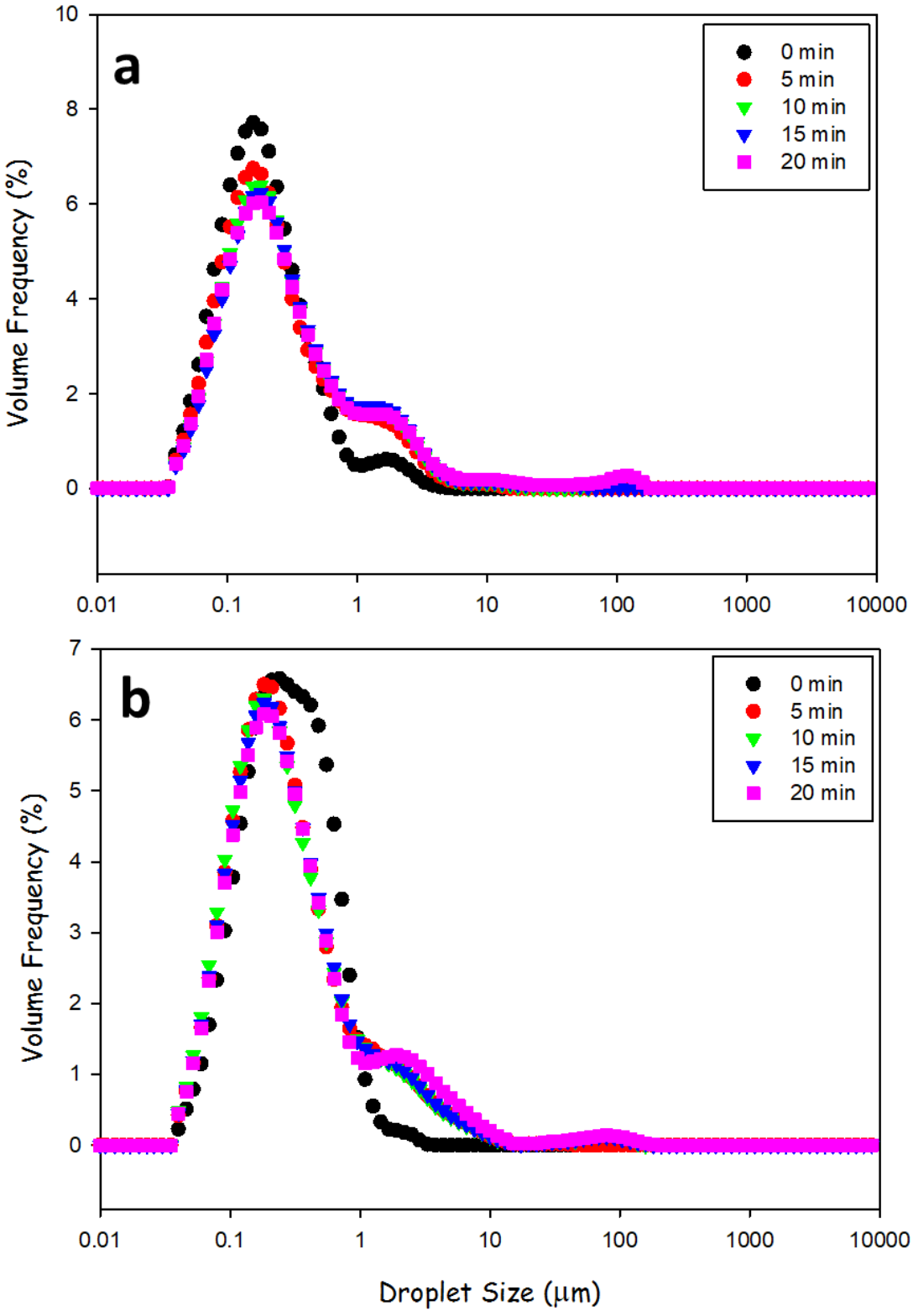


Figure 4-18 : Overtime evolution of droplet size distribution of oil-in-water) stabilised with NaCN (a) and CN-De (b) (~ 0.8% w/w protein, 20% w/w oil, pH 6.8) during storage at 95˚C.

Table 4-6: Droplet size parameters of NaCN and CN-De stabilised oil-in water emulsions (~0.8% w/w protein) as function of the heating time. All samples were measured in triplicate. Values are presented as mean ± standard deviation.

Time (min)	NaCN Emulsion			CN-De Emulsion		
	D3,2 (μm)	D4,3 (μm)	Span	D3,2 (μm)	D4,3 (μm)	Span
0	0.16 ±0.01	0.31 ± 0.03	2.57 ± 0.29	0.20 ± 0.01	0.37 ± 0.01	2.19 ± 0.08
5	0.17 ± 0.01	0.54 ± 0.02	5.88 ± 0.11	0.20 ± 0.01	0.67 ± 0.04	5.51 ± 0.78
10	0.19 ± 0.01	0.63 ± 0.02	6.18 ± 0.02	0.19 ± 0.01	1.36 ± 0.61	5.87 ± 1.15
15	0.20 ± 0.01	0.92 ± 0.07	6.74 ± 0.03	0.20 ± 0.01	1.42 ± 0.65	5.96 ± 1.12
20	0.19 ± 0.01	2.59 ± 1.28	7.82 ± 0.51	0.20 ± 0.02	1.75 ± 0.96	8.15 ± 2.32

The oil droplet diameter of WPI stabilised emulsions increased after exposure to high temperature (Figure 4-19). The surface and volume mean diameter increased respectively from 165 ± 4 nm and 713 ± 136 nm to 231 ± 11 nm and 7.809 ± 3.506 μm after 20 minutes at 95 ˚C. These results were consistent with the literature. The instability to heat of whey protein stabilised emulsions was expected.

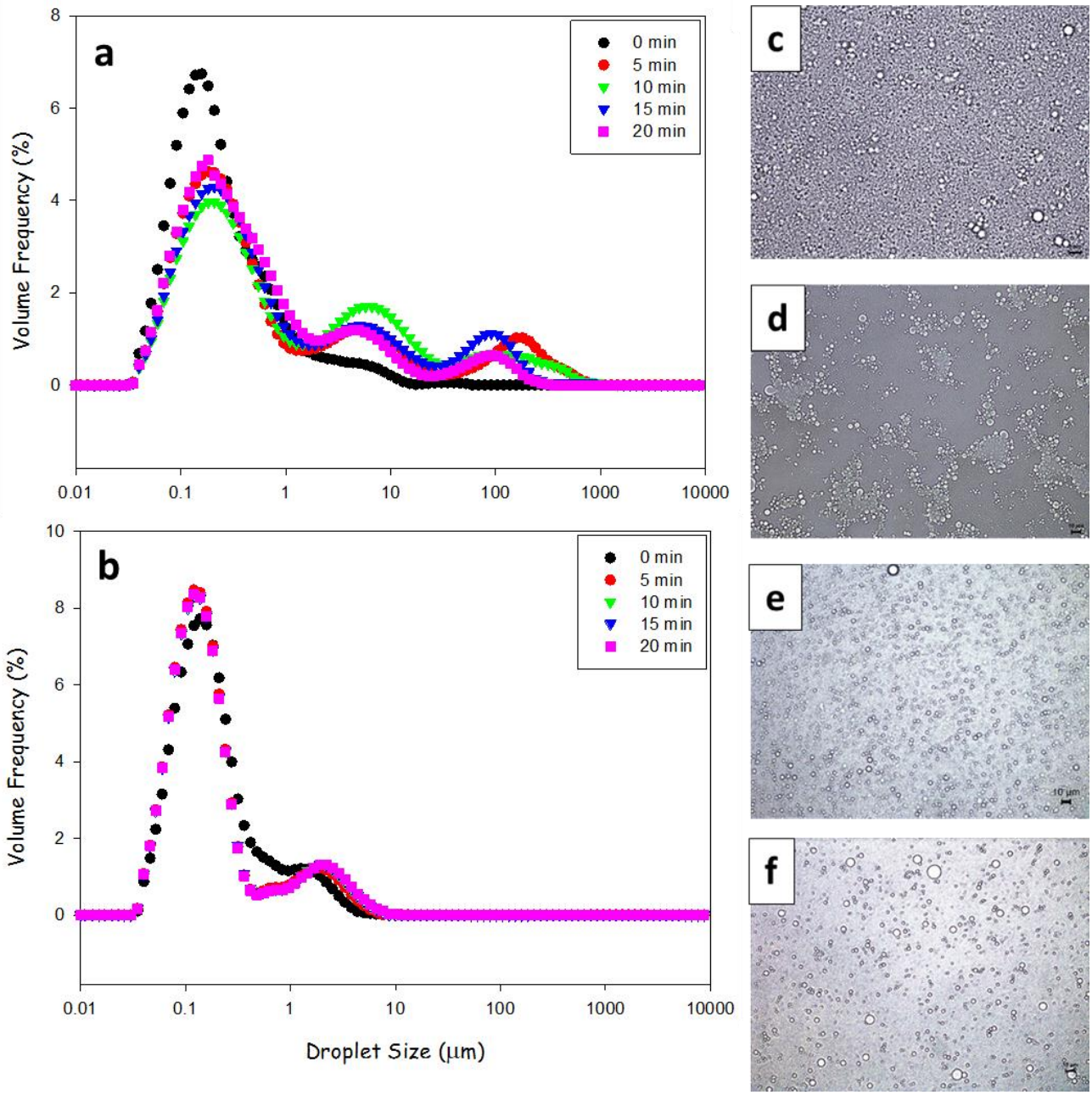


Figure 4-19: Overtime evolution of droplet size distribution of oil-in-water emulsions stabilised with WPI (a) and WPI-SBP (b) (20 % w/w sunflower oil, ~ 1.7% w/w protein) during storage at 95˚C. Microscope images represents the microstructure of a WPI stabilised emulsion before (c) and after (d) storage at 95˚C for 20 minutes; and the microstructure of a WPI-SBP stabilised emulsion before (e) and after (f) storage at 95°C for 20 minutes.

Similarly, the same experiments on WPI-SBP stabilised emulsions (Figure 4-19 b) confirmed the enhanced heat stability. There was no significant difference of droplet size after 5, 10, 15 or 20 minutes at 95 ˚C. The conjugation to SBP increase the heat stability of WPI preventing heat induced aggregation.

Table 4-7: Droplet size parameters of WPI and WPI-SBP stabilised oil-in water emulsions (~1.7% w/w protein) as function of the heating time. All samples were measured in triplicate. Values are presented as mean ± standard deviation.

Time (min)	WPI Emulsion			WPI-SBP Emulsion		
	D3,2 (μm)	D4,3 (μm)	Span	D3,2 (μm)	D4,3 (μm)	Span
0	0.17 ± 0.01	0.71 ± 0.14	5.19 ± 0.55	0.13 ± 0.01	0.38 ± 0.01	4.97 ± 0.47
5	0.24 ± 0.01	26.53 ± 10.16	239.27 ± 128.92	0.13 ± 0.01	0.42 ± 0.01	7.76 ± 0.09
10	0.28 ± 0.01	24.79 ± 8.34	105.77 ± 49.79	0.13 ± 0.01	0.47 ± 0.02	9.15 ± 0.25
15	0.26 ± 0.01	16.61 ± 6.04	122.95 ± 38.07	0.13 ± 0.01	0.47 ± 0.01	9.32 ± 0.10
20	0.23 ± 0.02	7.81 ± 3.51	25.84 ± 44.25	0.13 ± 0.01	0.49 ± 0.02	9.76 ± 0.25

4.3.4 Freeze-thaw stability

The freeze-thaw (FT) stability was studied to assess the ability of an emulsion to be reconstituted after freeze-drying and rehydration which is subsequently studied in Chapter 5. Instabilities caused by the freezing step have a major influence on the structure of the final freeze-dried product (Ghosh and Coupland 2008, Degner, Chung et al. 2014).

During freezing several events can lead to instability of the thawed emulsion and by extension of the freeze-dried and rehydrated emulsion. While expanding, the forming ice can force the oil droplets together leading to the fusion of the interfacial membranes of neighbouring droplets causing irreversible droplets aggregation. The same phenomenon can lead to coalescence if the interfacial membrane is disrupted and at least two droplets merge together. Freezing also makes

less water available to keep the emulsifier hydrated at the droplet surface, the emulsifier slowly desorbs from the surface and coalescence occurs. If the fat crystallises before the water freezes the fat crystals can protrude through the interfacial membrane causing its disruption, this can cause coalescence in the thawed emulsion (Degner, Chung et al. 2014).

Emulsions (80% w/w water, 20% w/w oil, pH 6-7) containing 2.5%, 5%, 7.5%, 10% and 15% w/w CN-De (~0.8%, ~1.7%, 2.5%, ~3.3% and 5% w/w NaCN) were inspected before and after freezing at -22°C and thawing at room temperature. The droplet size distributions before and after are shown in Figure 4-20.

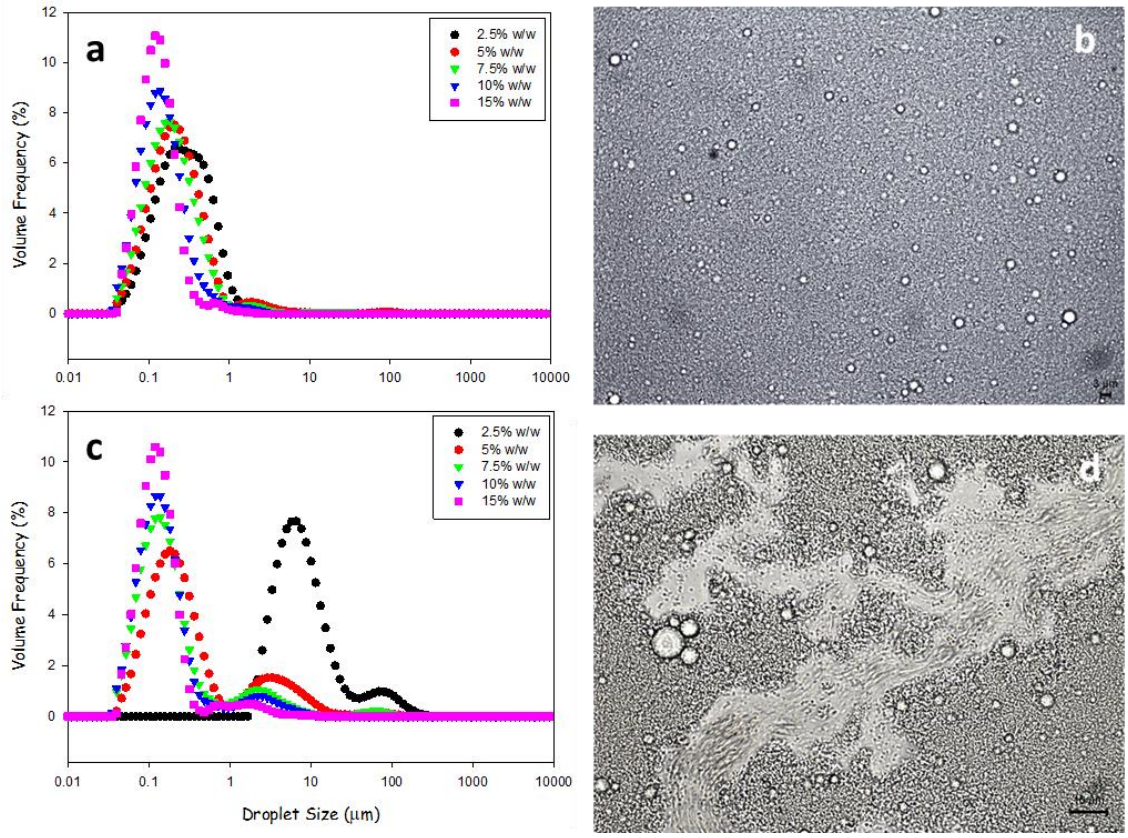


Figure 4-20: Droplet size distribution of CN-De stabilised oil-in-water emulsions (20% w/w sunflower oil) before (a) and after (c) freeze-thawing. Measurements were carried out at 2.5% w/w, 5% w/w, 7.5% w/w, 10% w/w and 15% w/w CN-De.

Microscope images were taken on an emulsion stabilised with 2.5% w/w CN-De before (b) and after (d) freeze-thawing.

The measurements showed that the emulsions from 5% w/w emulsifier (~1.7% w/w protein) displayed good stability to freeze-thawing.

At 2.5% w/w (~0.8% w/w NaCN) microscope images showed that aggregates formed over freeze-thawing (Figure 4-16). The ice forming while freezing forced the droplets together leading to the partial merging of their interfaces. The flocculated droplets were then detected as a single larger droplet by the instrument.

At higher concentrations the improved freeze-thaw stability was attributed to the difference of unfrozen water left after freezing. As the concentration increased, the amount of unabsorbed conjugate also increased in the continuous phase. Other researchers showed that the amount of unfrozen water after freezing was a factor for enhanced freeze-thaw stability (O'Regan and Mulvihill 2010). Upon freezing the difference of concentration of free hydrocolloids would be significant enough to increase the amount of unfrozen water and by extension influence positively the freeze-thaw stability. A slight difference in level of unfrozen water can lead to notable differences in freeze-thaw stability. Some authors assumed that unabsorbed protein in the continuous phase would increase the viscosity or form a transient gel that would provide a mechanical resistance to the deformation generated by the ice expansion (Ghosh, Cramp et al. 2006).

Emulsions stabilised with 2.5% w/w WPI-SBP (~1.7% w/w WPI, 20% w/w sunflower oil) were inspected before and after freezing at -22°C for 24 hours.

The increase of the volume mean diameter from 375 ± 7 nm to 2.88 ± 1.76 µm was the result of aggregates formation revealed by the DLS measurements and the microscope images (Figure 4-17).

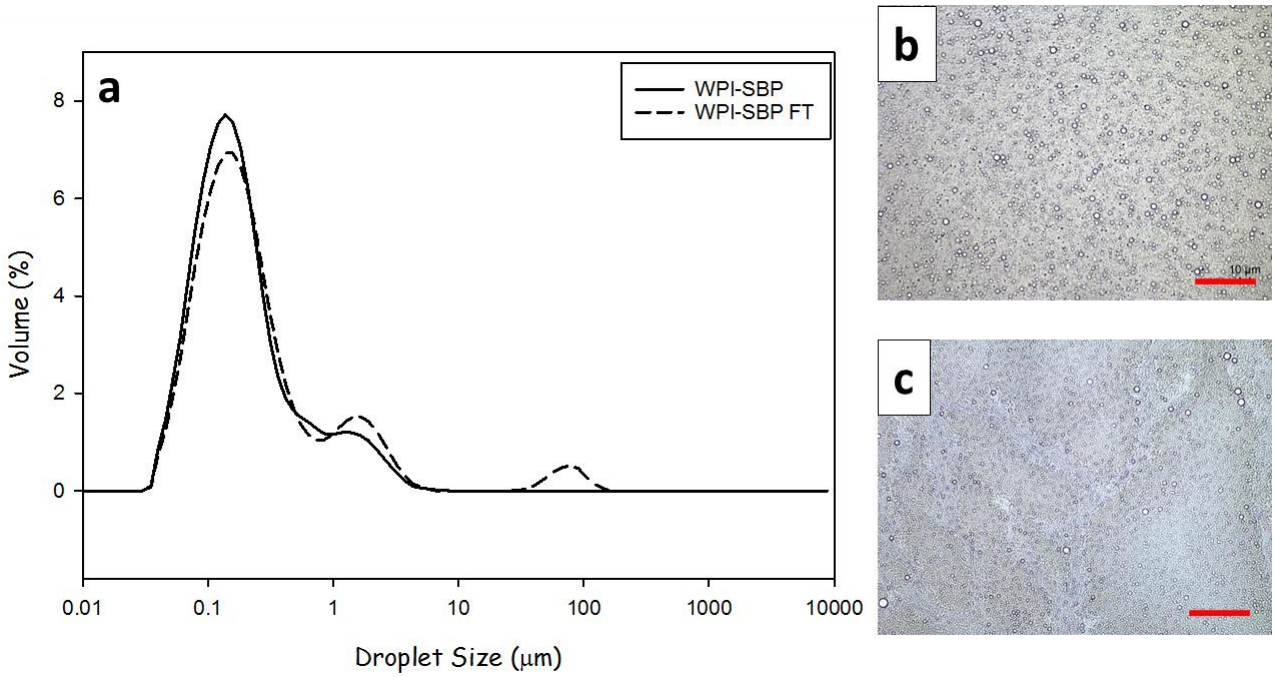


Figure 4-21: Droplet size distribution (a) and optical microscope images of WPI-SBP stabilised oil-in-water emulsions before and after freeze-thawing. Microscope images were taken on an emulsion stabilised with 2.5% w/w WPI-SBP before (b) and after (c) freeze-thawing. Scale bars represent 100 µm.

4.3.4.1 Effect on the pH and zeta potential

Measuring the zeta potential of the emulsions before and after freeze-thaw can be used as a means to evaluate the effect of the process on the composition the interfacial membrane (Xu, Yuan et al. 2010). The pH was also monitored before and after freeze-thaw.

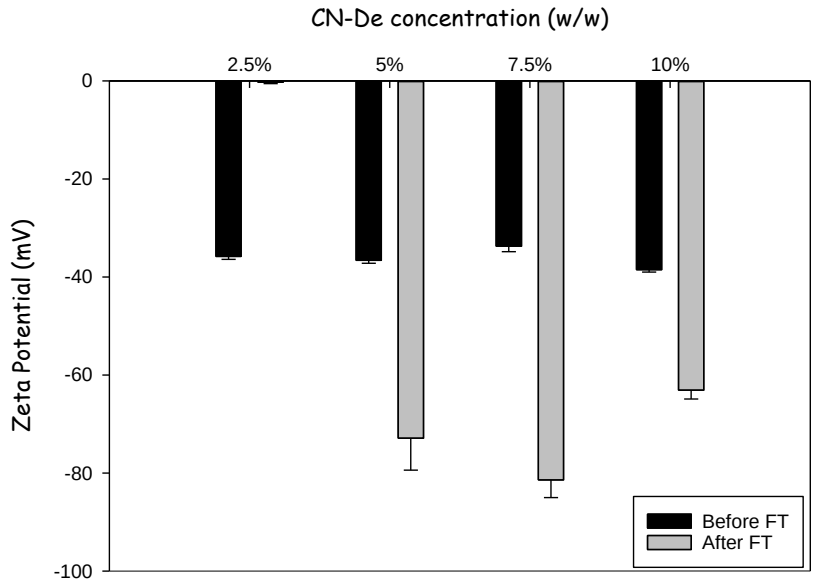


Figure 4-22: Evolution of zeta-potential of CN-De stabilised oil-in-water emulsions before and after freeze-thawing. All samples were measured in triplicate. Error bars represent standard deviation.

Before FT the ζ-potentials of all CN-De stabilised emulsions were similar independently of the conjugate concentration (Figure 4-18). Respectively -35.8 ± 0.6 mV, -36.6 ± 0.6 mV, -33.7 ± 1.1 mV and -38.5 ± 0.5 mV for 2.5%, 5%, 7.5% and 10% w/w conjugate. After FT the ζ-potential of the emulsions stabilised with 5% to 10 % wt. conjugate showed a drastic decrease. From ~ 37 mV to ~ 72 mV.

On the other hand, at 2.5% w/w conjugate, the negative charge decreased to zero. In their study *Xu et al.* (Xu, Yuan et al. 2010) attributed the ζ-potential decrease to a partial adsorption of the unconjugated polysaccharide that remained free in the continuous phase. In the present study the conclusion differed. Indeed, given the droplets aggregation triggered by the freeze-thaw process (Figure 4-16 c and d), the decrease of the negative charge for the emulsion was the direct result of the aggregation of the dispersed phase.

Nevertheless taking into consideration the assumptions made by these authors (Xu, Yuan et al. 2010), the drastic negative increase of the ζ-potential for 5%, 7.5% and 10% w/w CN-De could

be the result of the desorption of conjugate from the droplet surfaces or conformational changes of sodium caseinate caused by the freeze-thawing process.
The pH measurements revealed that FT had minimal to no effect on the pH of the emulsions.

For WPI-SBP stabilised emulsions before and after FT the ζ-potential of the emulsions had decreased by ~25%., from -40.1 ± 0.3 mV to -52.7 ± 7.1 mV. The same phenomenon was observed when using CN-De. Based on the observations made by *Xu et Al.* (Xu, Yuan et al. 2010), it was assumed that this decrease was the result of the partial adsorption at the droplet interface of unabsorbed SBP or conjugate free in the continuous phase prior to freezing.
Freezing and thawing the emulsions had no effect on the pH.

4.4 Conclusion

The stability to external stress of Maillard conjugates stabilised emulsions was investigated. Experiments showed that all conjugate stabilised emulsions had an enhanced stability at pH close to pI in comparison with protein stabilised emulsions.

However, the use of a pH sensitive polysaccharide limited the use of the conjugate on a wide range of pH. The conjugation had previously been identified as a means to increase emulsion stability at pH close the isoelectric point of the protein they contain (Chevalier, Chobert et al. 2001, Jiménez-Castaño, López-Fandiño et al. 2005, O'Regan and Mulvihill 2009, O'Regan and Mulvihill 2010). Nevertheless, the study of a pH sensitive polysaccharide showed that at pH close to the pKa the emulsions showed early signs of creaming.

The thicker interfacial membranes and steric hindrance provided by the glycoconjugates also increased the stability to salt induced droplet aggregation. The emulsions stabilised with both conjugates displayed enhanced stability when salt was added.

Before emulsification it indicated that the emulsifying properties of the conjugates stayed unaffected by the addition of salt.

After emulsification it highlighted the improved resistance to droplet aggregation.

In the case of WPI-SBP the addition of salt could also be effectively used as a means to increase the viscosity without affecting the stability of the system.

The emulsion systems produced with both conjugates displayed increase stability to temperature changes.

These results make Maillard conjugates potential ingredients for the formulation of food emulsions. The resistance to salt addition and pH changes of the emulsions they stabilise make them emulsifiers of interest for many food applications.

The resistance they displayed to temperature changes suggest promising results for drying applications using different processes.

Chapter 5

Drying and Rehydration of Oil-in-Water Emulsions Using Different Drying Processes

5.1 Introduction

In the previous chapter heat stability and freeze thaw stability experiments gave encouraging results for the use of Maillard conjugate stabilised emulsions for high and low temperature drying processes. Besides displaying better stability to temperature changes because of the thicker membrane they form at the interface, Maillard conjugates are interesting candidates for the stabilisation of emulsions destined to be dried.

Drying is commonly use in the food industry to increase the shelf life of emulsified products but also to reduce the storage and transport costs. The key challenge when drying a complex structure is retaining the said structure. The strength and the nature of the interface are two of the parameters that need to be taken into account when attempting to dry emulsions. Maillard conjugates have potential to be used to maintain structure.

Freeze-drying of emulsions has been less reported than spray drying. One of the potential limitations for the use of this process to produce re-dispersible emulsions resides in the quality of the dry powders. Indeed freeze-drying emulsions results in the formation of large flakes of dry products (Gejl-Hansen and Flink 1978). Yet the size of particles to be dispersed in water is a key parameter for optimum dispersion (Bockian, Stewart et al. 1957, Kissa 1999).

Considering it being a two steps process, instability can arise during freezing or during drying.

Spray drying is a widely used technique for the production of food powders (Fäldt and Bergenståhl 1995, Barbosa-Cánovas, Ortega-Rivas et al. 2005). However, the high processing temperatures make it a challenging technique to use when proteins are present in formulations. Sodium caseinate is resistant to moderately high temperatures in opposition with whey protein that denatures at around 60 ˚C. Over the past decade studies have permitted to established appropriate processing temperatures when drying emulsions stabilised with a range of proteins

(Young, Sarda et al. 1993, Bylaitë, Venskutonis et al. 2001, Baranauskienė, Venskutonis et al. 2006, Jayasundera, Adhikari et al. 2009).

The aim of this chapter was to study the suitability of the conjugates previously studied to stabilise re-dispersible emulsions dried using freeze-drying and spray drying. Emulsions were considered reconstituted if their microstructure, if their viscosity and if their mouthfeel remained the same after rehydration.

As previously, droplet aggregation (flocculation) and creaming were considered undesirable in view of food applications.

The storage stability of the dried powders was assessed as drying is mainly used to increase the shelf life of emulsions. Harsh storage conditions can not only affect the quality of dry food products in general but it can also affect the wetting, solubility and dispersibility of dry powders (Roos 1995, Fäldt and Bergenståhl 1996, Ortega-Rivas, Juliano et al. 2006, Fitzpatrick 2013).

Both emulsifiers were assessed as the nature of the emulsifier greatly influences the drying and rehydration and the storage stability.

5.2 Materials and Methods

5.2.1 Materials

5.2.1.1 Proteins

Casein sodium salt from bovine milk and egg white protein were purchased from Sigma Aldrich, UK. Whey protein isolate was ordered from Volac.

5.2.1.2 Polysaccharides

Dextrin from corn starch (DE ≤ 5. Mw = 7kDa) was purchased from Sigma Aldrich, UK. Sugar Beet Pectin (degree of esterification >50%, Mw = 45.3kDa) was purchased from Herbstreith & Fox KG (named Betapec RU 301).

5.2.1.3 Other materials

The sunflower oil (Solesta) used for the production of oil-in-water emulsions was purchased from a local store.

5.2.2 Methods

5.2.2.2 Emulsions preparation

All emulsions were prepared following the same protocol. Conjugates, protein or a mixture of protein and polysaccharide were dissolved in 80 g of distilled water; 2 g for CN-De and WPI-SBP, 1.33 g for WPI and 0.67 g for NaCN. The mixture was put under magnetic stirring for 1 hour after which 20 g of sunflower oil were added and the temperature increased to 50 ˚C. NaN_3 (0.01% w/w) was also added to prevent bacterial growth and increase long term stability. The mixture was stirred for another 30 minutes.

Pre-emulsions were made using a high shear mixer Silverson L5M (10 000 rpm for 15 s).

The final emulsions were produced by passing the pre-emulsions three times through a microfluidiser (Microfluidics model M-110S) at a pressure of 1250 bars.

The droplet size was measured using laser diffraction with a Malvern Mastersizer 2000.

5.2.2.3 Optical microscope

The microstructure of the emulsions was observed using an optical microscope Leica DM 2500 LED equipped with a camera LEICA DFC 450 C. The images were collected with the software Leica Application Suite V 4.8. The 40x or 100x objective lenses were mainly used for the obtainment of the images featured in this study.

5.2.2.4 Texture analysis

5.2.2.4.1 Shear viscosity measurements

The shear viscosity of the emulsions was measured at 25 ˚C, from 0.1 to 100 s^{-1} shear rate using a Kinexus rheometer equipped with a cylindrical double gap and cup geometry. The maximum sampling was set at 5 minutes and the integration time at 1 minute.

5.2.2.4.2 Traction coefficient

The traction coefficient of the emulsions was measured using a MTM2 (Mini Traction Machine) tribometer (PCS Instruments, London).

The load was set to 3 N and the ball speed was set between 1 and 1000 $mm.s^{-1}$.

5.2.2.5 Drying processes

5.2.2.5.1 Freeze-drying

Samples were kept in a commercial freezer at -22 ˚C before being freeze-dried for 5 days using a Scanvac CoolSafe freeze dryer. The temperature of the condenser was -110˚C and the pressure in the chamber around 0.1 hPa.

5.2.2.5.2 Spray drying

Powdered emulsions were prepared using a spray dryer Buchi Mini Spray Dryer B-290. Inlet temperature was set at the desired temperature. The feeding rate was set at 20% (10 $mL.min^{-1}$) unless stated otherwise and the aspiration power at 95%.

The final product was collected from the collection chamber and kept in a sealed container before further analyses.

5.2.2.5.3 Freezing point and glass transition temperature measurements

The freezing point and glass transition temperature were measured using a Differential Scanning Calorimetry (DSC 25 from TA Instruments). Data were analysed with the software provided (Trios V4 1.1.33073).

For freezing point measurements temperature ramps at 0.5 $°C.min^{-1}$ (unless state otherwise) were performed from 20 °C to -30 °C followed by an isothermal of 5 minutes at -30 °C. The temperature was then brought back to 20°C at a heating rate of 0.5 $°C.min^{-1}$.

For glass transition temperature measurements temperature ramps at 10 $°C.min^{-1}$ were performed from 20 °C to 200 °C followed by an isothermal of 1 minute at 200 °C. The temperature was then brought back to 20 °C at a cooling rate of 10 $°C.min^{-1}$.

5.2.2.6 Dry powder analysis

5.2.2.6.1 Wettability

The wettability of the powders was measured using the Washburn capillary rise method. A K100 Tensiometer from Krüss, Germany was equipped with a glass tube with a filter base filled with the powder to analyse. The contact angle was calculated by the software (Kruss Laboratory Desktop 3.1) using the following equation:

$$\frac{m^2}{t} = \frac{c \times \rho^2 \times \sigma \times \cos\theta}{\eta}$$

Equation 5-1

Where m is the mass, t the flow time, σ the surface tension of the liquid, c the capillary constant of the powder, ρ the density of the liquid, Θ the contact angle between the liquid and the powder and η the viscosity of the liquid.

5.2.2.6.2 Dispersibility

The dispersibility of the powdered emulsions was measured using a method inspired by *Klinkesorn et Al.* (Klinkesorn, Sophanodora et al. 2006). Dry emulsions were added to stirring water to allow re-dispersion. A sample of the re-dispersed emulsion was taken after 5, 10, 20, 30, 40, 50 and 60 minutes using a Pasteur pipette and the droplet size analysed using dynamic light scattering (Mastersizer 2000, Malvern UK).

5.2.2.6.3 Storage stability

The storage stability was assessed exposing the dry emulsion to different temperature and humidity for three weeks.

For room temperature/room humidity conditions, the emulsions were stored in a room at a temperature of ~22 °C and a relative humidity of ~35%.

For room temperature/high humidity conditions, the dry emulsions were stored at room temperature (~ 22°C) in desiccator over a saturated NaCl solution (relative humidity 76 %).

For room temperature/dry atmosphere conditions, the dry emulsions were stored in desiccator containing silica gel beads to ensure a moisture content of 0%.

For high temperature/room humidity conditions, the dry emulsions were placed in an oven at 40 °C. The relative humidity in the oven was ~45%.

The room temperature, room humidity and oven humidity were measured every day for a week. The reported value represents an average of these measurements.

5.2.2.6.4 Dry particle size measurements

The particle size of the dry emulsions was measured using a dry particle size analyser QicPic/R series from Sympatec.

5 2 2 6 5 Dry particle microstructure

Micrograph of the dry emulsions were obtained with scanning electron microscopy using an environmental scanning electron microscope Philips XL30 ESEM-FEG.

5.2.2.6.6 Moisture content and water activity

The moisture content was measured using a Ohaus moisture analyser MB25. The water activity was measured with Aqualab Dew Point Water Activity Meter 4TE.

5.2.2.6.7 Hygroscopicity

Hygroscopicity measurements were performed by placing 1 g of dry emulsions in a desiccator containing a saturated NaCl solution. The hygroscopicity represents the mass water absorbed per 100 g of sample after 10 days of storage.

$$\mathrm{Hp} = \left(\frac{w_1 - w_0}{w_0}\right) \times 100$$

Equation 5-2

Where *Hp* is the hygroscopicity, w_0 the initial weight of the powder and w_1 the weight after storage.

5.3 Results and discussion

5.3.1 Freeze drying and rehydration

5.3.1.1 Freeze-drying and rehydration of emulsions stabilised with a sodium caseinate-dextrin conjugate

The freezing point of a system is an important parameter to consider when freeze drying a product. If the product is exposed to a temperature higher than its freezing point its microstructure can be lost upon drying (Meister and Gieseler 2009). All solutes are converted to crystalline or amorphous solid below their glass transition temperature T_g during freezing to ensure a solid system and avoid a collapse during sublimation. To measure the freezing point of the present systems, Differential Scanning Calorimetry (DSC) was used. Emulsions (20% w/w sunflower oil, 80% w/w water) stabilised with 2.5% w/w CN-De, the standard concentration chosen in previous experiments, were studied for these experiments. The freezing point of these emulsions was measured at -16.9 ± 1.9 ˚C. The temperature of the freezer used for these experiments was -22 ˚C. The freezing point was measured above the freezing temperature meaning the product would be completely in its glassy state when lyophilised.

The emulsions stabilised with 5% w/w, 7.5% w/w, 10% w/w and 15% w/w CN-De conjugate showed good freeze-thaw stability (c.f. Chapter 4). For this reason, they were studied for freeze-drying and rehydration experiments.

All the selected emulsions gave compact powders that were easily dispersed in water after gentle grounding with a mortar and a pestle. The dispersions were stirred at 50˚C for 1 hour to allow full rehydration (Marefati, Rayner et al. 2013). Figure 5-1 shows the droplets diameter distribution of the emulsions before and after rehydration.

The average droplet size of the native emulsions (prior to drying) decreased with an increasing conjugate concentration.

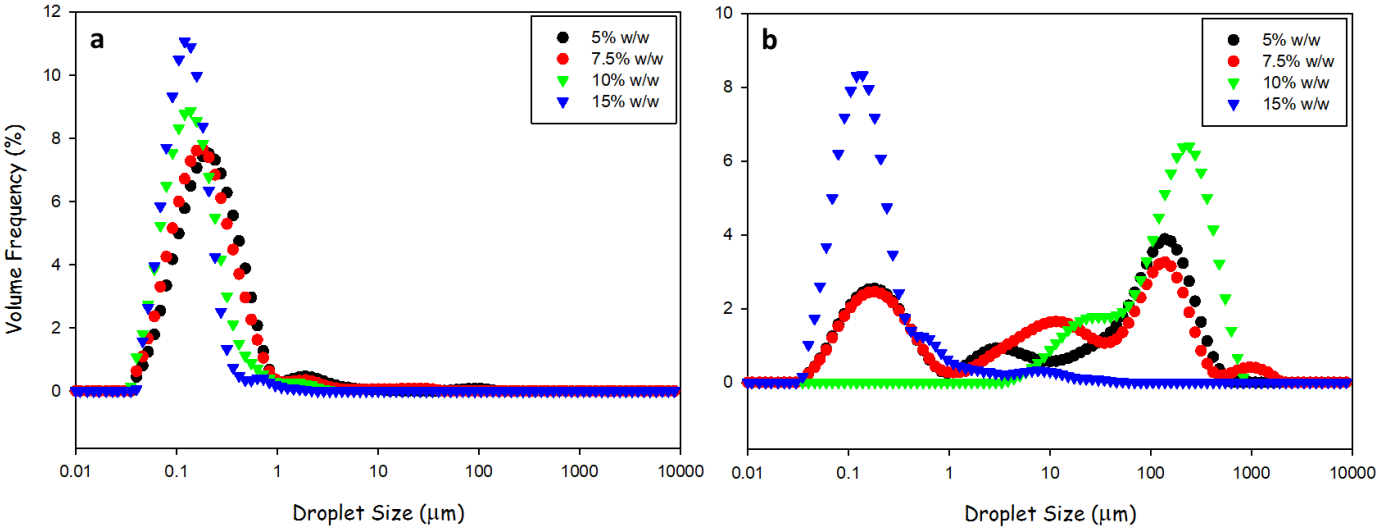


Figure 5-1 : Droplet size distribution of CN-De stabilised oil-in-water emulsions (20% w/w sunflower oil, 80% w/s water, pH 6-7) before (a) and after (b) freeze-drying and rehydration. Measurements were carried out at 5% w/w, 7.5% w/w, 10% w/w and 15% w/w CN-De.

Table 5-1 : Droplet size parameters of CN-De stabilised oil-in-water emulsions before and after freeze-drying and rehydration. All samples were measured in triplicate. Values are displayed as mean ± standard deviation.

Conjugate concentration (% wt.)	Native Emulsion			Freeze-Dried Redispersed Emulsion		
	D3,2 (µm)	D4,3 (µm)	SPAN	D3,2 (µm)	D4,3 (µm)	SPAN
5	0.18 ± 0.02	0.75 ± 0.35	2.23 ±0.60	0.43 ± 0.22	77.91 ± 29.97	9.69 ± 7.65
7.5	0.16 ±0.01	0.46 ± 0.16	2.32 ± 0.13	0.44 ± 6.22	87.56 ± 36.20	17.92 ± 10.45
10	0.12 ± 0.01	0.21 ± 0.02	1.90 ± 0.14	62.72 ± 1.07	206.43 ± 4.47	2.44 ± 0.02
15	0.12 ± 0.01	0.16 ±0.01	1.34 ± 0.10	0.13 ± 0.01	0.70 ± 0.21	3.84 ± 0.98

After rehydration a shift in the oil droplet size distribution to larger droplet sizes was observed for emulsions containing from 5% w/w to 10% w/w CN-De.

The most interesting result regarding the reconstitution was found at 15% w/w CN-De (5% w/w NaCN). At this concentration the surface mean diameter was the same before and after rehydration. However, the volume mean diameter ($D_{4,3}$) slightly increased from 163 ± 4 nm to 701 ± 200 nm after reconstitution. This difference was mainly attributed to the formation of aggregates.

These results revealed that using freeze-thawing to assess the freeze-drying stability was a disputable method. Indeed, despite showing the same microstructure before and after freeze-thawing emulsions from 5% w/w to 10% w/w CN-De presented droplet aggregation after freeze drying. This difference exposed that the sublimation of the ice was source of damages to microstructure of emulsions.

Rheology measurements were performed on the 15% w/w CN-De stabilised emulsion before and after drying and rehydration. The viscosity of emulsions is a key parameter in various applications. The successful reconstitution of a re-dispersible emulsion in the case of food application not only means retention of the droplet size but also retention of textural characteristics such as viscosity and mouthfeel.

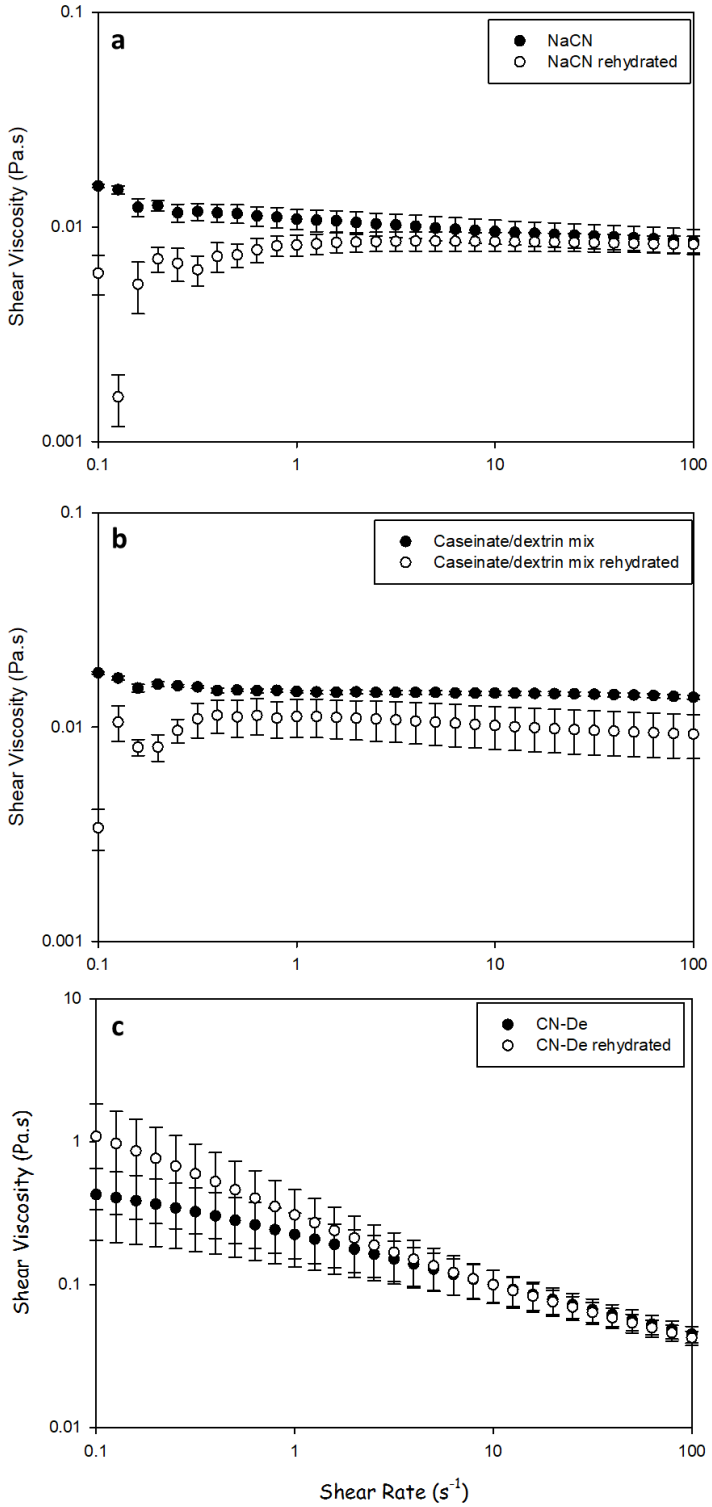


Figure 5-2: Steady state shear viscosity of oil-in-water emulsions (20% w/w sunflower oil, 80% w/w water, pH 6.8) stabilised with (a) 5% w/w sodium caseinate, (b) 5% w/w sodium caseinate in presence of 10% w/w dextrin and (c) 15% w/w sodium caseinate-dextrin conjugate (i.e. 5% w/w NaCN conjugated with 10% w/w dextrin) at 25°C. Samples were measured in triplicate. Error bars represent standard deviation.

The shear viscosity of the rehydrated emulsion showed an increase of over 100% at $0.1s^{-1}$ shear rate in comparison with the same emulsion prior to freeze-drying (Figure 5-2 c).

As the shear rate increased the viscosity reached that of the native emulsion.

At low shear rate that increase was the result of the presence of aggregates previously detected with the droplet size measurements.

In order to identify the effects of the conjugation of dextrin to NaCN, samples containing NaCN as emulsifier and others containing a mixture of NaCN and dextrin in the conjugated proportions were prepared. The shear viscosity of rehydrated emulsions from these experiments was measured and compared with that of the native emulsions (Figure 5-2 a, b).

Before and after freeze-drying and rehydration, $D_{3,2}$ stayed unchanged for both emulsions. From 113 ± 1 nm to 116 ± 2 nm for NaCN dextrin mixture stabilised emulsions and from 112 ± 0 nm to 120 ± 3 nm for NaCN stabilised emulsions.

The rheology measurements are shown in Figure 5-2.

The emulsions stabilised with NaCN and the mixture of both hydrocolloids displayed a Newtonian behaviour and similar viscosities before drying. Sodium caseinate stabilised emulsions typical display Newtonian behaviour (Dickinson and Golding 1997). After freeze-drying and rehydration, in both cases the viscosity varied by about 25% on average (23% for NaCN and 31% for the mixture) and the rheological behaviour remained the same.

Emulsions stabilised with CN-De and NaCN at the same protein concentration showed different viscosities and rheological behaviours; the emulsion stabilised with the CN-De had an average viscosity six times higher than the one stabilised with NaCN. The emulsion stabilised with the conjugate showed a shear thinning behaviour while the emulsion stabilised with NaCN displayed a Newtonian behaviour.

These results demonstrated that conjugation to dextrin increased the viscosity and changed the rheological behaviour of the emulsion produced. Nevertheless, the abilities to be freeze-dried

and rehydrated were comparable for the different systems tested both in terms of retention of the microstructure and physicochemical properties.

Similarly, the traction coefficient was measured before and after freeze drying and rehydration using tribology experiments. Friction forces can be used to evaluate the oral behaviour and mouthfeel of food products (Malone, Appelqvist et al. 2003, Dresselhuis, De Hoog et al. 2007). Results from these experiments are displayed in Figure 5-3.

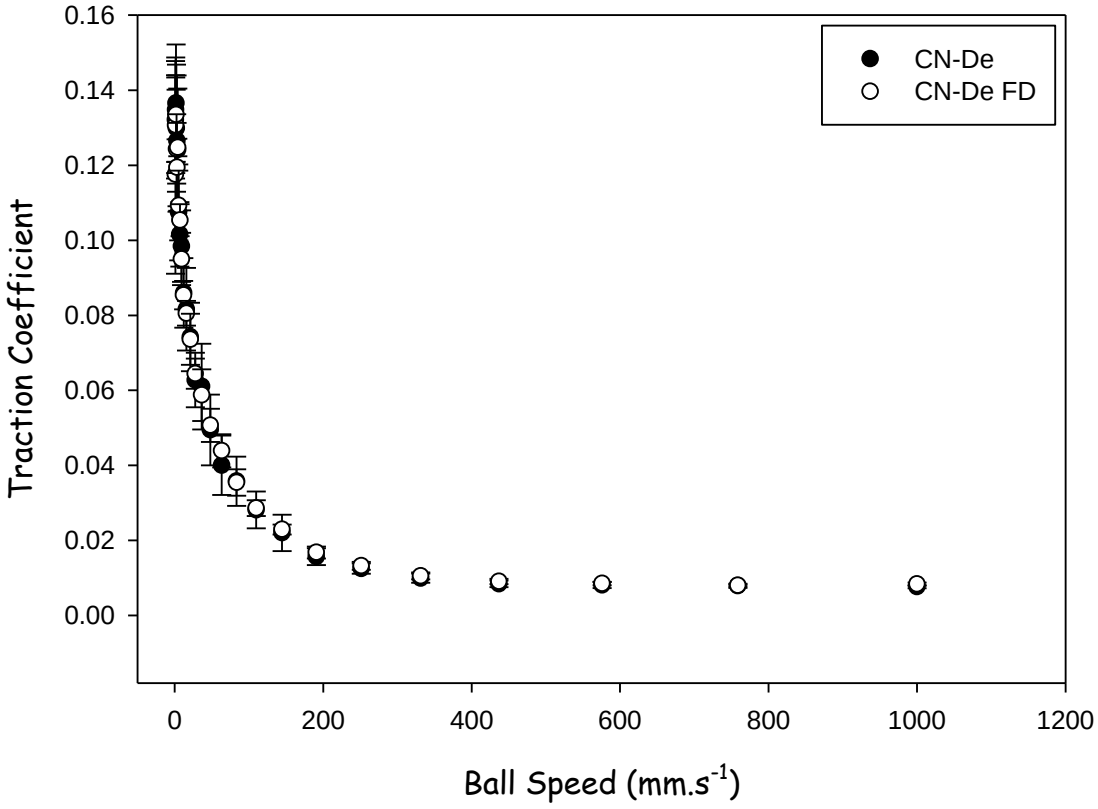


Figure 5-3: Traction coefficient as a function of the ball speed of 2.5% w/w sodium caseinate-dextrin conjugate stabilised oil-in-water emulsions (20% w/w sunflower oil, 80% w/w water, pH 6-7) before and after freeze-drying and rehydration at room temperature (22°C). Data points represent an average of measurements performed on three samples. Error bars correspond to the standard deviation of the average.

Before and after freeze drying and rehydration both samples displayed similar profiles for the evolution of the traction coefficient with increasing ball speed.

The long-term stability of the rehydrated 15% w/w CN-De stabilised emulsions (20% w/w sunflower oil) with added NaN_3 was evaluated by measuring the droplet size over time after 7 and 9 weeks of storage at 5 ˚C. The results displayed in Figure 5-4 show that the emulsions remained stable over the studies time period. The volume mean diameter showed a slight decrease from 931 ± 201 nm right after rehydration to 771 ± 63 nm after nine weeks of storage. The formation of aggregates was previously identified after the rehydration of the emulsions. This decrease was thus attributed to the disruption of the aggregates upon stirring possibly happening in the DLS equipment.

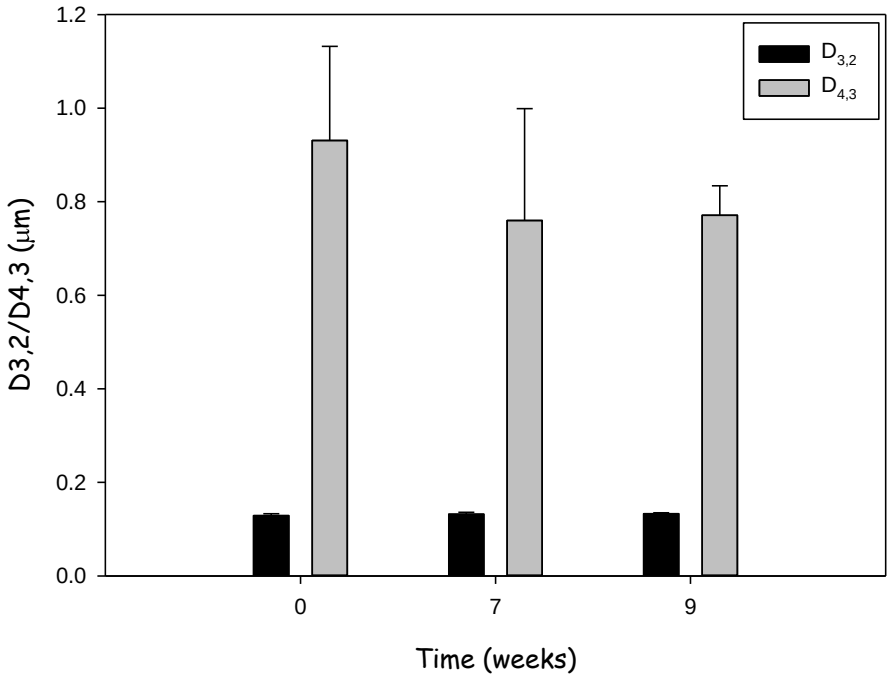


Figure 5-4: Droplet size evolution as function of time of a freeze-dried and rehydrated emulsion 20% sunflower oil, 80% water oil-in-water emulsions stabilised with 15% w/w CN-De. Samples were measured in triplicate. Error bars represent standard deviation.

5.3.1.2 Freeze-drying and rehydration of oil-in-water emulsions stabilised with a whey protein-sugar beet pectin conjugate

Based on the results obtained for the FT stability (Chapter 3) of the emulsions stabilised with WPI-SBP, emulsions stabilised with 2.5% w/w conjugate (~1.67% WPI, 20% w/w sunflower oil) were freeze-dried and rehydrated.

The freezing point for these systems was measured using DSC at -14.8 ± 0.5 °C.

The dry emulsions from these experiments were easily dispersed in water after gentle grounding with a mortar and a pestle. The droplet size measurements displayed in Figure 5-5 shows the evolution of the droplet diameter before and after drying and rehydration.

The majority of the droplets in the rehydrated emulsion had a diameter $D_{3,2}$ of 165 ± 4 nm in contrast with 144 ± 3 nm in the native emulsions (prior to drying). Based on these values the surface mean diameter $D_{3,2}$ could be considered constant. On the other hand, the volume mean diameter showed an increase from 375 ± 7 nm in the native emulsion to 2.196 ± 0.684 μm in the rehydrated emulsions. After examination with an optical microscope (Figure 5-6), this increase was attributed to the partial coalescence of the droplets.

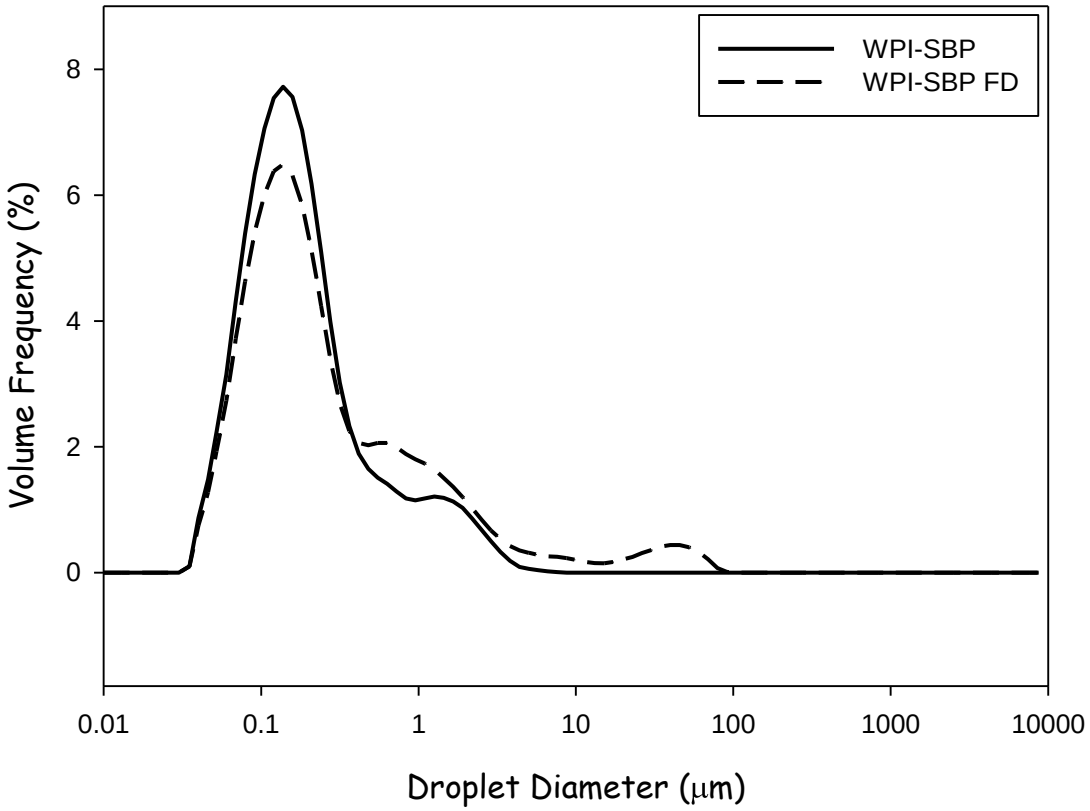


Figure 5-5: Droplet size distribution of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions (20% w/w sunflower oil, 80% w/w water, pH 5-6) before and after freeze-drying and rehydration at 50°C and cooling at room temperature (22°C).

The coalescence observed after rehydration could originate from both freezing and drying. Upon freezing, coalescence can be induced when two neighbouring droplets are forced together to the point of merging together forming a larger droplet (Degner, Chung et al. 2014). During drying the sublimation of ice crystal could have led to the partial desorption of the emulsifier from the droplet surfaces. When rehydrated two or more droplets with partially disrupted interfacial films can merge and form a larger droplet.

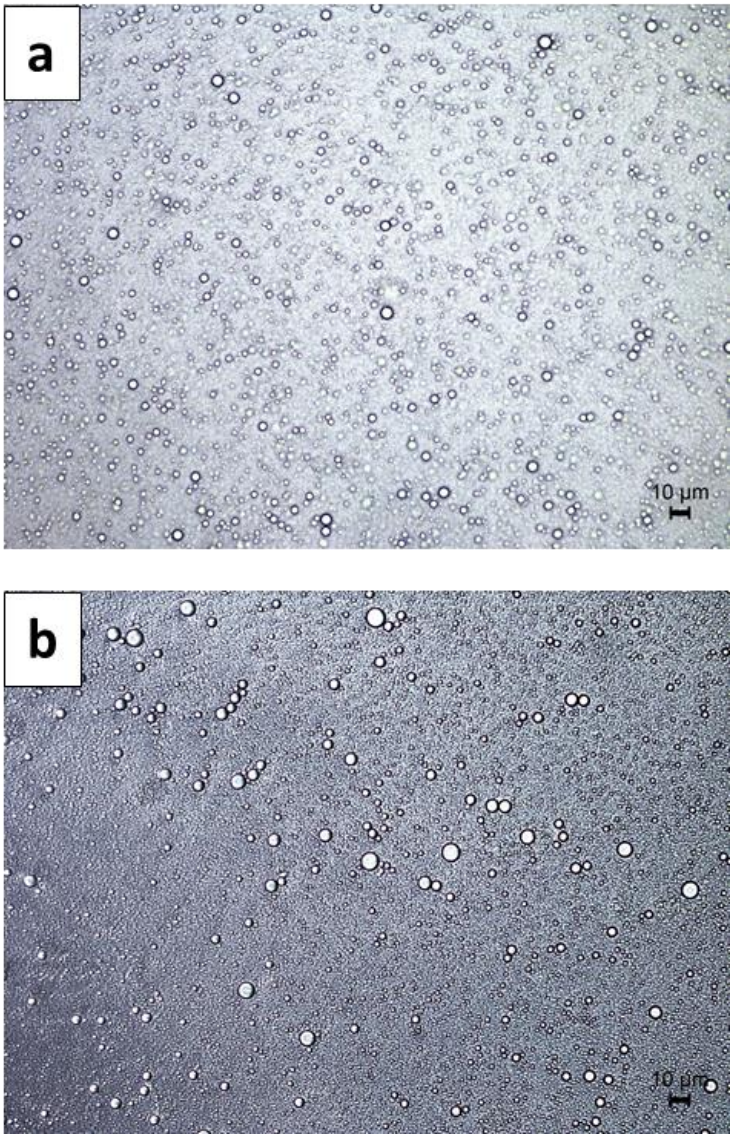
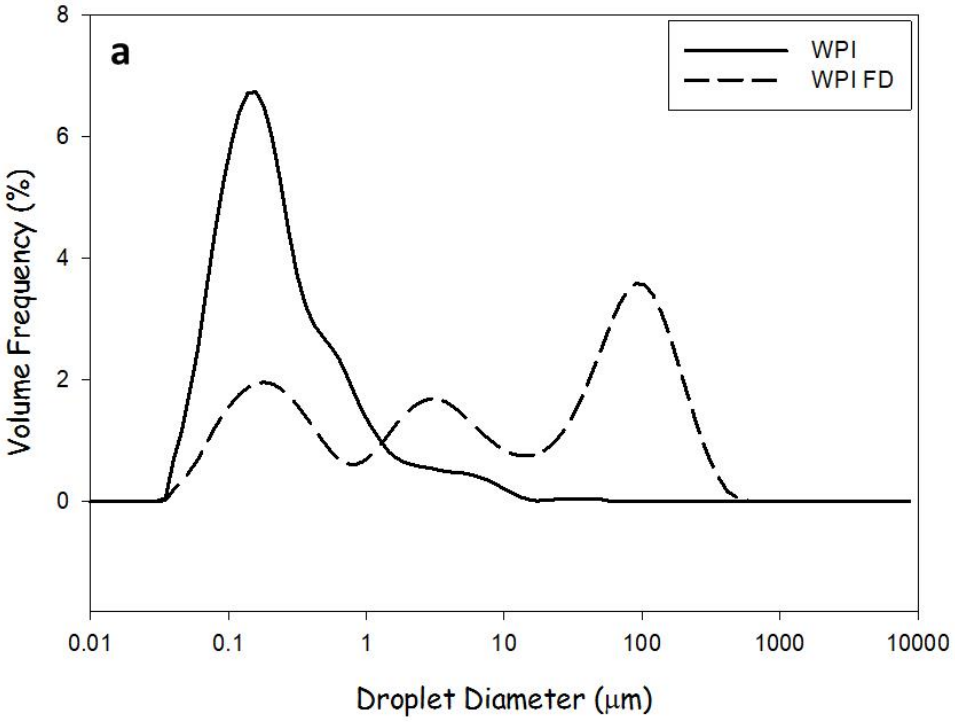


Figure 5-6: Optical microscope images of 2.5% w/w WPI-SBP stabilised oil-in water emulsions before (a) and after (b) freeze-drying and rehydration.

In order to spot the impact of conjugation of sugar beet pectin to whey protein, whey protein stabilised emulsions were produced. Emulsions stabilised with ~1.67% WPI (20% sunflower oil) were freeze dried and rehydrated. The droplet size distributions before and after FD and rehydration are displayed in Figure 5-7.



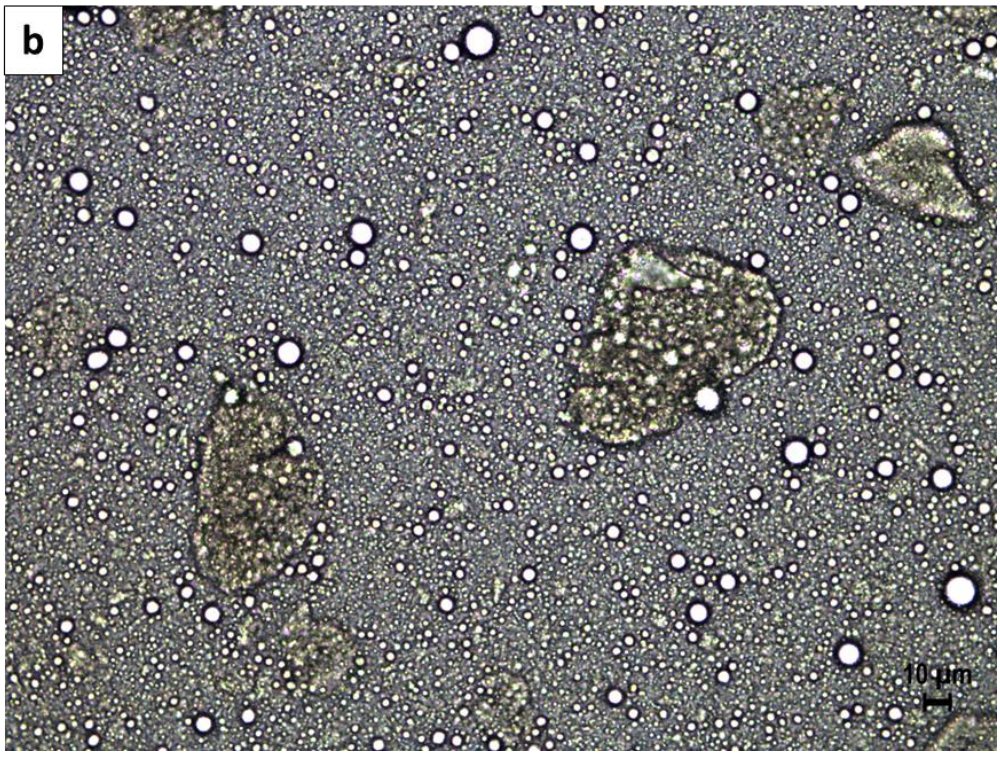


Figure 5-7: Droplet size distribution of 1.7% w/w WPI stabilised oil-in-water emulsions (20% w/w sunflower oil, 80% w/w water, pH 5-6) before and after freeze drying and rehydration at 50°C and cooling at room temperature (22°C) (a) and optical microscope images of a 1.7% w/w WPI stabilised emulsion after freeze-drying and rehydration (b).

After drying and rehydration the emulsions stabilised by whey protein alone presented multimodal distributions. The microstructure of the emulsions was affected by freeze-drying. Microscope images displayed in Figure 5-7 b revealed the formation of droplet aggregates.

It was previously observed that the conjugation of dextrin to sodium caseinate led to similar stability to freeze drying of the emulsions they stabilised in comparison with NaCN stabilised emulsions. In this case the conjugation of SBP to WPI significantly improved the ability of emulsions to be reconstituted after lyophilisation (Figures 5-5, 5-6).

The effects of drying on the viscosity and the traction coefficient of the emulsions were studied. Before and after FD and rehydration, the shear viscosity of the emulsion remained the same (Figure 5-8). Both the rheological behaviour and the shear viscosity as function of the shear rate were preserved in this case.

Before and after re-dispersion, the emulsions displayed a shear thinning behaviour. Nevertheless, after rehydration the shear viscosity showed a slight increase of about 12 % on average. This increase was mainly due to a higher viscosity in the rehydrated emulsion at low shear rate (Figure 5-8). As the shear rate increased the shear viscosity profile were similar for both emulsions. Previously coalescence was identified as the main cause of an increased droplet size in the re-dispersed emulsion. Yet fine droplet emulsions tend to display a minimum in rheological parameters at low shear rate. When fine droplets are replaced with larger droplets in the same emulsion (same formulation) viscosity, storage and loss moduli are higher at low shear rate (Pal 1996). Hence the lower viscosity at low shear rate of the native emulsion.

Emulsions containing an unconjugated mixture of WPI and SBP in the same proportions as in the conjugate were produced in order to assess the effects of the conjugation. Unfortunately the two phases separated after emulsification. The comparison with the previous results was thus impossible.

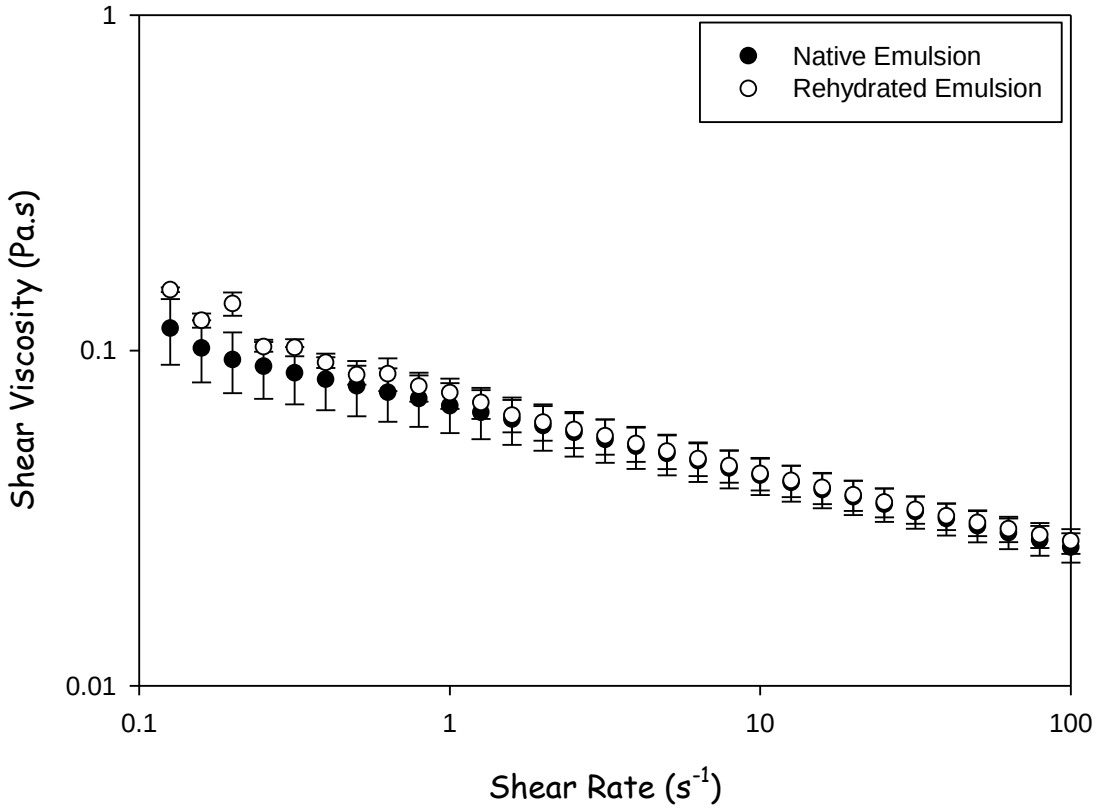


Figure 5-8: Steady state shear viscosity at room temperature (22°C) of 2.5% w/w WPI-SBP stabilised oil-in-water (20% w/w sunflower oil, 80% w/w water) before and after freeze-drying and rehydration. Samples were measured in triplicate. Measurements were carried out no more than 24 hours after rehydration. Error bars represent standard deviation.

In addition to viscosity measurement the traction coefficient was measured before and after freeze-drying and rehydration. Figure 5-9 shows a slight increase of the traction coefficient on the entirety of the ball speed range studied.

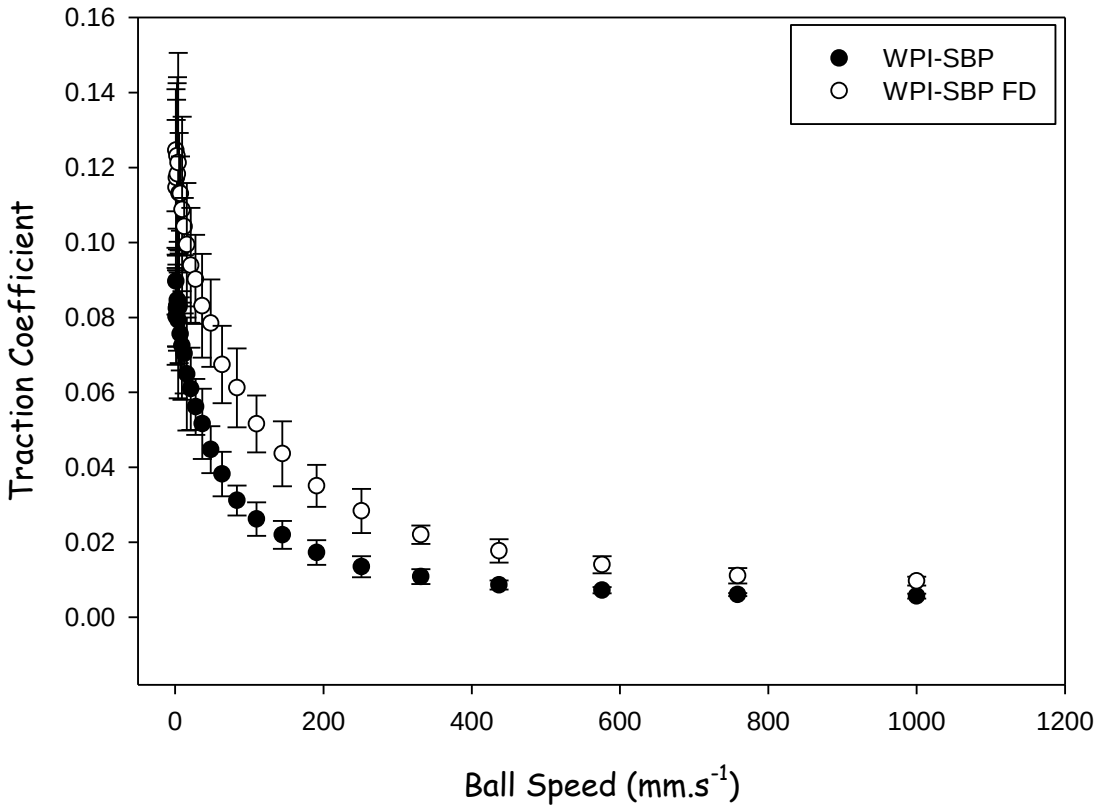


Figure 5-9: Traction coefficient as a function of the ball speed of 2.5% w/w whey protein isolate-sugar beet pectin conjugate stabilised oil-in-water emulsions before and after freeze-drying and rehydration. Data points represent an average of measurements performed on three samples. Error bars correspond to the standard deviation of the average.

5.3.2 Freeze-dried emulsion powders

The moisture content (MC) and the water activity (a_w) of the dry emulsions was measured right after FD. Moisture content and water activity are two value important to measure on dry products. High moisture contents and water activity lead to the development of bacteria and mould upon storage of dry product making them unsuitable for consumption in the case of food products (Scott 1957, Pitt and Hocking 2009). Most bacteria grow and develop at their maximum rates at moisture contents close to 1 (van den Berg and Rose 1959, SPERBER 1983). The development of most mould is inhibited at a water activity of 0.8 while microbial

proliferation is inhibited below a water activity of 0.6 (Barbosa-CÃ, Fontana Jr et al. 2008, Sabahelkhier, Yagoub et al. 2008).

Freeze drying has been recognised as a process permitting to attain low water activity values given that frozen water is sublimated during the process (Oetjen and Haseley 2004).

After freeze drying the CN-De oil filled powders exhibited low MC and a_w. Respectively 2.09 ± 0.63 and 0.0356 ± 0.0056. At these values both the development of bacteria and the formation of mould was inhibited in the dry products.

As mentioned in the literature review (c.f. Chapter 2), the existing knowledge on the dispersion of powders can be applied to dry emulsions as on the physical level dry emulsions behave like powders when dispersed into a liquid. For this reason, the wetting properties (wettability, sinkability) and dispersibility of the oil filled powders were studied (Pısecký 1997). The dry particle size was measured along with microstructure observations using Scanning Electron Microscopy (SEM).

Dry particle size measurements results, pictures of the particles and SEM micrographs are displayed in Figure 5-10.

From the particles size measurements coupled with the SEM micrographs it appeared that large particles formed during drying. The particles observed were believed to be fragments of CN-De conjugate matrix with entrapped oil droplets. Upon rehydration, the matrix would dissolve and the oil droplets would be dispersed into water.

This type of microstructure for freeze-dried emulsions has previously been reported by other authors (GEJL-HANSEN, FLINK et al. 1978). Indeed, one of the disadvantages of the freeze-drying process is the necessity for the final product to be grounded before being used or analysed. In the eventuality of dispersion of the dry solids, the inability to produce the smallest

particles possible can affect the dispersion time and dispersibility (Kim, Chen et al. 2002).

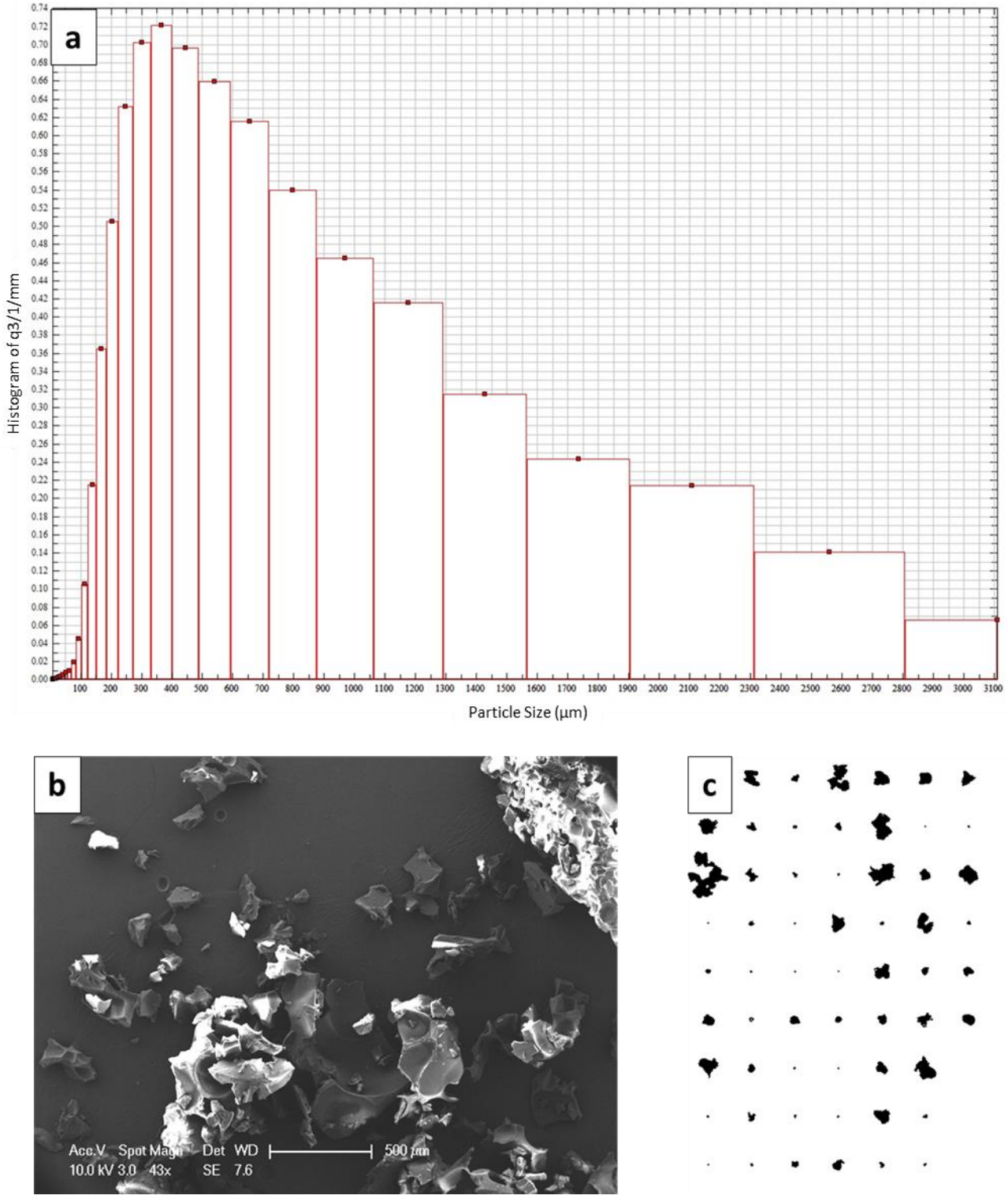


Figure 5-10: Particle size distribution (a), SEM micrographs (b) and particles shape (c) of CN-De sunflower oil filled powders (15% w/w CN-De, 20% w/w sunflower oil freeze dried oil-in water emulsions).

The surface mean diameter and volume diameter measured for the dry powders were respectively 670.56 μm and 1171.74 μm.

The wetting of a powder is the first step of the powder dispersion. This process is governed by the ability of molecules composing the powder to interact with the liquid in which it is dispersed. The contact angle formed between a liquid deposited and the surface of a solid is an indicator of the ability of that liquid to wet the surface.

The Washburn capillary rise method is a method frequently used to measure the contact angle of bulk powders (Heertjes and Kossen 1967, Buckton and technology 1993, Galet, Patry et al. 2010, Jaine and Mucalo 2015). In this study this method was used to measure the contact angle of the freeze-dried emulsions.

The oil filled powders displayed good wettability (i.e. contact angle below 90°) with a contact angle of 77 ± 1°.

The penetration of the solid was assessed measuring the wetting time also referred to as the sinkability when measured as a function of the surface area (Schober and Fitzpatrick 2005, Sharma, Jana et al. 2012). The wetting time is the time needed for a chosen mass of powder to disappear from the surface of the liquid (Fuchs, Turchiuli et al. 2006). The emulsions displayed a wetting time of about 6 minutes (3.45 ± 0.46 $mg.s^{-1}$) which could be considered a long time in comparison with most pharmaceutical powders penetrating the surface of water within seconds (Lerk, Schoonen et al. 1976).

Similar experiments to the ones performed CN-De powdered emulsions were carried out on WPI-SBP oil filled powders. Results from these experiments could give an understanding of how the nature of the emulsifier potentially affects the quality and re-dispersion of dry emulsions. The moisture content and water activity of the dry powders produced were measured

right after the end of the lyophilisation process. These values were respectively 1.20 ± 0.17% and 0.3853 ± 0.0011. As for the previous emulsifier at these values both the development of mould and bacteria was inhibited.

The physical structure of the powders was analysed. The particles obtained from these experiments were larger than with CN-De. The surface mean diameter and volume mean diameter measured were 988.08 μm and 1333.44 μm respectively (Figure 5-10). The same hypotheses and observations were made for this sample. The flakes observed after gentle grounding (Figure 5-11 b and c) of the freeze-dried samples were the result of the formation of a matrix of whey protein-sugar beet pectin conjugate around the oil droplets.

The powders obtained from these experiments displayed good wetting properties with a contact angle of 80 ± 2° and a sinkability of ~6 minutes (3.54 ± 0.53 $mg.s^{-1}$) comparable to the value obtained for CN-De dry emulsions.

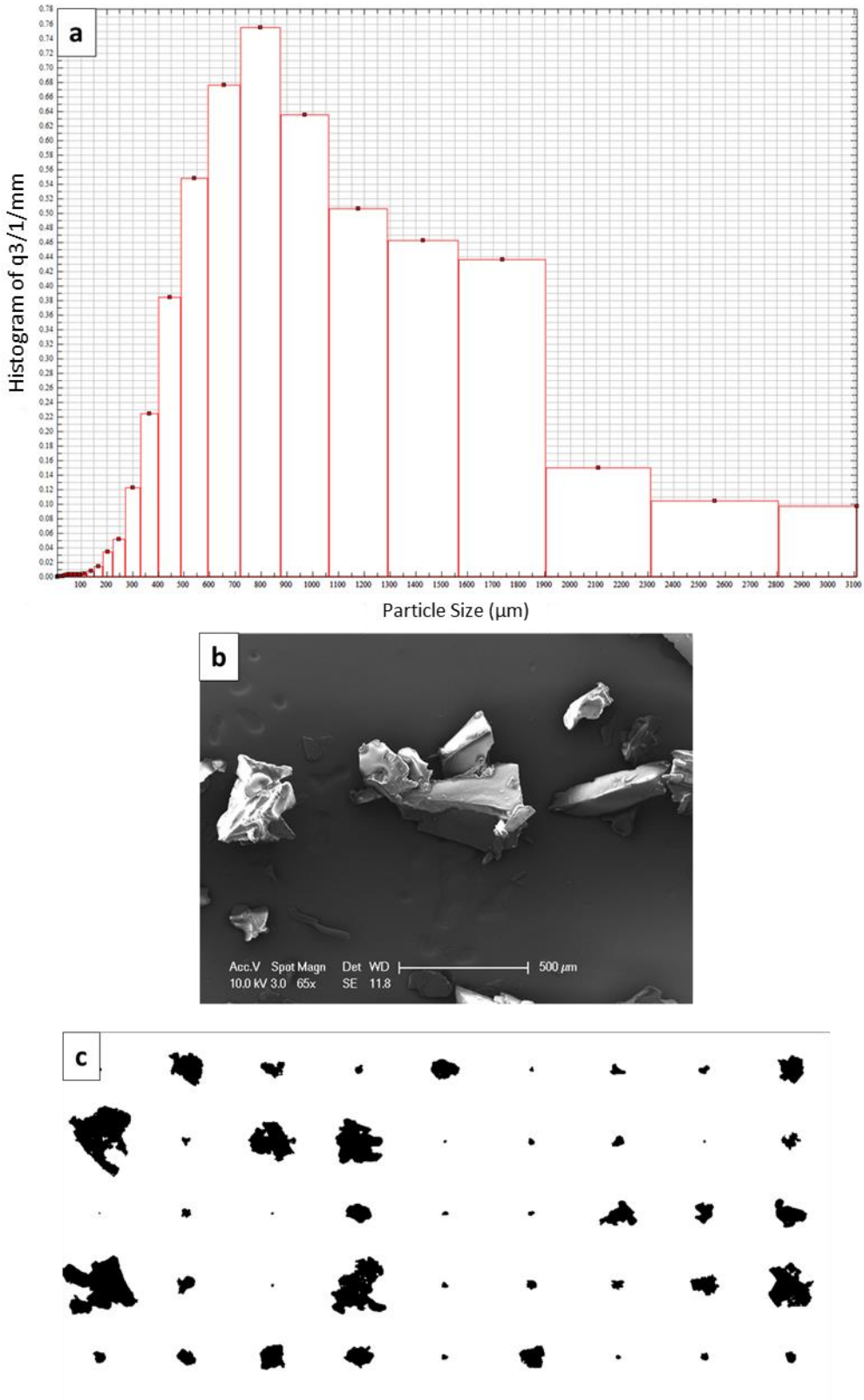


Figure 5-11: Particle size distribution (a), SEM micrographs (b) and particles shape (c) of WPI-SBP sunflower oil filled powders (2.5% w/w WPI-SBP, 20% w/w sunflower oil freeze dried oil-in water emulsions).

In order to quantify the dispersibility of the emulsions the evolution of the droplet size during rehydration was measured. The dry powder was dispersed into water and the droplet size measured using laser diffraction at different times (Klinkesorn, Sophanodora et al. 2006). The rehydration time was taken when no further evolution of the droplet size was noticed. Results are gathered in Figure 5-12.

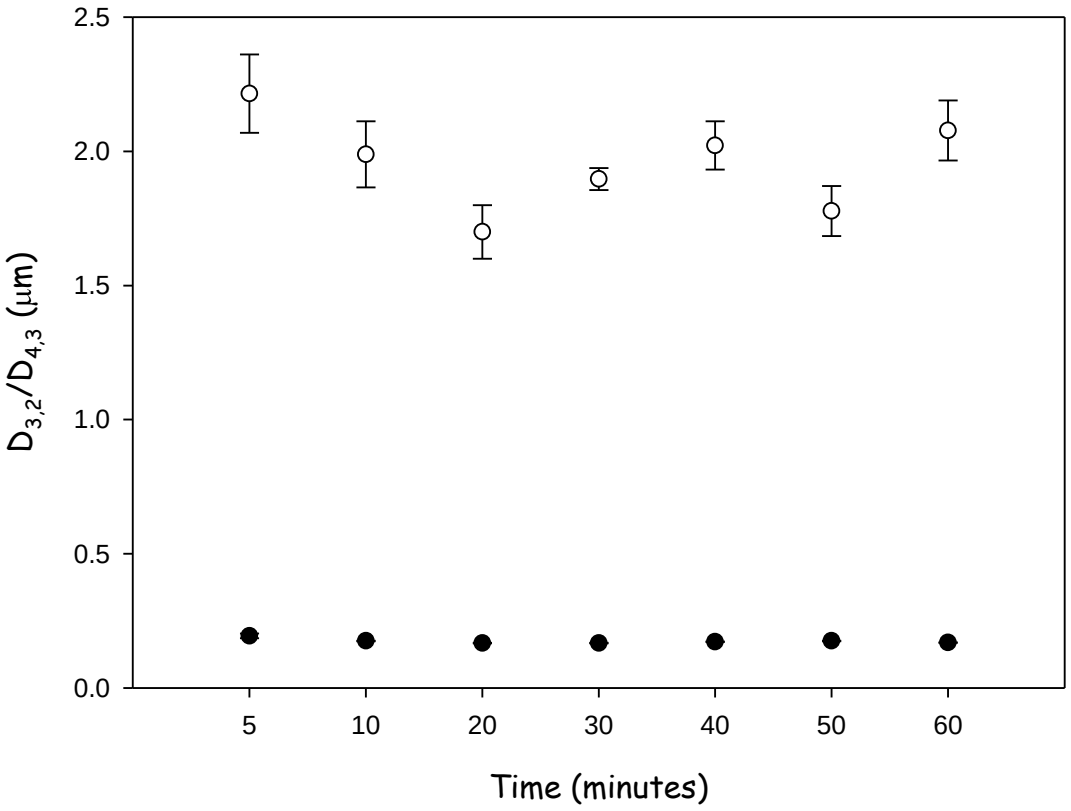


Figure 5-12: Evolution of the surface mean diameter ● and the volume mean diameter ○ as a function of dispersion time of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions (20% w/w sunflower oil, 80% w/w water, pH 5-6). Measurements were performed in triplicate at room temperature (22°C). Error bars correspond to standard deviation.

Results revealed that the emulsion was rapidly dispersed in water. Both the surface mean diameter and volume mean diameter displayed constant values from 5 minutes after dispersion. The rehydration of the emulsion was instantaneous.

5.3.2.1 Effects of the condition of storage on the reconstitution

One of the main reasons for drying emulsions is increasing their shelf life for transport and/or storage purposes. During storage dry products can be exposed to harsh conditions that can affect the long-term stability of product.

In the case of re-dispersible dry emulsions, the storage conditions can potentially affect the ability of the emulsions to be reconstituted after re-dispersion in water.

In this section the effects of different storage conditions on the ability of the emulsions to be reconstituted was studied. The powders were stored under different temperatures and humidity conditions for a prolonged period of time before rehydration.

5.3.2.1.1 Sodium caseinate-dextrin oil filled powders

In order to predict the stability of the dry powders to high levels of relative humidity, the hygroscopicity of the solids can be measured (Carstensen 1988, Cai and Corke 2000). When exposed to humidity, hygroscopic powders tend to form agglomerates (Murikipudi, Gupta et al. 2013).

The hygroscopicity of the dry emulsions was measured by keeping the powder at a relative humidity of 76% for a week. The variation of weight after that time was indicative of the amount water taken by the solid. The *Hygroscopicity classification* of the European Pharmacopeia classifies solids based on their mass variation after being stored for a week at a given relative humidity (Callahan, Cleary et al. 1982, Murikipudi, Gupta et al. 2013). After a week at 76% relative humidity, the dry emulsion displayed a mass variation of 1.37 ± 0.09%. According to the classification, the powder could be considered as a “slightly hygroscopic solid”, letting expect the formation of some agglomerates during storage in high relative humidity.

The dry emulsions remained visually identical before and after being stored at 40˚C (the relative humidity measured in the oven was ~45%), at room temperature/room humidity (the humidity

of the room was measured daily over a week at ~35%, the temperature of the room varied between 20 °C and 22 °C over a week) and at room temperature/0% humidity for three weeks. The aspect of the powder stored at room temperature/76% relative humidity changed after three weeks. As expected, the powders formed agglomerates under these conditions.

The moisture content and water activity were measured before and after storage. The values are displayed in Table 5-2.

Table 5-2: Moisture content and water activities of CN-De oil filled powders (freeze dried 15% w/w CN-De stabilised oil-in water emulsions, 20% w/w sunflower oil) before and after storage at different temperature and under different relative humidity conditions.

	Before		**After**	
	Moisture Content (%)	**Water Activity**	**Moisture Content (%)**	**Water Activity**
22°C/35%	3.46 ± 0.43	0.0338 ± 0.008	3.66 ± 0.80	0.3667 ± 0.1292
40°C/45%	1.14 ± 0.20	0.0306 ± 0.0007	1.89 ± 0.90	0.1737 ± 0.0844
22°C/76%	2.15 ± 0.32	0.0415 ± 0.0102	5.75 ± 0.85	0.6369 ± 0.0603
22°C/0%	1.52 ± 0.20	0.0236 ± 0.0030	0 ± 0	0.0343 ± 0.0044

Expectedly the highest increase for the moisture content was observed for the emulsions stored at high relative humidity. After storage the powder displayed a moisture content more than two times higher than the value before storage. Following the same trend, the water activity of the sample increased from 0.0415 ± 0.0102 to 0.6369 ± 0.0603.

Despite a higher humidity the sample stored in the oven at 40 °C (relative humidity ~45%) displayed no change of moisture content. However, its water activity increased.

Overall based on the previous values, the moisture contents and water activities of all samples studied remained below values at which bacterial growth and mould development are possible.

After rehydration the droplet size, shear viscosity, friction coefficient and microstructure of the emulsions were analysed

The droplet size distributions of all emulsions after three weeks of storage are displayed in Figure 5-13.

After re-dispersion in water the size distributions of the emulsions stored at room temperature under 76% relative humidity and the emulsions stored at 40 ˚C shifted to a larger size range. On the other hand, the droplet size of the emulsions stored in a room (at 22°C) and the ones stored at 0% RH remained approximately the same after rehydration.

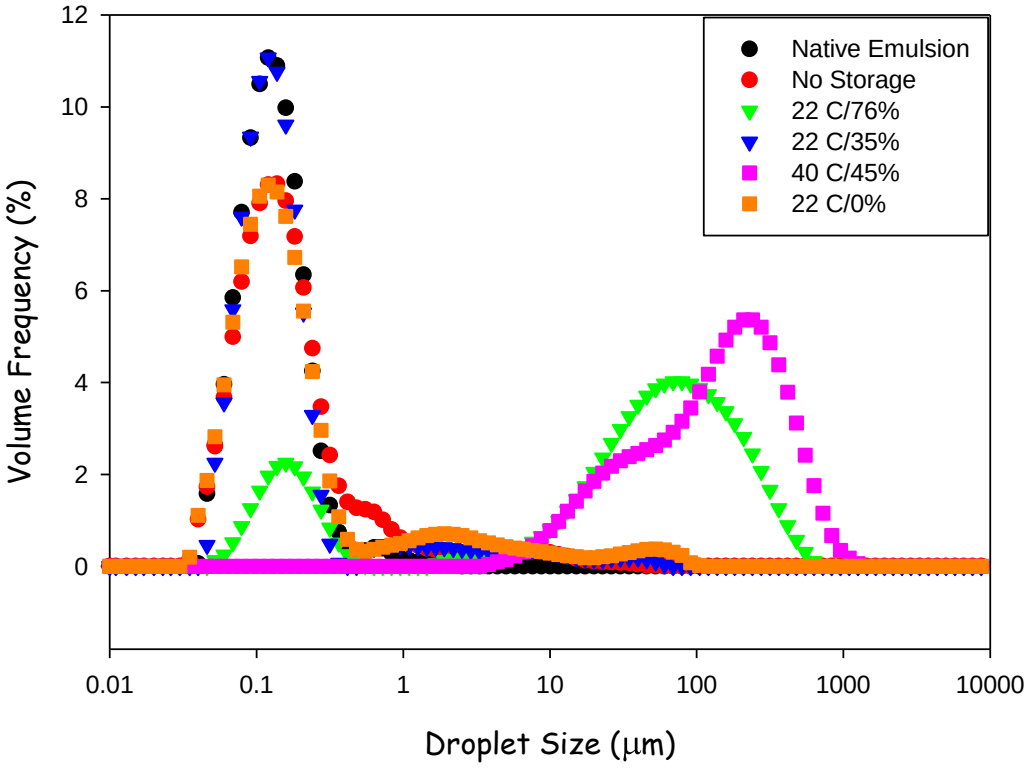


Figure 5-13: Droplet size distribution of 15% w/w CN-De stabilised oil-in-water before and after storage of the freeze-dried emulsions at different temperature and under different relative humidity conditions

Several hypotheses could explain the loss of microstructure of the emulsions after storage at high temperature and at high humidity.

The first hypothesis considered was formulated based on the study of *Al-Hakkak and Al-Hakkak*. When studying different incubation time for the conjugation of egg white and pectin *Al-Hakkak and Al-Hakkak* discovered that longer incubation times led to a decrease of the solubility of the conjugate without affecting the conjugation efficiency (Al-Hakkak and Al-Hakkak 2010).

The conjugate forming the outer layer on the dry emulsion particle, a decrease of its solubility could lead to a reduced ability of the dry emulsion to be dispersed in water. Therefore, this occurrence was assessed by incubating conjugate samples at 40˚C for three weeks. The solubility was measured and compared with the solubility of a non-incubated conjugate powder. The solubilities were measured at 50 ˚C to reproduce rehydration conditions.

The solubility of the non-incubated CN-De powder was 57.3 ± 0.7%. The solubility of the CN-De powder after three weeks at 40 ˚C was 57.41 ± 0.51%. These measurements showed that in this case, the extended period of time spent at 40˚C had no effect on the solubility of the conjugate. By extension it was assumed that the solubility of the dry emulsion stayed unchanged after storage at high temperature.

The other hypothesis was the plasticisation of the protein moieties present in the conjugate. For the emulsions stored at 40°C and those stored at high humidity the increase of the droplet size was attributed to plasticisation of the outer CN-De layer. Indeed, at high temperature or when exposed to high humidity levels proteins tend to form plastic films. When sodium caseinate powders are exposed to high temperature or water content (high relative humidity) the surface viscosity of the powder reduces allowing adhesion between the particles (Fox, Uniacke-Lowe et al. 2015). The formation of plastic films in dry emulsion powders led to formation of particle aggregates later resulting in aggregated droplets in the rehydrated emulsions.

The droplet size distribution of the emulsions stored at room temperature/room humidity and in a dry environment at room temperature remained the same after rehydration. In the other

conditions tested as pointed out previously, the droplet size distribution of the emulsions displayed a shift towards the larger range. Therefore, only the shear viscosities of the non-aggregated emulsions were measured.

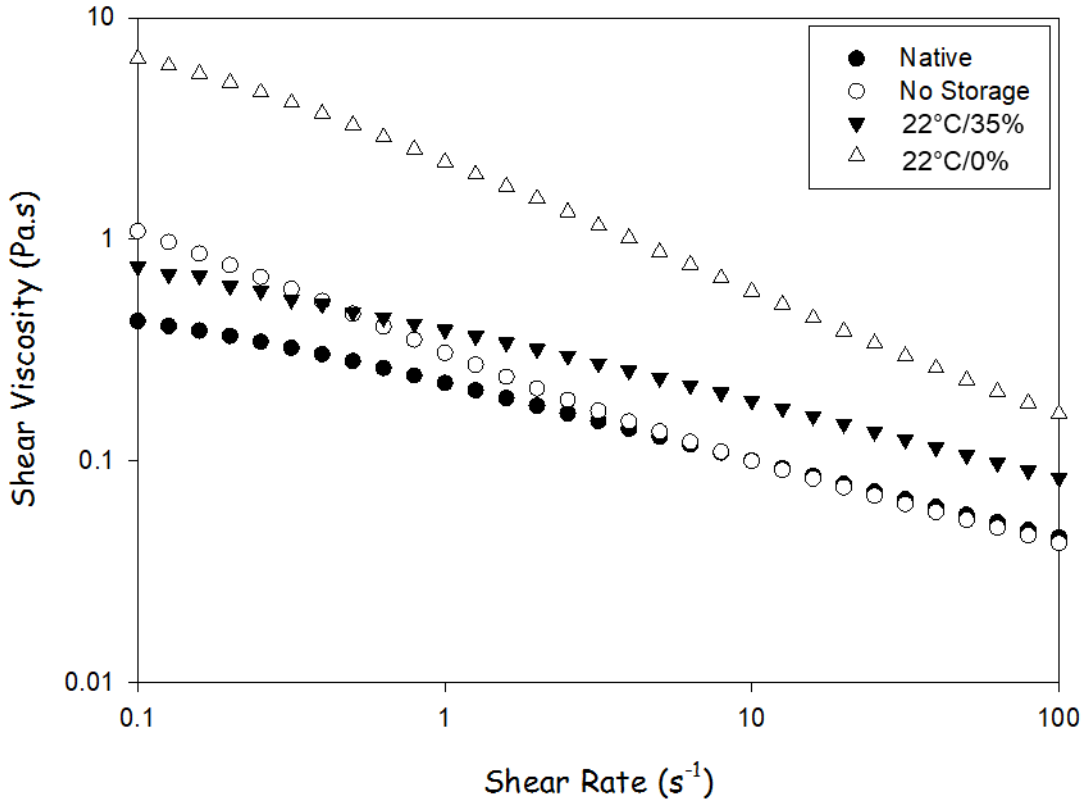


Figure 5-14: Steady state shear viscosity of 15% w/w CN-De stabilised oil-in-water emulsions before and after storage of the dry emulsions.

Before and after rehydration, the shear viscosity showed an increase on the entirety of the shear rate range tested for both storage conditions (Figure 5-14).

Keeping in mind that the main application for the present systems was for the production of food products, the friction forces of the samples were measured using tribology. In this study tribology was mainly used as a comparison tool to observe if the emulsions would have the same sensory properties (mouthfeel) before and after drying and rehydration depending on the storage conditions.

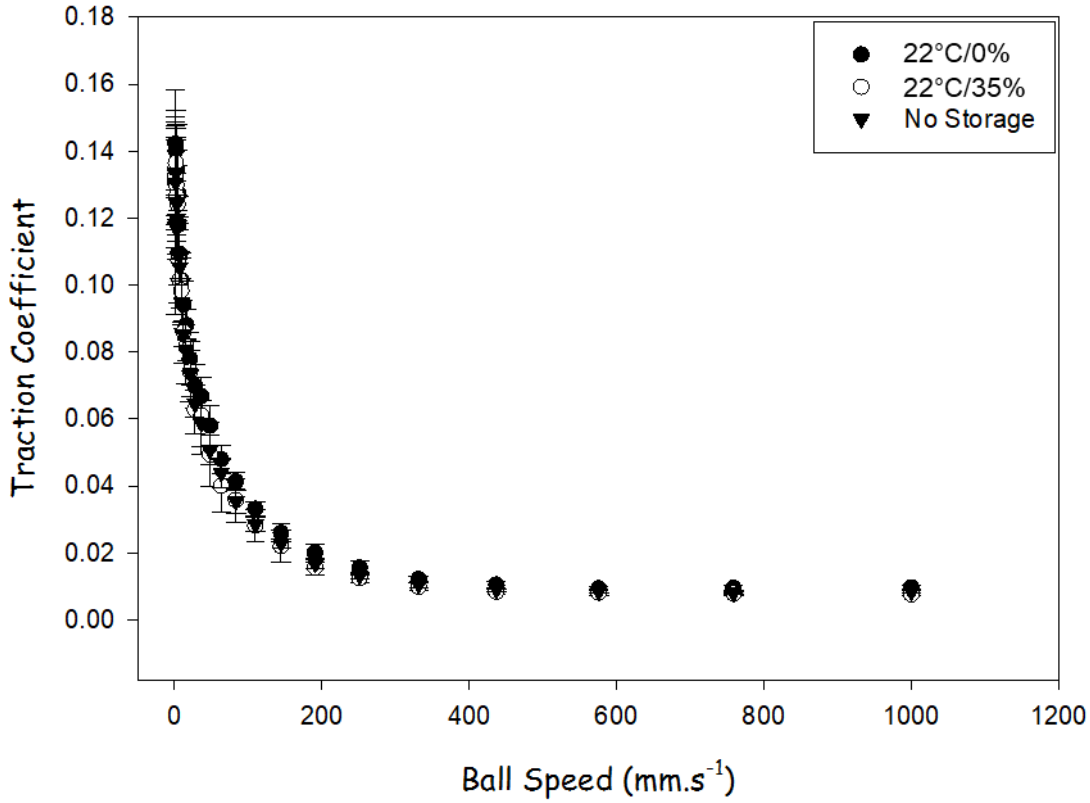


Figure 5-15: Traction coefficient as a function of the ball speed of 15% w/w CN-De stabilised oil-in-water emulsions before and after storage of dry emulsions. Data points represent an average of measurements performed on three samples. Error bars correspond to the standard deviation of the average.

Results displayed in Figure 5-15 show that the traction coefficient stayed the same under the storage conditions studied. Despite affecting the viscosity of the rehydrated emulsions, the storage of the powdered emulsions had no effect on their mouthfeel.

5.3.2.1.2 Whey protein isolate-sugar beet pectin oil filled powders

As for the previous emulsifier, the hygroscopicity of the dry emulsions produced with WPI-SBP was assessed by storing the powders for a week under a relative humidity of 76%. After a week under these conditions the weight of the dry powder had increased by 0.44 ± 0.15% on

average. Once more the solid could be classified as “slightly hygroscopic” according to classification given by the European Pharmacopeia.

The storage stability of the dry emulsions was tested by storing the emulsions at different temperature and/or relative humidity as described for the CN-De. The dry emulsions were stored at room temperature/ room humidity, at room temperature under a relative humidity of 76% and at room temperature in a dry atmosphere. The emulsions were rehydrated after three weeks of storage.

After storage the MC and a_w were measured. An increase of both values was expected for the powders stored at room temperature/room humidity and at a relative humidity of 76%.

Table 5-3 summarises the measurements made on the powders.

For these experiments the same powder was divided to create the different samples. Before storage the moisture content and water activity were respectively 1.20 ± 0.17% and 0.3853 ± 0.0011.

Table 5-3: Moisture content and water activities of WPI-SBP oil filled powders (freeze dried 2.5% w/w WPI-SBP stabilised oil-in water emulsions, 20% w/w sunflower oil) after storage at different temperature and under different relative humidity conditions.

	After	
	Moisture Content (%)	**Water Activity**
22°C/35%	1.64 ± 0.69	0.3949 ± 0.0035
22°C/76%	4.56 ± 0.29	0.6935 ± 0.0072
22°C/0%	0 ± 0	0.2142 ± 0.0008

As for CN-De dry emulsions, the water activity stayed constant after storage in a room while the moisture content slightly increased. Under high humidity, the moisture content showed the greatest increase. As expected, the hygroscopic powder absorbed the atmospheric water leading to the increase of the moisture content.

Once again after storage in dry environment the moisture content was 0%. The water activity stayed constant in these conditions.

The oil droplet size was measured after rehydration (Figure 5-16).

All emulsions showed signs of droplet aggregation after storage. Microscope images revealed the formation of large aggregates (Figure 5-17).

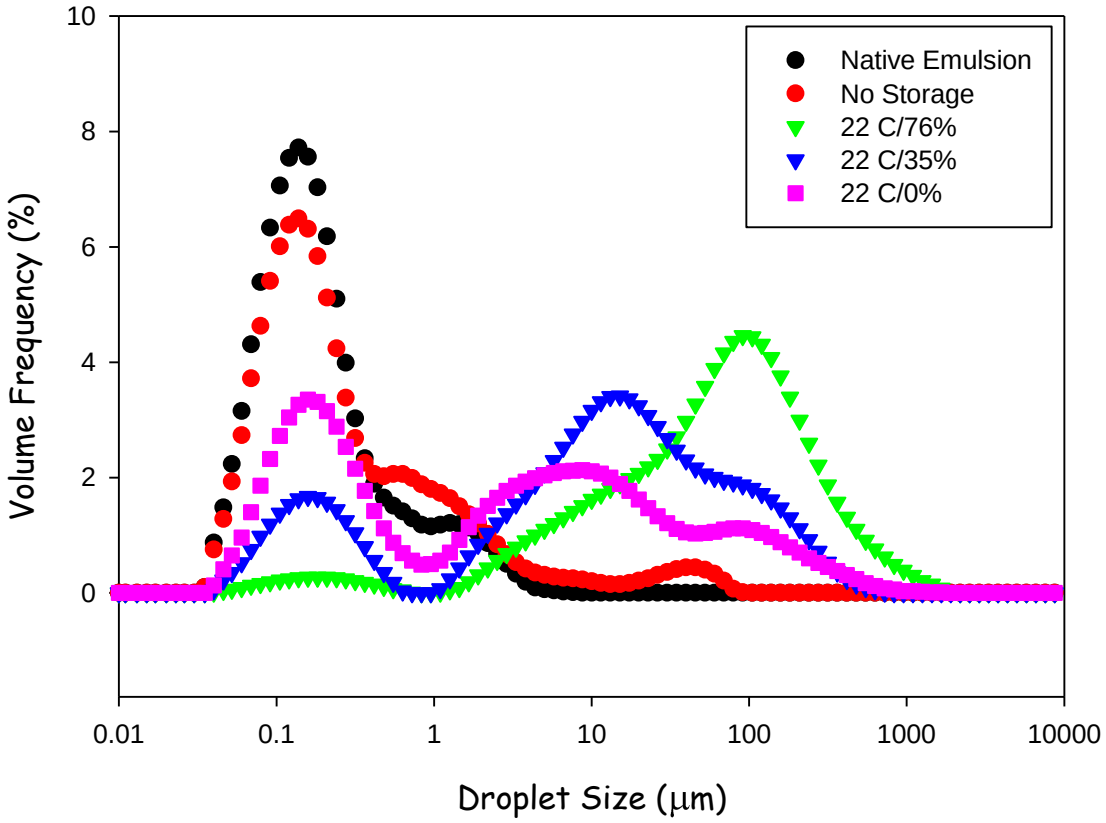


Figure 5-16: Droplet size distribution of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions before and after storage of the freeze-dried emulsions at different temperature and under different relative humidity conditions.

The formation of droplet aggregates in the re-dispersed emulsions was attributed to the properties of pectin. Indeed, the interaction of pectin with atmospheric water can cause agglomeration of particles in pectin powders. Particle agglomeration can alter the wetting,

dispersion and dissolution abilities of pectin (Mathlouthi and Rogé 2003, Einhorn-Stoll, Benthin et al. 2015, Einhorn-Stoll 2018).

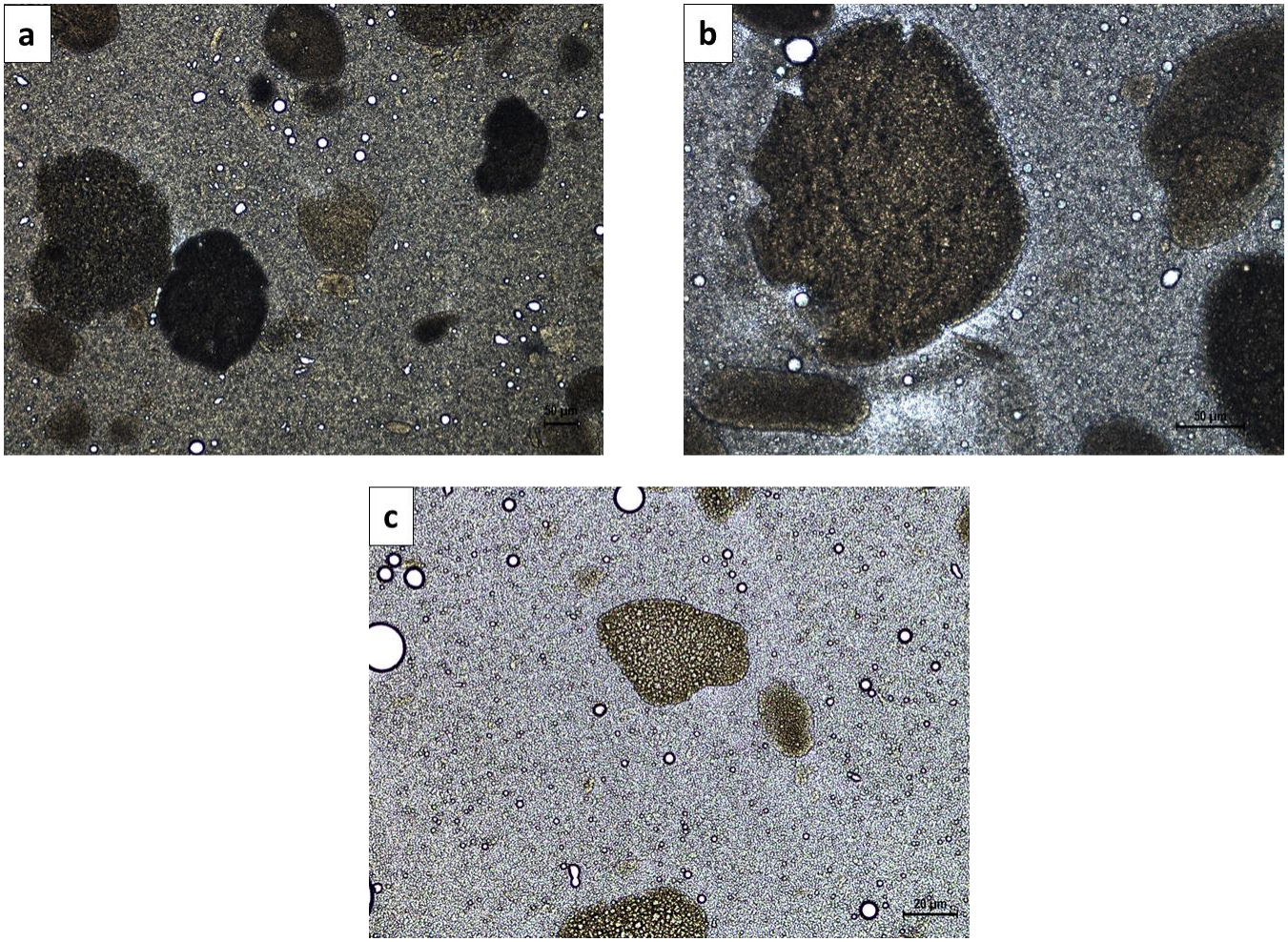


Figure 5-17: Optical microscope images of 2.5% w/w WPI-SBP stabilised emulsions (20% w/w sunflower oil, 80% w/w water, pH 5.8) after storage at room temperature (22°C) and 0% relative humidity (a), room temperature (22°)/room humidity (~35%) (b) and room temperature (22°C) and 76% relative humidity (c).

5.3.3 Spray drying and rehydration

5.3.3.1 Spray drying and rehydration of oil-in-water emulsions stabilised with a sodium caseinate-dextrin conjugate

Emulsions stabilised with CN-De at concentration ranging from 5% to 10% w/w (20% w/w sunflower oil) were spray dried and rehydrated. Increasing concentrations of conjugate were chosen in order to compare the effect of the material concentration on reconstitution.

The inlet temperature of the equipment was chosen according to DSC data giving the glass transition temperature of the emulsion systems. The aim was to obtain as little particle aggregation and stickiness as possible in the final powders in order to facilitate the re-dispersion later. Indeed sticky powders are generally obtained when the product is dried at temperatures lower than 20˚C above the T_g of the emulsion system (Labuza 1995). In other experiments stickiness was completely avoided in the final dry powder by working at a temperature 20˚C above the T_g confirming the previous observation made (Bhandari, Datta et al. 1997).

To determine the T_g at the different CN-De concentrations DSC was used. The T_g was found independent of the concentration of conjugate with values ~160°C. Based on this result and information available in the literature (Labuza 1995, Jayasundera, Adhikari et al. 2009) the optimum inlet temperature range for this emulsifier was between 190 °C and 210 °C. However other results showed that the optimum inlet temperature to spray dry milk proteins was between 185-195 °C (Baranauskienė, Venskutonis et al. 2006). For sodium caseinate it was found at 180 °C (Hogan, McNamee et al. 2001).

From these different data the lowest inlet temperature for this study was set at 180 °C and the highest 205 °C (the highest temperature was limited by the equipment maximum temperature 210°C).

The first set of spray drying experiments was performed at an inlet temperature of 180 °C.

The outlet temperature was left uncontrolled in this study and varied between 85-100 °C. The outlet temperature could have been controlled by adapting the aspiration rate of the equipment (Drapala, Auty et al. 2017). However, this method is detrimental to the yield of drying. The outlet temperature directly affects the moisture content and water activity of the final dry products (Fleming 1921). In this study experiments were carried out at earlier stages to study the effects of the outlet temperature on the powders. The effects on the moisture content and water activity were found negligible enough to disregard this parameter.

After drying, all emulsions gave powders displaying limited particles agglomeration (visually assessed). The powders were easily dispersed in water and gave visually homogenous emulsions. The droplet size and microstructure of the rehydrated emulsions were studied.

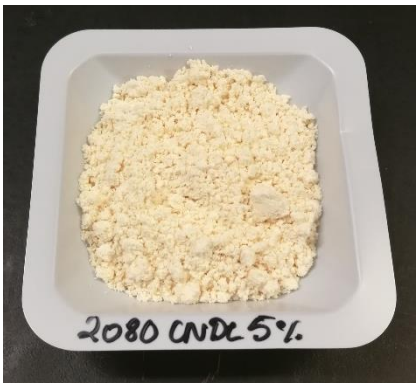


Figure 5-18: Photograph of a 5% w/w CN-De stabilised oil-in-water dry emulsion (20% w/w sunflower oil) immediately after spray drying at 180 °C.

The microstructure of all emulsions was lost through spray drying (Figure 5-18). The DLS measurements revealed a shift towards the larger droplet distribution in comparison with the native emulsions. The microscope images displayed in Figure 5-18 confirmed the formation of droplet aggregates but also showed partial coalescence of the oil droplets.

In normal conditions coalescence is rarely observed in hydrocolloid stabilised emulsions. The electrostatic repulsion between the droplets and thick interfacial membrane offers low possibility respectively for the droplets to get close to each other and for the interfacial film to rupture. However under increased stresses of drying, the dehydration of the interfacial

membrane can cause the protein to “lift” from the interface due to the change of solubilisation causing partial rupture of the interfacial film (Elversson and Millqvist-Fureby 2005). Additionally the droplets are forced in close proximity during the process favouring on one hand aggregation and on the other coalescence (Walstra 2003).

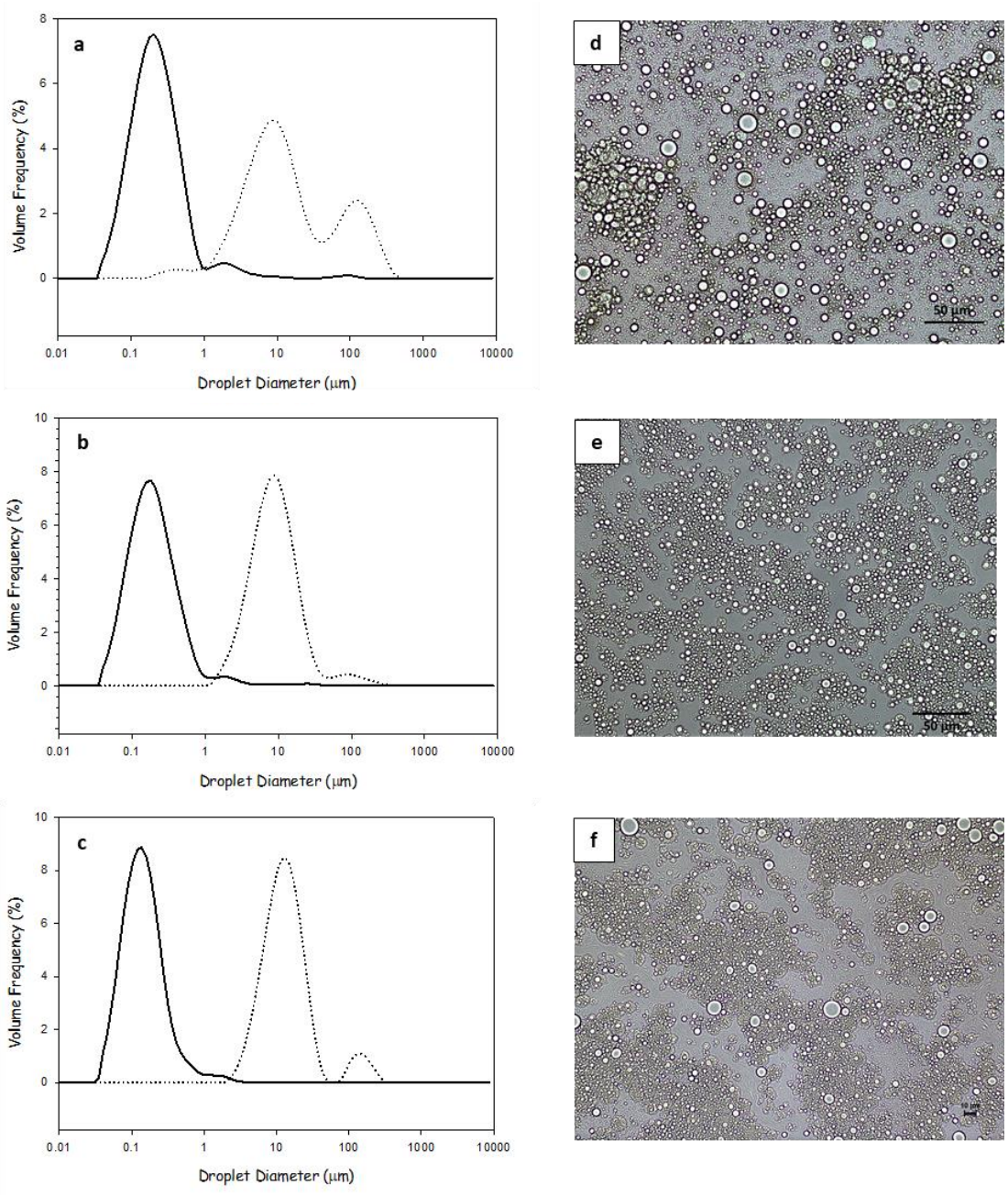


Figure 5-19: Droplet size distribution of CN-De stabilised oil-in-water emulsions (20% w/w sunflower oil, pH 6.8) before (——) and after (·····) spray drying at 180°C and rehydration. Measurements were carried out at 5% w/w (a), 7.5% w/w (b) and 10% w/w (c) CN-De.

Optical microscope images of rehydrated spray dried emulsions stabilised with 5% w/w (d), 7.5% w/w (e) and 10% w/w (f) CN-De.

The effect of additional shear to break down the aggregated droplets was assessed on 5% w/w CN-De stabilised emulsions.

The samples were sheared for a fixed amount of time (10 minutes) at increasing shear rates. To avoid re-emulsification the shear rate was kept low at values between 1500 and 3000 rpm using a high shear homogeniser.

The droplet size measurements performed using DLS (Figure 5-19) revealed that shear had no effect on the droplet size distribution. Irreversible aggregates had formed during spray drying.

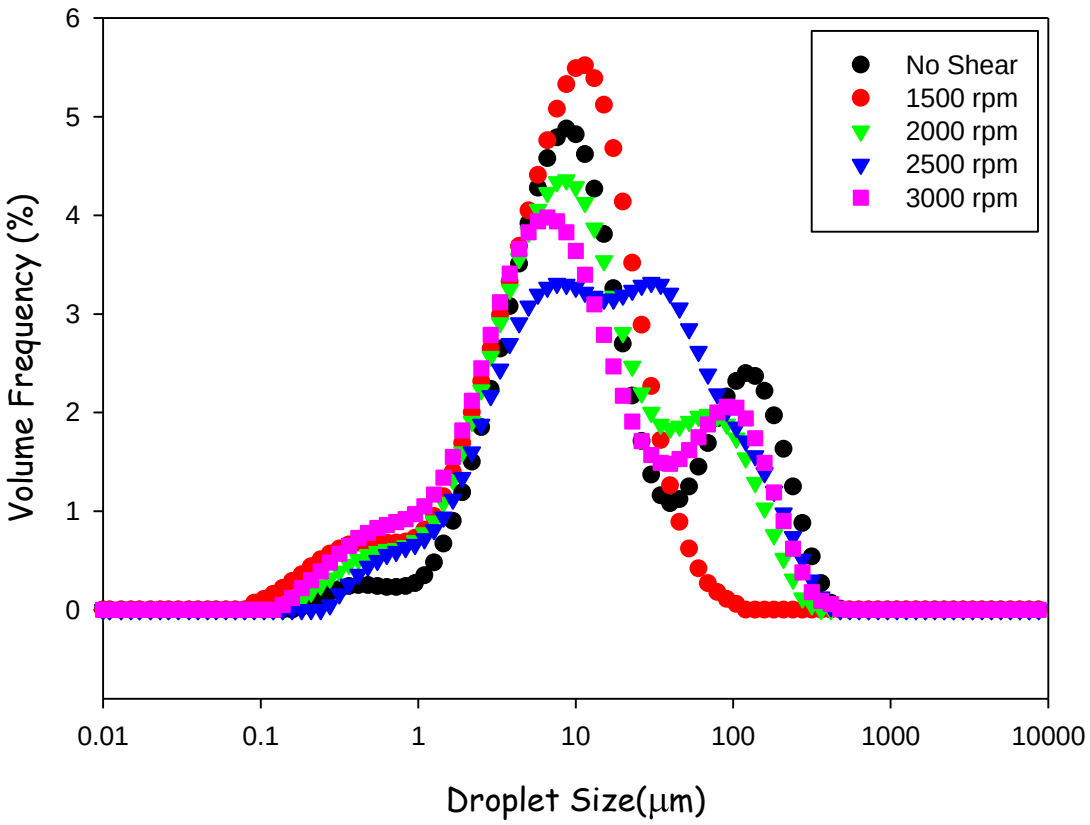


Figure 5-20: Droplet size distribution of spray dried 5% w/w CN-De stabilised emulsions (20% w/w sunflower oil, 80 % water, pH 6.8) before and after application of shear. Measurements were carried out at room temperature (22°C).

For further experiments 7.5% w/w CN-De was kept as a standard concentration. Indeed, upon rehydration, at this concentration the volume mean diameter displayed the smallest variation.

Furthermore, the concentration of emulsifier directly affects the particle size of the dry product (c.f. Chapter 2, Figure 2-9). In an attempt to favour the re-dispersion of the emulsions after SD, this concentration was chosen over 10% w/w CN-De.

Emulsions stabilised with 7.5% w/w CN-De were produced, spray dried at 190 ℃ and 205°C and rehydrated. These experiments were carried out to evaluate the effects of the inlet temperature on the reconstitution of the emulsions. The droplet size distribution and microstructure were studied. For both inlet temperatures tested a shift towards the larger droplet size distribution occurred in the rehydrated emulsions. Similar to when drying the emulsions at 180 °C, aggregation and coalescence happened during atomisation.

5.3.3.2 Spray dried oil filled powders

The particle morphology of powdered emulsions dried at 180 °C and 190°C was observed using electron microscopy. Micrographs displayed in Figure 5-20 show that the shape of the powders was independent of the drying temperature. At both temperatures the powered emulsions were made of agglomerated particles of various sizes. In both cases the surfaces of the sphere shaped particles presented hollows.

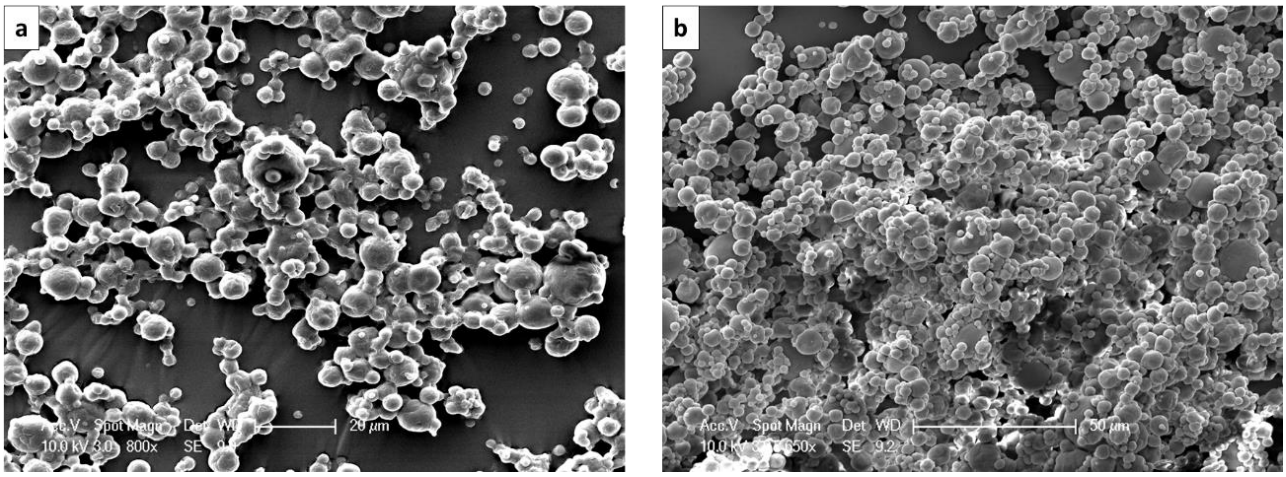


Figure 5-21: SEM micrographs of CN-De sunflower oil filled powders (7.5% w/w CN-De, 20% w/w sunflower oil spray dried oil-in water emulsions) dried at 180 °C (a) and 190 °C (b).

5.3.3.3 Spray drying and rehydration of oil-in-water emulsions stabilised with a whey protein isolate-sugar beet pectin conjugate

Similarly, DSC was used to determine the suitable inlet temperature to dry WPI-SBP stabilised emulsions. The glass transition temperature was measured at ~135 °C using this technique. Considering previous studies (Labuza 1995) the inlet temperatures were chosen at values starting from 30 °C above the glass temperature measured. *Baranauskiene et al.* demonstrated an optimal inlet temperature of 185 °C for whey proteins (Baranauskienė, Venskutonis et al. 2006). While *Drusch*'s experiments proved that the suitable drying temperature for sugar beet pectin was 170 °C (Drusch 2007). Thus, inlet temperatures of 165 °C, 175 °C and 185 °C were investigated.

Emulsions stabilised with 2.5% w/w WPI-SBP were spray dried at 165 °C and rehydrated. Results from these experiments are gathered in Figure 5-21. Before and after spray drying and rehydration the droplet size shifted towards larger diameters. Microscope analyses revealed that this shift was the result of coalescence in the rehydrated emulsions rather than aggregation or

flocculation (Figure 5-21 b). Coalescence of droplets in re-dispersed spray dried emulsions has previously been reported (Hogan, McNamee et al. 2001, Hogan, McNamee et al. 2001, Jafari, Assadpoor et al. 2008). Experiments on protein stabilised spray dried emulsions showed the stability to spray drying is dependent on the amount of unadsorbed protein in the continuous phase (Taneja, Ye et al. 2013, Taneja 2016). It was proved that unadsorbed protein stabilise powder particles during spray drying. Meanwhile a low concentration of unadsorbed protein led to the migration of protein from the interfacial film to the air/water interface upon drying. This displacement caused gaps in the interfacial membrane that would lead to partial coalescence upon rehydration of the spray dried emulsion powders. In the present experiments this phenomenon was likely the cause of the coalescence observed in the re-dispersed emulsions.

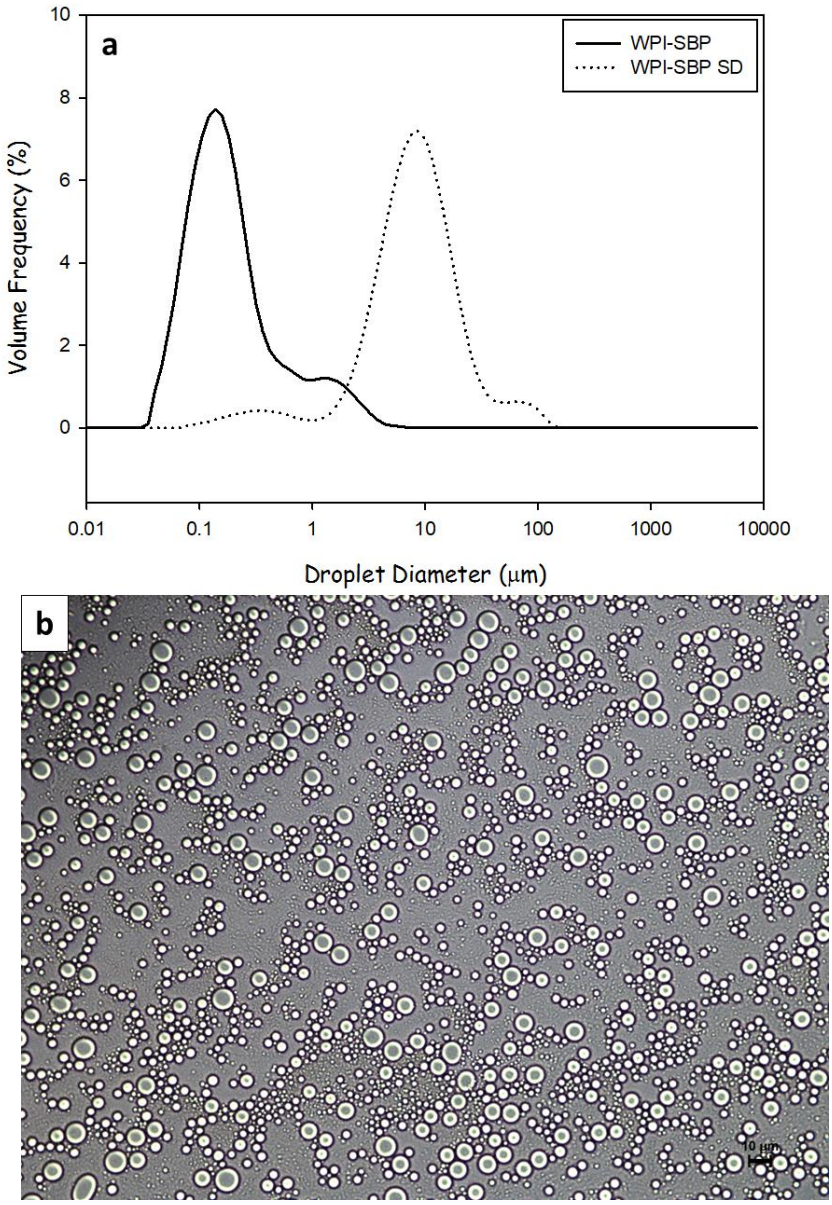


Figure 5-22: Droplet size distribution of a 2.5% w/w WPI-SBP stabilised emulsion before and after spray drying at 165 °C and rehydration (a). Optical microscope image of a 2.5% w/w WPI-SBP stabilised emulsions after spray drying at 165 °C and rehydration.

The concentration of WPI-SBP was increased in an attempt to increase the spray drying stability of the emulsions. Emulsions stabilised with ~8% w/w WPI-SBP (7.875% w/w) were produced and rehydrated after spray drying at 165 °C, 175 °C and 185 °C.

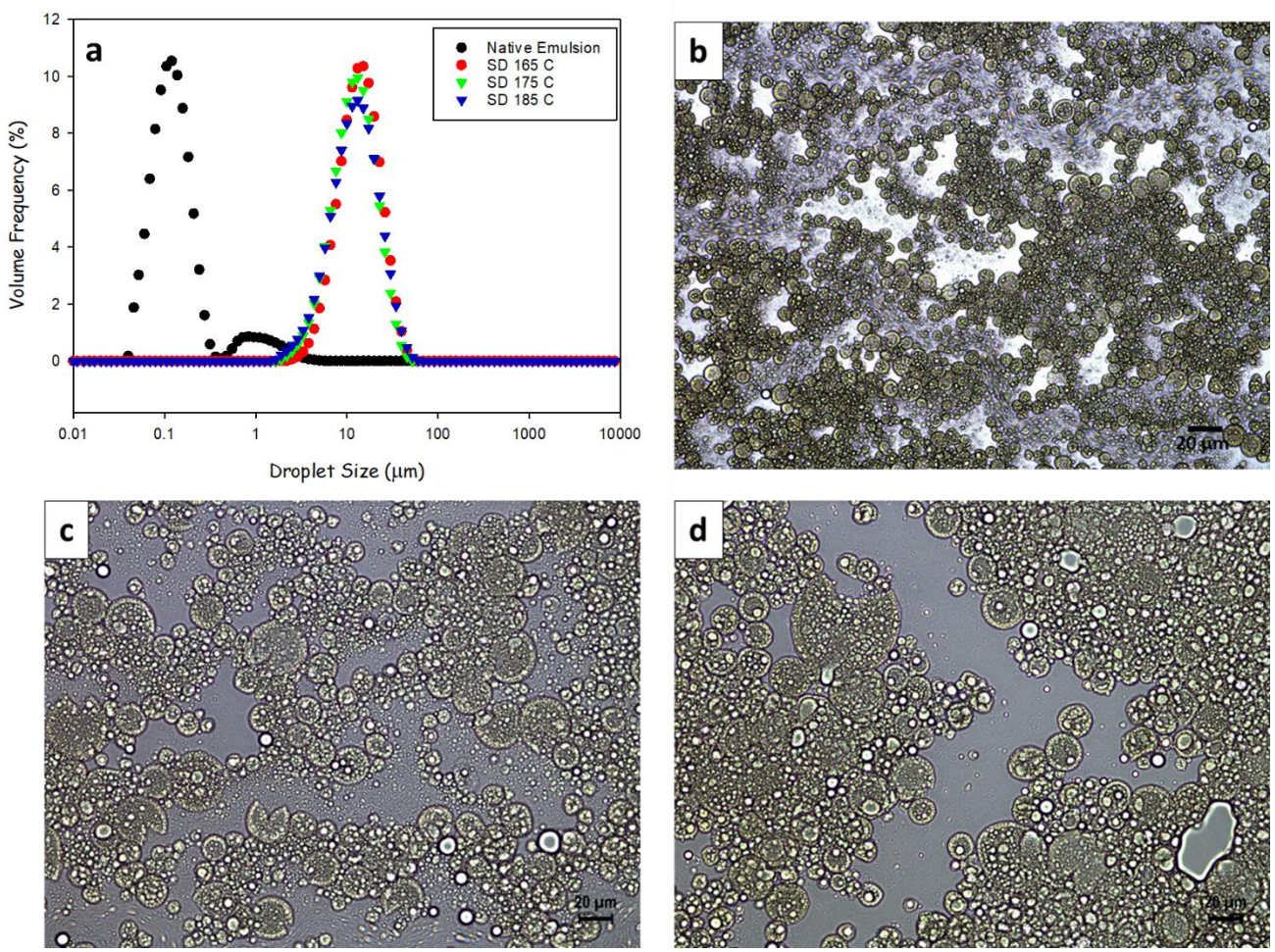


Figure 5-23: Droplet size distribution of ~8% w/w WPI-SBP stabilised emulsions before and after spray drying and rehydration (a). Optical microscope images of ~8% w/w WPI-SBP stabilised emulsions after spray drying at 165 °C (b) at 175 °C (c) and 185 °C (d) and rehydration.

Figure 5-22 shows that after rehydration the same profile as previously was witnessed for the droplet size distribution. A shift towards the larger sizes was observed. Unlike for emulsions stabilised with 2.5% w/w WPI-SBP (20% w/w sunflower oil, 80% w/w water), microscope pictures showed that this increase was the result of the formation of large poorly soluble particles during spray drying. The heat stability of the system is directly linked to the insolubility of the final powder (Sharma, Jana et al. 2012, Sadek, Schuck et al. 2015, Schuck, Jeantet et al. 2016, Toikkanen, Outinen et al. 2018). Insolubility arises when proteins unfold

during drying (Straatsma, Van Houwelingen et al. 1999, Baldwin 2010, Sharma, Jana et al. 2012, Toikkanen, Outinen et al. 2018).

5.4 Conclusion

The present chapter aimed to assess the suitability of two Maillard conjugates for the stabilisation of emulsions to be dried. Freeze-drying and spray drying were chosen as the drying methods.

Experiments showed that both conjugates could be used for the production of re-dispersible freeze-dried emulsions. The rheological analyses comparing emulsions produced with the CN-De, NaCN and a mixture of NaCN and dextrin revealed that the conjugation altered the rheological behaviour and increased the viscosity of the emulsions. However, there was no evidence of an improved the resistance of the CN-De stabilised emulsions to freeze-drying in comparison with NaCN stabilised emulsions.

When using WPI-SBP smaller amount of conjugate were needed to produce re-dispersible emulsions. Not only the microstructure and viscosity of the emulsions was preserved but also the conjugation of SBP to WPI proved to have enhanced the freeze-drying stability. Indeed, WPI stabilised emulsions showed poor stability to freeze-drying characterised by droplets flocculation after rehydration.

Emulsions stabilised with both conjugated emulsifiers showed no stability to spray drying. Droplet aggregates believed to be the result of poor heat stability of the systems were observed in the rehydrated emulsions. In some sample, coalescence was the main destabilisation mechanism observed suggesting that the conjugate was displaced from the oil/water interface during drying.

Chapter 6

Influence of The Processing Parameters and Emulsion Formulation on Dry Powder Characteristics and Reconstitution Ability After Rehydration

6.1 Introduction

The previous chapter treated the drying and rehydration of emulsions stabilised with two different Maillard conjugates. Emulsions were successfully reconstituted (droplet size and texture properties) after freeze-drying and rehydration. On another note the systems showed no spray drying stability

In the present chapter different process parameters and formulations were studied. Process parameters can affect the final structure of a dried product. Changing these parameters can affect the pore size in the case of freeze-drying or the particle size for spray drying.

Modification of the formulation on the other side can improve the resistance of emulsion systems to high and low temperature.

When using freeze drying several studies have explored the effect of increased freezing rate or of the effect of the addition of cryoprotectants to the formulation (Thanasukarn, Pongsawatmanit et al. 2004, Ghosh and Coupland 2008, Degner, Chung et al. 2014, Iyer, Cayatte et al. 2017).

Varying the spray drying parameters has also been identified as a means to affect the quality of the final dry products.

In this chapter WPI-SBP stabilised emulsions (2.5% w/w conjugate, 20% w/w sunflower oil, 80% water) with added salt (0.1% to 1% w/w NaCl or $CaCl_2$) presented in Chapter 4 were freeze-dried and rehydrated in order to gauge the effects of salt addition on the reconstitution. Results from these experiments were also valuable data for application to real emulsified food products.

With the same aim in mind, emulsions formulated at different pH (2, 5.2 and 12) that showed no signs of microstructure changes were freeze-dried and rehydrated.

The effect of higher freezing rate was investigated using liquid nitrogen to freeze the samples prior to freeze-drying.

Subsequently cryoprotectants were added to the formulations to reduce the impact of freezing on the microstructure. Indeed, freezing has been confirmed in the fourth chapter of this study as a cause for droplet aggregation in freeze-thawed emulsions.

For spray drying and rehydration experiments, the process parameters were investigated. A different feeding rate and its impact on the powdered emulsions and their reconstitution were studied.

Lastly, addition of lactose to the emulsions was explored. Lactose along with other small polysaccharides has been used to improve spray dried powders quality by helping emulsifiers stay at the oil/air interface during evaporation (Fäldt and Bergenståhl 1996, Vega and Roos 2006, Vignolles, Jeantet et al. 2007, Toikkanen, Outinen et al. 2018).

In this study, lactose was incorporated in emulsions using two methods; as an additive, incorporated after emulsification in the bulk prior to spray drying and through conjugation with the production of protein-lactose conjugates used to stabilise the systems.

6.2 Materials and Methods

6.2.1. Materials

6.2.1.1 Proteins

Casein sodium salt from bovine milk and egg white protein were purchased from Sigma Aldrich, UK. Whey protein isolate was ordered from Volac.

6.2.1.2 Polysaccharides

Dextrin from corn starch (DE ≤ 5, Mw = 7 kDa), maltodextrin (DE = 4.0-7.0, Mw = 3.6 kDa), lactose and sucrose were purchased from Sigma Aldrich, UK. Sugar Beet Pectin (degree of esterification >50%, Mw = 45.3 kDa) was purchased from Herbstreith & Fox KG (named Betapec RU 301).

6.2.1.3 Other materials

The sunflower oil (Solesta) used for the production of oil-in-water emulsions was purchased from a local store.

6.2.2 Methods

6.2.2.1 Preparation of the conjugates

All conjugates were prepared as follow; protein and polysaccharide were dissolved separately in deionized water at a concentration of about 10% (w/v) and stirred overnight. The two solutions were then mixed together under magnetic stirring for an hour. The mixture was then frozen at -22˚C for at least 24 hours and lyophilised for 4 days.

To carry out the Maillard reaction, the dried mixture was placed in a desiccator over a saturated solution of KBr to ensure a relative humidity of 79%, and heated at 60˚C for 96 hours. After which the conjugates were placed in a freezer at -22˚C for 24 hours to stop the reaction. The conjugate was then freeze dried for 24 hours before being used for experiments.

Table 6-1: List of protein and polysaccharides used for the preparation of conjugates

Protein	Polysaccharide
Sodium Caseinate	Lactose
Whey Protein Isolate	Dextrin
	Sugar Beet Pectin

6.2.2.2 Characterisation of the conjugates

6.2.2.2.1 O-pthaldialdehyde assay

The conjugation efficiency was assessed by comparing the amount of free amino groups in conjugated samples with a protein sample, using the OPA assay *(Pan, Mu et al. 2006)*. A mixture of 80 mg of o-phtaldialdehyde (OPA) (dissolved in 2 mL of a 95% ethanol solution), 50 mL of borate buffer pH 9.5, 5 mL of a 20% sodium dodecyl sulphate solution and 0.2 mL β-mercaptoethanol was prepared and adjusted to a final volume of 100 mL. Conjugates solutions were prepared with a 3 $mg.mL^{-1}$ protein concentration. The samples were made by

adding 0.1 mL of conjugate solution to 2.70 mL of OPA solution, and allowed to incubate at room temperature for one minute.

An absorbance scanning between 330 and 350 nm was then performed on the samples using a UV-Visible Spectrometer Orion Aquamate 8000 from Thermo Scientific. The conjugation efficiency was calculated with the absorbance at 340 nm using the following equation:

$$\%\ conjugation = \left(1 - \frac{\text{A after conjugation}}{\text{A before conjugation}}\right) \times\ 100 \qquad \textit{Equation 6-1}$$

Where A is the absorbance.

6.2.2.2 Emulsion preparation

All emulsions were prepared following the same protocol. Conjugates, protein or a mixture of protein and polysaccharide were dissolved in 80 g of distilled water. The mixture was put under magnetic stirring for 1 hour after which 20 g of sunflower oil were added and the temperature increased to 50 ˚C. NaN_3 (0.01% w/w) was also added to prevent bacterial growth and increase long term stability. The mixture was stirred for another 30 minutes.

Pre-emulsions were made using a high shear mixer Silverson L5M (10 000 rpm for 15 s).

The final emulsions were produced by passing the pre-emulsions three times through a microfluidiser (Microfluidics model M-110S) at a pressure of 1250 bars.

The droplet size was measured using laser diffraction with a Malvern Mastersizer 2000.

For the study of the effect of lactose on the stability to spray drying, lactose was incorporated in the emulsions after emulsification.

6.2.2.3 Optical microscope

The microstructure of the emulsions was observed using an optical microscope Leica DM 2500 LED equipped with a camera LEICA DFC 450 C. The images were collected with the software Leica Application Suite V 4.8. The 40x or 100x objective lenses were mainly used for the obtainment of the images featured in this study.

6.2.2.4 Texture analysis

6.2.2.4.1 Shear viscosity measurements

The shear viscosity of the emulsions was measured at 25 ˚C, from 0.1 to 100 s^{-1} shear rate using a Kinexus rheometer equipped with a cylindrical double gap and cup geometry. The maximum sampling was set at 5 minutes and the integration time at 1 minute.

6.2.2.4.2 Traction coefficient

The traction coefficient of the emulsions was measured using a MTM2 (Mini Traction Machine) tribometer (PCS Instruments, London).

The load was set to 3 N and the ball speed was set between 1 and 1000 $mm.s^{-1}$.

6.2.2.5 Drying processes

6.2.2.5.1 Freeze-drying

Samples were kept in a commercial freezer at -22 ˚C before being freeze-dried for 5 days using a Scanvac CoolSafe freeze dryer. The temperature of the condenser was -110˚C and the pressure in the chamber around 0.1 hPa.

6.2.2.5.2 Spray drying

Powdered emulsions were prepared using a spray dryer Buchi Mini Spray Dryer B-290. Inlet temperature was set at the desired temperature. The feeding rate was set at 20% (10 $mL.min^{-1}$) unless stated otherwise and the aspiration power at 95%.

The final product was collected from the collection chamber and kept in a sealed container before further analyses.

6.2.2.5.3 Freezing point and glass transition temperature measurements

The freezing point and glass transition temperature were measured using a Differential Scanning Calorimetry (DSC 25 from TA Instruments). Data were analysed with the software provided (Trios V4 1.1.33073).

For freezing point measurements temperature ramps at 0.5 °C.min^{-1} (unless state otherwise) were performed from 20 °C to -30 °C followed by an isothermal of 5 minutes at -30 °C. The temperature was then brought back to 20°C at a heating rate of 0.5 °C.min^{-1}.

For glass transition temperature measurements temperature ramps at 10 °C.min^{-1} were performed from 20 °C to 200 °C followed by an isothermal of 1 minute at 200 °C. The temperature was then brought back to 20 °C at cooling rate of 10 °C.min^{-1}.

6.2.2.6 Dry powder analysis

6.2.2.6.1 Dry particle microstructure

Micrograph of the dry emulsions were obtained with scanning electron microscopy using an environmental scanning electron microscope Philips XL30 ESEM-FEG.

6.2.2.6.2 Hygroscopicity

Hygroscopicity measurements were performed by placing 1 g of dry emulsions in a desiccator containing a saturated NaCl solution. The hygroscopicity represents the mass water absorbed per 100 g of sample after 10 days of storage.

$$\mathrm{Hp} = \left(\frac{w_1 - w_0}{w_0}\right) \times 100$$

Equation 6-2

Where *Hp* is the hygroscopicity, w_0 the initial weight of the powder and w_1 the weight after storage.

6.2.2.6.3 Bulk density

The bulk density was measured according to the following method. Powders were inserted into a burette placed on tared scale. The burette was then filled with powder up to ~ 20 mL. The mass of powder was taken and the density calculated using the Equation 6-1.

$$\rho = \frac{w}{V}$$

Equation 6-3

With w the mass of powder weighted and V the volume occupied by the powder in the burette.

6.3 Results and discussion

6.3.1 Influence of the processing parameters

6.3.1.1 Effects of the freezing rate on the freeze-drying stability of sodium caseinate-dextrin conjugate stabilised emulsions

The rate of freezing of emulsified product prior to FD is one of the parameters that can be controlled in order to enhanced the stability to the process (Degner, Olson et al. 2013). Controlling the freezing rate can affect both the stability to freezing and the stability to drying. In the section treating of the freeze-drying process (Chapter 2), a description of how the freezing rate affects the microstructure of freeze-dried products was given. The rate of freezing is a parameter directly affecting the number and the size of ice crystals of a final frozen product (Degner, Olson et al. 2013, Degner, Chung et al. 2014). The size of the pores in a freeze-dried product and the rate of sublimation of the ice are directly linked to the size of the ice crystals of the frozen phase. Yet, the size of the ice crystal can be controlled by controlling the freezing rate. Commercial freezers generally have a freezing rate of ~0.9 °C.min^{-1}.

Controlling the freezing rate to reduce the ice crystal size when freezing can be used as a way to help preserving fragile emulsion structures (Degner, Olson et al. 2013).

Smaller ice crystals are in theory less likely to disturb the interfacial membrane and result in unstable emulsion after thawing or drying.

Higher freezing rate was used in an attempt to reduce the amount of conjugate needed for the production of an oil-in-water emulsion resistant to freeze-drying. Emulsions stabilised with 2.5% w/w CN-De were used for these experiments.

6.3.1.1.1 Freezing point

The freezing rate of 2.5% w/w CN-De stabilised emulsions was increase using liquid nitrogen (N_2). The freezing rate and the freezing point are two values dependent on each other. Increasing the freezing rate of a system provoke a decrease in the freezing point (Reid, Kerr et al. 1994). DSC was used to gauge the effect of higher freezing rates on the freezing point of emulsions. Three different freezing rates were studied. The standard emulsion stabilised with 2.5% w/w CN-De was frozen in the instrument at different freezing rate. Table 6.1 shows that the freezing point decreased with an increasing freezing rate. This result was consistent with other studies (Reid, Kerr et al. 1994).

Table 6-2: Effect of the freezing rate on the freezing point of 2.5% w/w CN-De stabilised emulsions.

Freezing rate (˚C/min)	Freezing point (˚C)
0.5	-16.9±1.9
5	-17.7±0.7
10	-18.3±0.9

6.3.1.1.2 Freeze-thaw stability

As previously the FT stability was used a mean to assess potential instabilities brought about by the freezing step. Emulsions produced with 2.5% w/w CN-De were frozen using N_2 and allowed to melt at room temperature. The droplet size was measured before and after FT and the microstructure observed.

After being frozen using liquid nitrogen and being thawed at room temperature the emulsions showed visual signs of creaming. The droplet size of the emulsions displayed a shift towards larger size (Figure 6-1 a). This was indicative of droplets aggregation or coalescence. The optical microscope images (Figure 6-1 b) revealed that the dispersed phase aggregated upon freezing.

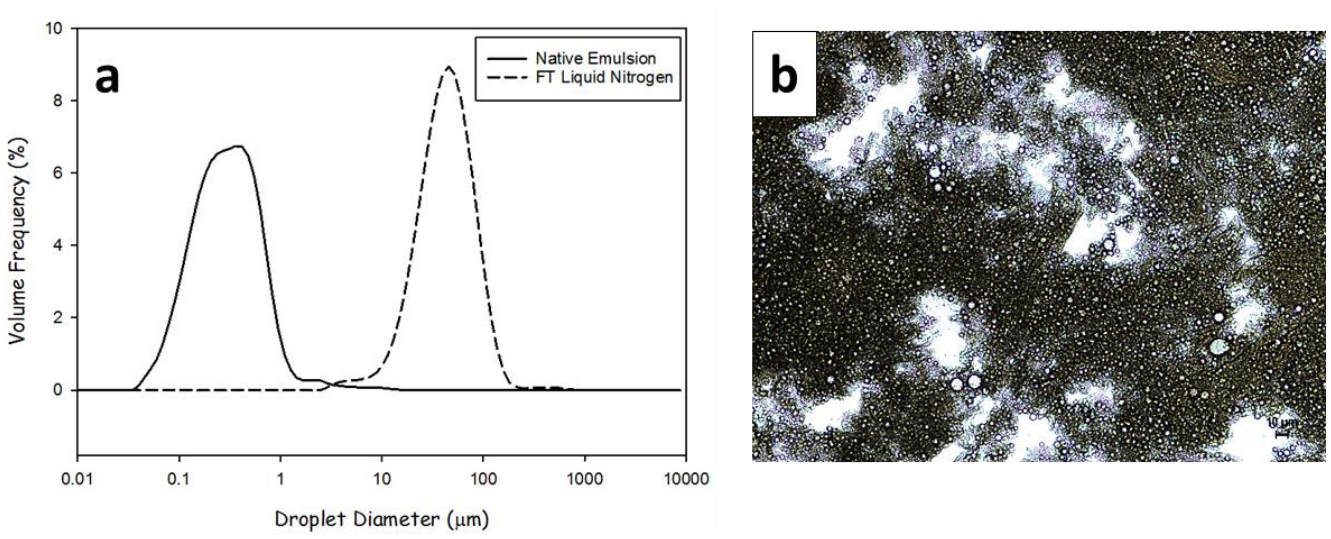


Figure 6-1: Droplet size distribution (a) of a 2.5 % w/w (~0.8% w/w NaCN) CN-De stabilised oil-in-water emulsion before and after freeze-thaw using liquid nitrogen. Microscope image (b) represents the microstructure of the emulsion after freeze-thaw using liquid nitrogen.

6.3.1.1.3 Freeze-drying and rehydration

Emulsions stabilised with 2.5% w/w CN-De were tested for freeze-drying and rehydration experiments in spite of the droplet aggregation after freeze-thawing. The droplet diameter and microstructure were observed before and after the process (Figure 6-2).

After drying and rehydration the droplet size measurements performed using DLS revealed a multimodal distribution. Figure 6-2 compares the droplet size distribution of the different emulsions. When put in comparison with the droplet size distribution of the native emulsions a portion of droplet remained the same size in the freeze-dried emulsions.

Experiments at lower freezing rate (c.f. Chapter 3) revealed that upon freeze-thawing droplets irreversibly aggregated. The microscope images from experiments using liquid nitrogen displayed in Figure 6-2 b show that the larger droplets detected with the instrument were the result of coalescence as no aggregation was observed.

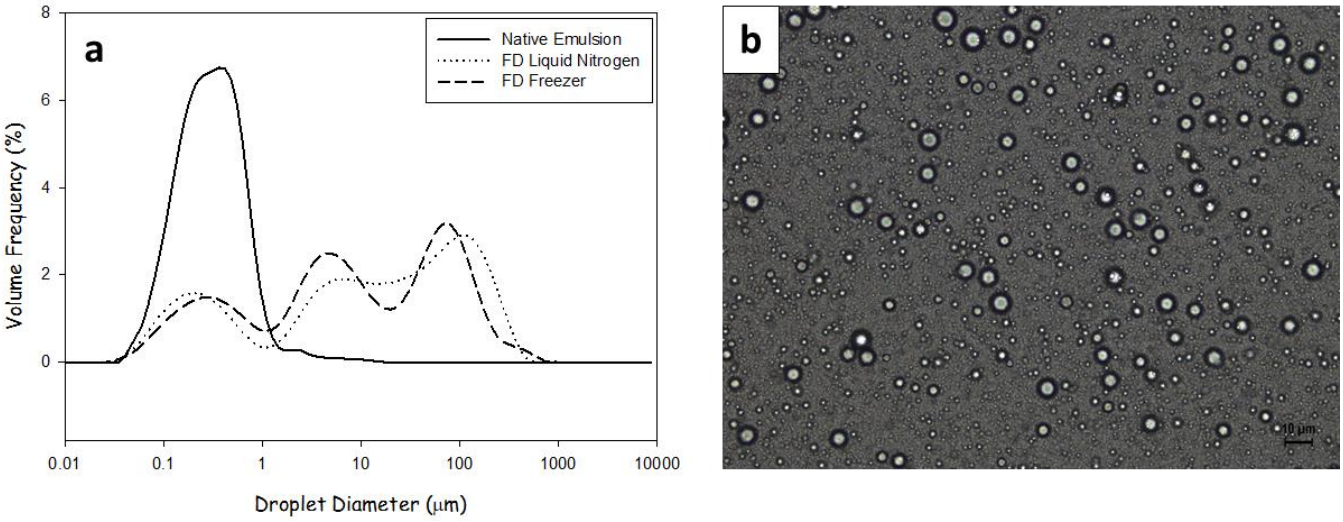


Figure 6-2: Droplet size distribution (a) of a 2.5 % w/w (~0.8% w/w NaCN) CN-De stabilised oil-in-water emulsion (20% w/w sunflower oil, 80% w/w water, pH 7) before and after freeze-drying. Emulsions were frozen using liquid nitrogen or a freezer. Microscope image (b) represents the microstructure of the emulsion after freeze-drying using liquid nitrogen.

Coalescence occurs when the interfacial film surrounding oil droplets is at least partially disrupted. In this case the hypothesis was that the formation of smaller ice crystal around the droplet increased the number of sites of potential disruption. Upon sublimation of the ice a hole would form in the interfacial membrane. Indeed, instability triggered by the drying part of the freeze-drying process can be the result of ice sublimating in the vicinity of sensitive structures. Considering that using a commercial freezer led to the formation of larger ice crystal this phenomenon was less likely to occur. Instability would be mainly caused by the forced gathering of the oil droplets during freezing.

6.3.1.2 Effects of spray drying feeding rate on powder quality

As depicted in the second chapter of this study, the spray drying parameters can affect the dry product differently (Chapter 2, Figure 2-9). Previous studies have shown that a more complex series of factors can influence the quality of the powders obtained through spray drying (Figure

6-3) (Verdurmen and de Jong 2003, Taneja 2016). For the present experiments the simpler set of parameters discussed in the Literature Review of this study was considered.

Keeping in mind the rehydration the focus was made on all parameters capable of potentially affect the re-dispersion or rehydration of the dry emulsions. The powders properties have been identified as the main parameters controlling the dispersion of a powder (Idf 1979, Schubert 1987, Pısecký 1997, Schober and Fitzpatrick 2005, Sharma, Jana et al. 2012). The spray drying parameters are the main influences on the values of these powder properties (Birchal, Passos et al. 2005, Koç, Sakin-Yılmazer et al. 2014). The only spray drying parameters affecting the quality of the powder produced (i.e. the particle size) have been identified as; the air flow, the feeding rate, the solvent and the material concentration.

For this study the airflow was left unchanged as it also affects the outlet temperature. The choice was made to disregard factors affecting powder parameters other than the particle size.

The concentration of material had been investigated previously in this study when different conjugate concentrations were tested (emulsions stabilised with 5% w/w, 7.5% w/w and 10 % w/w CN-De and emulsions stabilised with 2.5% w/w and 8% w/w WPI-SBP, 20% w/w sunflower oil, 80% w/w water).

For these reasons, the effect of the feeding rate on the rehydration was the only other parameter investigated.

The final objective was to decrease the size of the particle. Indeed smaller particles tend to have better dispersion in solution (Hogekamp and Schubert 2003). The lower the particle size, the lower the solubility (Kurozawa, Morassi et al. 2009). The solubility of particles has been identified as a major factor for the overall dispersibility of a powder (Koç, Sakin-Yılmazer et al. 2014). For this purpose, the feeding rate was decreased from 10 $mL.min^{-1}$ to 2.5 $mL.min^{-1}$. Indeed, decreasing the amount of material to dry at once decreases the size of the final particles.

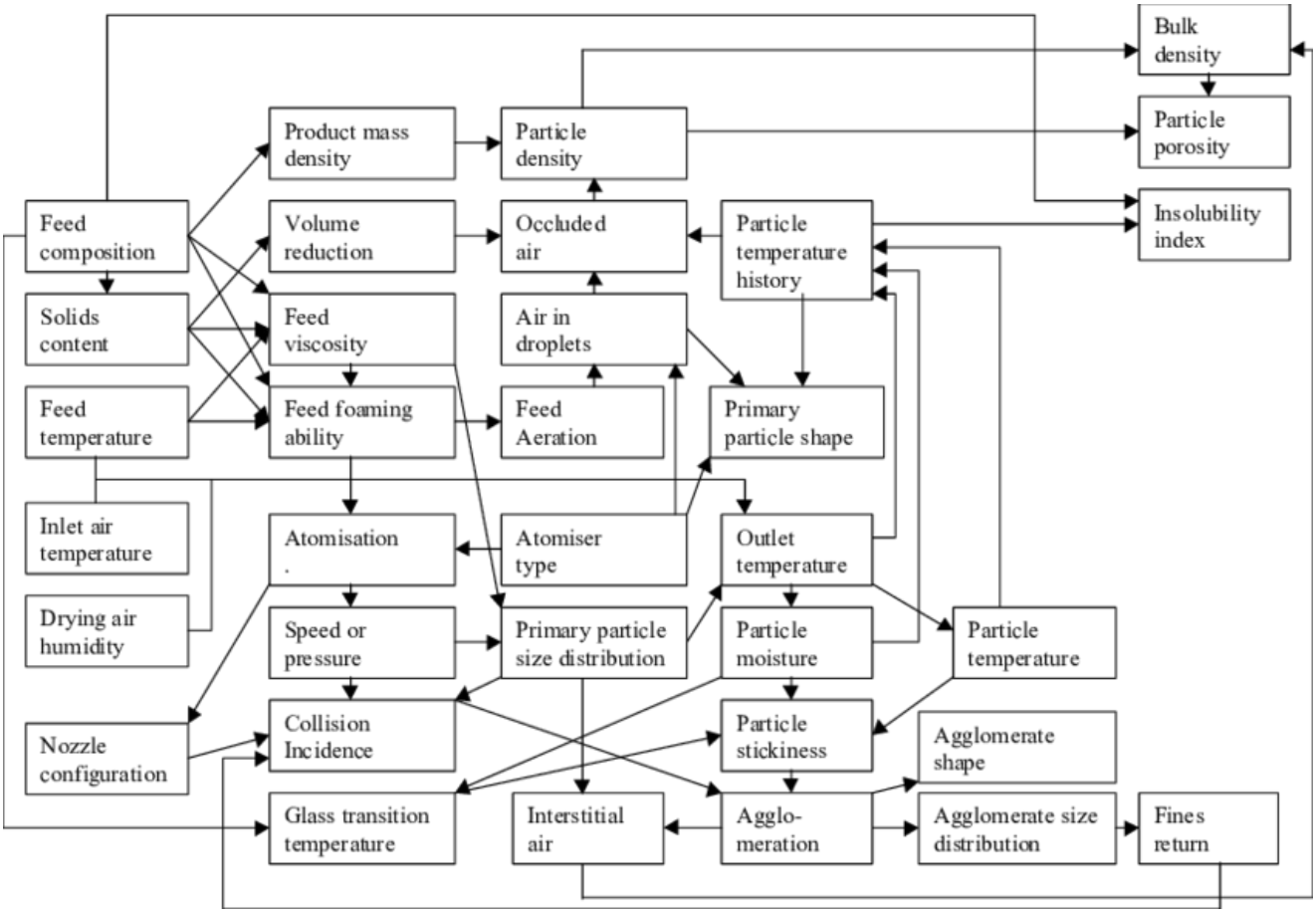


Figure 6-3: Representation of the influence of spray drying parameters on the characteristics and properties of the final powders (Verdurmen and de Jong 2003).

Emulsions stabilised with 7.5% w/w CN-De were spray dried at 180 °C at a feeding rate of 2.5 $mL.min^{-1}$. Droplets size measurements of the rehydrated emulsions from these experiments are gathered in Table 6-2 along with results from experiments at higher feeding rate (Chapter 5) (10 $mL.min^{-1}$).

Table 6-3: Droplet size parameters of 7.5 % w/w CN-De stabilised oil-in-water emulsions after spray drying as a function of the feeding rate. All samples were measured in triplicate. Values are displayed as mean ± standard deviation.

Feeding Rate (mL/min)	Rehydrated Emulsion		
	D3,2	D4,3	Span
2.5	21.43 ± 0.17	66.64 ± 0.81	4.81 ± 0.12
10	7.25 ± 0.01	15.00 ± 0.59	2.13 ± 0.20

Reducing the feeding rate led to the formation of larger aggregates in the rehydrated emulsions. The $D_{4,3}$ increased from 15.002 ± 0.580 µm at a feeding rate of 10 mL.min^{-1} to 66.693 ± 0.804 µm when the rate was reduced to 2.5 mL.min^{-1}. This result was unexpected as a lower feeding should have reduced the size of the spray dried particles.

The shape and size of the dry emulsions from these experiments were analysed using SEM. Micrographs displayed in Figure 6-4 revealed a similar shape and size as the one obtained at a higher feeding rate (Chapter 5, Figure 5-20).

A greater difference between the two feeding rates studied would have probably revealed structural differences in the powders. Their ability to be rehydrated would have been affected simultaneously.

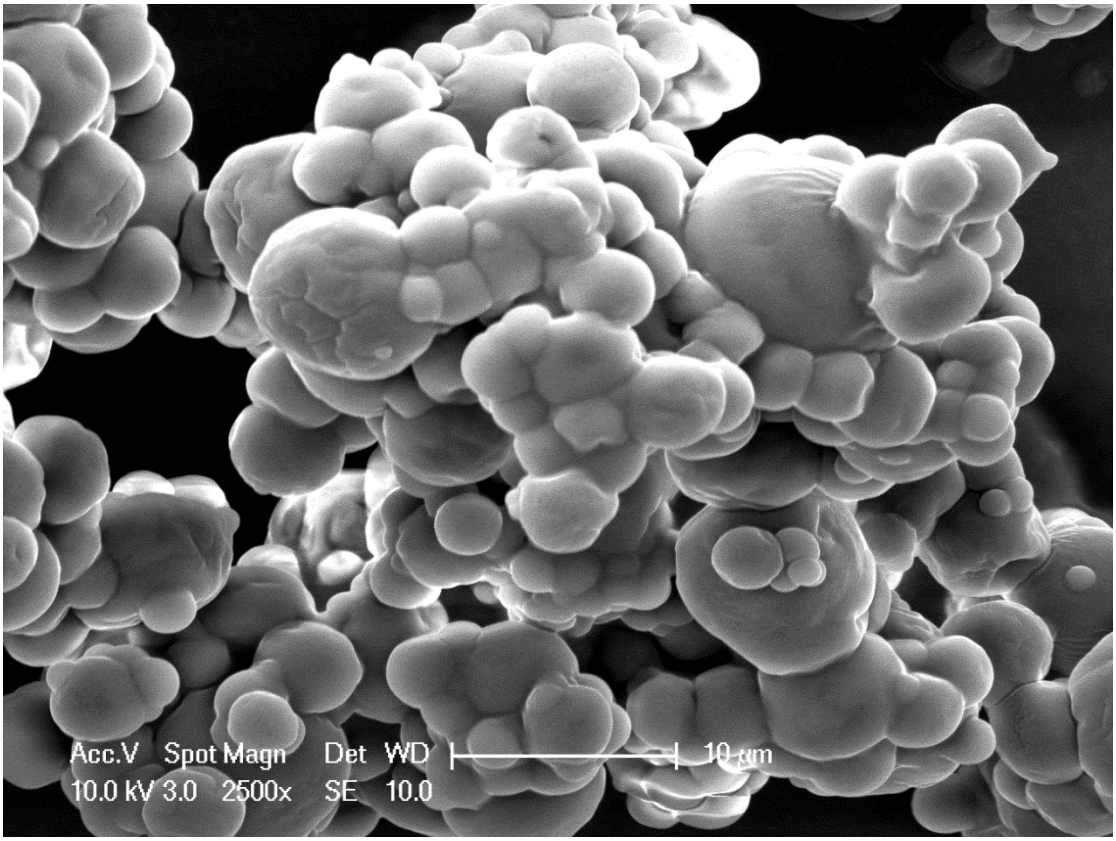


Figure 6-4: SEM micrograph of CN-De spray dried powders (7.5% w/w CN-De, 20% w/w sunflower oil) at a feeding rate of 2.5 mL/min.

6.3.2 Influence of the formulation

6.3.2.1 Effect of the formulation on the reconstitution of freeze dried emulsions

6.3.2.1.1 Effect of the use of a cryoprotectant

In the section of this study describing the freeze-thaw stability of the CN-De stabilised emulsions (Chapter 4, Section 4.3.4), freezing had been identified as a step causing instability in the emulsions. Freezing emulsions can be the source of many destabilisation mechanisms (Degner, Chung et al. 2014). To enhance the stability to freezing and by extension to freeze-drying, a cryoprotectant can in theory be used. Cryoprotectants slow down the ice formation

process leading to smaller ice crystals (Tsvetkova, Phillips et al. 2002) or they can act on the glass transition (freezing point) temperature of a system (Ghosh, Cramp et al. 2006). Furthermore, they are believed to replace the water bonded through hydrogen bond at the surface of the protein. Thus they prevent the fusion of the neighbouring interfacial membranes inhibiting the formation of droplet aggregates during freezing (Crowe, Leslie et al. 1994, Jorgensen and Nielson 2009).

"Cryoprotectant" is the term used to describe a compound used to increase the freezing stability of a system. When used to increase the drying stability, this one is called *"lyoprotectant".* Sugars and polyols are often used to increase the freeze-thaw stability of emulsified systems (Thanasukarn, Pongsawatmanit et al. 2004, Ghosh, Cramp et al. 2006, Cornacchia and Roos 2011, Degner, Chung et al. 2014).

The effects of the use of cryoprotectants were studied on emulsions stabilised with 2.5% w/w. sodium caseinate-dextrin conjugate (~0.8% w/w NaCN). At this concentration, the emulsions showed no stability to both freeze-thawing and freeze-drying with formation of aggregates after both processes (Chapter 4 & 5).

Three different type of sugars were used as cryoprotectants; maltodextrin (DE 4-7, Mw 3.6 kDa), sucrose and glucose. They were added after emulsification to avoid any interference during the emulsification process.

After addition of the chosen cryoprotectant, the droplet size was measured using DLS.

Emulsions showed signs of aggregation upon addition of maltodextrin. As the concentration increased the volume mean diameter ($D_{4,3}$) of the emulsions displayed greater values. The flocculation induced by maltodextrin was suspected to be mainly due to bridging flocculation. Consequently, maltodextrin was dismissed as a potential cryoprotectant for drying and rehydration experiments.

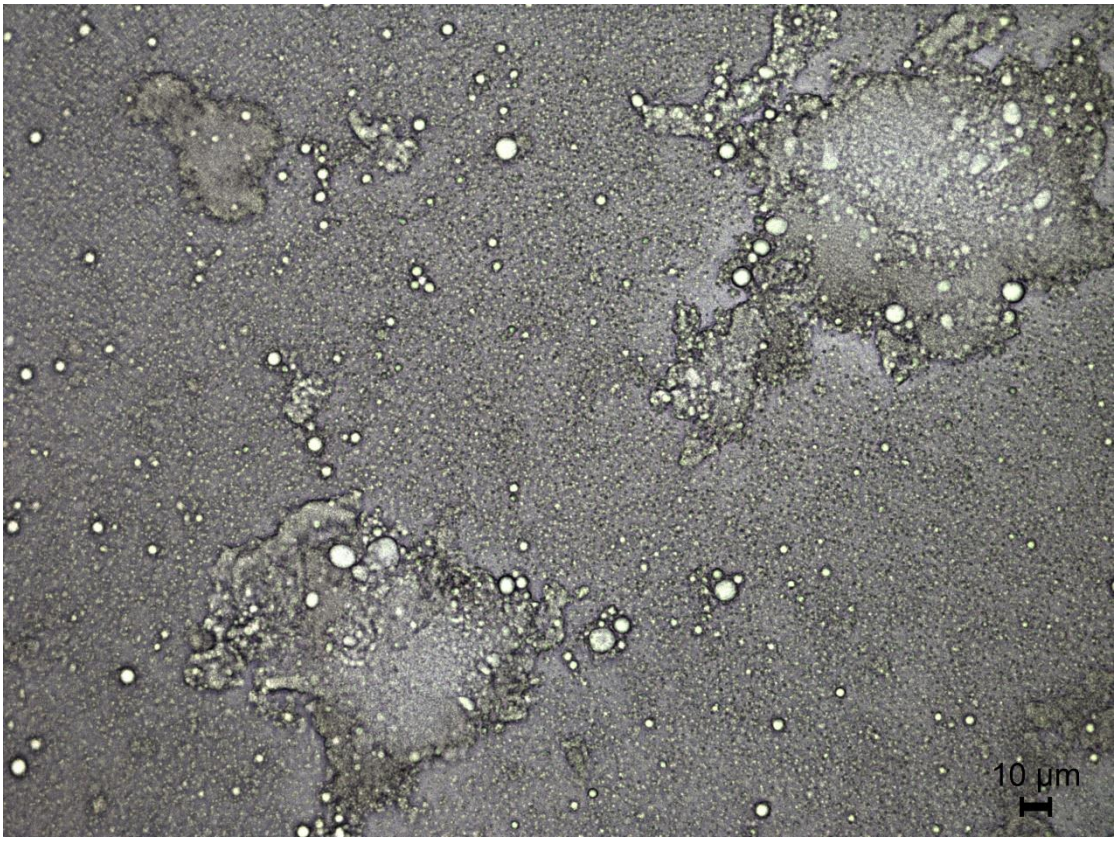


Figure 6-5: ***Optical microscope images of a 2.5% w/w CN-De stabilised oil-in-water emulsions (20 % w/w sunflower oil) with 10 % w/w added maltodextrin.***

Freezing point

Considering the theory on cryoprotectants, the glass transition temperature also called freezing point at low temperature is an important parameter to consider.

Below the freezing point both the continuous phase (water) and the dispersed phase (oil) of emulsions are in a glassy state.

Above the freezing point, the system is in a soft rubbery state. If freeze-dried as so the system will lose its structure (micro and macrostructure) (Meister and Gieseler 2009).

To allow the system to be completely frozen (solid) for freeze-drying the freezing temperature should be lower than the freezing point.

Freezing point measurements on emulsions containing sucrose (20% w/w) revealed a decrease of the freezing point from -16.9 ± 1.9˚C, measured previously, to -19.6 ± 0.1˚C.

This effect was expected considering that in theory cryoprotectants decrease the freezing point and at the same time increase the volume of unfrozen water (Ghosh and Coupland 2008, Petzold and Aguilera 2009, Degner, Chung et al. 2014). In previous study authors demonstrated that the more unfrozen water the better the freeze-thaw stability (Ghosh, Cramp et al. 2006, O'Regan and Mulvihill 2010).

Despite being lowered the freezing point stayed above the freezing temperature (i.e. -22˚C). Freeze drying experiments could be undertaken without risking the collapse of the microstructure.

Freeze-drying and rehydration

Several emulsions containing concentrations ranging from 5% w/w to 20% w/w (5%, 7.5%, 10%, 15% and 20%) of sucrose or glucose were studied for freeze-drying and rehydration experiments.

Optical microscope images (Figure 6-5) revealed that for both sugars the increase in droplet size distributions (Table 6-3) was the results of droplet aggregation and partial coalescence.

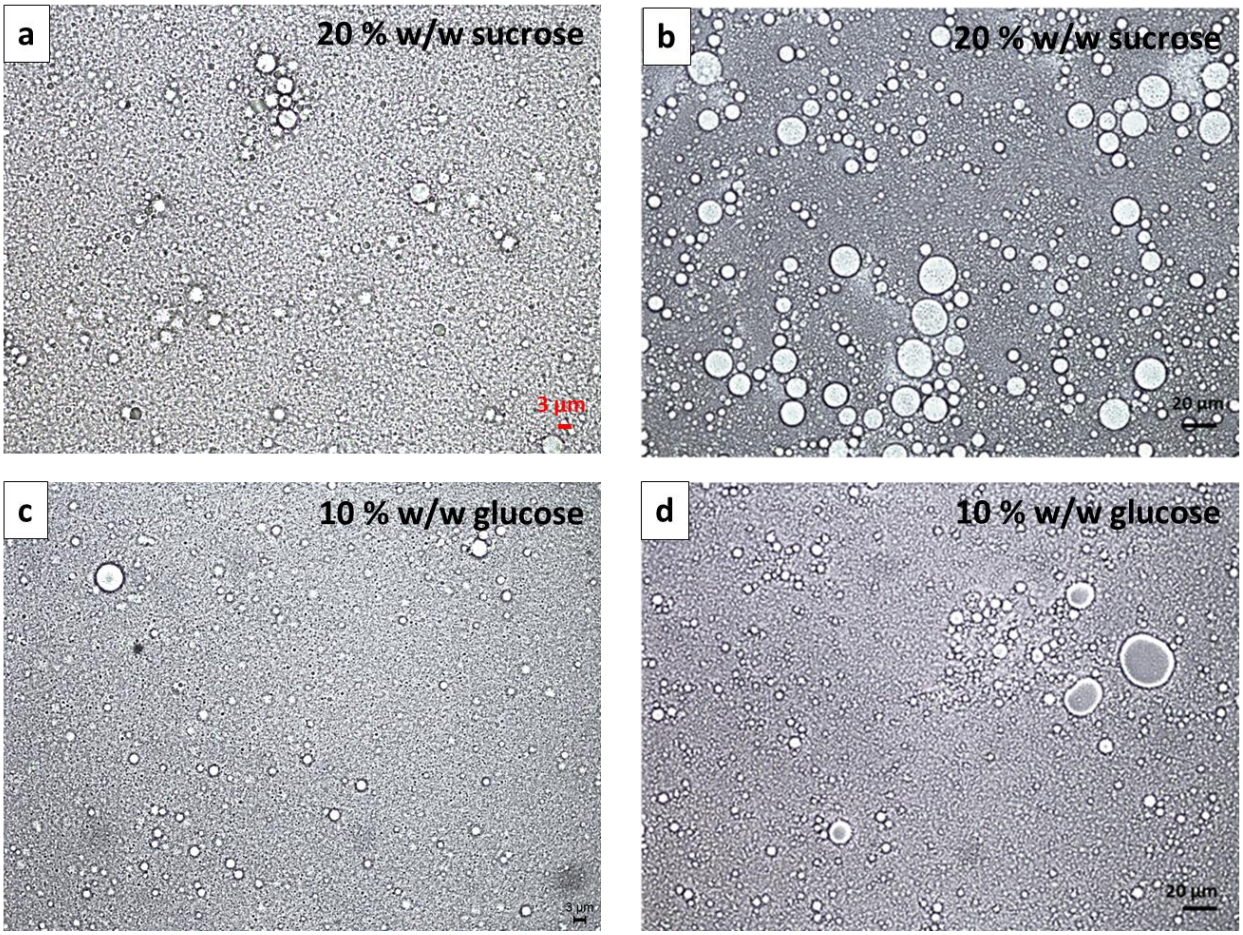


Figure 6-6: Optical microscope images of 2.5% w/w CN-De stabilised oil-in-water emulsions (20 % w/w sunflower oil) with 20% w/w added sucrose (a,b) and 10% w/w added glucose (c,d) before (a,c) and after (b,d) freeze-drying and rehydration.

For sucrose and glucose as the concentration of sugar increased, flocculation and coalescence were limited (Table 6-3). This result revealed that the addition of sugar enabled a better retention of the microstructure after lyophilisation.

From 5% w/w glucose a drastic change was noted. When looking at the volume mean diameter to evaluate the formation of aggregates and the extent of coalescence; without cryoprotectant $D_{4,3}$ increased from 457 ± 59 nm to 43.943 ± 12.464 µm after drying and rehydration; with 5% w/w glucose the volume mean droplet size was 2.035 ± 0.146 µm after rehydration.

At the highest concentration tested, 10% w/w., both the surface mean diameter and the volume mean diameter showed the smallest variation; respectively from 194 ± 30 nm to 216 ± 18 nm and from 645 ± 164 nm to 2.921 ± 1.044 μm before and after freeze drying and rehydration. When using sucrose, higher concentrations were needed to notice changes of the same magnitude.

Table 6-4: Droplet size parameter of 2.5% w/w CN-De stabilised oil-in-water emulsions (20% w/w sunflower oil, 80% w/w water) as function of cryoprotectant concentration before and after freeze-drying and immediate rehydration at 50°C. Experiments were carried in triplicate at room temperature (22°C). Values are presented as mean ± standard deviation.

Cryoprotectant Concentration (%)	Native Emulsion			Rehydrated Emulsion		
	D3,2 (μm)	D4,3 (μm)	SPAN	D3,2 (μm)	D4,3 (μm)	SPAN
Without Cryoprotectant	0.24 ± 0.01	0.46 ± 0.06	2.10 ± 0.10	0.76 ± 0.57	43.94 ± 12.47	14.22 ± 3.94
Sucrose 5	0.20 ± 0.02	0.45 ± 0.06	2.55 ± 0.09	6.93 ± 0.43	53.79 ± 20.41	21.02 ± 7.54
Sucrose 7.5	0.22 ± 0.01	0.75 ± 0.10	2.84 ± 0.21	0.25 ± 0.03	18.68 ± 10.84	114.67 ± 68.61
Sucrose 10	0.24 ± 0.01	1.91 ± 0.66	3.17 ± 0.21	0.38 ± 0.07	52.87 ± 19.66	164.77 ± 30.33
Sucrose 15	0.16 ± 0.01	0.59 ± 0.08	2.51 ± 0.10	0.23 ± 0.01	8.86 ± 4.15	72.38 ± 34.27
Sucrose 20	0.20 ± 0.03	1.64 ± 0.83	4.28 ± 1.13	0.19 ± 0.02	6.99 ± 2.75	12.1 ± 26.7
Glucose 5	0.21 ± 0.05	0.71 ± 0.33	2.56 ± 0.16	0.31 ± 0.03	2.04 ± 0.15	7.76 ± 0.33
Glucose 7.5	0.20 ± 0.05	0.35 ± 0.08	2.24 ± 0.01	0.25±0.02	6.29 ± 3.35	13.85 ± 19.21
Glucose 10	0.19 ± 0.04	0.65 ± 0.16	2.97 ± 0.19	0.22 ±0.02	2.92 ± 1.04	10.38 ± 11.40

At 20% w/w sucrose, both the $D_{4,3}$ and the $D_{3,2}$ showed the smallest variation after freeze drying and rehydration. The surface mean diameter increased by 4% and the volume mean diameter was multiplied by four.

In their study *Tang et Al.* demonstrated that insufficient concentrations of cryoprotectant lead to an incomplete coating of the liposomes nanoparticles favouring the aggregation and increasing the particle size after FD (Tang, Song et al. 2012).

At all the concentrations tested, glucose showed a better ability to improve the retention of the microstructure than sucrose, by limiting the formation of aggregates. Glucose and sucrose are both low molecular weight sugars, but sucrose is formed of two monosaccharides. It was assumed that the sucrose interacted with the dextrin at the surface of the oil droplets and form bridges between droplets which will later lead to localised flocculation. This hypothesis was confirmed when comparing $D_{4,3}$ of the native emulsions with increasing concentrations of sucrose (Table 6-3). The volume mean diameter showed measurements ranging from 454 nm to 1.913 µm after addition of sucrose.

In both cases the increase of the volume mean diameter was mainly the result of droplet aggregation.

Glucose showed the best ability to help maintaining the microstructure overall.

Shear viscosity measurements were performed applying a shear rate ranging from $0.1s^{-1}$ to 100 s^{-1} at room temperature (22°C) before and after freeze-drying and rehydration. The measurements are displayed in Figure 6-6. Both emulsions displayed a shear thinning behaviour before and after the process. For the two sugars the viscosity decreased after drying and rehydration. The emulsion containing glucose showed the greatest variation after rehydration (81%). When using sucrose, the average viscosity varied by 26%.

The addition of sugar helped the retention of the structure of the emulsions, with limited droplet aggregation after rehydration. However, the viscosity was altered in both cases, with glucose showing the largest variation.

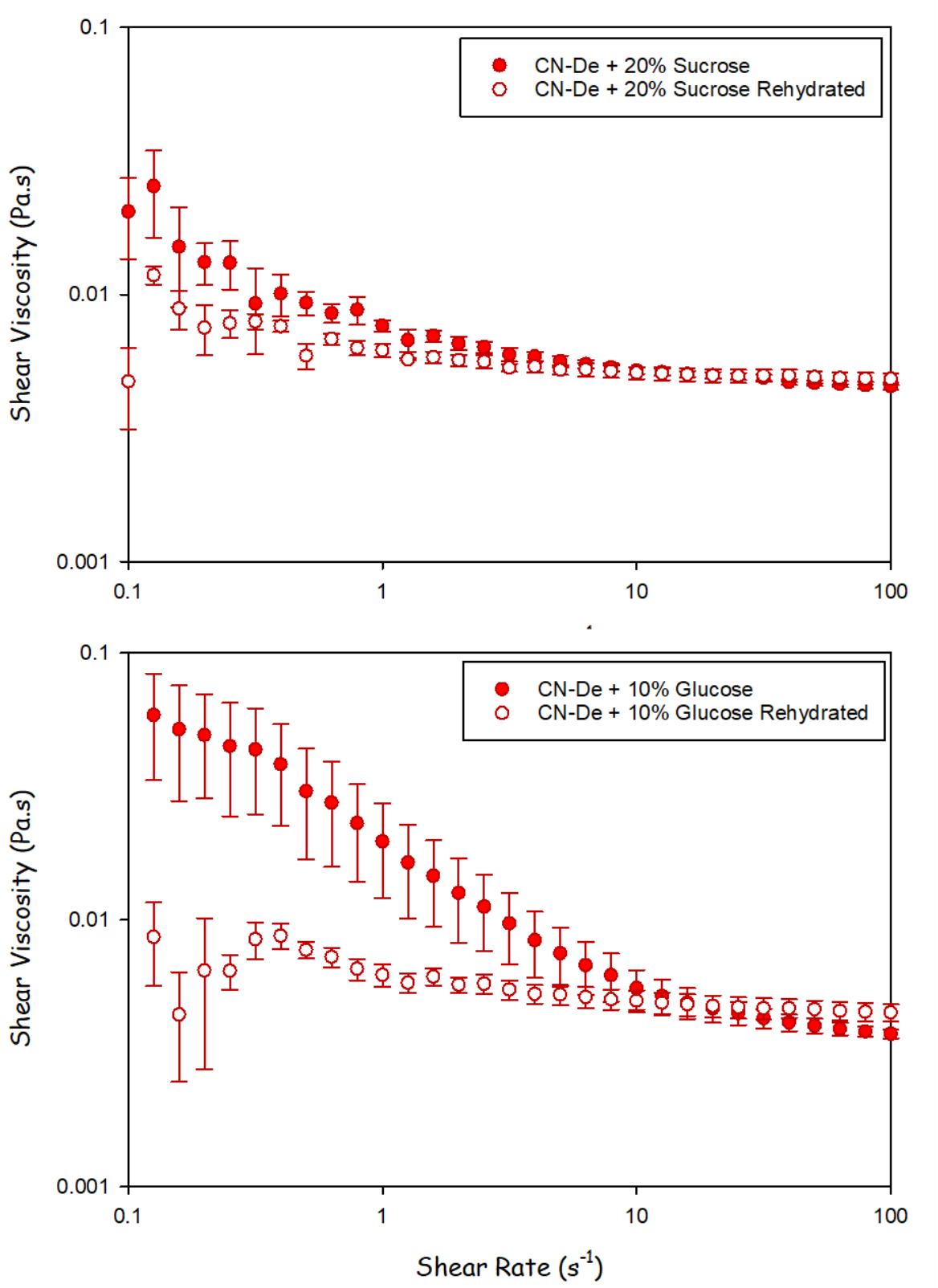


Figure 6-7: Steady state shear viscosity of 2.5% w/w CN-De stabilised oil-in-water emulsions with 20% w/w added sucrose (a) and 10% w/w added glucose (b) before and after freeze-drying and rehydration. Samples were measured in triplicate. Error bars represent standard deviation.

6.3.2.1.2 Effect of Salt addition

Real food emulsions containing a range of ingredients, studying the effects of salt addition on the rehydration was relevant for this study. The emulsions stabilised with WPI-SBP displayed a good stability to the addition of salt before and after homogenisation (Chapter 4). For this reason, they were tested for freeze-drying stability.

Effect of the addition of salt before emulsification on the reconstitution of the freeze-dried emulsions

Emulsions that previously showed no signs of microstructural changes (i.e. droplet aggregation, creaming) after addition of salt before emulsification (2.5% w/w WPI-SBP, 20% w/w sunflower oil, 80% w/w water, 0.1%, 0.25% and 0.75% w/w NaCl or 0.1%, 0.25%, 0.5%, 0.75% and 1% w/w $CaCl_2$) were freeze-dried for 4 days and rehydrated at 50°C.

Droplet size was measured before and after rehydration. Results from these experiments are gathered in Figure 6-7. After rehydration all droplet distributions were multimodal with the appearance of droplets in the larger size range. The later were identified as aggregated droplets after optical microscope observations (Figure 6-8).

The droplet aggregation triggered by freeze-drying was attributed to the change of salt concentration during ice formation. Upon freezing the expanding ice forces every solute in reducing portions of unfrozen water consequently their concentration locally increases (Banin and Anderson 1974). In the case of salt local increase of the concentration can disturb the interaction between hydrocolloid coated droplets. Indeed, by partially screening the charges around the droplets the electrostatic repulsion is suppressed. The droplets of the still unfrozen emulsion portion can get closer and interact, encouraging aggregates formation.

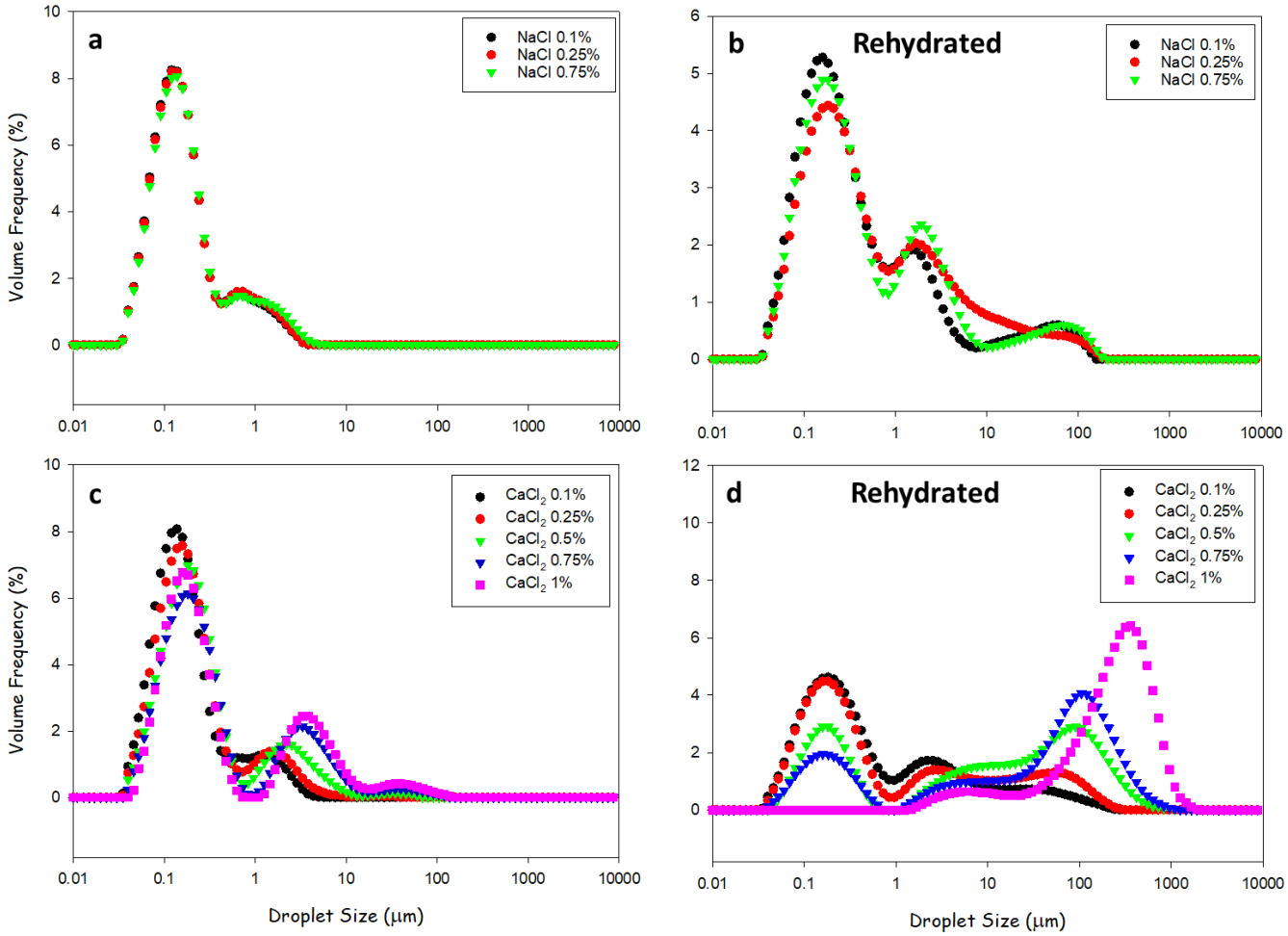


Figure 6-8: Droplet size distribution of WPI-SBP stabilised oil-in-water emulsions before (a,c) and after (b,d) freeze-drying and rehydration in the presence of NaCl (a,b) and $CaCl_2$ (c,d).

Table 6-5: Droplet size parameters of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions as a function of salt concentration before and after freeze-drying and rehydration.

Salt Concentration (%)	Native Emulsion			Rehydrated Emulsion		
	D3,2	D4,3	SPAN	D3,2	D4,3	SPAN
NaCl 0.1	0.13 ± 0.01	0.31 ± 0.01	4.69 ± 0.01	0.20 ± 0.01	4.94 ± 0.10	15.72 ± 0.19
NaCl 0.25	0.13 ± 0.01	0.32 ± 0.01	4.92 ± 0.03	0.24 ± 0.01	5.4 ± 0.3	25.87 ± 0.75
NaCl 0.75	0.14 ± 0.01	0.36 ± 0.01	5.51 ± 0.07	0.22 ± 0.01	6.22 ± 0.24	18.47 ± 0.65
$CaCl_2$ 0.1	0.14 ± 0.01	0.34 ± 0.01	4.97 ± 0.08	0.23 ± 0.01	7.09 ± 0.44	46.33 ± 3.09
$CaCl_2$ 0.25	0.15 ± 0.01	0.65 ± 0.11	6.75 ± 0.21	0.25 ± 0.01	14.32 ± 0.92	129.48 ±8.40
$CaCl_2$ 0.5	0.18 ± 0.01	1.30 ± 0.13	10.15 ± 0.42	0.48 ± 0.02	58.91 ± 13.08	11.91 ± 1.65
$CaCl_2$ 0.75	0.20 ± 0.01	2.62 ± 0.12	17.73 ± 0.86	0.59 ± 0.03	92.58 ± 19.22	4.96 ± 0.54
$CaCl_2$ 1	0.20 ± 0.01	3.72 ± 0.04	25.60 ± 1.14	45.79 ± 4.93	324.29 ± 41.40	2.48 ± 0.05

However, for NaCl the droplet size increase after rehydration stayed independent of the concentration. For all salt concentrations tested the size variation stayed consistent between the native and rehydrated emulsions. The volume mean diameter varied from 375 ± 7 nm to ~5 μm for every salt concentration (Table 6-4).

With calcium chloride on the other hand, the microstructure was lost upon rehydration. The proportion of larger droplets increased with the salt concentration (Figure 6-7, c & d).

At 0.1% w/w $CaCl_2$ a group of droplets in the size range 1-1000 μm was detected by the instrument. Based on microscope observation, this occurrence was the result of coalescence (Figure 6-8 c).

As the concentration of $CaCl_2$ increased the volume frequency of the droplets in this size range increased.

However, at higher salt concentration this increase was the result of droplet aggregation rather than coalescence as shown in Figure 6-8.

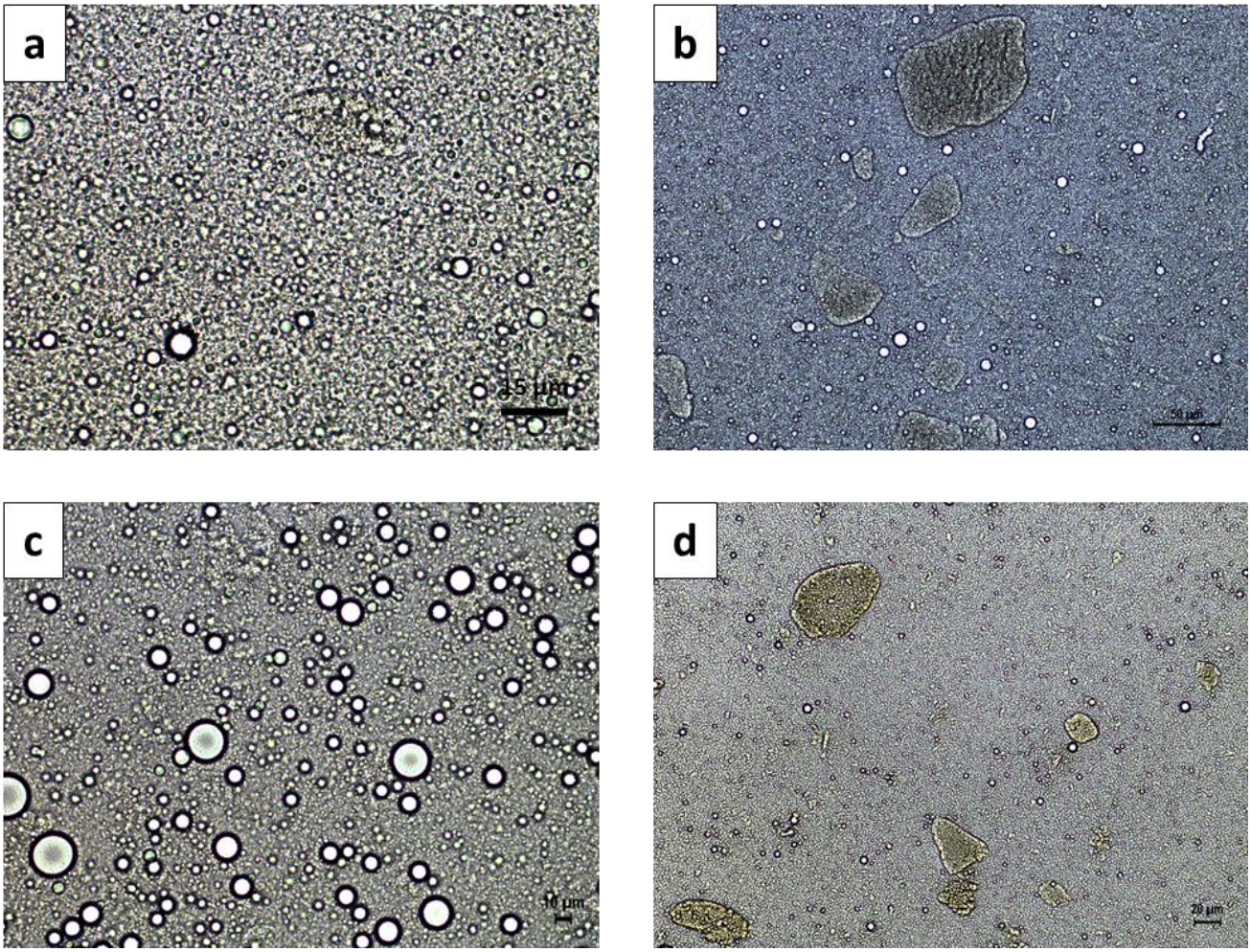


Figure 6-9: Optical microscope image of 2.5% w/w WPI-SBP stabilised emulsions (20% w/w sunflower oil) containing 0.1% w/w NaCl (a), 1% w/w NaCl (b), 0.1% w/w $CaCl_2$ (c) and 1% w/w $CaCl_2$ (d) added before emulsification. Images were taken after freeze-drying and rehydration.

Effect of the addition of salt after emulsification on the reconstitution of the freeze-dried emulsions

The same trend was observed in the emulsions when salt was added after emulsification. The emulsions containing low salt concentration (0.1% w/w) showed the best resistance to droplet aggregation or coalescence after freeze-drying and rehydration for both salts (Figure 6-9). Emulsions with added NaCl displayed a better stability to freeze-drying. Despite a change in the microstructure the emulsions were visually homogenous.

Emulsions with added $CaCl_2$ displayed a shift towards the larger droplet size at lower concentration in comparison with the same emulsion already containing salt during homogenisation studied in the previous section (Figure 6-7).

At 0.25% w/w salt the majority of the droplets detected by the instrument were in the large size. A small number of droplets remained detectable in the smaller size range.

The difference observed between salt addition before and after emulsification could be explained by the availability of the salt in both systems.

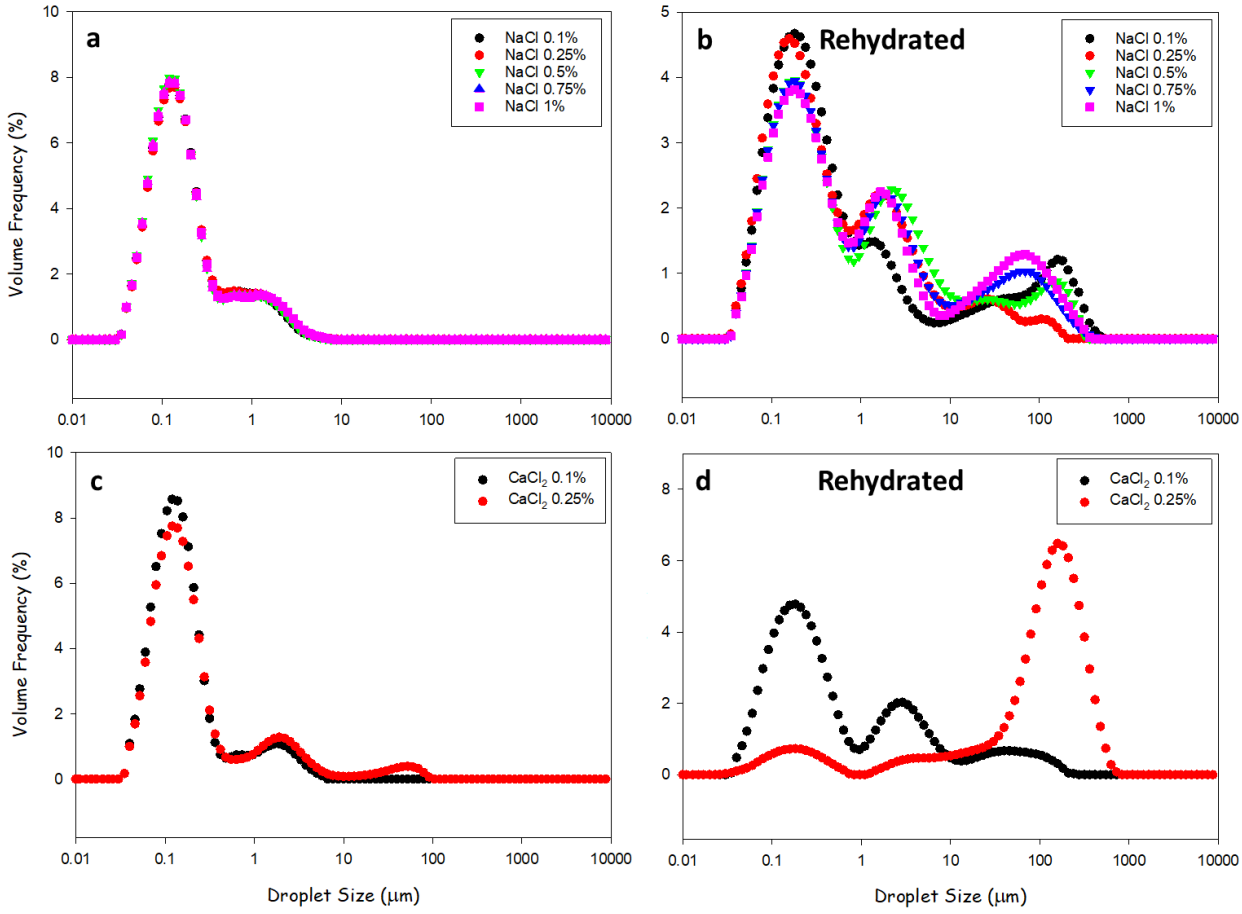


Figure 6-10: Droplet size distribution of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions (20% w/w sunflower oil) with added NaCl (a,b) and $CaCl_2$ (c,d) before (a,c) and after (b,d) freeze-drying and rehydration.

When added before emulsification the salt interacted with the conjugate affecting its emulsifying properties and at the same time reducing the amount of free salt in the continuous phase.

When added after emulsification the binding of the salt to the conjugates was limited by the amount of charged groups protruding in the continuous phase leaving more free ions in the bulk.

The freezing step led to localised increase of the concentration of solute. At the same salt concentration more free ions were present in the continuous phase to screen the charged groups coating the droplets. Lower salt concentrations were enough to trigger flocculation.

6.3.2.1.3 Effect of pH

The effect of pH studied in Chapter 4 on emulsions stabilised with CN-De and WPI-SBP showed increased stability at different pH; at acidic pH (pH 2), around the pI of WPI (pH 5.2) and at basic pH (pH 12).

With CN-De despite displaying droplet aggregation the emulsions were visually homogenous at pH 2 and 12.

WPI-SBP stabilised emulsions showed good stability at pH 5.2 (the isoelectric point of WPI) and at pH 12.

Amongst the emulsions previously produced, WPI-SBP stabilised emulsions at pH 5.2 were chosen for freeze-drying and rehydration experiments. This emulsion was chosen due to the limited droplet aggregation observed at this pH.

As for previous freeze-drying experiments the freezing of point the emulsion was measured beforehand. The influence of the pH on the freezing point was assessed using DSC.

The pH has no effect on the freezing point of the emulsions.

The freezing point of the emulsion was found at -15.5 ± 0.3 °C.

Emulsions formulated without pH adjustment (pH measured at ~6) showed a freezing point of -14.8 ± 0.5 °C.

Emulsions stabilised with 2.5% w/w WPI-SBP at pH 5.2 displayed good resistance to freeze drying. The shift towards larger droplets size was limited (Figure 6-10). The surface mean diameter and volume mean diameter increased from 140 ± 1 nm to 203 ± 2 nm and from 416 ±34 nm to 2.362 ± 0.103 μm respectively.

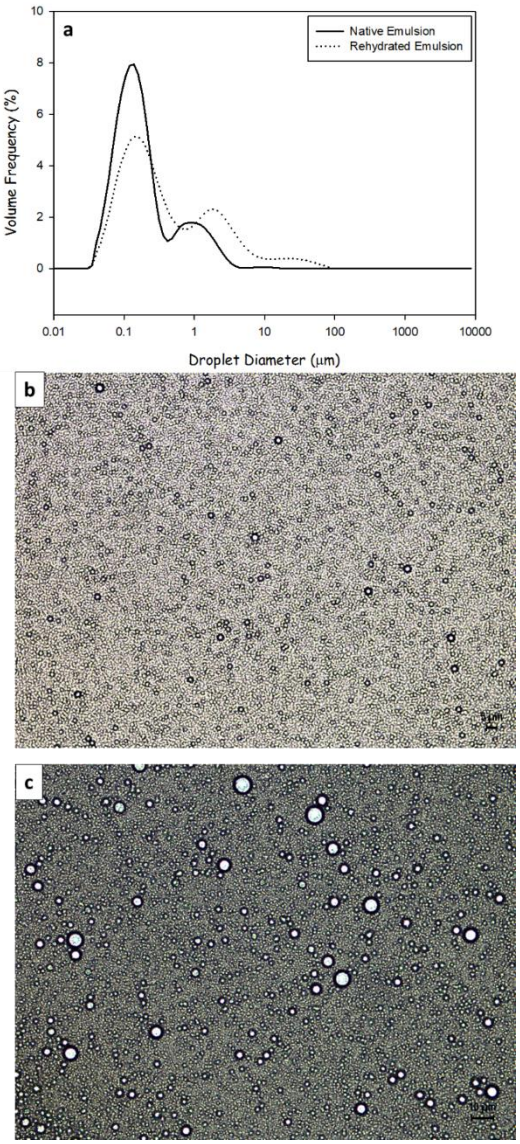


Figure 6-11: ***Droplet size distribution of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions pH 5.2 before and after freeze drying and rehydration (a) and optical microscope images of a 2.5% w/w WPI-SBP stabilised emulsions pH 5.2 before (b) and after (c) freeze-drying and rehydration.***

Microscope images (Figure 6-10, b and c) revealed that the increase of $D_{4,3}$ was mainly the result of partial coalescence in the rehydrated emulsions. This occurrence was the signed of partial disruption of the interfacial membrane. Coalescence could have arisen during freezing or during ice sublimation. Freeze-thawing experiments could have been a way to identify at which step of the process the coalescence occurred.

6.3.2.2 Effect of the formulation on the reconstitution of spray dried emulsions

6.3.2.2.1 Effect of the addition of lactose

The positive effects of lactose on the encapsulation efficiency of oil-in-water sodium caseinate stabilised emulsions has been proved (Fäldt and Bergenståhl 1995, Rennie, Chen et al. 1999, Vega and Roos 2006, Vignolles, Jeantet et al. 2007). Small sugars have shown an ability to improve the quality of dry emulsions (Jayasundera, Adhikari et al. 2009). However, the mechanism of the stabilisation of protein by sugars during drying is not well understood. Some believe that a change of solubilisation occurs during rehydration (Fäldt and Bergenståhl 1995, Cicerone and Douglas 2012). Indeed, small sugars like lactose could to some extent replace the water molecules that are slowly removed from the interfacial protein film thus reducing or inhibiting protein destabilisation. Leading both to an increase stability of the structure and a better encapsulation efficiency (Hogan, McNamee et al. 2001). In the case of re-dispersible emulsions, in another study *Faldt and Bergenstahl* demonstrated that adding lactose to whey protein-stabilised emulsions limited the increase of the droplet size upon rehydration in comparison with emulsions without lactose (Fäldt and Bergenståhl 1996).

In addition to this theory others mention that some water remains at the interface helped by the highly hydrophilic sugar and maintain the hydration of the interfacial protein preventing its loss of conformation (McClements and technology 2010, Cicerone and Douglas 2012).

In the present study the effects of lactose were first studied to increase the spray drying stability of the emulsions.

Additionally, the potentiality of synergistic effects with the conjugate were investigated.

Later lactose was incorporated directly in the conjugate, with the production of a protein-lactose conjugate.

For both emulsifiers the same inlet temperatures as set previously were used as the addition of lactose had no effect on the T_g of the systems.

Effects of the addition of lactose on sodium caseinate-dextrin conjugate stabilised oil-in-water emulsions stability to spray drying

Emulsions stabilised with 7.5% w/w CN-De (20% w/w sunflower oil) were produced. This concentration was chosen as it was the lowest concentration tested in chapter 5 that showed the smallest droplet size variation after spray drying and rehydration. Increasing concentrations (20, 30 and 40% w/w) of lactose were added after emulsification. The droplet size was measured after addition of lactose, before drying for all emulsions produced.

The emulsions resulting from these drying experiments were all visually homogenous after rehydration. DLS measurements displayed in Figure 6-11 show that the shift toward larger droplet sizes was considerably limited from 20% w/w. The volume mean diameter however increased from 184 ± 1 nm to 7.091 ± 0.385 µm. This increase was later identified with optical microscopy and attributed to partial coalescence.

This result confirmed previous observations made on spray dried NaCN stabilised emulsions in the presence of lactose (Hogan, McNamee et al. 2001). Lactose effectively limited the droplet aggregation and assumedly the protein denaturation.

When the concentration of lactose was increased to 30% w/w, the variation of $D_{4,3}$ was even smaller. The volume diameter increased from 265 ± 16 nm to 1.629 ± 0.061 µm.

At 40 % w/w lactose an increase similar to one observed for 20% w/w was noted.

Consequently, for further experiments, the concentration of 30% w/w lactose was kept as a standard.

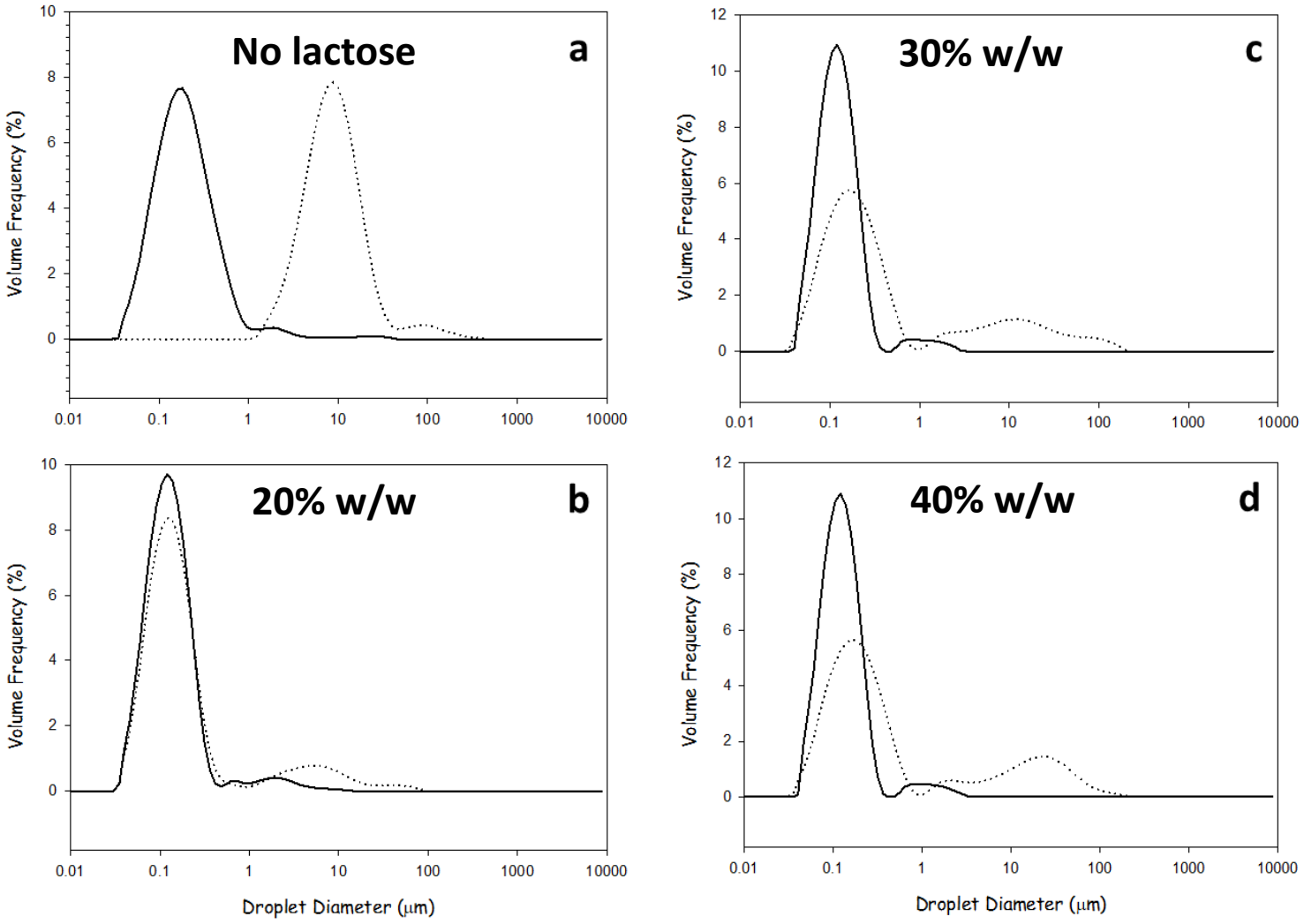


Figure 6-12: Droplet size distribution of 7.5% w/w CN-De stabilised oil-in-water emulsions before and after spray drying and rehydration without lactose (a) or with 20% w/w (b), 30% w/w (c) and 40% w/w (d) added lactose.

Table 6-6: Droplet size parameters of 7.5% w/w CN-De stabilised oil-in-water emulsions as a function of lactose concentration before and after spray drying and rehydration.

Lactose concentration (% w/w)	Native Emulsion			Spray-Dried Emulsion		
	D3,2 (µm)	D4,3 (µm)	SPAN	D3,2 (µm)	D4,3 (µm)	SPAN
0	0.19 ± 0.01	0.46 ± 0.16	2.32 ± 0.13	7.25 ± 0.02	15.00 ± 0.59	2.13 ± 0.20
20	0.12 ± 0.01	0.18 ± 0.01	1.35 ± 0.01	0.19 ± 0.01	7.09 ± 0.39	73.79 ± 4.17
30	0.11 ± 0.01	0.27 ± 0.02	1.62 ± 0.02	0.13 ± 0.01	1.63 ± 0.07	20.63 ± 0.76
40	0.12± 0.01	0.19 ± 0.01	1.37 ± 0.01	0.19 ± 0.01	7.17 ± 0.44	99.42 ± 5.56

In order to highlight a possible synergistic effect between lactose and the conjugate, NaCN stabilised emulsions with added lactose were produced.

In case of a confirmed synergistic effect between CN-De and lactose, NaCN stabilised emulsions with added lactose would display poor spray drying stability.

The protein was introduced in the same proportion as in the conjugate at 7.5% w/w CN-De (i.e. 2.5% w/w NaCN).

Emulsions stabilised with 2.5% w/w NaCN and 30% w/w added lactose were spray dried. The droplet size measurements from these experiments are shown in Figure 6-12.

The measurements were compared with the measurements carried out on the same emulsions in absence of lactose.

Results revealed that the addition of lactose increased the stability to spray drying of NaCN stabilised emulsions.

Results from these experiments brought to light that no synergistic effect existed between the conjugate and the lactose. Indeed, the emulsions stabilised with NaCN in presence of lactose displayed similar stability to spray-drying.

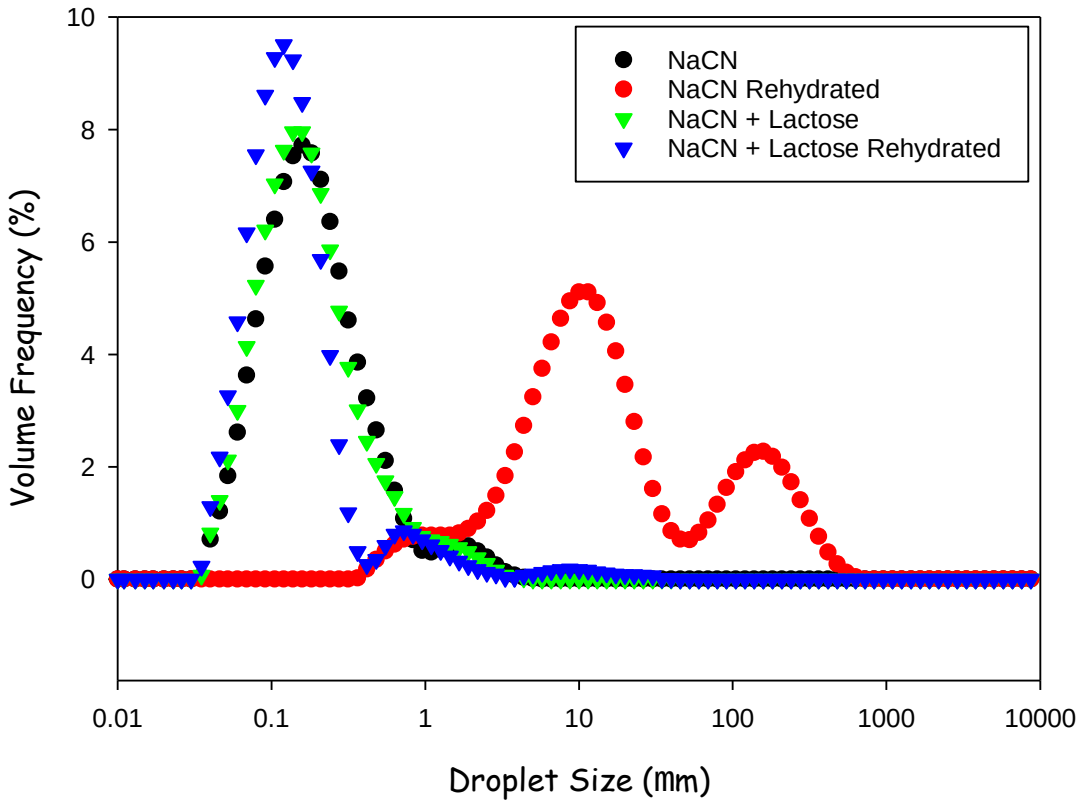


Figure 6-13: Droplet size distribution of 2.5% w/w NaCN stabilised emulsions before and after spray drying and rehydration with and without addition of 30% w/w lactose.

Effects of the addition of lactose on whey protein-sugar beet pectin conjugate stabilised oil-in-water emulsions stability to spray drying

Similarly, the effects of addition of lactose to WPI-SBP stabilised emulsions were investigated. Emulsions stabilised with 2.5% w/w WPI-SBP with 30% w/w added lactose were spray dried at the inlet temperatures fixed in Chapter 5.

Figure 6-13 shows the droplet size measurements of the emulsions before and after drying at different inlet temperatures.

Emulsions dried at 165 °C and 175 °C showed a slight increase of the volume mean diameter; respectively from 570 ± 120 nm to 1.254 ± 0.295 µm and 1.581 ± 0.037 µm.

Optical microscope observations revealed that the increase of $D_{4,3}$ was mostly due to partial coalescence in the rehydrated emulsions. Partial coalescence had previously been attributed to poor heat stability of the systems (Chapter 5).

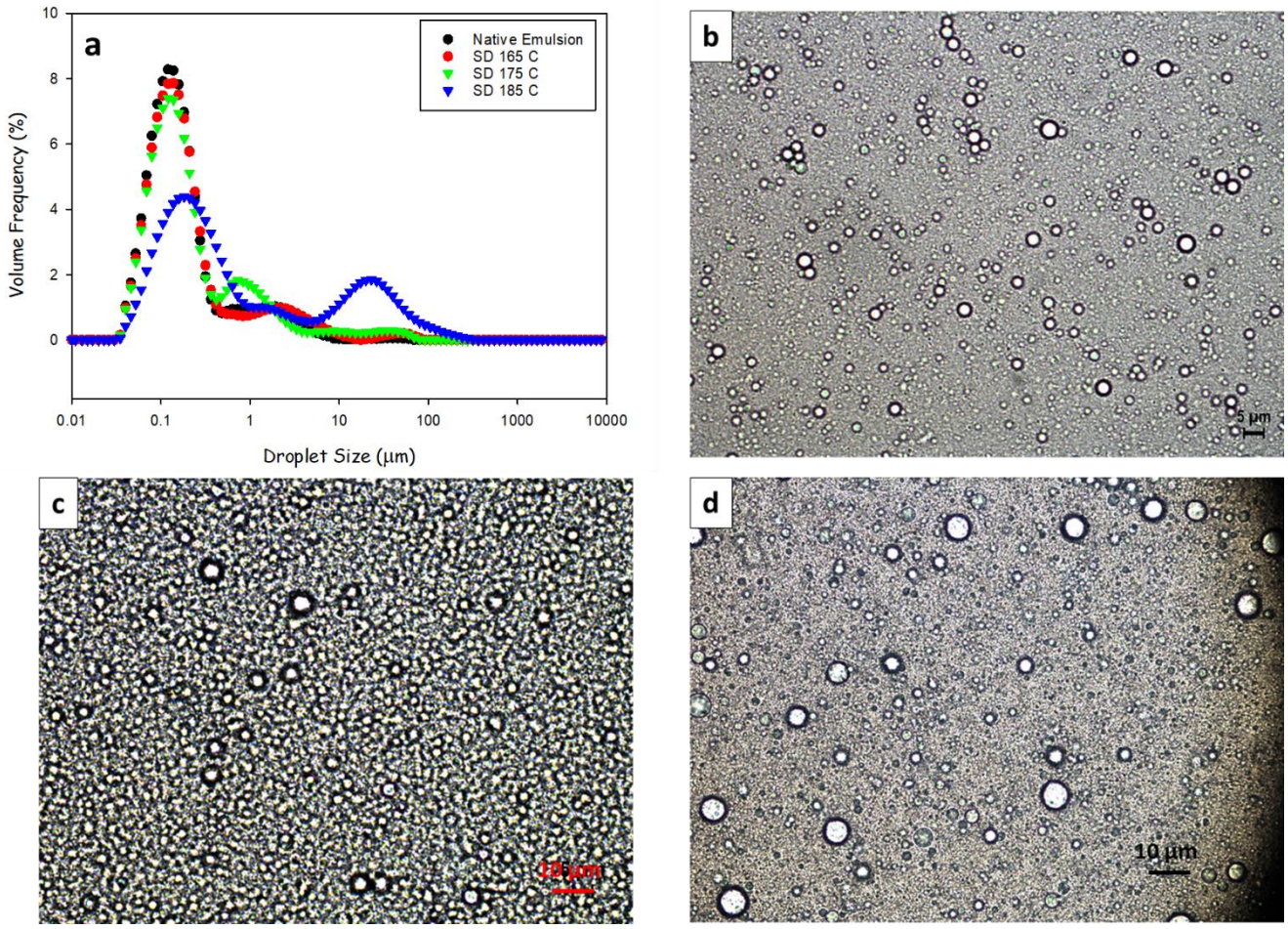


Figure 6-14: Droplet size distribution of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions with 30% w/w added lactose before and after spray drying and rehydration (a) and optical microscope images of a 2.5% w/w WPI-SBP stabilised emulsions after spray drying and rehydration at 165 °C (b), 175 °C (c) and 185 °C (d).

Emulsions were reconstituted after spray drying at 165 °C and 175 °C.

Their viscosity and traction coefficient were analysed before and after rehydration.

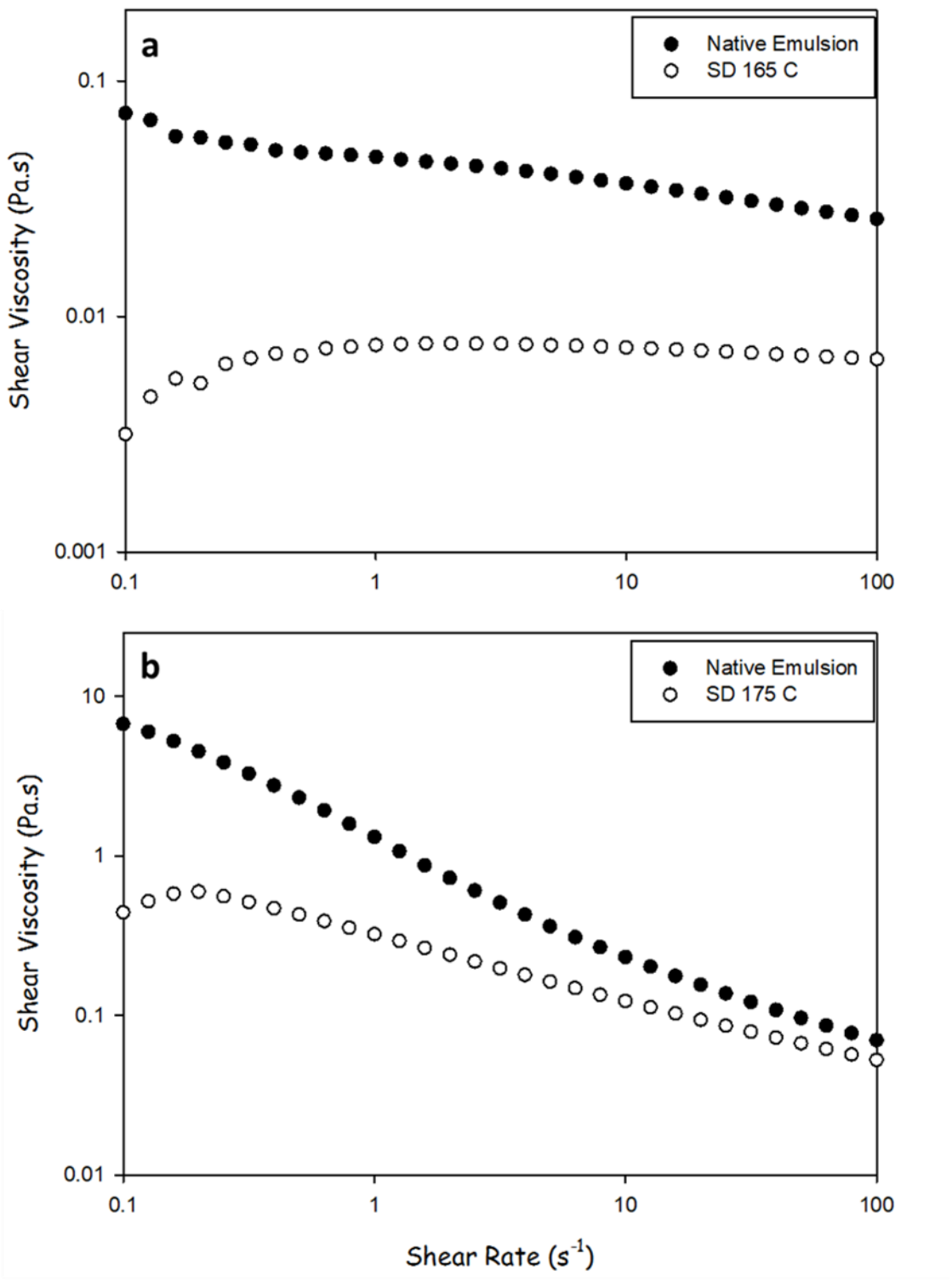


Figure 6-15: Steady state shear viscosity of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions (20% w/w sunflower oil) with 30% w/w lactose added before and after spray drying at 165 °C (a) and 175 °C (b) and rehydration.

After spray drying and rehydration the emulsions displayed an increase in the shear viscosity. Examination of the microstructure of the rehydrated emulsions revealed that the increase in droplet size was mainly due to partial coalescence. The shear viscosity of a system is highly dependent on the composition of the bulk. Fine droplets tend to increase the viscosity of emulsions. Larger droplets generally result in emulsions with lower shear viscosity (Derkach 2009). In the present systems the decrease in viscosity noted after rehydrated could be attributed to the partial droplet coalescence.

Despite an increase of the shear viscosity after rehydration the traction coefficient stayed the same for the rehydrated emulsions. Rheological analyses give information on the texture of the bulk emulsion whilst tribology analyses a thin film of this emulsion. Therefore the composition of this film can be completely different from the bulk (Stokes, Boehm et al. 2013). Furthermore rheology measurements strongly rest on the mechanics of the dispersed phase and its interactions with surfaces (Davies and Stokes 2008, Stokes, Davies et al. 2008). Interactions between droplets and interactions between hydrocolloids can differ on this microscopic scale. In the present case from a tribological point of view the emulsions remained the same.

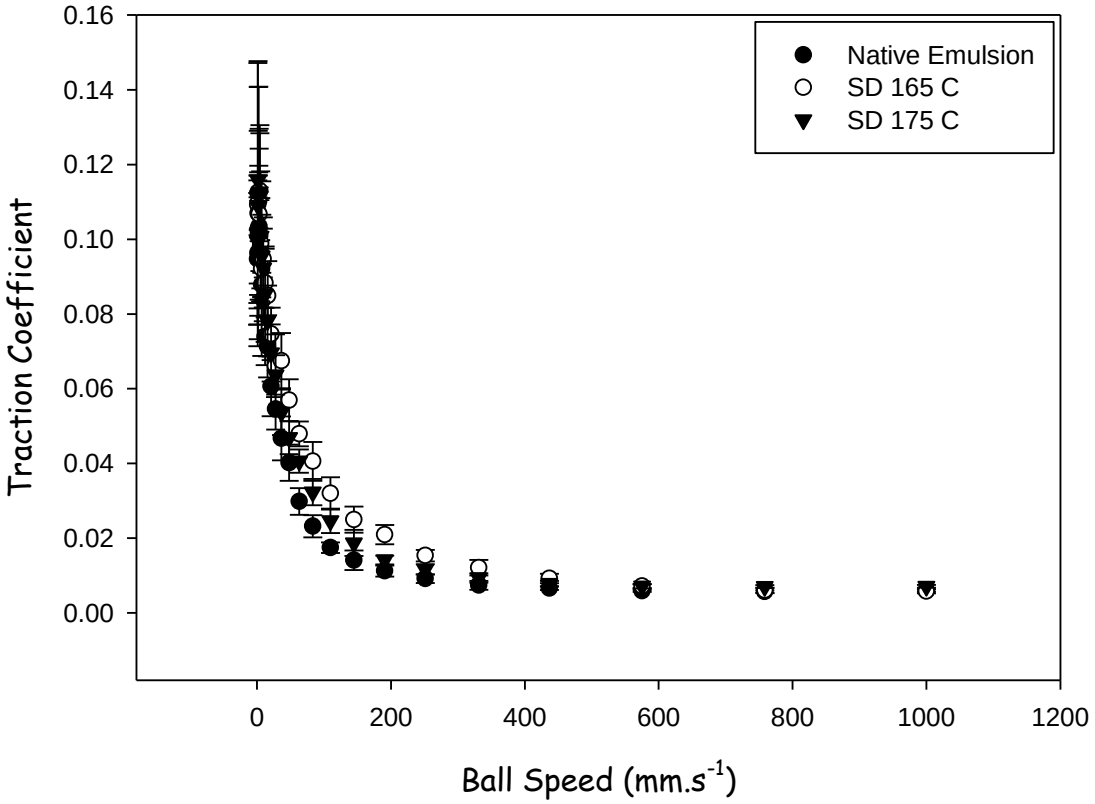


Figure 6-16: Traction coefficient as a function of the ball speed of 2.5% w/w WPI-SBP stabilised oil-in-water emulsions with 30% w/w added lactose before and after spray drying at 165 °c and 175 °C and rehydration. Data points represent an average of measurements performed on three samples. Error bars correspond to the standard deviation of the average.

The droplet size of the reconstituted emulsions from the previous experiments was monitored over time. Results are displayed in Figure 6-16.

For both emulsions the droplet size stayed constant over the period of time studied. There was no progression of the droplet coalescence.

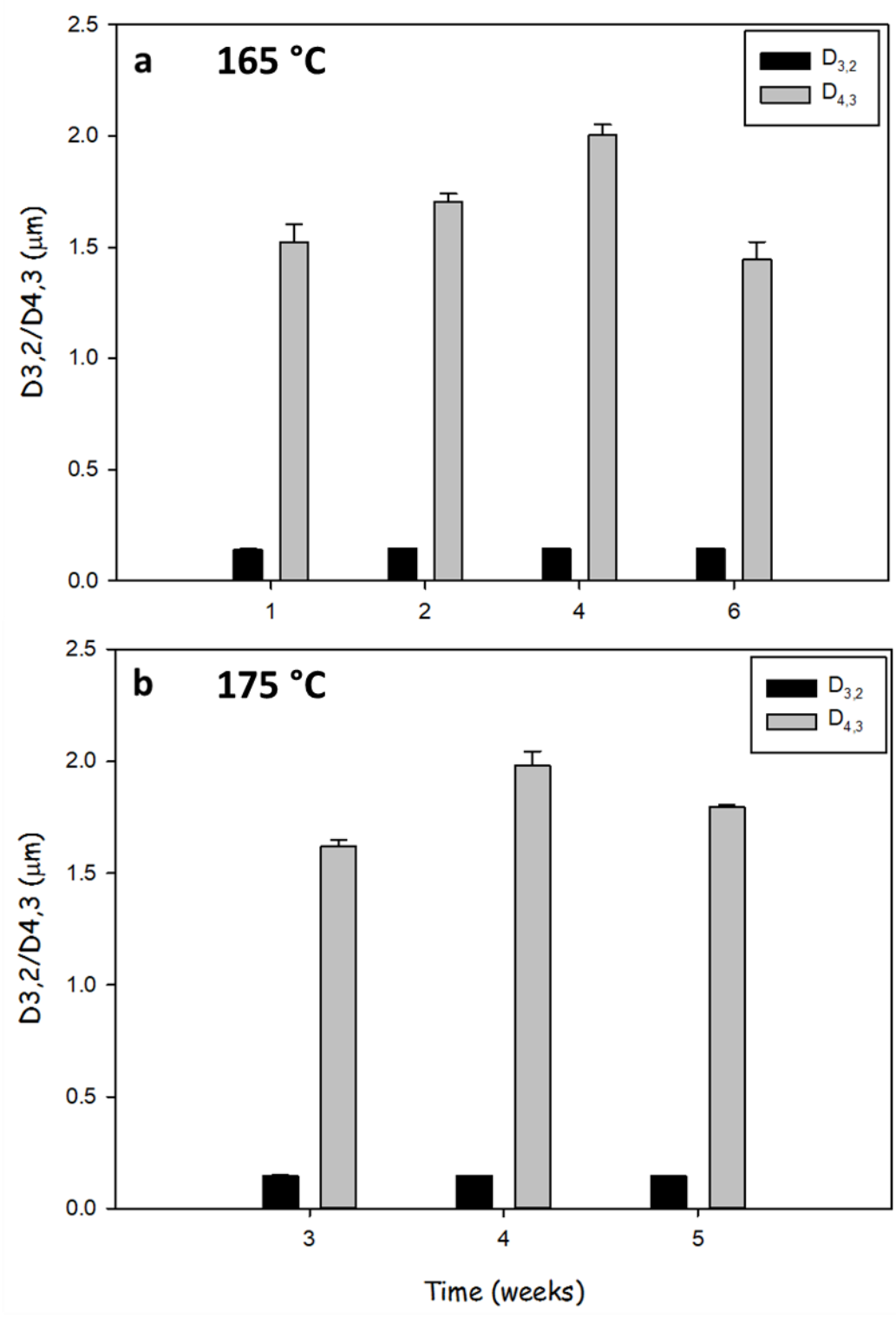


Figure 6-17: Droplet size evolution as function of time of spray dried and rehydrated emulsion 20% sunflower oil, 80% water oil-in-water emulsions stabilised with 2.5% w/w WPI-SBP containing 30% w/w added lactose. Emulsions were spray dried at 165 °C (a) and 175 °C (b). Samples were measured in triplicate. Error bars represents standard deviation.

Synergistic effects between the conjugate and lactose were investigated.

Emulsions stabilised with 1.7% w/w WPI and 30% w/w added lactose were produced and spray dried at 165 °C. The droplet size was measured before and after drying and rehydration. The same experiments were performed on a 1.7% w/w WPI stabilised emulsion for comparison purposes.

DLS measurements are displayed in Figure 6-17. WPI stabilised emulsions with added lactose showcased a better spray drying stability than their WPI-SBP stabilised equivalent. Results were similar to ones obtained with CN-De. Once again no synergistic effect existed between WPI-SBP and lactose.

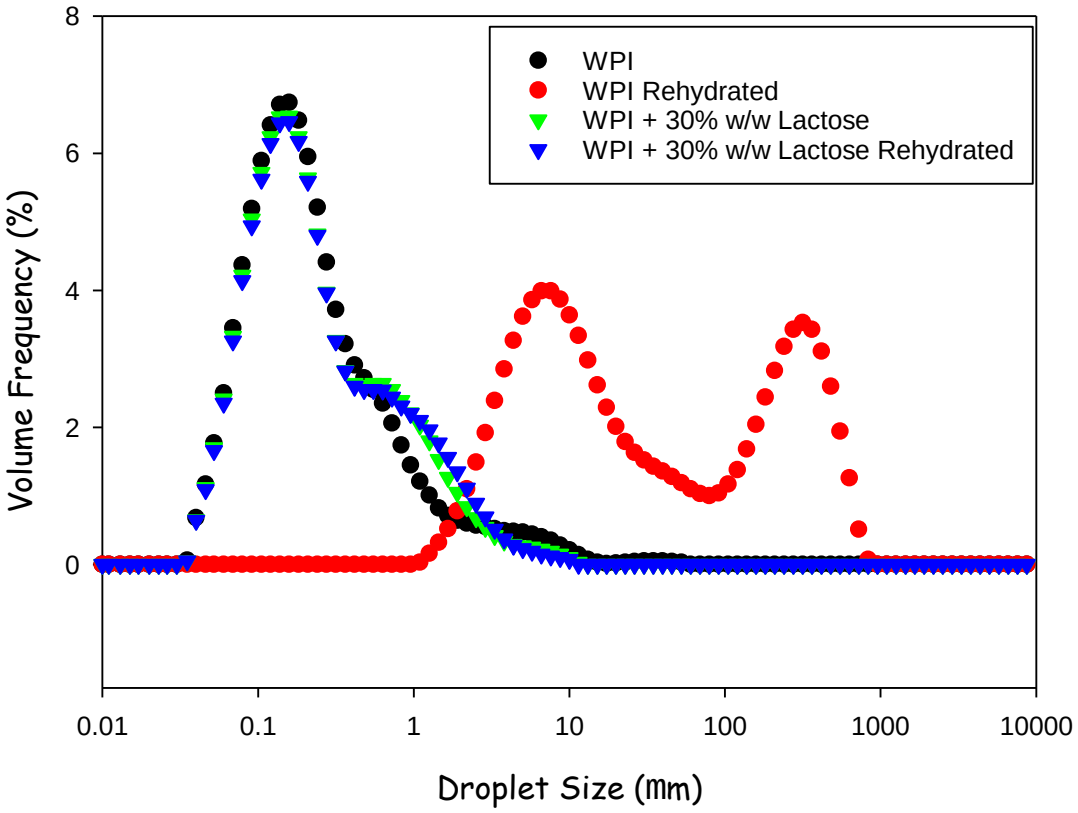


Figure 6-18: Droplet size distribution of ~1.7% w/w WPI stabilised emulsions before and after spray drying and rehydration with and without addition of 30% w/w lactose.

Dry Emulsions

Reconstituted emulsions were obtained from drying experiments performed at different inlet temperatures. Consequently, the quality of these two powders was studied and compared.

The hygroscopicity and bulk density of the powders were measured.

Table 6-6 gathers all the powders properties.

Table 6-7: Moisture content, water activity, hygroscopicity and bulk density of WPI-SBP lactose oil filled powders (spray dried 2.5% w/w WPI-SBP stabilised oil-in-water emulsions, 20% w/w sunflower oil with 30% w/w added lactose).

Inlet Temperature (°C)	Moisture Content (%)	Water Activity	Hygroscopicity (%)	Bulk Density, ρ (g/mL)
165	3.09 ± 1.35	0.1418 ± 0.0061	0.09 ± 0.05	0.321 ± 0.005
175	1.81 ± 0.02	0.1423 ± 0.0264	1.53 ± 0.05	0.305 ± 0.006

To qualitatively gauge the extent of agglomeration at the different temperature the bulk density ρ was calculated. The bulk density of powders corresponds to the volume occupied by a given mass of powder. It can be a good indicator of powder agglomeration since it directly linked to the porosity (Zuurman, Riepma et al. 1994). High density equals to lower porosity. The porosity depends on the way the particles of a powder stack up. Agglomerated powders tend to have higher porosity than single grains powders.

The bulk densities of the powders resulting from spray drying at different inlet temperature were similar.

Hygroscopicity measurements displayed an increase for the dry emulsions produced at 175 °C. Hygroscopicity can be directly linked to the structure and to the arrangement of the particles constituting a solid. Amorphous solids tend to have greater hygroscopicity (Downton, Flores-Luna et al. 1982). Crystalline solid are characterised by low hygroscopicity resulting in better storage stability(Jayasundera, Adhikari et al. 2009).

Lactose often lead to the formation of amorphous structure. The hygroscopicity measured at 165 °C could be an indication of a more crystalline structure.

However, these results were arguable considering the moisture content measured after rehydration. Indeed, the moisture content of powdered emulsions produced at 165 °C was 3.09 ± 1.35 % in opposition with 1.81 ± 0.02 % at 175 °C.

Assuming that the powder produced at 165 °C already held in a high amount of water it was possible that further water adsorption was limited.

6.3.2.2.2 Effect of the use of a protein-lactose conjugate

Spray-drying and rehydration of sodium caseinate-lactose conjugate stabilise oil-in-water emulsions

Lactose was covalently linked through Maillard reaction to sodium caseinate (heated at 40°C for 96 hours) to produce sodium caseinate-lactose conjugates (CN-La).

In order to obtain the highest conjugation efficiency several conjugates at different NaCN/lactose ratios (w/w) were produced. The results from conjugation efficiency measurements are displayed in Table 6-7.

Table 6-8: Conjugation efficiencies of CN-La conjugates at diffrent sodium caseinate/lactose ratios

NaCN:lactose (w:w)	Conjugation efficiency (%)
2:1	83.36 ± 3.57
1:1	88.03 ± 0.42
1:2	79.33 ± 5.77
1:3	76.81 ± 6.75
1:4	89.29 ± 0.61

Every ratio tested gave high conjugation efficiencies (>70%).

The conjugation efficiencies of the different ratios decreased according to the following order: 1:4>1:1>2:1>1:2>1:3. The ratio 1:4 was disregarded in order to avoid potentially high concentration of unconjugated lactose that could distort the final result. Consequently, the ratio 1:1 was selected instead to produce oil-in-water emulsions.

Instantaneous droplet aggregation was observed in the emulsion stabilised with the selected conjugate. Production of emulsion with the other conjugates led to the same result. It was mentioned previously that low solubility proteins could lead to early instability in the emulsions they stabilise (c.f. Chapter 2 and 3). To identify the cause of the instability observed, the solubility of the 1:1 NaCN/lactose conjugate was measured. The conjugate exhibited a solubility of 49.03 ± 4.88%. The solubility of the conjugate was slightly below 50%. Yet the initial solubility of a protein is a factor influencing its emulsifying properties (Crenwelge, Dill et al. 1974, Kinsella, Melachouris et al. 1976, De Wit 1989). The more protein in solution the more protein available to stabilise the oil/water interface. The causes of flocculation were uncertain. However, the solubility was a suspected factor.

Conjugates with the different NaCN/lactose ratios were assessed as emulsifiers. Flocculation arose during emulsification.

NaCN-lactose conjugates were dismissed.

Spray-drying and rehydration of whey protein-lactose conjugate stabilised oil-in-water emulsions

Conjugation of lactose to WPI was carried out at 40°C for 96 hours. The conjugation efficiency of the different conjugates produced was measured after dry heating. At all ratios tested the conjugation efficiency was measured ~70%.

The same rationale as previously mentioned was used to choose the adequate conjugate. The conjugation as a function of the WPI/lactose ratio decreased according to the following order: 1:3>1:4>1:1>2:1>1:2. The conjugate giving the highest conjugation efficiency was chosen for further experiments.

Emulsions stabilised with 8% w/w (2% WPI) whey protein isolate- lactose conjugate (WPI-La with a ratio 1:3) were produced.

The T_g was first determined using DSC. The T_g for the present systems was ~165 °C.

Based on this value the inlet temperature was set at 195 °C.

Droplet size measurements after rehydration are displayed in Figure 6-18.

The microstructure of the emulsion was successfully reconstituted after rehydration. The surface mean diameter and volume mean diameter both increased respectively from 131 ± 1 nm to 165 ± 1 nm and from 382 ± 3 nm to 1.043 ± 0.019 μm.

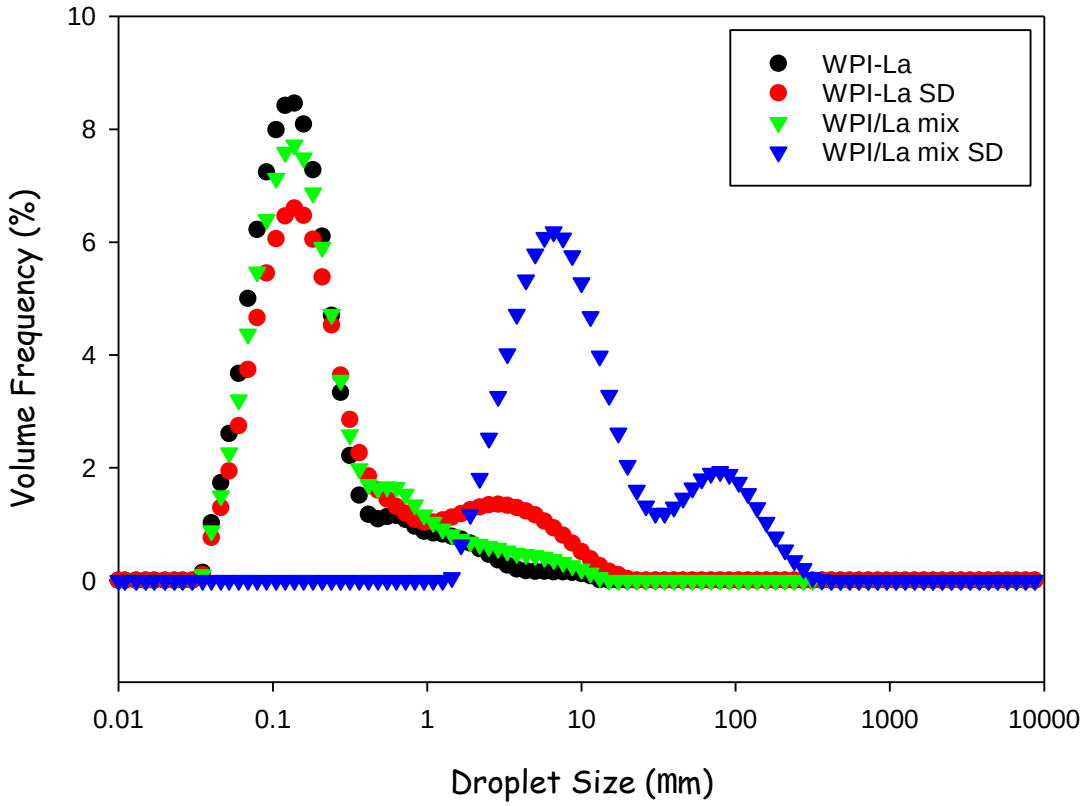


Figure 6-19: Droplet size distribution of 2% w/w WPI-La stabilised emulsions and 2% w/w WPI stabilised emulsion with 6% w/w added lactose before and after spray drying and rehydration.

In order to highlight the effect of conjugation an emulsion containing unconjugated WPI and lactose in the same proportions as in the conjugate was produced. Emulsion stabilised with 2% w/w WPI and 6% w/w added lactose was produced, spray dried and rehydrated.

The structure of the emulsion was lost after rehydration. Both the surface mean diameter and volume mean diameter increased in the rehydrated emulsion. From 144 ± 1 nm to 7.429 ± 0.245 µm and from 541 ± 10 nm to 27.975 ± 1.986 µm. These results suggested that conjugation of lactose to whey protein isolate increased the heat stability of the emulsions produced.

These results were compared with previous results where 30% w/w lactose was added to a ~1.7% w/w WPI stabilised emulsion.

At similar protein concentration the amount of lactose needed to preserve the microstructure of the emulsion during spray drying was reduced by 5 times when using a whey protein isolate–lactose conjugate.

6.4 Conclusion

The influence of drying process parameters (feeding rate, inlet temperature) and emulsion formulation on the reconstitution of dry emulsions was explored.

Freezing rate showed no effect on the freeze-drying stability of emulsions. At high and low freezing rate the microstructure of the emulsions was lost after rehydration.

For spray drying on the other hand, the impact of the feeding rate was investigated. The feeding rate was reduced in an attempt to decrease the particle size in the final powder. Rehydrated emulsions from these experiments displayed similar structures as the ones obtained when spray drying at higher feeding rate.

Experiments varying the formulation of the emulsions gave interesting results.

The use of a cryoprotectant in the formulation considerably improved the freeze-drying stability of emulsions.

The amount of emulsifier was reduced by a factor six to get reconstituted emulsions in comparison with emulsions without cryoprotectant.

Changing the pH of the emulsions had no effect on their freeze-drying stability. The emulsions were successfully reconstituted.

When salt was added before emulsification its impact on the freeze-drying stability of the emulsions was limited. Furthermore, sodium chloride negatively affected the reconstitution to lesser extent than calcium chloride.

Enhanced spray drying stability provided by lactose was confirmed with these experiments. When added to emulsions, lactose decreased the formation of droplet aggregates in rehydrated emulsions. Emulsions with added lactose were successfully reconstituted.

The incorporation of lactose through conjugation with protein led to the production of systems with an excellent spray drying stability.

Chapter 7

General Conclusions and Future Recommendations

7.1 General conclusions

This study has been carried out with the aim of contributing to the existing knowledge on Maillard conjugates and their properties in oil-in-water emulsions. Additionally, the glycoconjuagtes were used for the production of re-dispersible dry emulsions.

Different protein/polysaccharide couples were produced and tested to stabilised oil-in-water emulsions. The selected hydrocolloids displayed the highest conjugation efficiencies.

The conjugation efficiency was highly dependent on the hydrocolloid weight ratio.

In the third chapter it was also established that the particle size of conjugates containing a pH sensitive polysaccharide could be controlled by adjusting the pH of reaction. The conjugation efficiency was unaffected in this case.

The thickness of the interfacial membrane provided by the conjugates was measured and compared to the layer provided by proteins. The conjugates indeed provided interfacial layer five time thicker that the proteins.

The conjugation of polysaccharide to protein had no effect on the interfacial tension. This result was the sign that the gradient of polarity between protein and polysaccharide was negligible.

The conjugation also showed no effect on the surface coverage. The surface coverage of the protein and the conjugate were similar. However, based on the previous studies this result was attributed to the size of the polysaccharide used. It can be concluded that the surface coverage of proteins is unaffected by conjugation to low molecular weight polysaccharides.

Oil-in-water emulsions stabilised with the glycoconjugates displayed good stability to addition of salt, to pH changes and to high and low temperature exposure.

It was found that the viscosity could effectively be tailored by addition of salt without disturbing the microstructure of the emulsions.

However, experiments revealed that at a pH equal to the pK_a of a pH sensitive polysaccharide the emulsifying properties of the conjugate were affected.

The heat stability of whey protein was significantly improved through conjugation to a polysaccharide.

The emulsions were successfully freeze dried and reconstituted after rehydration. The viscosity and the traction coefficient remained the same for both conjugates tested.

The dry emulsions showed good stability to storage at room temperature and moderate relative humidity (≤35 %). However, exposure to high humidity and to moderate temperature (40°C) led to the plasticisation of the protein moieties present in the conjugate.

On the other hand, the conjugate stabilised emulsions displayed poor stability to spray drying. Protein denaturation occurred despite the conjugation.

Nevertheless, processing parameters and formulation could be changed to get re-dispersible emulsions.

Addition of cryoprotectant to the formulation led to reconstitution of freeze-dried emulsions at low conjugate concentration.

The freezing rate however had no effect on the freeze-drying stability of the systems.

The stability to spray drying was considerably improved by addition of lactose to the emulsions.

7.2 Future Recommendations

This project stands at the crossroads of two fields; formulation and engineering. This particularity opens a range of possibilities for further experiments.

However future recommendations are made based on the experiments carried out in this work.

Whey protein isolate-sugar beet pectin conjugate: effect of the pH of conjugation

In this study two whey protein isolate-sugar beet pectin conjugates with high conjugation efficiencies were produced at different pH. For this study the conjugate giving highest conjugation efficiency was selected. This way the number of unconjugated hydrocolloids in the continuous phase of emulsions was kept to a minimum. However, the two conjugates could be compared in terms of emulsifying properties and in term of functional properties in oil-in-water emulsions. If differences arise, the pH of conjugation could be adjusted for desired outcomes.

Interfacial rheology

In addition to interfacial thickness measurements, interfacial rheology would add further information on the interfacial behaviour of the conjugates. Drying processes put droplet interfaces under high stress. Trying to establish a relationship between the conjugate composition, its interfacial thickness and its interfacial rheology could help developing systems suitable for drying. Highly deformable droplets would in theory have a better resistance to drying.

Factor influencing the coverage of the conjugates

In this study the surface coverage was investigated on basic emulsions containing oil, water and emulsifier. Nevertheless, the coverage of proteins can be affected by the formulation of the emulsions they stabilise. Salts and pH changes can affect the surface coverage of proteins

(Srinivasan, Singh et al. 1996). High surface coverage can help in the protection of emulsion structures during drying. Furthermore, the effect of salts and pH changes on the interfacial rheology could be investigated.

Surface oil and encapsulation efficiency

In this study the conjugation efficiency and surface oil were not investigated. The surface oil can affect the dispersibility of the powdered emulsions. In Chapter 5 the dispersilibty of the dry emulsions was studied. Investigated the effects of surface oil on the rehydration time would give a whole understanding of the re-dispersion of the dry emulsions.

Oil oxidation

Freeze-dried emulsions showed a good stability to storage at room temperature and moderate humidity (≤35 %). Measuring the oil oxidation would add complementary information on the storage stability of the dry emulsions.

Conjugates have the potential to enhance the oil oxidation stability.

Storage stability of dry emulsions containing sugars

Emulsions with added cryoprotectant showed good stability to freeze-drying. Sugars are highly hydrophilic and can be highly hygroscopic compounds (Dittmar 1935). The storage stability of dry powders containing sugars could be affected by high concentration of the later.

Similarly, lactose was added to emulsions before spray drying. The impact of this addition to the storage stability needs investigation.

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