Physical Stability of High Protein Bars During Shelf-life

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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September 2016

The present work was performed during 6 months (February to July) at *Nizo food research* (Ede, The Netherlands) under the supervision of Inge Gazi (Project Manager) and Thom Huppetz (Principal Scientist).

Acknowledgments

I would like to thank to my supervisors at Nizo food research, Dr. Thom Huppertz, not only for keeping me continuously motivated and for giving new challenges almost every day. To Inge Gazi, that always supported me, answering all my questions and always ready to discuss all the ideas and doubts that I had in my mind. I also want to thank everybody at Nizo food research who taught me how to understand and use new equipment and techniques, especially to Franklin Zoet, who taught me how to make protein bars, and to Jan Klok, for all the support at the lab. To professor Marília Mateus, not only for giving the opportunity to have this internship but also for the continuous support and care, despite the distance.

I would like to thank to my parents for all the support during these 5 years and for all the sacrifices they made to give me the possibility to be an engineer. To my boyfriend for staying by my side every day and for not letting me down.

I am grateful to everyone that helped me during this internship. I learnt a lot over these 6 months, not only professionally but also personally and now I know that I am ready to face any challenge.

Abstract

One of the major problems with this bars is that they become harder over time. The aim of this study was to investigate the effects of milk protein ingredients on the physical stability of HPB. Bar hardening was studied under accelerated shelf-life conditions at 37ºC during 30 days. Model bars comprising of 45% carbohydrate syrup, 45% milk protein ingredient and 10% glycerol were prepared using a range of milk protein ingredients. Texture analysis, water activity measurements and color changes were correlated with the properties of the protein powders (moisture sorption, particle size and structure, protein composition).

It was possible to identify two extremes in texture: crumbly and dry texture given by acid and rennet caseins, showing increased hardness over storage time, and viscous liquid and sticky texture given by casein hydrolysates, that formed the softest bars. The degree of solubility and the fraction of powders particles in the bar matrix were found to be the major factors that contribute to differences in texture. Hardness of the bars was also found to be influenced by the viscosity granted by the dissolved protein powder particles in the bar matrix.

Correlation of bar hardness with protein ingredient properties indicate the following driving forces for development of bar hardness: (1) water-sorption by proteins; (2) sorption of carbohydrate syrup by protein powder particles, (3) (partial) dissolution of proteins/peptides. Based hereon, protein type and powder particle size and density were identified as the most important properties of protein ingredients determining their behaviour in nutritional bars.

Keywords: carbohydrate, hardness, milk proteins, moisture content, nutritional bar, texture

Resumo

Um dos maiores problemas com barras de proteína é o seu endurecimento. O objetivo deste estudo passou por investigar os efeitos das proteínas do leite na estabilidade das barras, sobre condições de tempo de prateleira acelerado a 37ºC durante 30 dias. Barras com 45% de solução de hidratos de carbono, 45% de proteínas de soro de leite e 10% de glicerol foram preparadas usando uma diversa gama de proteínas. A textura, atividade da água e alterações na cor foram correlacionadas com as propriedades das proteínas (absorção de água, tamanho e estrutura das partículas, composição das proteínas).

Foi possível identificar dois extremos na textura: quebradiça e seca dada pela caseína ácida e caseína de coalho, mostrando um aumento na dureza com o tempo, e viscosa e espessa dada pelos hidrolisados, que formaram as barras com menos endurecimento. O grau de solubilidade e a fração de partículas na composição da barra foram consideradas como os principais fatores que contribuem para a diferença na textura. Foi também possível concluir que o endurecimento das barras é influenciado pela viscosidade atribuída pelas proteínas dissolvidas.

A correlação do endurecimento das barras com as propriedades do ingrediente proteico indica que os principais motivos para o desenvolvimento de dureza são: (1) absorção de água pelas proteínas; (2) absorção da solução de hidratos de carbono pelas proteínas; (3) dissolução (parcial) das proteínas/péptidos. Baseado na investigação decorrida, o tipo de proteína e o tamanho das partículas e densidade foram identificados como as propriedades determinantes na escolha dos ingredientes proteicos, influenciando o comportamento das barras nutricionais.

Palavras-chave: barra nutricional, dureza, hidratos de carbono, proteínas do soro de leite, teor de humidade, textura

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α-lac – α-lactalbumin

β-lac – β-lactoglobulin

p – Water vapor pressure

p_0 – Saturated vapor pressure of pure water

ρ_p – Powder particle density

ρ_T – True density

AMP – Advanced Maillard reaction products

a_w – Water activity

BSA – Bovine serum albumin

CaCas – Calcium caseinate

CaCas R – Calcium caseinate roller dried

CH – Casein hydrolysate

CPP – Casein phosphopeptides

DH – Degree of hydrolysis

DM – Dry matter

EMC – Equilibrium moisture content

EMR – Equilibrium humidity

HFCS – High fructose corn syrup

HPB – High protein bars

IMF – Intermediate moisture foods

MCC – Micellar casein concentrate

MPC – Milk protein concentrate

NaCas – Sodium caseinate

OPA – o-phthalaldehyde

RH – Relative humidity

RP-HPLC – Reverse phase high-performance liquid chromatography

v_{oa} - Specific volume of occluded air

WP – Whey proteins

WPH – Whey protein hydrolysate

WPI – Whey protein isolate

1. Introduction

High protein bars (HPBs) were primarily used by athletes and body builders. However, trough the past few years the market for nutritional and health bars has been reflecting the concern of people to have a healthier life style. As a result, this type of products that were previously meant for muscle building, are now being marketed as products that promote good health. Presently, sports and energy bars are some of the fastest growing segments, as consumers are turning busier day-by-day and less reticent towards purchases of meals and meal replacements. As all the food products, besides the nutritional value, protein bars should be appealing to the consumer, promoting a satisfying experience. The flavor is one of the more important attributes. However, texture is also a crucial point to have in consideration. One of the major problems related with the texture of HPBs is that they became too hard over time, making them unacceptable for the consumer (McMahon et al. 2009).

Typically, commercial protein bars are composed of two main ingredients: powdered proteins from soy or dairy sources and sugar- or polyol-based syrups (Li et al. 2008). The ingredient distribution is normally 20-40% protein, 10-50% carbohydrates and 10-15% fats with a water activity between 0.5 to 0.7 (Dan & Labuza 2010). HPBs usually also contain other components including flavors, stabilizers, and inclusions such as peanuts and dried fruits (McMahon et al. 2009). Whey protein isolate (WPI) is one of the most common sources of protein, but caseins can also be used. The carbohydrates are usually a blend of high fructose corn syrup (HFCS) and a polyol syrup (glycerol, sorbitol or maltitol) which has the function to provide the water needed to form a dough. HPBs also have a source of fat, which can be vegetable shortening, cocoa butter or vegetable oil (McMahon et al. 2009).

1.1. Literature review

Commercial high protein bars can contain a wide range of proteins, e.g., dairy proteins (caseins and/or whey proteins), soy proteins, egg proteins and gelatins. In some cases, a mixture of proteins can be used in HPBs formulation. When proteins are blended together they can have synergistic or antagonistic effects. This means that a bar can have a better or worse texture than the expected texture when only the pure proteins are used (Imtiaz et al. 2012). The present study investigates the influence of milk protein ingredients in the formulation of HPBs and their influence on texture. Milk proteins can be divided into caseins and whey proteins. The milk protein ingredients are commercially available either as mixtures of caseins and whey proteins in e.g., MPCs and MCCs, or as protein isolates in e.g., caseinate and WPCs and WPIs (McSweeney & O'Mahony 2013).

1.1.1. Dairy Proteins

Whey proteins

Whey proteins are globular proteins that have a high level of secondary, tertiary and quaternary structure. There are four main groups of whey proteins: β-lactoglobulin, α-lactalbumin, BSA and immunoglobulins (Nehete et al. 2013). After removing the casein from milk whey proteins can be obtained. They are very similar to milk serum (milk from which casein and fat has been removed) (McSweeney & O'Mahony 2013).

Whey proteins are preferably used in the production of HPBs since they confer a smooth flavor and contribute to a chewy and caramel-like texture (Liu et al. 2009). Whey proteins can come in the form of protein isolates (>90% protein), protein concentrates (<90% protein) or protein hydrolysates. The major difference between WPC and WPI is that WPI has a higher content of protein than WPC and has less lactose and minerals (McSweeney & O'Mahony 2013).

- **Whey protein concentrate (WPC)** is made of delactosed and desalted whey. The process involves the whey concentration by evaporation to obtain lactose crystals followed by the process of desalting the liquid. The remaining liquid is dried, generally by spray-drying, which results in highly soluble powders. t (Walstra et al. 2005).
- **Whey protein isolates (WPI)** are produced from a relatively pure whey and the process is similar to the one used to WPC. For the production of WPI it is necessary to do at least one step of diafiltration (Walstra et al. 2005)
- **Whey protein hydrolysates** are obtained through the cleavage of peptides bonds, which results in peptides of different sizes and free amino acids (Sinha et al. 2007). Proteins can be hydrolyzed using enzymes, acids or alkali. The process is used to obtain a product with different molecular, nutritional, functional and sensory properties from the starting proteins (McSweeney & O'Mahony 2013). Hydrolysates have some advantages when compared to the native whey protein: higher solubility, lower viscosity, improved water adsorption and emulsifying capacity and higher thermal stability (McSweeney & O'Mahony 2013).

Caseins

Caseins can be defined as the proteins precipitating from milk at pH 4.6 (Walstra et al. 2005). They can be subdivided in four main subgroups, all of which are phosphoproteins: α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, representing 38%, 10%, 36% and 13% of the total casein composition (Nehete et al. 2013). Caseins are flexible with no rigid α-helix and β-pleated sheet structures. The structural arrangement of casein micelles is not yet fully understood and

there is no structure unanimously accepted. However, the concept that appears to be universally accepted is the casein micelle is electrostatically and sterically stabilized by a “hairy” surface of κ-casein (McSweeney & O’Mahony 2013). Caseins are made from skimmed milk (Walstra et al. 2005). There are several types of casein preparations that can be made: rennet casein, acid casein, caseinates and micellar casein.

- **Rennet casein** is the resulting casein after the addition of calf rennet that cleaves the crucial peptide bond (Phe_{105}-Met_{106}) in κ-casein. This casein is composed of calcium paracaseinate-calcium phosphate and some impurities and is insoluble in water (Walstra et al. 2005);
- **Acid casein** is also insoluble in water and is obtain by the acidification of skim milk. The milk pH is changed from 6.7 (normal milk pH) to about 4.6, which causes caseins to precipitate. The decrease in pH can be carried out by mineral acids, e.g., HCl or through fermentation of the lactose in the skim milk with production of lactic acid.
- **Caseinates** are obtained dissolving acid casein at neutral pH, with the NaCas being the most common. Ca and K caseinates can also be obtained. They are all highly soluble in water and almost flavorless (Walstra et al. 2005).
- **Micellar casein** ingredients such as as **MPC** (milk protein concentrate) or **MCC** (micellar casein concentrate) are obtained from skim milk by membrane processes. The resulting micelles have similar properties to the ones of natural casein micelles. Micellar caseins are powdered milk products produced by ultrafiltration, in case of the MPC, and by microfiltration in the case if MCC, of skim milk to concentrate casein and whey proteins in the retentate with lactose permeating out (Banach et al. 2013). MPCs in general are composed by 80% caseins and 20% whey proteins, the same ration that is found in milk.
- **Casein hydrolysates can be** obtained by enzymatic hydrolysis, acids or alkali. These hydrolysates have the same advantages mentioned before for whey protein hydrolysates.

1.1.2. Texture

Hardness of the bars can be attributed to a various number of mechanisms. These include aggregation of proteins following formation of intermolecular disulphide bonds and non-covalent interactions, Maillard reactions (non-enzymatic browning) (Zhou et al. 2013), moisture migration, phase separation phenomena (Hogan et al. 2012) and fat oxidation (Dan & Labuza 2010). In fact, hardening of HPB does not occur due to a single mechanism, it is a combination of various chemical, physical, thermodynamic and process related factors (Hogan et al. 2012).

Disulphide bonds and non-covalent interactions

The formation of insoluble aggregates induced by the thiol-disulfide intercharge reaction is one of the mechanisms reported by Zhou et al. 2008 for bar hardening. If the thiol-disulfide reaction could be prevented, the hardening of the bars could be inhibited, resulting in a longer shelf life (Dan & Labuza 2010).

Zhou et al. 2008 suggested that an increase of storage temperature would result in increased flexibility and mobility of protein peptides. This would cause the tertiary structure loss of proteins stored at high temperatures, with strong effect in the components that have lower denaturation temperatures (bovine serum albumin – BSA and α-lactalbumin). The high mobility and change in the protein structure would increase the accessibility of sulfhydryl group and disulfide bonds which contribute to the formation of more intermolecular disulfide bonds for samples stored at high temperatures. The majority of whey proteins are globular proteins that have relatively high hydrophobicity and compactly folded peptide chains (Walstra et al. 2005) During storage these proteins can undergo denaturation and form soluble or insoluble aggregates (Zhou et al. 2008) . The formation of these aggregates depends not only on the composition and concentration of whey proteins but also on other factors such as pH, ionic composition and temperature (Walstra et al. 2005). Moreover, Zhou et al. 2008 showed that in the early stage, the formation of the aggregates does not imply a significant change in texture. When the formed aggregates start to interact with each other, a more global network forms and hardening of the protein bar can be observed (change in texture).

Maillard Reactions

With a strong effect on food quality, Maillard reaction can be described as the chemical reaction between amino groups and reducing sugars. High protein bars contain, in most of the cases, both reducing sugars and proteins, which would result in the occurrence of Maillard reaction.

Van Boekel 1998 mentioned that Maillard reaction occurs much faster in dried milk products than in milk. The drying process will provide a lower water activity, but should be emphasized that the reason behind the change in the rate of the reaction is the increase in the reactants concentration. Several types of products can be formed during the three different stages of Maillard reaction. The first stage consists in the formation n of the Amadori product, if the sugar is an aldose sugar, or the Heyns product, if the sugar is a ketose (Van Boekel 2006). The product is formed through the condensation of a reducing sugar with an amino group. The second stage consists in the formation of the Advanced Maillard reaction Products (AMP) (Chevalier et al. 2001). The process starts from the Amadori/Heyns product, leading to numerous essential fission sugar-amino compounds, with the release of the amino group. The last stage involves dehydration, fragmentation, cyclization and polymerization reactions in which the amino groups participate again. One of the last products to be formed are the melanoidins (Wang et al. 2011). These heterogeneous, nitrogen-containing brown pigments are the result of the condensation and polymerization of proteins (Chevalier et al. 2001). For some foods, like

cocoa and coffee, the browning is a desirable effect. For other products, like milk products or dried milk products (milk powder or whey powder), the brown color is not a desirable effect.

The various paths of the Maillard reaction, and consequently, the products formed, depend strongly on temperature, pH, nature of reactants (type of sugar, type of amino acid, or protein) (Van Boekel 2006) and also on water activity and moisture content (Morales & Van Boekel 1998). Van Boekel 2006 reports that amino acids have a different behavior when compared to proteins or peptides due to the availability of the reactive amino. In the case of proteins and peptides the reactive amino group is lysine because the α-amino groups are tied up in the peptide bond, and so they are not available. Maillard reaction induces the formation of crosslinks between the proteins and the brown pigments are for a large part covalently attached to proteins.

Zhou et al. 2013 showed that these modifications in high protein food bars are relatively slow if stored at room temperature, and might take weeks or months to develop some undesired effects. Nevertheless, high storage temperatures would accelerate the progress of deterioration, indicating the instability of HPBs. As a result, some proteins could undergo glycation and aggregation (Chevalier et al. 2001). Zhou et al. 2013 verified that a large amount of insoluble protein particles were formed when exposed to high temperatures and in presence of fructose (reducing sugar), suggesting the formation of protein aggregates. The non-disulfide covalent cross-links formed through Maillard reaction could lead to the formation of insoluble protein aggregates, leading to the hardening of HPBs.

Moisture content and water activity

HPBs belong to a class of food products denominated as intermediate moisture foods (IMF). IMF have a moderate moisture content, generally in the range of 10-40%. Humectants/plasticizers are also part of the protein bar formulations and they are used to control the water activity and texture. They include a group of polyols such as glycerol, sorbitol, maltitol and propylene glycol (Liu et al. 2009). The water present in the protein bars acts as a plasticizer, increasing the molecular mobility of proteins. Polyols have higher molecular weight and higher glass transition temperature, which make them less effective plasticizers. Hereupon, the replacement of water with polyols would decrease the molecular mobility of proteins.

Liu et al. 2009 showed that the lower the glass transition temperature of the polyol the more effectively it will work as a plasticizer, with glycerol being the most effective in providing the softest texture, followed by sorbitol and maltitol. Glycerol also has the lowest molecular size and in consequence it would be less excluded in the local domain around the protein (McMahon et al. 2009). Liu et al. 2009 also revealed that propylene glycol causes changes in protein conformation and stability resulting in aggregation of whey proteins and hardening during storage, so it should not be in whey protein-based high-protein intermediate-moisture foods.

An increase of water activity should result in softer bars but this phenomenon is not always observed. McMahon et al. 2009 verified that for some bars, an increase of the water activity during storage time does not imply a softer bar. Actually, they became harder and this

can be due to losing the capacity of water to act as plasticizer around the protein particles. Therefore, the elevated rate of bar hardening can be explained by the decreasing content of water available to act as plasticizer, as the water became free increasing the water activity (Li et al. 2008).

Phase Separation Phenomena

McMahon et al. 2009 reported that the phase separation phenomena usually occurs when there are multiple polymers present in solution, which results in a reduction of the possible configurations. Usually, HPBs only have proteins as polymers and some polysaccharides in the formulation. Sugar crystallization can induce phase separation, by causing high stress. McMahon et al. 2009 also concluded that phase separation into large protein-rich and protein-depleted aqueous regions is one of the mechanisms that initiates bar hardening and increases protein-protein interactions.

Protein type

Caseins

Rao et al. 2016 affirmed that caseins have two characteristics that can make them a better candidate for the formulation of protein bars rather than whey proteins. The first one is that they do not have a characteristic denaturation temperature, not becoming insoluble by heating at temperatures below 100ºC. Furthermore, caseins cannot or can hardly be denatured because they have little secondary and tertiary structure (Walstra et al. 2005). The second one is that they have no free sulfhydryl group and only two disulfide groups. Hence, the effect of disulfide-induced protein aggregation in casein during storage should be smaller (Rao et al. 2016). MPC are not often used in the formulation of HPB because they tend to give a crumbly texture and lack cohesiveness (Li et al. 2008).

Whey

Hogan et al. 2012 mentioned that the use of whey protein hydrolysates has some advantages, as bars produced therewith remained softer throughout shelf life. However, they can give a bitter flavor to the HPB. McMahon et al. 2009 hypothesized that one of the causes for the preference of hydrolysates rather than isolates or concentrates is the increase in the interfacial area between protein/co-solvent, resultant from the higher number of proteins/peptides molecules. As mentioned before, hydrolysates have an increased hydrophilicity brought about by hydrolysis of peptide bonds, which allows more interactions between the carbohydrate phase and the protein particles.

1.1.3. Techniques

Texture analyses

The changes in texture over time can be measured using a texture analyzer. The principle behind this equipment is simple and passes by measuring the mechanical deformation of the sample. The magnitude of the force (N) and deformation as well as the shape of the curve are related to different properties and can be directly related to the sensory perception of texture (Lal Dar & Light 2014).

Water activity

Water activity is a measure of the energy status of the water in a system and thus of its availability to act as a solvent and participate in chemical or biochemical reactions. a_w can also be defined as the relative humidity of air in equilibrium with a sample in a sealed measurement chamber (Fontana 2002).

$$aw = \frac{water\ vapour\ pressure\ over\ sample}{saturation\ vapour\ pressure\ of\ pure\ water} = \frac{p}{p_0} = equilibrium\ humidity \quad (1)$$

The method used for measuring water activity is based on the dew point which is defined as the temperature to which air must be cooled at constant pressure and water content to reach saturation. The dew point sensor inside the water activity equipment measures the dew point temperature of the air and an infrared thermometer measures the sample temperature (Fontana 2001). With this value is possible to calculate the relative humidity by the equation (2).

$$RH = \frac{dew\ point\ temperature\ at\ saturarion\ vapor\ presure}{saturation\ vapor\ pressure\ at\ the\ sample\ temperature} \quad (2)$$

The sample humidifies or dehumidifies the air volume inside the chamber until the equilibrium humidity is reached. This phenomenon takes place due to the partial water vapor pressure difference between the sample and the air (Fontana 2001). When the water activity of the sample and the relative humidity of the air are in equilibrium, the measurement of the headspace humidity gives the water activity of the sample (Fontana 2001).

Water activity is influenced by temperature and therefore changes in temperature can cause a water migration between different components. For this reason, temperature is a variable of extreme importance in the production high protein bars and should be tightly controlled. As mentioned before, protein bars have a water activity value between 0.5 and 0.7

which make them microbiologically stable since there is no microbial growth in this range of water activity.

Powder Particle Size

The main characteristic that defines a powder and its physical properties is the state of subdivision of its individual particles. The size, distribution and shape of the individual particles of the powder will have influence in all the basic powder inherent characteristics, such as flowability, ease of dispersion in fluids, dissolution rate, mouth feel, etc (Bhandari et al. 2013). The techniques to measure particle size can be divided in three different categories: methods based on direct measurement (each particle is examined individually), methods based on classification of particles (particles are separated by size and the classification is based on the measurement of the amount in each size class) and methods based on secondary measurements (involves the measurement of some property of the powder that depends on particle size). In the present work, the method used is based on secondary measurements, and thus it involves the measurement of the diffraction pattern created by laser light passing through powder particle dispersion.

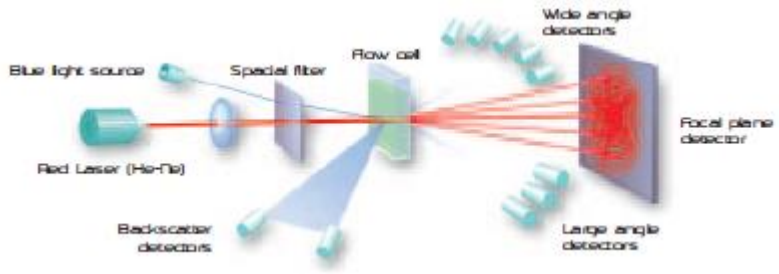

Figure 1 Mechanism behind dynamic light scattering from (Malvern Instruments Ltd 2000)

Dynamic light scattering is a technique used to predict size distribution of small particles in suspension. The working principle is based on the light that is diffracted by the powder particles which is inversely proportional to the particle size. The sample is prepared and dispersed to the correct concentration and then delivered to the optical bench that captures the diffraction pattern that is created by making particles pass through a focused laser beam. The detection is made by a series of photosensitive light detectors, and each detector collect the light scattering from a particular range of angles. The measurement (the capturing of the scattering pattern) is represented by a histogram, and each bar represent the light scattering from one of the detectors. The final result is based on a combination of many snapshots, in order to have a representative reading of the scattering pattern (Malvern Instruments Ltd 2000). The particle size distribution is calculated by the Mie theory which is a general theory for the interaction of light with a spherical homogeneous particle that takes into account backscattering,

diffraction, refraction and light absorption of the light by the particles (Malvern Instruments Ltd 2007). For particles with irregular shapes, the Mie theory devolves the diameter of the sphere that has the same diffraction pattern in terms of mass (or volume) distribution (Bhandari et al. 2013). This particle size technique is currently one of the most used techniques for measuring the size distribution of powder particles.

RP-HPLC

In the dairy industry, RP-HPLC has been widely used due to its versatility, short analysis time and high resolution (Elgar et al. 2000). In RP-HPLC, the stationary phase, usually based on silica particles, has a lower hydrophobicity than the mobile phase that contains mixture of water and organic solvents. In RP chromatography, particle separation is the result of adsorption forces (Skoog & West 1980). Interactions of more hydrophobic compounds with the solid support are stronger than that of hydrophilic ones. When the mobile phase (polar) is delivered in isocratic or gradient elution mode, the hydrophobic molecules are absorbed onto the solid support and the hydrophilic ones are eluted first and detected at a short retention time (Nollet & Toldrá 2013). The hydrophobic compounds will require a higher concentration of organic solvent to promote desorption. The resulting order of elution is detected by UV detection and a chromatogram is plotted. The order of peaks on the chromatogram is a function of the order of elution and the quantification of each peak gives the concentration of the compounds in the sample (Skoog & West 1980).

The percentage of denatured whey proteins can be determined by RP-HPLC by isolating whey proteins and caseins from undenatured whey proteins by isoelectric precipitation at pH 4.6 (Parris & Baginski 1991). The extension of denaturation can then be quantified by comparing the expression of whey proteins in 4.6 pH soluble fraction to a relative control sample (Ferreira et al. 2001). This method is extremely efficient for the major whey proteins α-lactalbumin and β-lactoglobulin, however is not easy to quantify minor whey proteins such as BSA and immunoglobulin (Elgar et al. 2000).

Sorption Isotherms

The absorption of water by milk powders is generally one of the main causes behind changes in the properties of milk powders. Swelling, gelling, emulsifying, foaming and organoleptic properties are some of the critical characteristics that are influenced by the water sorption behavior of milk powders. Therefore, is important to measure about the hydration as a function of water activity. The typical water sorption curves are called water sorption isotherms and represent the powder hydration as a function of water activity at constant temperature (McSweeney & O'Mahony 2013). Casein is the main water absorber at low moisture contents ($a_w < 0.2$) and for intermediate values ($0.2 < a_w < 0.6$), sorption is dominated by the transformation of the physical state of lactose (Bhandari et al. 2013).

The equilibrium moisture content is obtained by exposing the powder material to a define RH and bringing it to equilibrium. The sorption isotherms are then assembled by the representation of the equilibrium moisture content as function of the defined RH.

Free amino residues (OPA method)

Free lysine residues in milk proteins are able to participate in a large range of reactions (Goodno et al. 1981). In Maillard reaction, these available lysine residues react with a reducing sugar and become blocked or glycated (Mehta & Deeth 2016). The extent of the Maillard reaction can be evaluated by measuring the free amino groups that were not involved in the non-enzymatic browning. These free amino groups can be quantified by the OPA method: reaction between the o-phthalaldehyde, a thiol compound and the reactive free amine (Goodno et al. 1981).

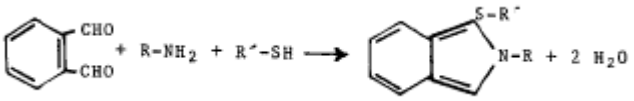

Figure 2 Reaction between an o-phthaldehyde, a thiol coumpound and a reactive free amine from (Goodno et al. 1981)

The reaction mentioned above forms an adduct that absorbs strongly at 340nm. These OPA-amino acid adducts are used in the spectrophotometric determination of primary amines. The OPA method is rapid, sensitive, simple and applicable to all proteins. Moreover, the nature of the side chain of the an amino-acid does not significantly affect absorptivity of the resulting adduct and peptide bonds do not affect the molar absorptivity for OPA adducts of peptides (Church et al. 1983). The quantification of the free amino groups over time can be used to determine the progress of the Maillard reaction.

2. Materials and Methods

2.1. Carbohydrates

Fructose and sucrose were produced by Galam Group and glucose by Cargill. Maltose was provided by Merck KGaA and maltitol by Tokyo Chemical Industry co, LTD.

2.2. Protein Powders

Sodium caseinate (NaCas), calcium caseinate 1 (CaCas 1), calcium caseinate 2 (CaCas 2), calcium caseinate roller dried (CaCas R), casein hydrolysate 1 (CH 1), casein phosphopeptides (CPP), MCC80, casein hydrolysate 2 (CH 2), whey protein isolate 1 (WPI 1), whey protein isolate 2 (WPI 2), whey protein isolate 3 (WPI 3), acid and rennet casein, MPC90 and whey protein hydrolysate (WPH).

2.2.1. Protein Powder Fractions

CaCas 1 and WPI 2 were fractionated using a Vibratory Sieve Shaker AS 200 (Retsch, Germany). The sieves used to fractionate CaCas 1 had the sizes of 32, 71, 125, 150 and 250 µm and for WPI 2 of 63, 125, 150, 250 and 400 µm.

2.2.2. Pre-equilibrated powders

NaCas, WPH and WPI 2 powders were equilibrated at an a_w of 0.65 (average water activity of commercial protein pars) and then used in the formulation of protein bars. The powders were equilibrated in desiccators at room temperature during 20 days, time after no change in weight was observed.

2.2.3. Coated Powders

NaCas was coated using a powder coated granulator GPCG 1.1 (Glatt GmbH, Germany). The container was filled with the powder to be processed and the coating agent was sprayed by a nozzle inside the container. The amount of coating agent was adjust depending on the amount of NaCas inserted in the container in order to have concentrations of 5 and 10% coating.

2.2.4. Insolubilizing MPC90

MPC90 was equilibrated at a_w=0.4 in a climate chamber set to 20ºC. The powder was divided into plastic containers and sealed with parafilm. Half of the powder was placed in an oven at 60ºC and the other half in a cold room at 5ºC. After one week of storage, a part of the powder at 5ºC was microwaved for 5 min and maximum power. The three MPC90 powder samples (the fresh/control powder stored at 5ºC, the powder stored at 60ºC and the microwaved powder) were tested for solubility. To test the solubility of the powders, solutions of 4% powder were made by adding 2 g of each MPC90 to 48 g of pre-heated water at 30ºC, with continuous stirring. The solutions were stirred for 10 minutes and then 40 g of each solution was added to a 50 mL conical bottom centrifuge tube and centrifuged for 15 minutes at 700×g and 20ºC.

2.3. Powder Characterization

2.3.1. Dry Matter Content

Dry matter content was determined by oven drying the protein powders as follows: ~2g of sample was weighted into metal cups (without lid) and placed into the oven for 2 hours at 102ºC. After that time, the cups were placed into desiccators (with lid) for 30 min to cool down. The samples were weighted and placed into the oven for 1 hour. After cooling down in the desiccators, the weight was measured again. This step was repeated until the difference between weights was less than 0.0015 g. The water content was determined by the weight loss. The free moisture content was also determined by Infrared Moisture Analyser MA 150 (Sartorius, Germany)

2.3.2. Powder Particle Density

Powder particle density (ρ_p) was determined by using a Beckman 930 pycnometer (Beckman Instruments Inc., USA).

The true density of the powder (ρ_t) was estimated by calculation and taking into account the composition of the different powders (protein, fat, moisture, lactose and others). The component density values were found in literature (Walstra et al. 2005) and they represent the values for apparent density in aqueous solution, not the density of the components in dry state. The theoretical true density of the powder material was calculated using the following formula:

$$\rho_t = \frac{100}{\frac{\%protein}{\rho_{protein}} + \frac{\%lactose}{\rho_{lactose}} + \frac{\%fat}{\rho_{fat}} + \frac{\%water}{\rho_{water}} + \frac{\%other}{\rho_{other}}} \quad (3)$$

Where:

$$\%other = 100 - (\%protein + \%lactose + \%fat + \%water) \quad (4)$$

And:

%protein, %lactose, %fat, %water, %other = concentrations of protein, lactose, fat, moisture and other components, respectively in powders.

ρ_t= true density, g/cm^3

$\rho_{protein}$ = 1.400, g/cm^3

$\rho_{lactose}$ = 1.780, g/cm^3

ρ_{fat} = 0.918, g/cm^3

ρ^{20}_{water} = 0.998, g/cm^3

ρ_{other} = 1.850, g/cm^3

The occluded air was calculated by using the equation (5), as described in the GEA Niro analytical method A 11 (Niro 2006).

$$v_{oa} = \frac{100}{\rho_p} - \frac{100}{\rho_t} \qquad (5)$$

Where:

v_{oa} = specific volume of occluded air, $cm^3/100\ g$

Powder particle size was measured by laser light scattering using a Mastersizer 2000 and a HYDRO 2000S (Malvern Instruments Ldt, UK).

Shape and state of agglomeration was also tested using a REICHERT-JUNG POLYVAR microscope (Leica Microsystems, Switzerland). The powder was fixed in a microscope slide with parafin oil and the results were collected using bright field and different magnifications.

2.3.3. RP-HPLC

The protein samples were prepared by dissolving 0.4 g of powder into 9.6 g of MilliQ water at 50ºC for 2 hours. The 4.6 pH soluble fractions were prepared as follows: 100 µl of acetic acid 10% was added to 1000 µl of protein sample and vortexed. After 15 min 100 µl of 1 M Na-acetate was added to the samples and vortexed. The resulting solutions were centrifuged at 15000×g for 30 min. The supernatant was removed for HPLC analysis.

The samples for HPLC were prepared by adding 300 µl of buffer E+ (buffer containing urea, Bis (2-hydroxyethyl)amino-tris(hydroxymethyl)methane – (Bis-Tris), sodium citrate and hydrochloric acid (HCl) supplemented with 20 mg/ml of dithiotreitol (DDT)) to 100 µl of sample and vortex. After 1 hour, 1500 µl of buffer D (buffer containing urea, acetonitrile (CH_3CN) and trifluotoacetic (TFA)) were added to the sample and vortexed again. The samples were frozen until the HPLC analysis.

Before HPLC analysis the samples were filtered using Millex SV 0.22 µm syringe filters (Millipore, Ireland). Reversed phase high performance liquid chromatography with UV detection at 220 nm was used for quantitative analysis of the caseins, whey proteins and peptides in the protein samples. The equipment used was the UltiMate 3000 Series Thermostatted Column Compartments (TermoFisher Scientific, USA) composed by a solvent rack, pump, autosampler, column compartment and detector. The separation was done using a reverd-phase column Aeris WIDEPORE 3.6µ XB-C18, size 250 x 2.10 mm (Phenomenex, Torrance, USA). Solvent A was a mixture of 2% acetonitrile, 98% water and 0,1% TFA and the solvent B contained 60, 40 and 0.8% of the same components, respectively. The flow rate was 0.4mL/min and the column temperature was maintained at 40ºC.

The areas beneath the peaks in each chromatogram were obtained by integration using the CHROMELEON 7.2 software (ThermoFischer Scientific, USA). The amount of each protein was directly related to the corresponding peak area in the chromatogram. The percentage of denaturated whey protein was determined using the following formula:

$$\% \, denaturated \, protein = \frac{PA_{total} - PA_{sup}}{PA_{total}} \times 100 \quad (6)$$

Where:

PA_{total} = peak area of total protein in solution;

PA_{sup} = peak area of protein in supernatant.

2.3.4. Sorption Isotherms

The moisture sorption behavior of protein powders was determined by weighing about 1g of each protein powder onto plastic cups with 7 cm length and 3 cm diameter and then holding at room temperature in desiccators containing saturated solutions of P_2O_5, K_2CO_3, $Mg(NO_3)_2$, $NaNO_3$, NaCl and KCl, with water activities of ~0.0, 0.432, 0.532, 0.65, 0.75 and 0.85 respectively. Powders were equilibrated until no change in weight was observed.

2.3.5. Solubility test

The solubility of caseinates, WPI and CH in solutions with 45 g of carbohydrate syrup (HFCS 70%), 10 g glycerol and 10 g protein powder was tested. The solution was stirred for 15 min at 800 rpm and then centrifuge for 20 min, at 4500×g and 20ºC.

2.4. Preparation of High Protein Bars

Bar material was made in 80 g batches using a formulation of 45, 45 and 10% m/m of protein powder, carbohydrate syrup and glycerol, respectively, which corresponds to 36 g protein powder, 36 g carbohydrate syrup and 10 g glycerol. The ingredients were mixed manually using a spatula. The carbohydrate syrup was first mixed with the glycerol for 1 min and then half of the protein ingredient was added. After mixing for 30 s, the other half of the protein was added to the mixture and mixed for another 30 s, scraping down the sides of the container to ensure even mixing and mixed for a final 30 s. The resulting mixture was molded into plastic cups (~8 g for hardness measurements and ~4 g for water activity measurements) and the lids were sealed with parafilm to prevent moisture loss. Samples were left to equilibrate for about 1 day at room temperature and then they were stored at 37ºC and ambient humidity to accelerate thermodynamic changes in bars.

2.4.1. Texture analysis

The firmness of the protein bars was measured using a Texture Analyser XT Plus from Stable Micro Systems (Godalming, UK) with 30 kg load cell, which was calibrated with a 5 kg weight or with a 5 kg load cell, which was calibrated with a 1 kg weight. A cylindrical probe with 5 mm diameter (see Figure 3, probe B) was used to penetrate the sample at the test-speed of 1 mm/s. The target mode selected was distance which was set to 6 mm. The experiments were performed with “auto-trigger” which had the value of 0.1 N (for the 30 kg load cell) or 0.001 N (for the 5 kg load cell). Measurements were taken for freshly produced bars and subsequent intermittent measurements following storage at 37ºC. Samples were equilibrated at room temperature prior to hardness measurements and the results were expressed as maximum peak force (N) as function of distance (mm).

The hardness of the carbohydrate syrups was measured with probe A (see Figure 3) using the same method as described for protein bars. The hardness value (N) correspond to the force needed to overcome the surface tension of the syrup after one day of equilibration.

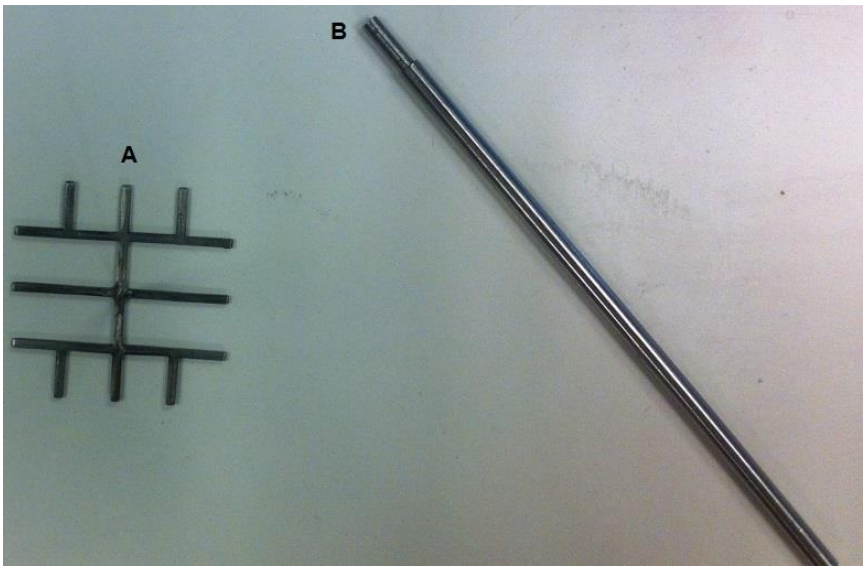

Figure 3 Probes used in the texture analyzer (A-probe used to measure the hardness (N) of the carbohydrate syrups; B-probe used to measure the hardness (N) of the protein bars)

2.4.2. Water Activity

Water activity, a_w, was determined using an AQUA LAB serie 3 (Decagon Devices, USA). Samples were measured in AQUA LAB water activity cups (Decagon Devices, USA) and were considered as equilibrated if no change in a_w occurred for 1 min.

2.4.3. Free amino residues (OPA method)

The samples were prepared by diluting the protein bars 100 times. For this purpose, 1 g of protein bar in was dissolved 9 g of MilliQ water using a magnetic stirrer. And 1 g of the resulting solution was taken and diluted in 9 g MilliQ.

The preparation of the OPA solution required the preparation of a 0.1 M Borax solution by dissolving 3.814 g di-sodium tetraborate-decahydrate ($Na_2B_4O_7.10H_2O$) in 100 mL of MilliQ water and a 20% SDS solution by dissolving 20 g of sodiumdodecylsulfate in 100 mL of MilliQ water. The OPA reagent was prepared freshly by dissolving 16 mg of OPA (Sigma, P-0657) in 0.4mL methanol, followed by the addition of 2.5 mL of 0.1 M Borax buffer, 88 mg DTT (Aldrich) and 0.25 mL of 20% SDS. The volume was adjusted to 20 mL with MilliQ water.

Volumes of 200 µL of each sample were titrated manually to a 96 well plate, all to different wells. The addition of the reagents and the following procedure was done automatically by a robot into a 384 well plate. The final 384 well plate contained 8 µL of sample and 72 µL of reagents in each well.

The calibration curve was determined by adding the same volume of different concentrations of leucine solution (0.0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0 mM) and measuring the absorbance at 340nm by a M3 spectrophotometer (Molecular Devices, USA).

3. Results

3.1. Carbohydrates

The different sugar solutions are described in Table 1. Variations of -10, -5, +5 and +10 of the solubility limit at 20ºC were tested.

Table 1 Carbohydrate solubility limit at 20ºC expressed in g carbohydrate / 100g of solution and the mass values for each carbohydrate variation (-10, -5, +5 and +10%) in the solubility limit. Sucrose solubility from (Cook & Shinners 2011), *glucose solubility from* (Alves et al. 2007) *and fructose solubility from* (Crestani et al. 2013)

g carbohydrate / 100 g of solution						
	Glucose	**Sucrose**	**Fructose**	**Glucose + Sucrose**	**Glucose + Fructose**	**Sucrose + Fructose**
-10%	43	60	71	22+30	22+36	30+36
-5%	46	64	75	23+32	23+38	32+38
Solubility at 20ºC	48	67	79	24+34	24+40	34+40
+5%	50	70	83	25+35	25+41	35+41
+10%	53	74	87	26+37	26+43	37+43

The higher values registered by the texture analyzer correspond to the force needed to break the surface tension of the carbohydrate solutions. The results are represented in Figure 4 and show that increasing the carbohydrate concentration resulted in an increase in the force needed to break the surface tension. Fructose made the hardest carbohydrate solutions, with a significant increase in hardness when +10% of solubility at 20ºC was tested. No experiments were done to confirm the veracity of the results, therefore the value for fructose could be a consequence of some experimental error.

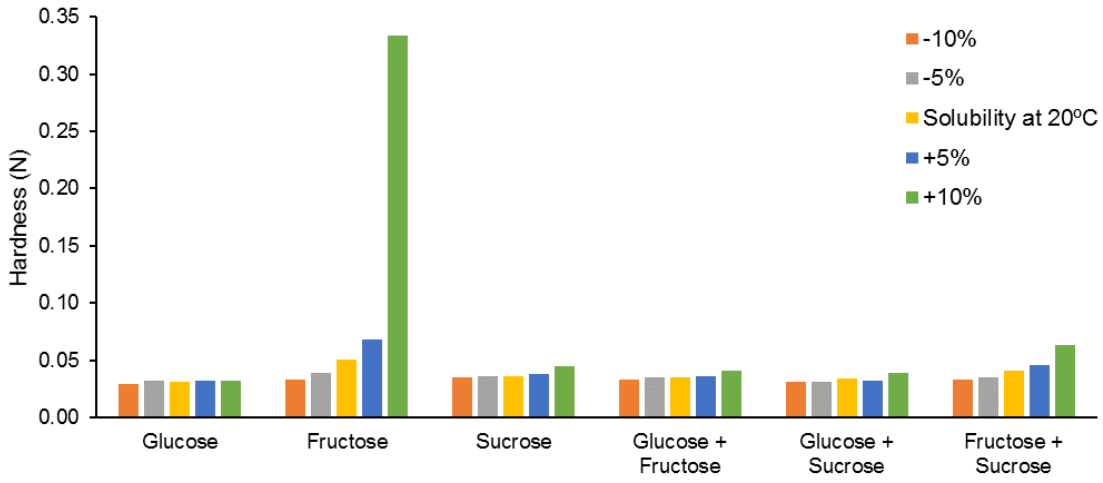

Figure 4 Hardness (N) of the carbohydrate solutions.

The values for water activity are presented in Figure 5. Increasing the percentage of carbohydrate in carbohydrate solution decreased the water activity.

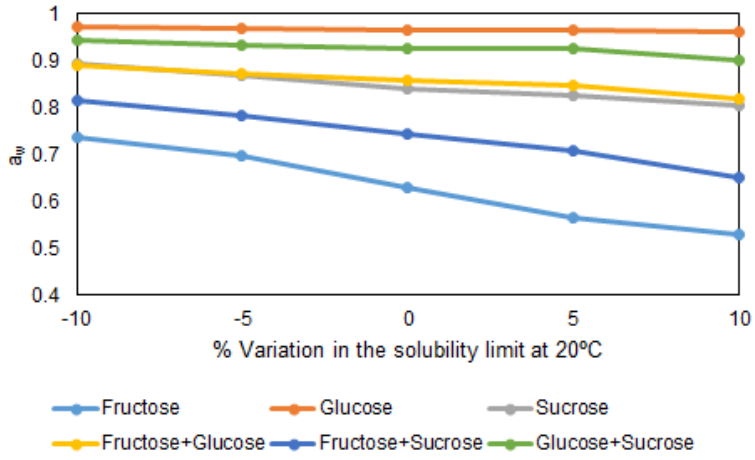

Figure 5 Water activity of the carbohydrate solutions.

3.2. Powder Properties

3.2.1. Whole Powders

Powder Density

The results obtained from measuring powder particle density and from calculating true density and volume of occluded air in the 15 proteins ingredients are presented in Figure 6. The values of powder particle density and volume of occluded air have a direct correlation, with a powder with a higher powder particle density having a lower volume of occluded air. The air present inside the particles will contribute to lowering the powder particle density.

The calculated true density was based in the assumption that all the powders had a protein content of 90% which resulted in a similar value for all of the powders, with the exception of MCC80 that has 80% of protein content.

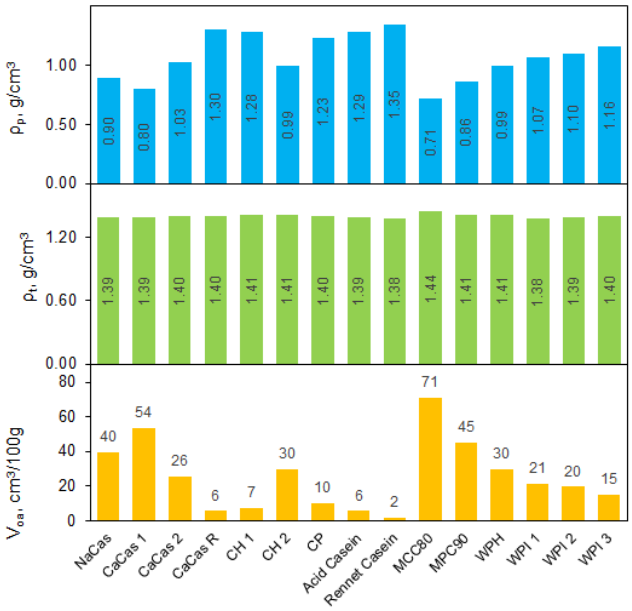

Figure 6 Powder Particle Density (ρ_p), True Density (ρ_T) and Specific Volume of Occluded Air (V_{oa}) in the different proteins.

According to the values for the different CaCas tested it can be concluded that the same type of protein powder could have different properties. CaCas 1 and CaCas 2 are both spray dried powders, but from different suppliers, and CaCas R is from the same supplier as CaCas 1 but it was roller dried. Powder structure and properties are extremely dependent on the drying technique. A roller dried powder will have an irregular shape and structure and a low volume of occluded air.

Powder Particle Size

The results obtained from measuring particle size and the resulting values for the exterior particle surface area are presented in Table 2. The value used as the powder particle size for calculating the exterior particle surface area is the median powder particle diameter, d(0.5), expressed in µm, that is the value which 50% of the powder particles are smaller than.

Table 2 Results obtained from powder particle size for the proteins in study

	d(0,1) (µm)	d(0,5) (µm)	d(0,9) (µm)	D[3,2] - Surface weighted mean (µm)	D[4,3] - Volume weighted mean (µm)	Exterior particle surface area (m^2/100g)
NaCas	48.3	126.8	289.7	87.8	151.8	5.3
CaCas 1	31.0	74.9	192.7	56.2	99.6	10.1
CaCas 2	22.1	61.7	174.7	17.9	86.5	9.5
CaCas R	25.0	118.9	427.0	28.2	179.2	3.9
CH 1	14.2	53.6	108.5	15.7	58.3	8.8
CH 2	90.8	231.2	487.0	130.5	262.4	2.6
CPP	11.1	39.8	79.1	14.7	43.1	12.3
WPH	122.6	247.4	452.8	154.2	267.9	2.4
Acid Casein	49.2	130.8	252.3	59.0	141.8	3.6
Rennet Casein	28.4	115.5	247.6	34.7	128.6	3.9
WPI 1	21.7	64.8	138.9	39.4	74.0	8.7
WPI 2	61.6	142.6	307.1	94.2	167.2	3.8
WPI 3	23.9	74.0	169.6	44.8	87.8	7.0
MCC 80	19.8	63.3	161.6	42.5	81.3	13.3
MPC 90	10.9	31.8	68.0	10.2	36.0	22.0

The differences in CaCas powders are also reflected in the powder particle size and concomitant exterior particle surface area. Roller dried powders have a larger particle size which results in a lower exterior surface area of the powder particles.

The differences in CaCas powders can be also seen in the particle size distribution (See appendix A). Spray dried products have a narrow particle size interval due to the precise drying method, that only makes particles in a small range of sizes. Roller dried powder particles have an irregular shape and size, which will result in a powder with a wide particle size distribution.

Microstructure of Powder Particles

The resulting images of the protein powders observed under the microscope are present in the Figure 7 (casein-based powders) and Figure 8 (whey-based powders).

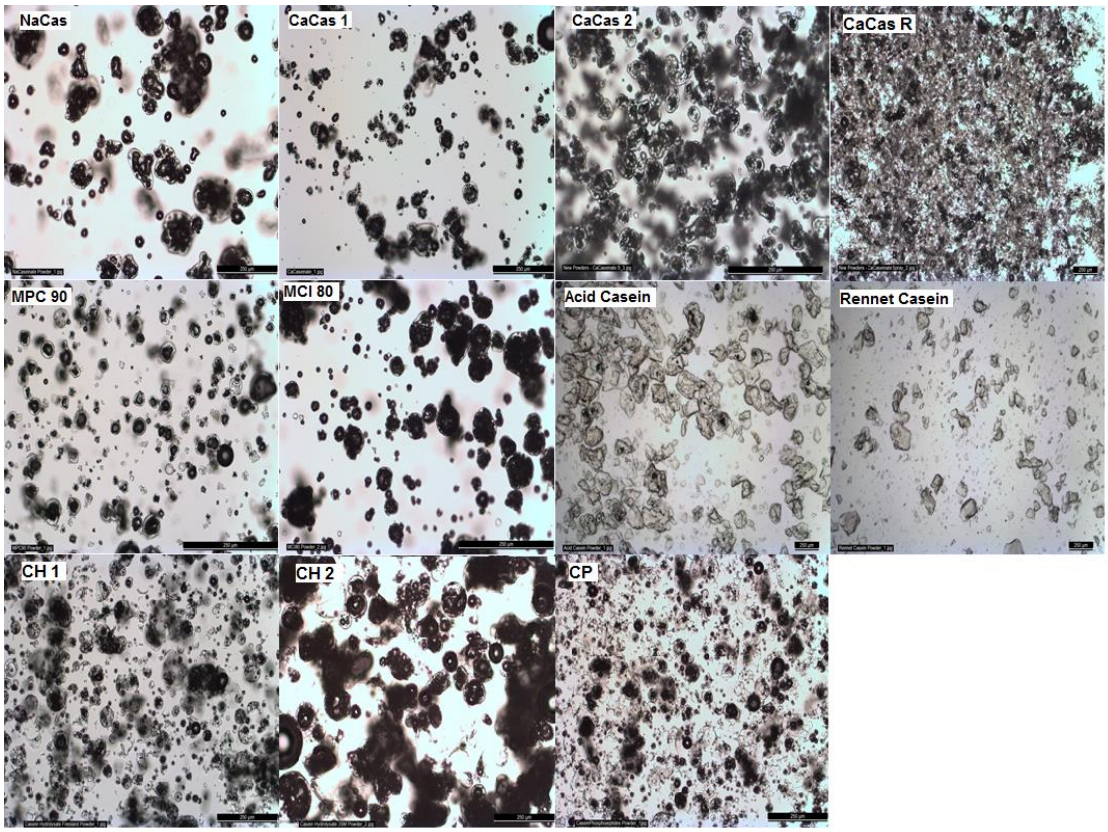

Figure 7 Microstructure of casein-based powders under light microscope (scale 250 µm).

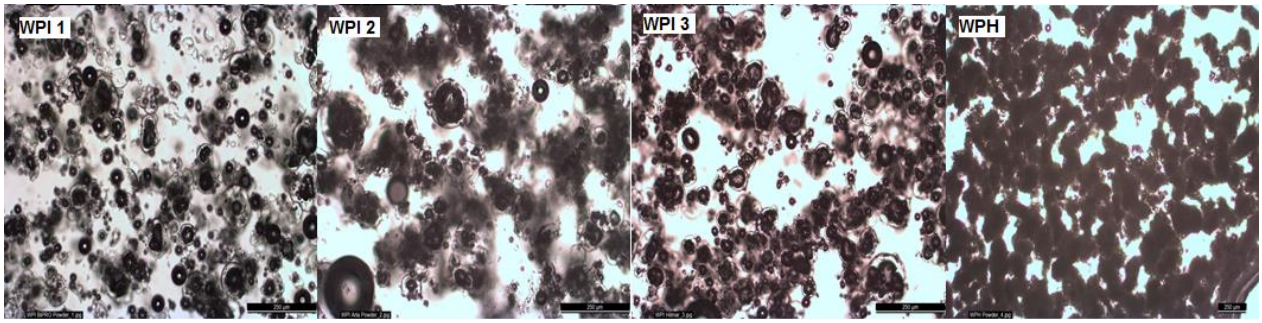

Figure 8 Microstructure of whey-based powders under light microscope (scale 250 µm).

With the exception of acid and rennet casein and CaCas Roller, all the powders had a regular and spherical shape. The irregular shape of the acid and rennet casein are similar to CaCas Roller, which could indicate that those powders were also roller dried.

Moisture Content and Initial Water Activity

As it can be seen in the figure below, the initial water activity of each powder is directly related with the moisture content: higher moisture content is followed by higher water activity. Acid and rennet casein are the powders with the highest values for moisture content and initial a_w and MCC80 the one with the lowest values.

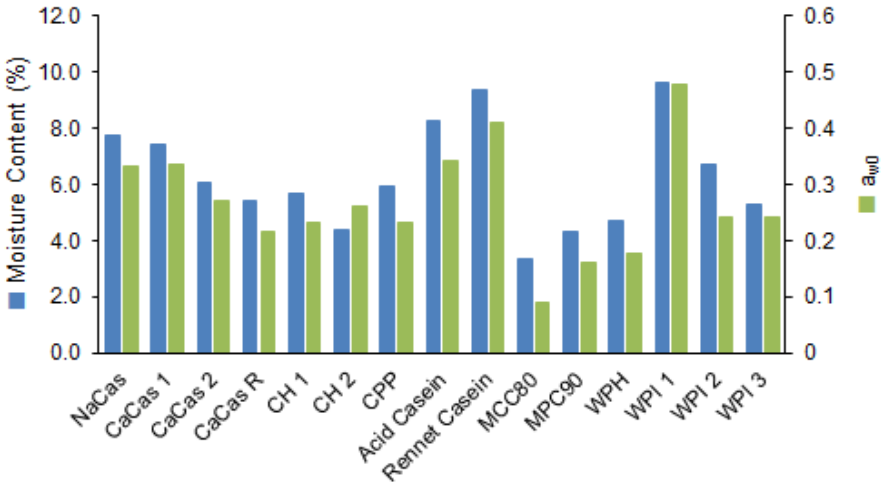

Figure 9 Moisture content (%) and initial water activity for each protein powder.

RP - HPLC

Whey Protein Denaturation

The calculated values for the denatured whey protein showed a high percentage of denatured α-lac for the MCC80, as well as a high amount of denatured β-lac compared to the other whey protein-containing ingredients.

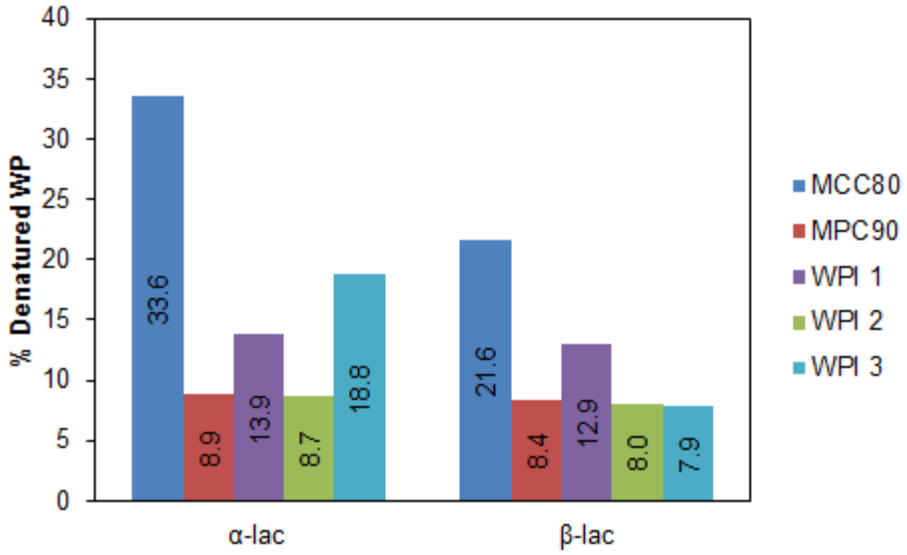

Figure 10 Percentage of denatured WP (whey protein) in MCC 80, MPC 90, WPI 1, WPI 2 and WPI 3.

Degree of hydrolysis

The chromatogram shown in Figure 11 represents the degree of hydrolysis of the different casein hydrolysates in study. Through the observation of the peaks in the chromatogram it was found that CH 2 had the highest DH (degree of hydrolysis), followed by CH 1 and CPP with the lowest DH.

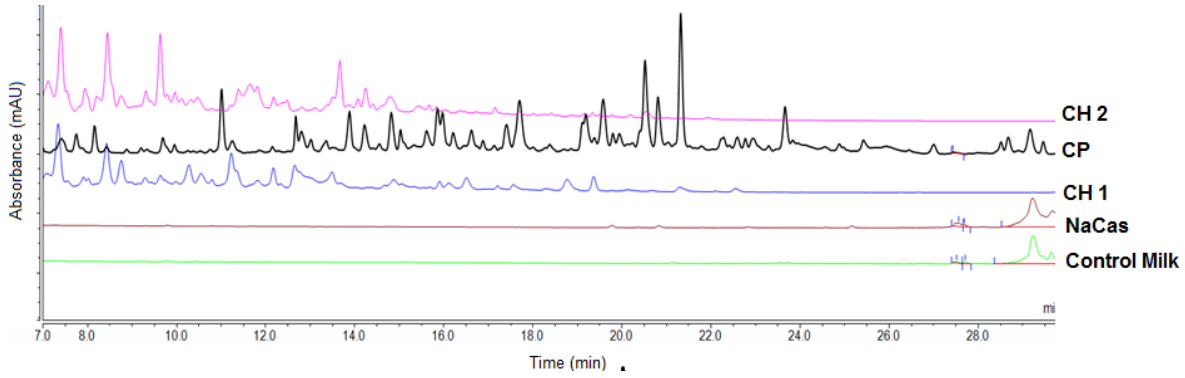

Figure 11 Degree of hydrolysis of the casein hydrolysates.

In Figure 12 is presented the chromatogram for WPI 1 and WPH. WPH showed to have a higher DH than CH 1 and CH 2, and a similar DH of CPP.

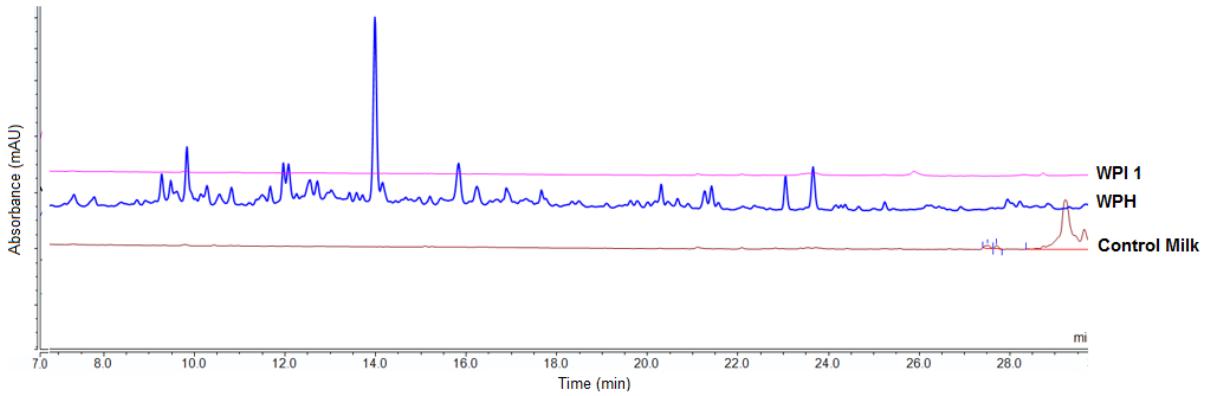

Figure 12 Degree of hydrolysis of WPH compared with WPI 1.

Sorption Isotherms

The sorption isotherms of the different proteins were obtained by equilibrating the powders at defined water activities until no changes in weight were observed. As it can be seen in Figure 13, CH 1 is the protein ingredient that showed the highest water sorption, followed by CH 2 and CPP.

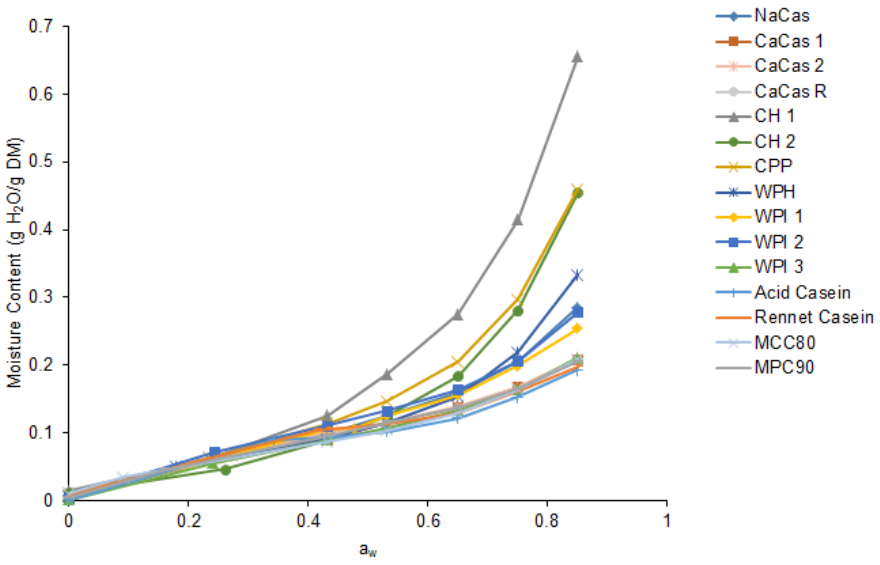

Figure 13 Sorption isotherms of the different protein powders.

The sorption isotherms for each protein is presented in the figure below. All the whey-based powders were found to have a relatively similar water sorption behavior, with the exception of WPH. Also, the non-hydrolyzed casein based-powders had a similar water sorption behavior.

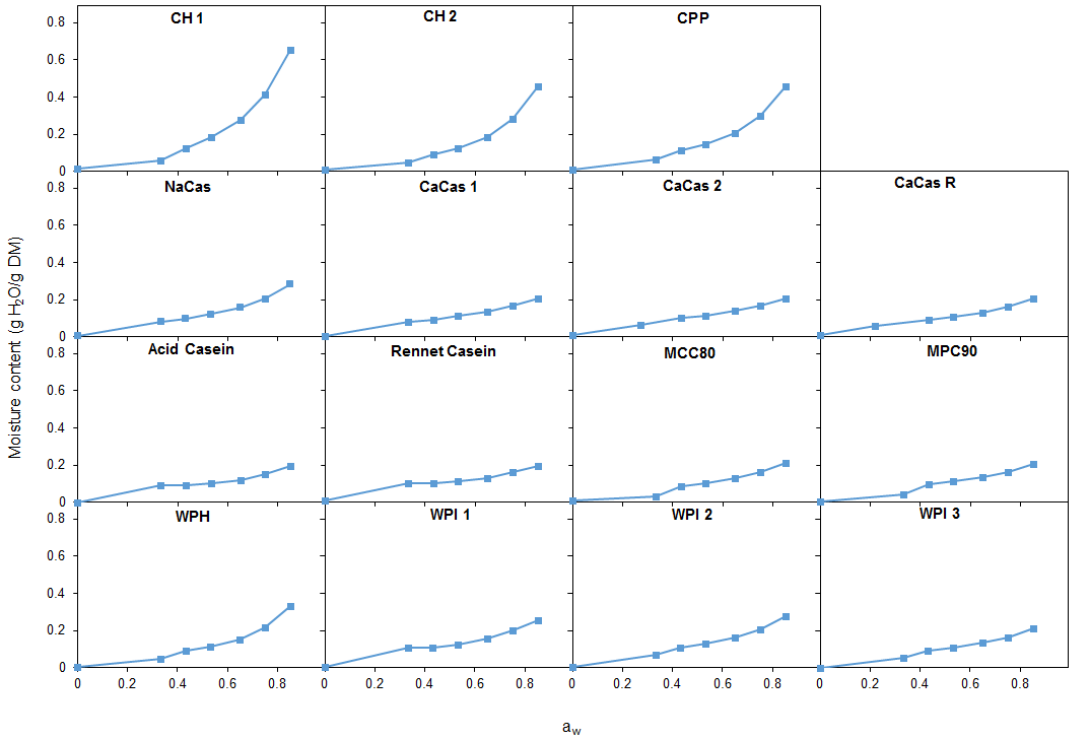

Figure 14 Sorption isotherms of the individual protein powders.

Solubility test

The results of the solubility test for NaCas, WPI 2 and CH 2 are shown in Figure 15. After 1 day of storage at 37ºC, the centrifuged WPI 2 and CH 2 solutions were completely translucent, which is a result of the dissolution of the powder particles in the syrup. In the case of NaCas, the solution was comparable to a gel in texture, and it was not possible to conclude if the powder particles were completely and/or partially dissolved in the syrup.

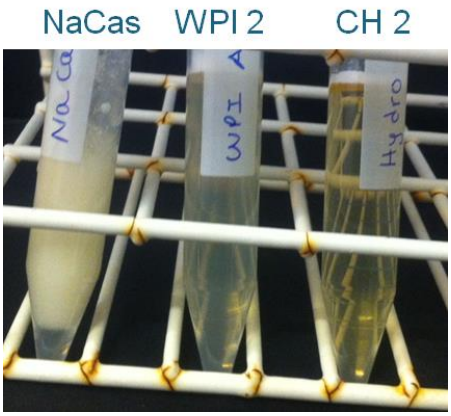

Figure 15 Solubility teste for NaCas, WPI 2 and CH 2 after 1 day of storage at 37ºC.

3.2.2. Fractionated Powders

In order to investigate the influence of the powder particle size on the final product, CaCas 1 and WPI 2 were fractionated into different powder particle size classes. The fraction F1 contained the smallest particles and the following ones had larger particle sizes, with F4 being the fraction with the largest particles. The powder particle size distribution of the fractions is presented in Table 3.

Table 3 Fractions of the CaCas 1 and WPI 2 and respective size ranges (µm)

	CaCas 1	WPI 2
F1	[32-71]	[63-125]
F2	[71-125]	[125-150]
F3	[125-150]	[150-250]
F4	[150-250]	[250-400]

Powder Density

The results for powder particle density, true density and specific volume of occluded air for the fractionated powders are shown in Figure 16. It was found that, within the same protein powder, the powder particle density and the calculated true density and volume of occluded were similar to all fractions.

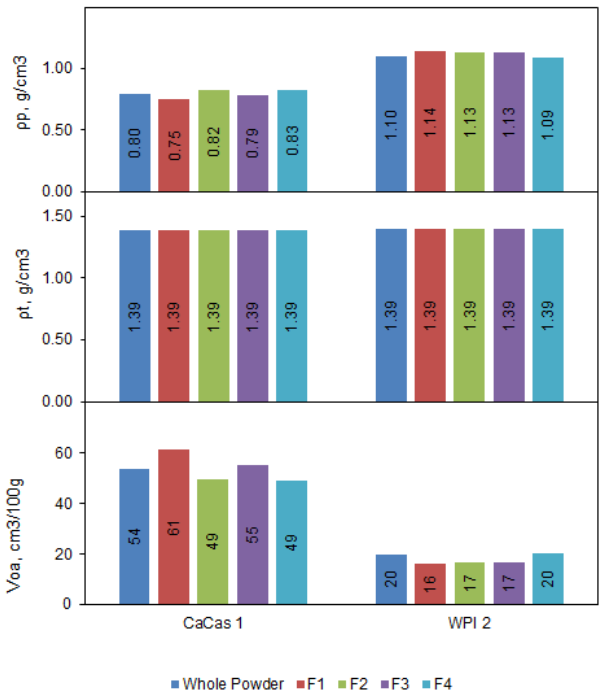

Figure 16 Powder Particle Density (ρ_p), True Density (ρ_T) and Specific Volume of Occluded Air (V_{oa}) in the fractionated protein powders.

Powder Particle Size

The results obtained from measuring the particle size and the resulting values for the exterior particle surface area are presented in Table 4.

Table 4 Powder particle size distribution and powder particle surface area of the fractionated protein powders

		d(0,1) (µm)	d(0,5) (µm)	d(0,9) (µm)	D[3,2] - Surface weighted mean (µm)	D[4,3] - Volume weighted mean (µm)	Exterior particle surface area (m^2/100g)
CaCas 1	**Whole Powder**	31.0	75.9	192.7	56.2	99.6	10.1
	F1	24.5	48.1	84.7	38.7	51.6	16.6
	F2	32.9	71.3	134.2	54.5	78.2	10.2
	F3	26.4	61.5	123.3	18.4	69.1	12.4
	F4	37.2	91.7	217.6	66.4	112.8	7.9
WPI 2	**Whole Powder**	61.6	142.6	307.1	94.2	167.2	3.8
	F1	43.2	86.2	151.0	60.7	91.7	6.1
	F2	73.0	122.7	193.4	87.6	127.3	4.3
	F3	94.7	166.3	281.6	138.1	178.0	3.2
	F4	213.0	327.8	503.1	310.4	345.2	1.7

Sorption Isotherms

The changes in mass of the powder samples are expressed as a percentage of initial sample mass in Figure 17. It was found that, within the same powder type, all the fractions have similar moisture uptakes during the storage time.

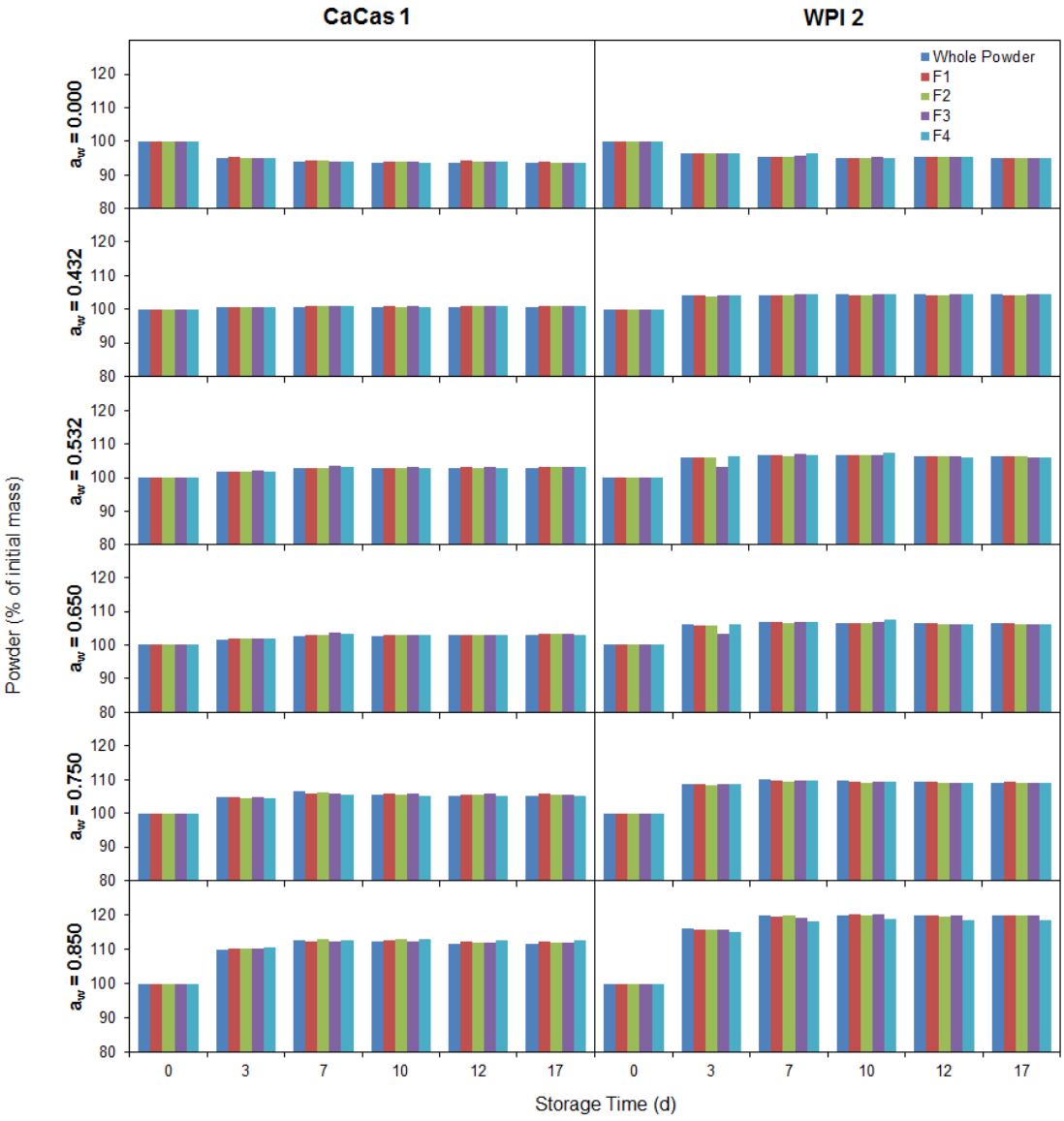

Figure 17 Changes in mass of the powder samples expressed as a percentage of initial sample mass of the fractionated powders during storage time

The sorption isotherms are also presented in Figure 18. All the 4 fractions are overlapped with the sorption isotherm of the whole powder which confirms that the 4 fractions absorb the same amount of water for each value of a_w as the whole powder.

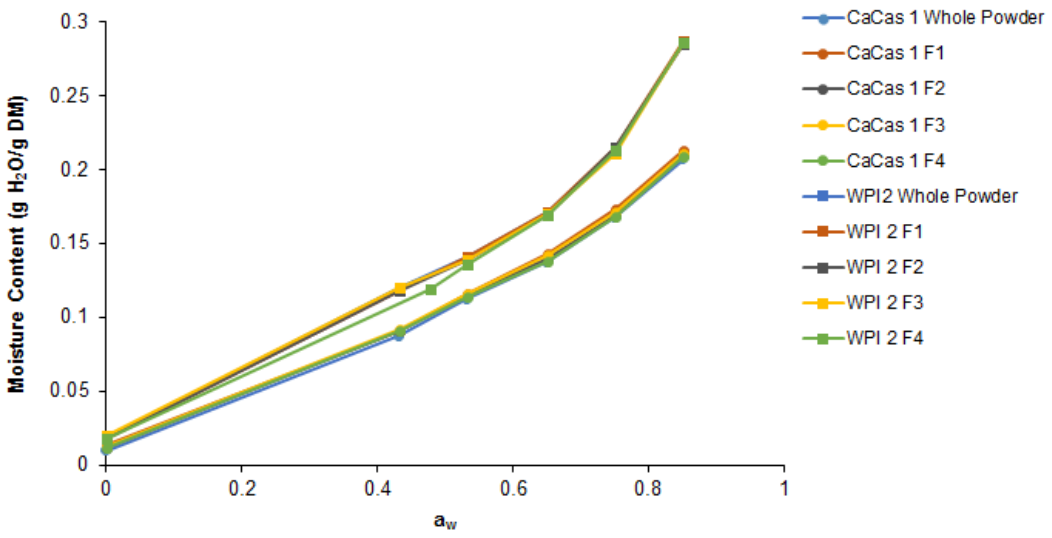

Figure 18 Sorption isotherms of the fractionated powders.

3.2.3. Coated Powders

NaCas powder was coated with 5 and 10% palm oil, lecithin or a mixture of thereof (75% palm oil and 25% lecithin).

Sorption Isotherms

The EMC for the coated powders was calculated in terms of g total water in the coated powder per g total dry matter. The coating agent is not 100% dry solids and therefore the initial water present in the coated powder is the sum of the water present in the protein powder and the water in the coating agent. The moisture content of the coated powders and the non-coated powder was calculated by infrared drying and the results are present in Table 5.

Table 5 Moisture content (%) for each coated powder measured by infrared drying

	5%	**10%**
Palm Oil	6.63	6.04
Lecithin	6.95	7.93
Palm Oil/Lecithin	6.64	6.63

The moisture content of the non-coated powder was also measured and it had a value of 5.52%. The resulting sorption isotherms are presented in Figure 19.

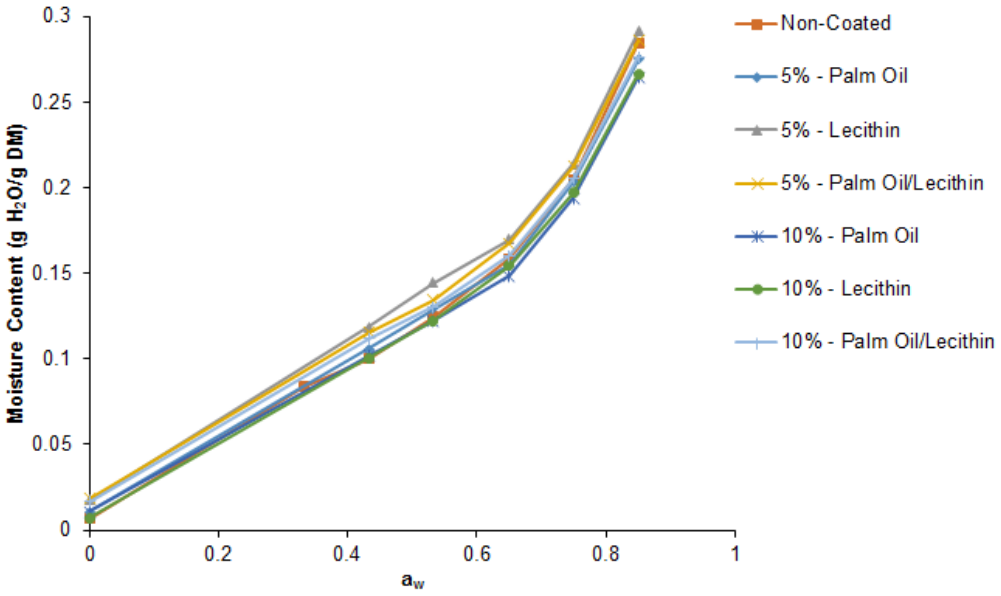

Figure 19 Sorption Isotherms of the coated powders.

All the powders have similar EMC for each water activity. Moreover, there is no difference in the sorption behavior between the coated and non-coated powders. However, as can be seen in Figure 20, the 10% coated powders have less water sorption capacity than the 5% coated powders.

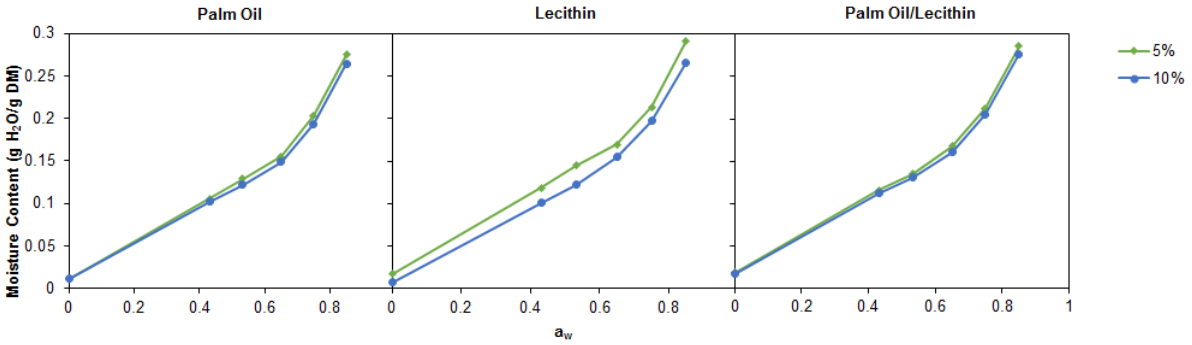

Figure 20 Sorption Isotherms of the coated powders: comparison between the 5 and 10% coated powders.

3.2.4. Insoluble MPC90

Solubility of pre-treated MPC90

The solubility of the pre-treated MPC90 was tested and the results are shown in Figure 21. The pellet that can be seen for the MPC90 stored at 60ºC and for the microwaved MPC90 confirm that these treatments resulted in the formation of insoluble powder particles.

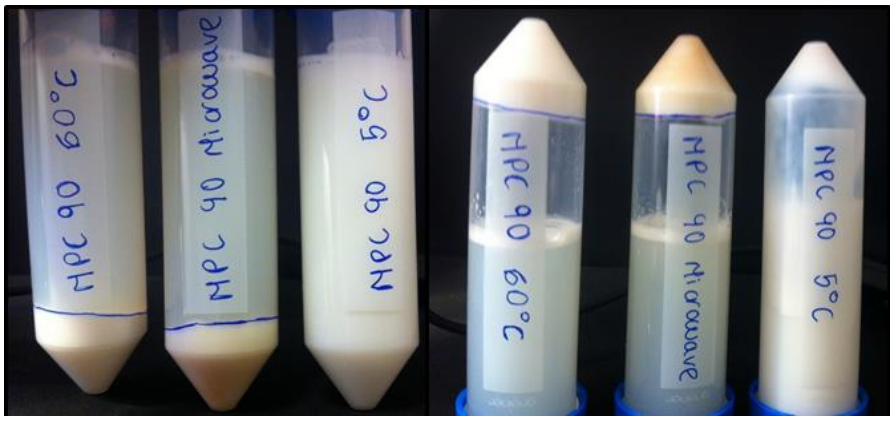

Figure 21 MPC90 reconstituted at 4% powder content following centrifugations for 15 min at 700×g; from left to right: MPC90 pre-treated at 60ºC, MPC90 pretreated in the microwave, and fresh MCP90.

3.3. High Protein Bars

3.3.1. High Fructose Corn Syrup – Carbohydrate concentration

Texture

To investigate how the hardening of the bars is influenced by the concentration of the carbohydrate syrup, three sugar solutions with different content of solids were made. Table 6 shows the amount of each component in 100 g of sugar solution. The sugar solution is considered as a high fructose corn-like syrup (HFCS) as it is composed of fructose and glucose dissolved in water.

Table 6 Composition of the carbohydrate solutions

	65%	**70%**	**75%**
Fructose	32.5	35.0	37.5
Glucose	32.5	35.0	37.5
Water	35.0	30.0	25.0

The texture results acquired over 4 weeks of storage at 37ºC are shown in Figure 22. The 13 protein ingredients where separated into 3 different groups: casein-based, whey-based and hydrolysates (WPH and casein hydrolysates). Casein hydrolysates resulted in softer bars with a texture comparable to a viscous solution. The results for the casein hydrolysates are presented in Figure 23 as they had lower values of hardness, and therefore a smaller scale was required. Hardness increased as a function of time in all bars and the extent of hardness was different between the protein bars. The hardness is expressed as the force needed applied by the probe at 3 mm distance into the bar as a function of storage time at 37ºC.

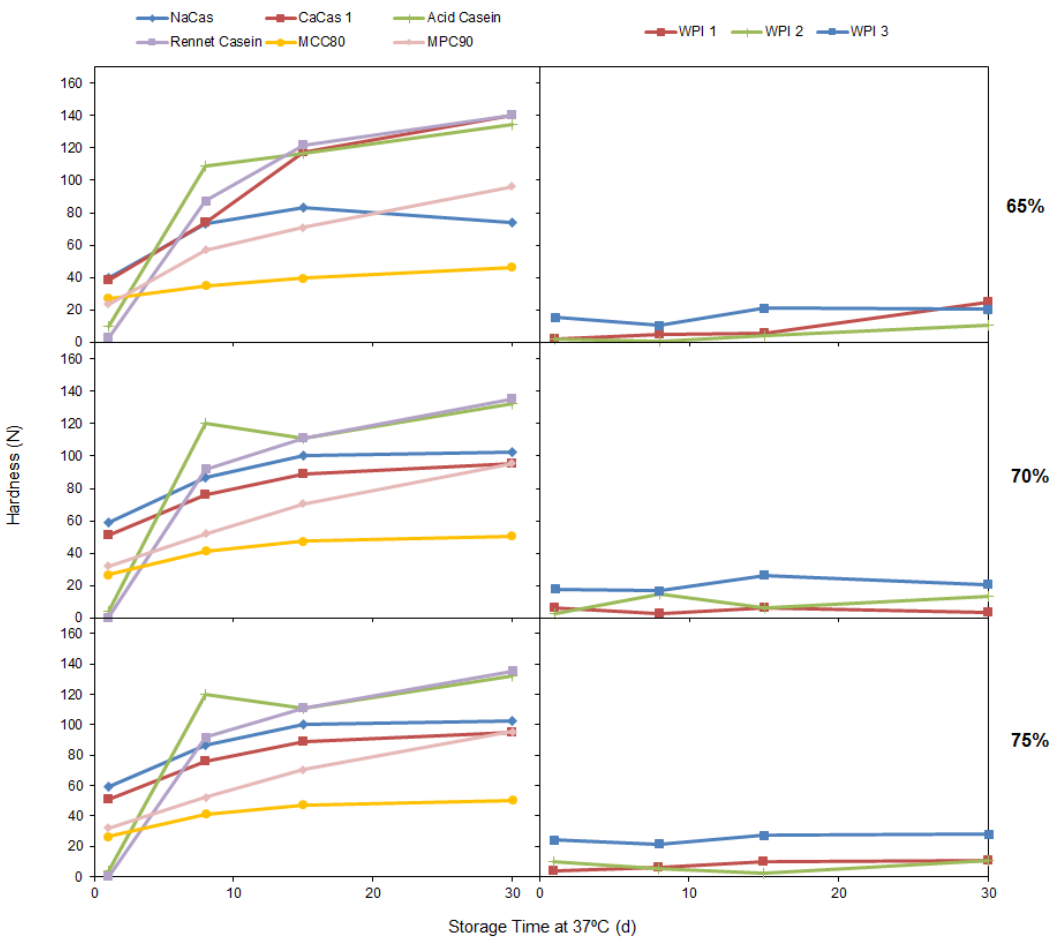

Figure 22 Hardness (N) over 30 days of storage at 37ºC of bars prepared with different protein ingredients and with different carbohydrate concentrations.

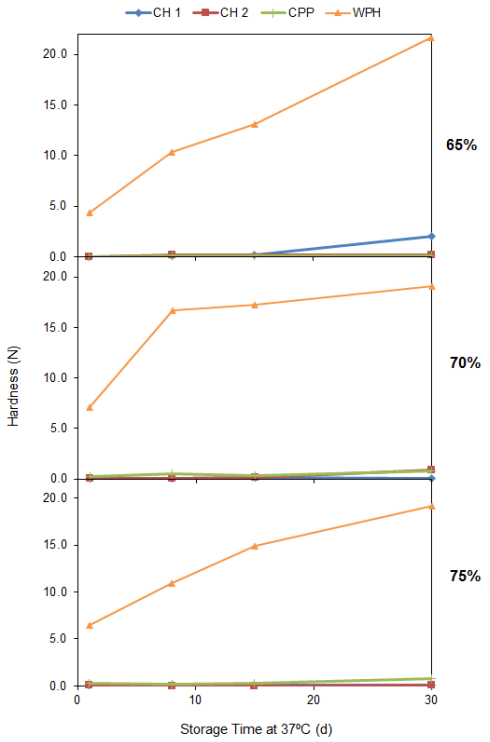

Figure 23 Hardness (N) over 30 days of storage at 37ºC of bars prepared with different hydrolysates and with different carbohydrate concentrations.

From the intact protein ingredients, WPI resulted in the formation of the softest bars. On other hand, WPH resulted in the hardest bars out of all the hydrolysates. In the group of the casein-based protein ingredients, MCC80 made the softest bars for all the concentrations of carbohydrate solution. Although all the bars showed an increasing in hardness over time, if a longer distance is considered, it can be observed a decrease in the force value. This happens because the fracture point is reached. In Figure 24 is the graphic representation of the force depending on the distance measured for acid casein, rennet casein, MCC80 and MCP90. The maximum of the curve indicates the fracture point of the sample, that is around 4 mm.

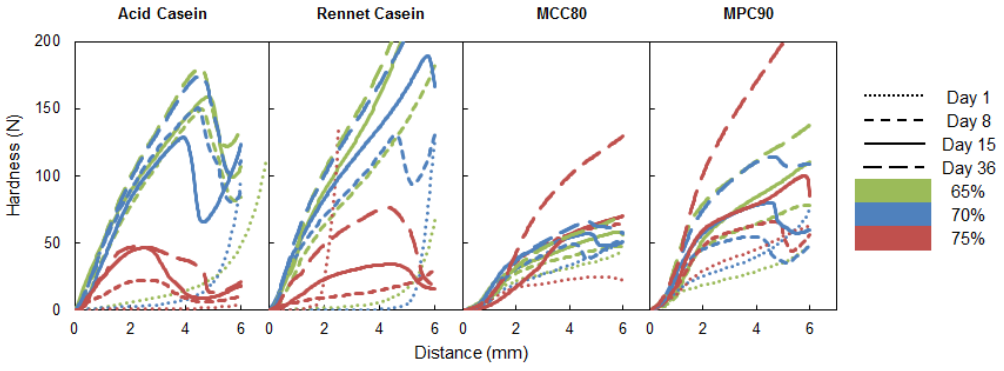

Figure 24 Force applied to penetrate the protein bar samples down to a distance of 6mm for acid and rennet casein, MCC80 and MPC90.

Water Activity

Water activity depends, amongst others, on the carbohydrate syrup concentration. The bars prepared with 65% carbohydrate syrup had higher water activity, due to the higher amount of water present in the bars. Also, a_w decreased with storage time for most the protein powders used.

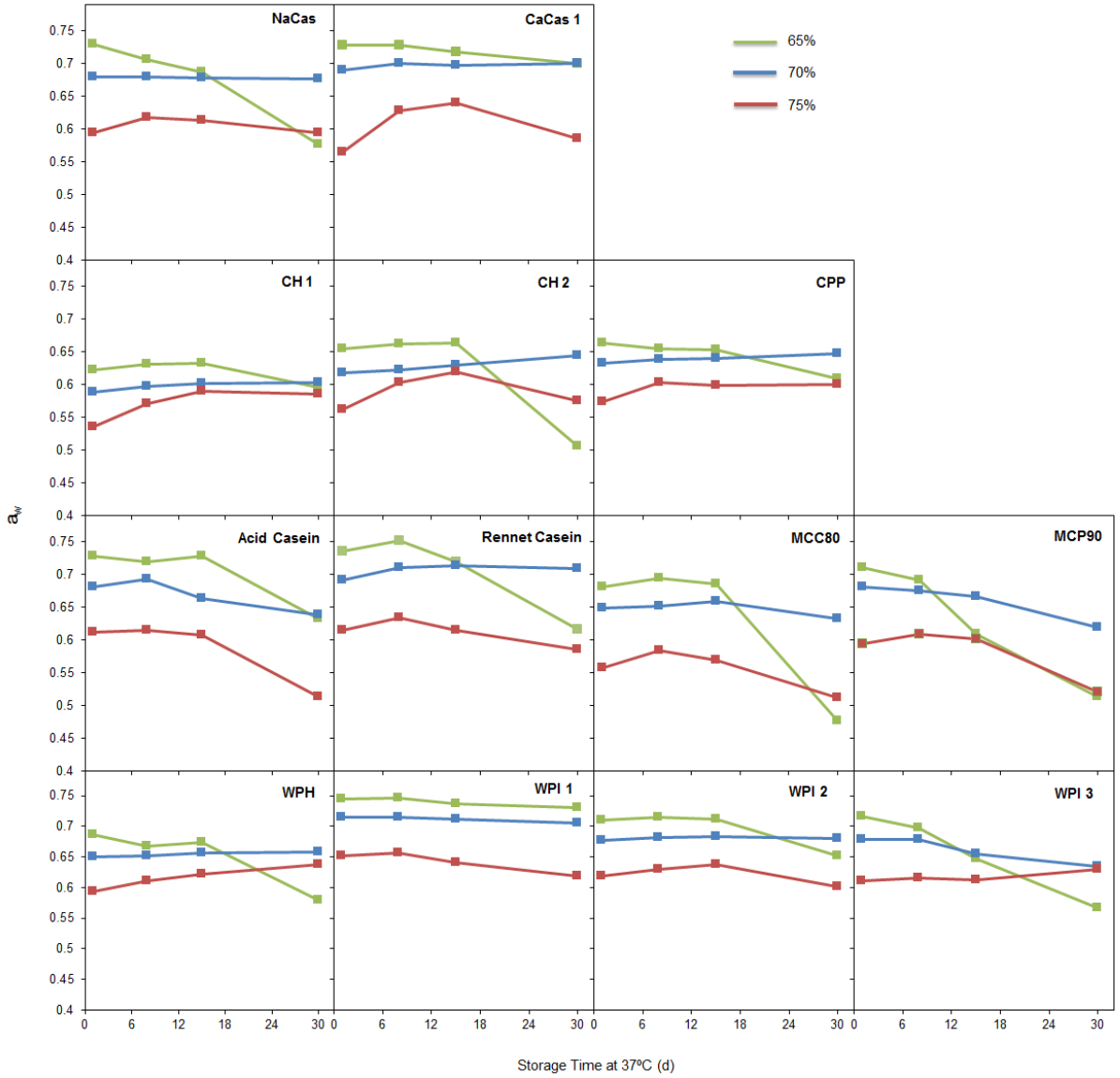

Figure 25 Water activity of the bars prepared with HFCS and with 13 protein ingredients over 30 days of storage at 37ºC.

Color Changes

The changes in color are caused by Maillard reaction, specifically noticeable in the case of the bars prepared with casein hydrolysates, Figure 26. Some changed color from white to dark/black in the in the 30th day of storage. The comparison between the different casein hydrolysates,

carbohydrate concentrations and storage time is presented in Figure 26. The differences in color for the casein and whey protein-based bars (with the 70% carbohydrate syrup) are presented in Figure 26 and Figure 27, respectively.

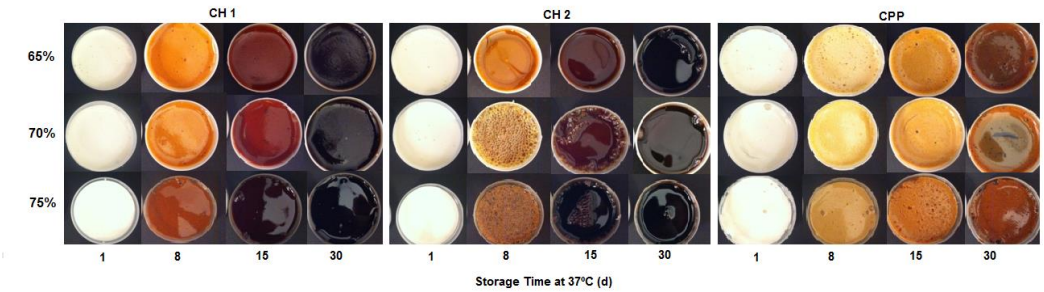

Figure 26 Color changes in the bar prepared with casein hydrolysates over 30 days of storage at 37ºC.

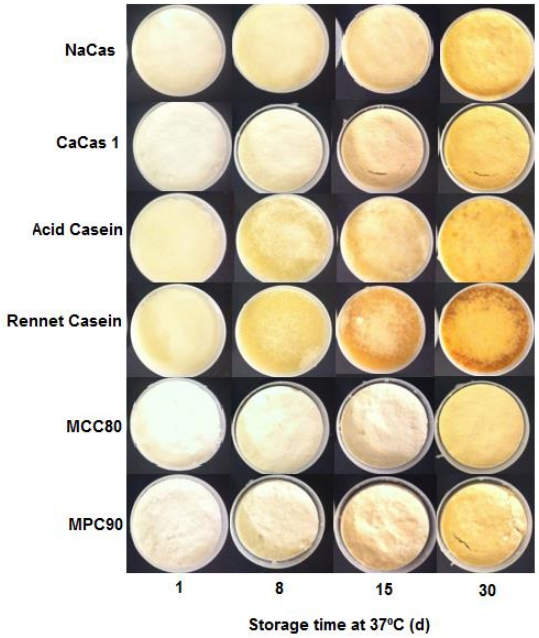

Figure 27 Color changes in the bars prepared with casein-based powders and a 70% carbohydrate syrup over 30 days of storage at 37ºC.

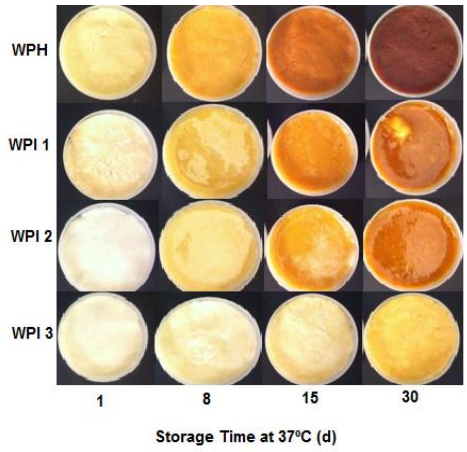

Figure 28 Color changes in the bars prepared with whey-based powders and a 70% carbohydrate syrup over 30 days of storage at 37ºC.

All the syrups used in the next experiments were made using a 70% carbohydrate concentration (70 g carbohydrate / 100 g solution).

3.3.2. Effect of reducing and non-reducing carbohydrate, and of sugar alcohol on protein bar hardness

Maltose and sucrose are reducing and non-reducing sugars, respectively, with similar solubility in water. Maltitol is the equivalent sugar alcohol of maltose and it also have similar solubility in water to sucrose and maltose.

Texture

The hardness (N) of NaCas bars is expressed as the force needed applied by the probe at 3 mm distance into the bar as a function of storage time at 37ºC. Hardness started at similar values and increased with time at the same rate for all the bars, regardless of the type of sugar used. The results are presented in Figure 29.

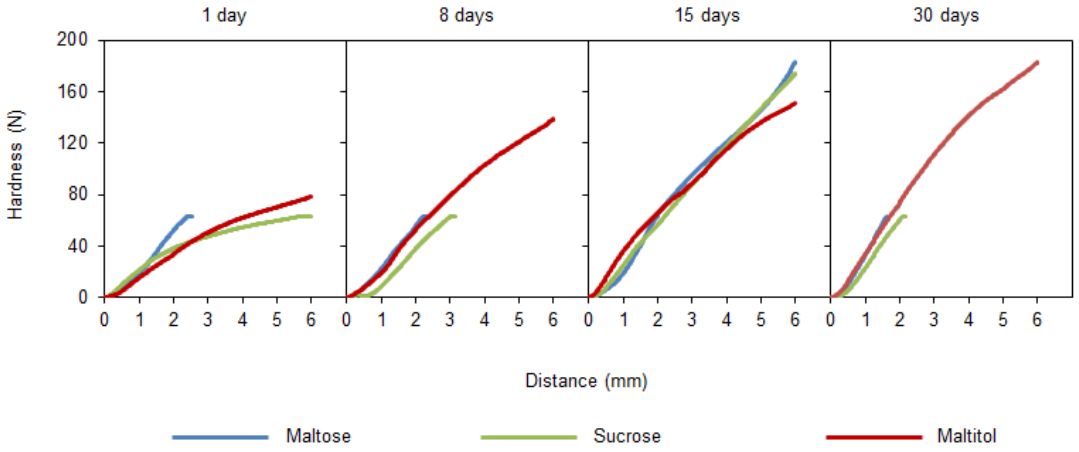

Figure 29 Hardness (N) of the bars prepared with a 70% maltose, sucrose and maltitol syrups as a function of storage time (d) at 37 ºC.

Water Activity

Water activity showed a decrease with the storage time and had higher value for the bars prepared with maltose syrup, probably due to the formation of water through Maillard reaction.

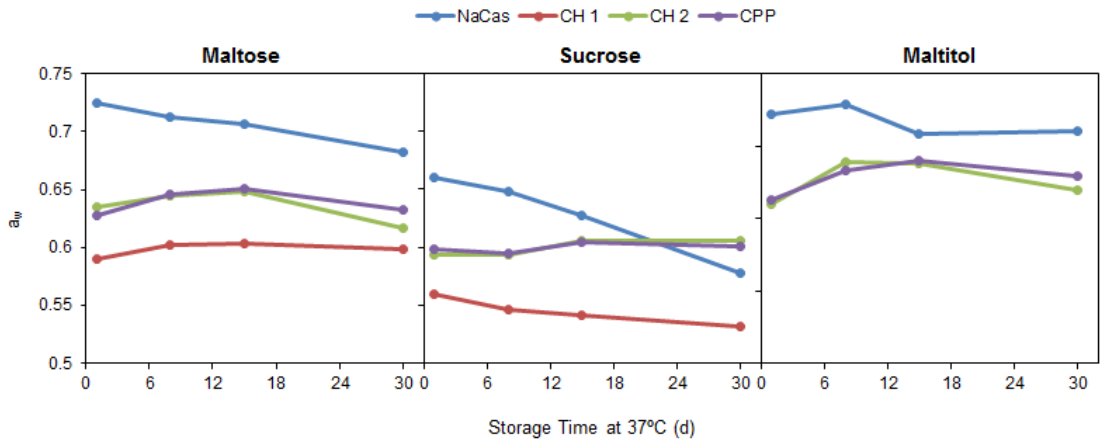

Figure 30 Water activity of the bars prepared with maltose, sucrose and maltitol syrups, and with NaCas, CH 1, CH 2 and CPP over 30 days of storage at 37ºC.

Free amino residues (OPA Method)

In order to investigate the extent of the Maillard reaction, the free amino groups residues in the protein bars prepared with NaCas, CP, CH 1 and CH 2, and sucrose or maltose syrup, were quantified with OPA method during storage of the bars at 37ºC. As it was expected, samples prepared using maltose syrup showed a decrease in the concentration of leucine equivalent over time, whereas the bars prepared with sucrose syrup the leucine equivalent concentration remained almost constant. Maltose and sucrose are both disaccharides. Unlike maltose, sucrose is a non-reducing disaccharide and therefore it does not participate in Maillard reaction. The results are shown in Figure 31.

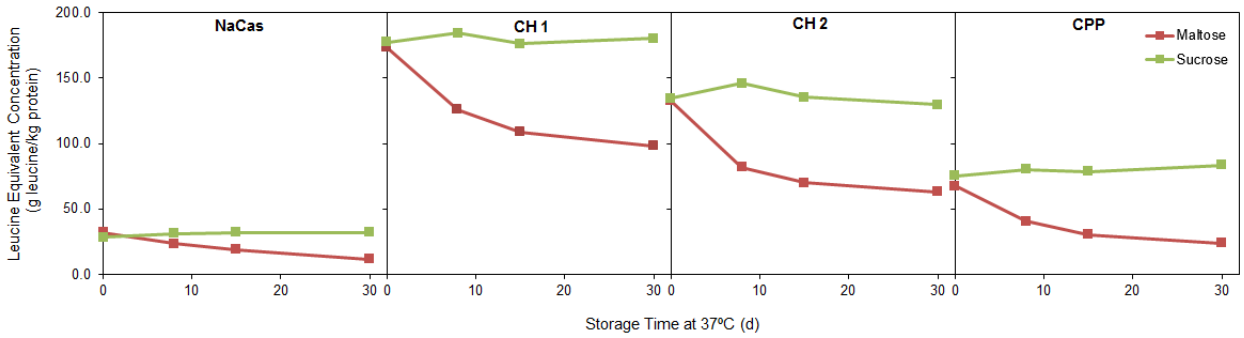

Figure 31 Changes in the leucine equivalent concentrations as a function of storage time (d) at 37ºC.

Color Changes - Maltose and Sucrose

Bars prepared with maltose and sucrose syrups underwent different color changes. Discoloration is highly perceptible over time in the samples prepared with maltose, which is the result of the non-enzymatic browning. In the samples made with maltose is highly perceptible the browning over time, which is a result of the enzymatic browning. Sucrose, in the other hand, a non-reducing disaccharide, produced samples with small changes over the 30 days of storage at 37ºC. The discoloration in bars made with sucrose can be associated with the presence of lactose, a reducing sugar, in the protein powder ingredients. The results are presented in Figure 32.

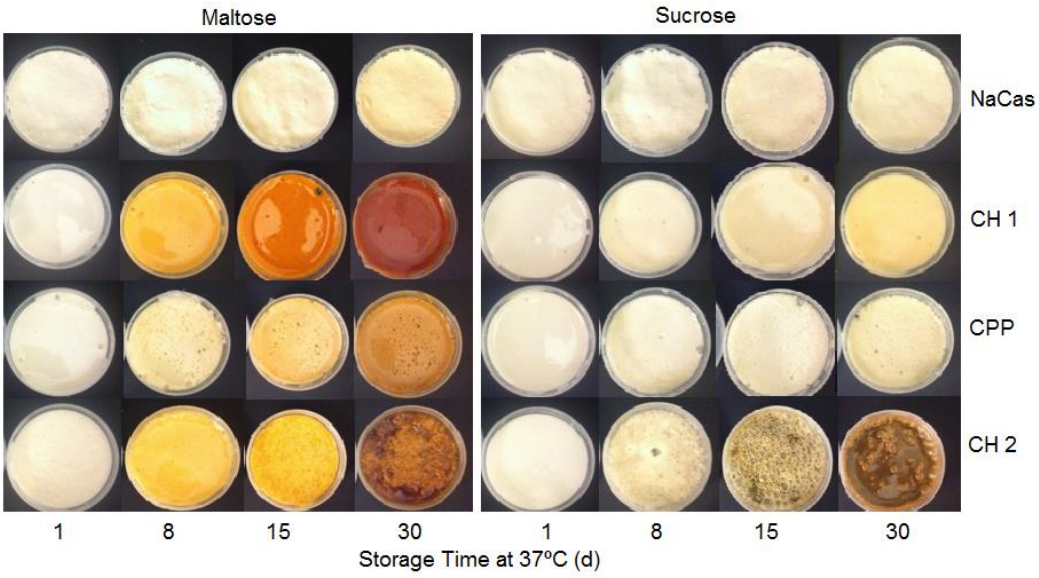

Figure 32 Color changes in bars prepared with maltose and sucrose syrups during storage for 30 days at 37º.

3.3.3. Effect of powder properties on the texture of protein bars

High protein bars were prepared using 3 types of CaCas powders, i.e., 2 spray-dried powders from different suppliers, and 1 roller-dried powder. The hardness over time of the bars prepared with the different CaCas powders is shown in Figure 33. The results presented showed that hardness of the bars prepared with the same type of protein ingredient can depend on the powder properties of the protein ingredient. The bars prepared with CaCas 2 were harder than the ones prepared with CaCas 1 and CaCas R and these differences can be associated with the properties of each protein powder (powder particle size, powder particle density and occluded air).

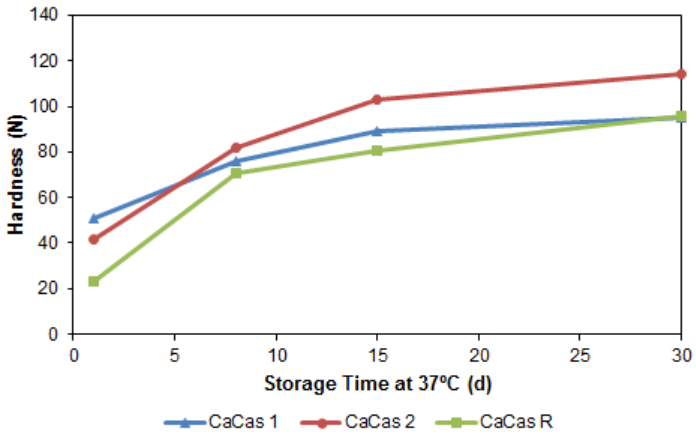

Figure 33 Hardness (N) of protein bars prepared with different CaCas powders, i.e., CaCas 1, CaCas 2 and CaCas R.

3.3.4. Pre-equilibrated powders

The water gained in the pre-equilibration was calculated for each powder and an 80% carbohydrate syrup was then diluted with the specific amount of water needed for each protein bar. Also, it was assured that all bars had the same amount of protein material: the water absorbed by the powders was compensated in the carbohydrate syrup. The final composition of the bars (protein powder, carbohydrate, water and glycerol) was the same for all the powders used.

Texture

The texture results are presented in Figure 34. It was found that pre-equilibration of protein powders prior to bar manufacture result in an efficient softening of the bars. The effect was more pronounced for NaCas than for WPH or WPI 2. Although softer, NaCas remained more susceptible to bar hardening than WPH and WPI 2.

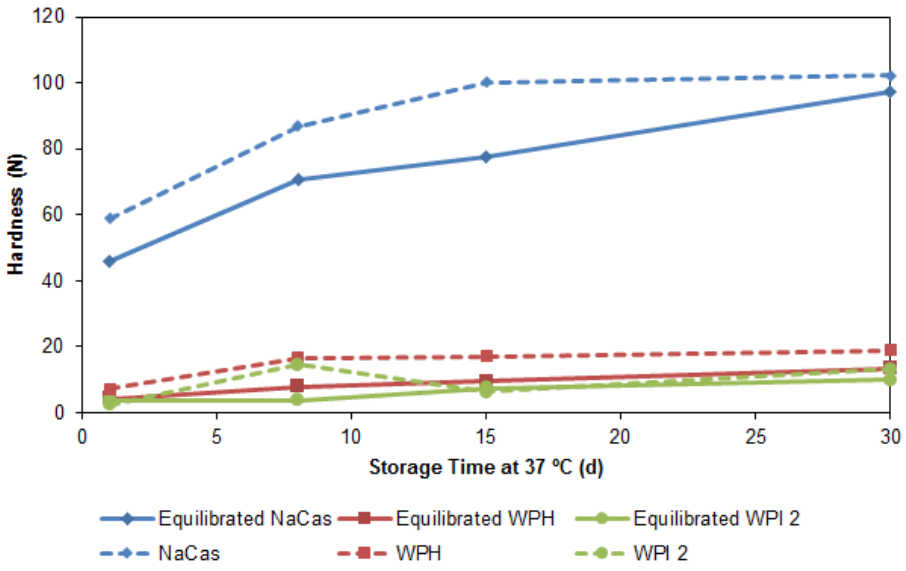

Figure 34 Hardness (N) over 30 days of storage at 37ºC for the pre-equilibrated powders.

Water Activity

Once again, water activity stayed almost constant over the 30 days of storage, ranging between 0.64 and 0.7. The results are presented in Figure 35.

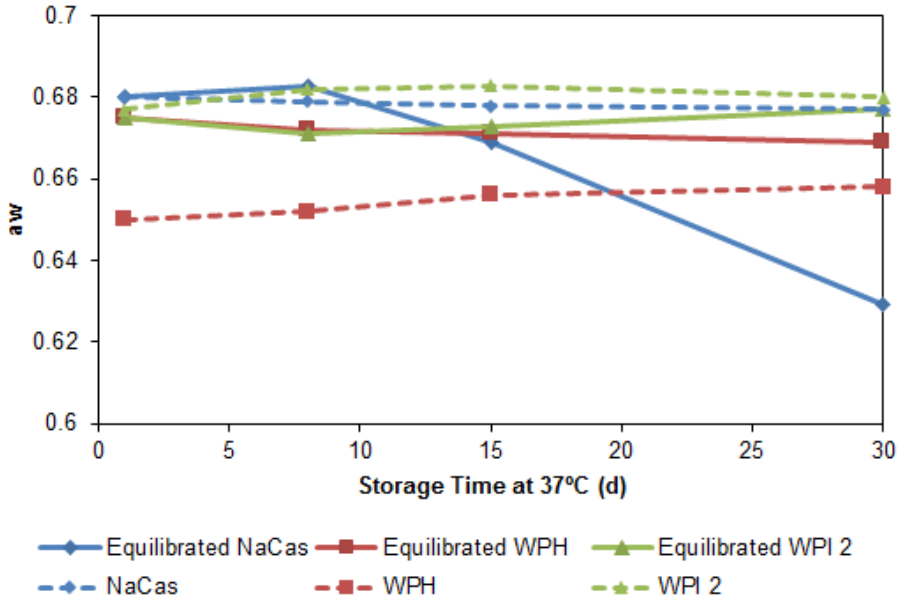

Figure 35 Water activity over 30 days of storage at 37ºC for the pre-equilibrated powders.

3.3.5. Fractionated Powders

Texture

The results of hardness over time for the bars prepared with fractionated powders are presented in Figure 36. As would be expected, the bars prepared with CaCas 1 were harder to begin with, and developed higher hardness over time than the bars prepared with WPI 2.

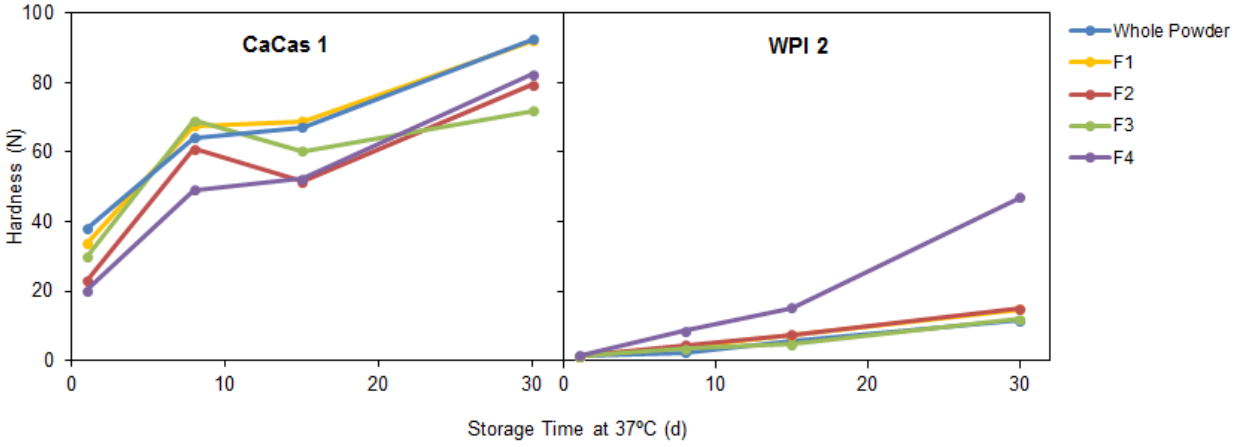

Figure 36 Hardness (N) over 30 days of storage at 37ºC of the bars prepared with fractionated powders.

Water activity

The water activity values of the bars prepared with CaCas 1 and WPI 2 fractions are shown as a function of storage time at 37ºC in Figure 37. The decrease in water activity was higher for the bars prepared with CaCas 1 fractions, compared to the ones prepared with WPI 2 fractions. The fraction with the lowest final value for activity was F3 for CaCas 1 and F4 for WPI 2.

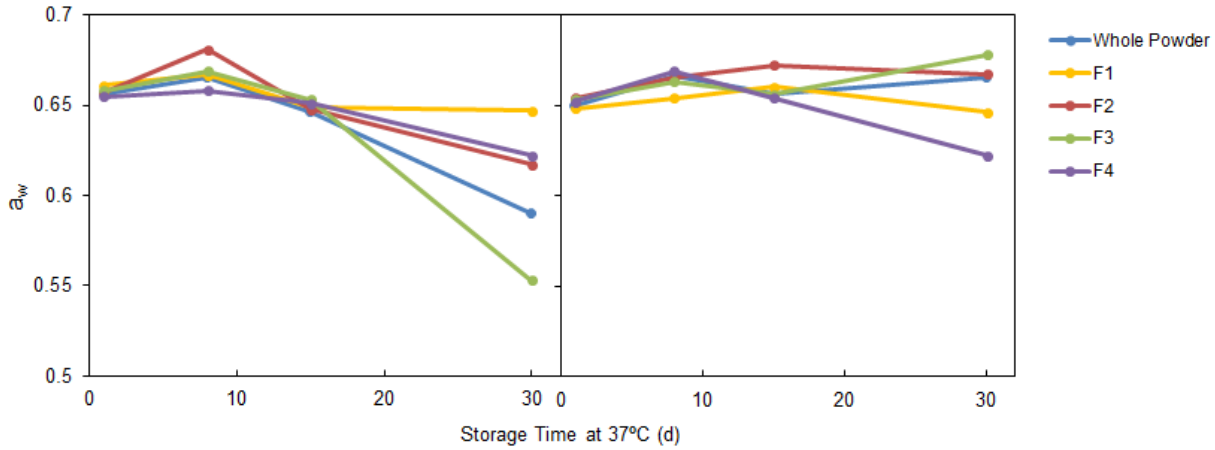

Figure 37 Water activity over 30 days of storage at 37ºC of the bars prepared with fractionated powders.

3.3.6. Protein Blends

When mixed together, some protein materials can have synergistic effects and produce a softer bar than the actual single ingredients. Protein blends of NaCas with CH 2 and WPH with WPI 2 were prepared using different ratios (Table 7).

Table 7 Ratios of the protein ingredients in blends and percentages of each protein powder in mixture

Ratio	NaCas (% in NaCas : CH 2) WPI 2 (% in WPI 2 : WPH)	CH 2 (% in NaCas : CH 2) WPH (% in WPI 2 : WPH)
0:1	0	100
1:4	20	80
1:2	33	67
1:1	50	50
2:1	67	33
4:1	80	20
1:0	100	0

Texture

The mixtures were then used to prepare bars that were stored during 30 days at 37ºC. The texture results are presented in Figure 38. For the mixture with WPI 2 and WPH it was found that the hardness of the single ingredients is higher than the hardness measured for the mixture of both protein ingredients, which shows that these protein powders together can develop synergistic effects and produce a softer bar.

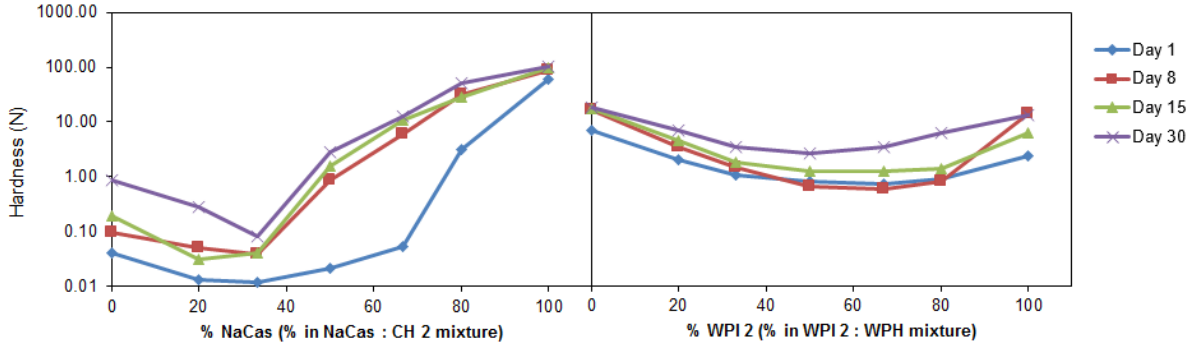

Figure 38 Hardness (N) over 30 days of storage at 37ºC of the bars produced with the protein blends. The values of hardness are represented in a logarithmic scale.

Water Activity

The water activity values were also measured and the results can be seen in Figure 39. Water activity showed a small variation over the 30 days of storage and it remained always between 0.6 and 0.7. The water activity of the bars produced with protein blends mostly remained in between the water activity levels of the bars produced with the individual protein ingredients.

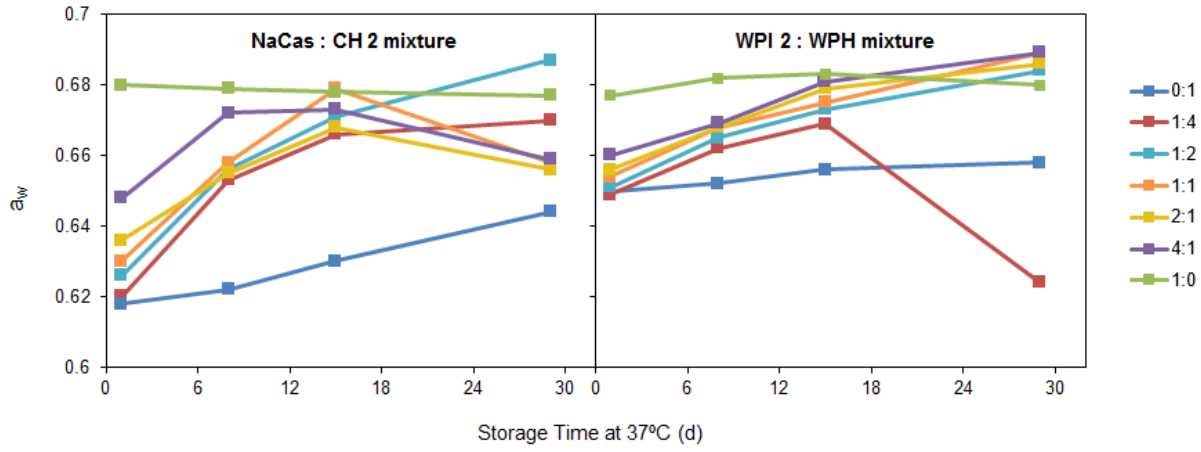

Figure 39 Water activity over 30 days of storage at 37ºC of the bars produced with the protein blends.

3.3.7. Coated Powders

In order to evaluate the influence of a coated particle surface on protein bars hardness, NaCas powder was coated with palm oil or lecithin, or with a mixture of both (75% palm oil and 25% lecithin). Two concentrations coating material were tested (5 and 10%). Two types of control samples were prepared: bars with non-coated NaCas where the coating material was added to the bar formulation, and bars with no lecithin and no palm oil. Table 8 and Table 9 represent the formulation of the bars.

Table 8 Formulation with coated and non-coated powders with 5 or 10% of single coating material and with no lecithin or palm oil added

	NaCas coated 5%	NaCas coated 10%	Non-coated NaCas 5%	Non-coated NaCas 10%	NaCas
Palm oil/Lecithin (g)	0	0	2.25	4.5	0
Protein Powder (g)	47.25	49.5	45	45	45
Glycerol (g)	10	10	10	10	10
Sugar Syrup (g)	45	45	45	45	45
Total (g)	102.25	104.5	102.25	104.5	100

Table 9 Formulation with coated and non-coated powders with 5 or 10% of a 75% palm oil and 25% lecithin mixture and with no lecithin or palm oil added

	NaCas coated 5%	NaCas coated 10%	Non-coated NaCas 5%	Non-coated NaCas 10%	NaCas
Palm oil (g)	0	0	1.69	3.38	0
Lecithin (g)	0	0	0.56	1.13	0
Protein Powder (g)	47.25	49.5	45	45	45
Glycerol (g)	10	10	10	10	10
Sugar Syrup (g)	45	45	45	45	45
Total (g)	102.25	104.5	102.25	104.5	100

Texture

The hardness of the bars with coated and non-coated powder particles was plotted against the storage time and compared to the hardness of a NaCas bar with no coating agent added to the formulation. The results are shown in Figure 40 and revealed a softening effect of the coated particles that gave softer bars than the pure NaCas bars. In the 30th of storage all the powders reached a hardness close to the NaCas bars with no coating agent added. Lecithin (coated and non-coated) remained softer than the others. However, there is no difference in using fat (palm oil) or an amphiphilic molecule (lecithin). Moreover, the difference between the coated and non-coated powders was not noteworthy.

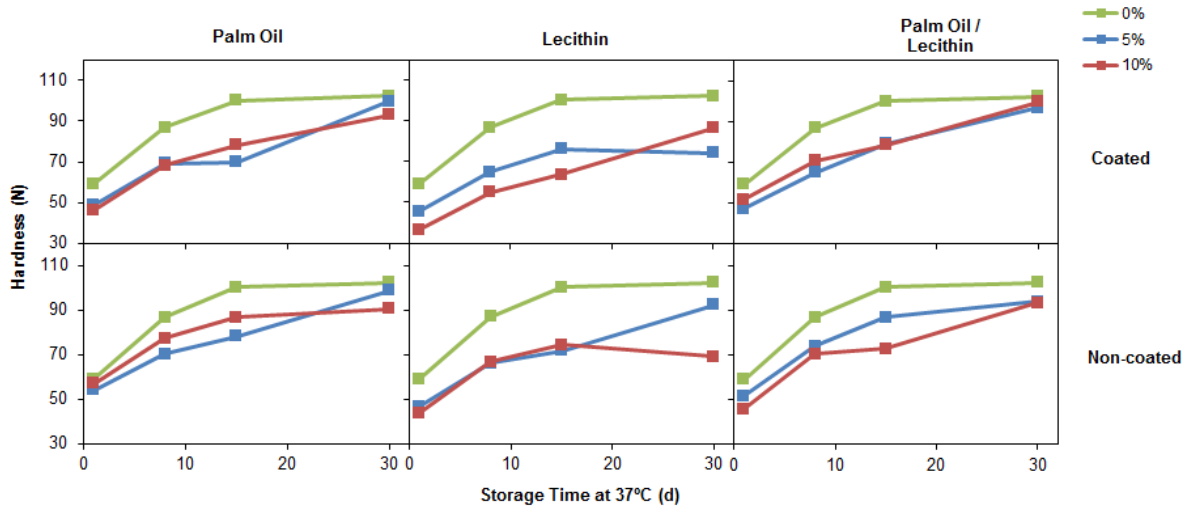

Figure 40 Hardness (N) over 30 days of storage at 37ºC of the bars with produced with the coated and non-coated powders, and for the bars with no lecithin or palm oil added.

Water Activity

The results for water activity are present in Figure 41. Water activity of the coated and non-coated powders stayed almost constant over time, ranging between 0.65 and 0.7 for all the powders tested.

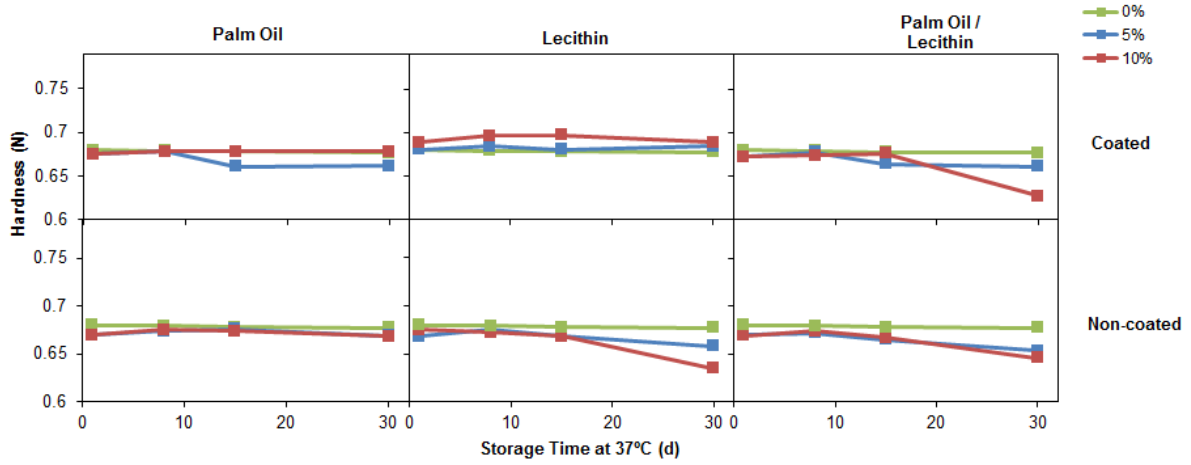

Figure 41 Water activity over 30 days of storage at 37ºC of the bars with produced with the coated and non-coated powders, and for the bars with no lecithin or palm oil added.

3.3.8. Insoluble MPC 90

The three powders tested for solubility were used to make protein bars in to order to evaluate the influence of an insoluble powder in the bar formulation. The texture and water activity were measured on the day after making the bars (day 1), 8th and 15th day. The results are presented below.

Texture

Insoluble MPC90 (stored at 60ºC or microwaved) resulted in harder bars than the soluble/control MPC90 with an increase in hardness over the storage time. The results can be seen in Figure 42.

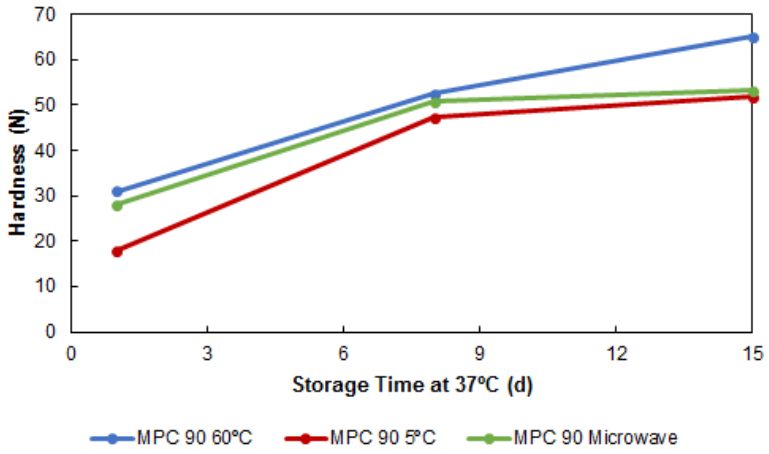

Figure 42 Hardness (N) over 15 days of storage at 37ºC for the MCP90 powders.

Water Activity

Water activity remained constant over the storage time and it is presented Figure 43.

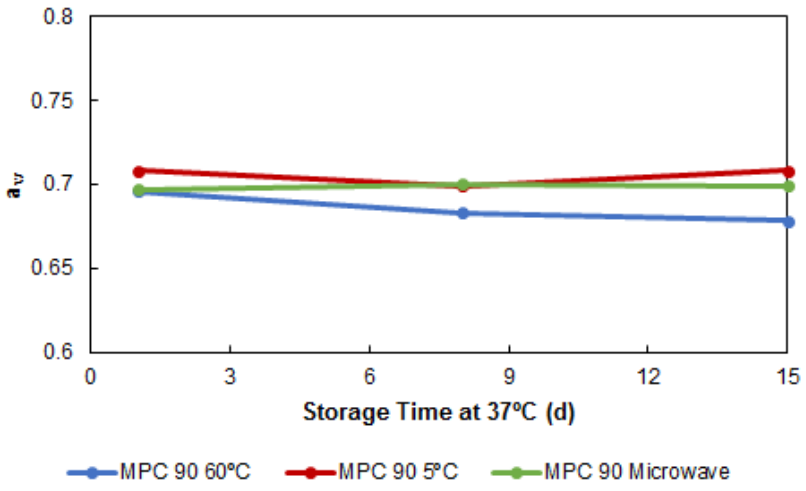

Figure 43 Water activity of protein bars prepared with the different MPC90 powders over 15 days of storage at 37ºC.

4. Discussion

4.1. Carbohydrates

The carbohydrates are one of the main ingredients present in the formulation of protein bars and they will therefore have an influence over the texture of the final product. Each carbohydrate has a characteristic solubility limit and this limit is a determinant factor to the final texture of the carbohydrate syrup and, as a consequence, for the protein bar. The use of different concentrations of carbohydrate in solution, varying from -10 to +10% of the solubility limit for each carbohydrate showed a gradual increase in the surface tension of the resulting solution. Fructose is the carbohydrates that has the major increase, from 0.05N (-10% of the solubility limit) to 0.33N (+10% of the solubility limit). Fructose is also the one which has the higher solubility limit. When mixtures of the different carbohydrates were tested the trend was the same: an increase in hardness with the increase in the solubility limit (Figure 4). The water activity values are directly related with the amount of water added in each solution and they therefore decreased with the increase of carbohydrate in the solution.

The three different carbohydrate syrup concentrations tested (65, 70 and 75%) revealed that the final hardness of the bars was not influenced by the carbohydrate concentration used in the bars formulation. The discussion and the conclusions will be based on the 70% sugar concentration.

As mentioned before, maltose is reducing sugar and therefore is able to participate in non-enzymatic browning. Maillard reaction has been reported as one of the possible contributors for the hardening of the bars. Although the small difference in texture between this two disaccharides, Maillard reaction were found to have less or no influence in the texture of bars.

The extent of Maillard reaction is confirmed by the decrease in the leucine equivalent over time presented in Figure 31 for maltose. This leucine equivalent corresponds to the free amino groups that reacted with the reducing sugar through non-enzymatic browning, which lead to a decrease in their concentration. The same is confirmed by the color changes observed during storage. Bars with maltose underwent a noticeable browning, unlike the bars with sucrose that remained light-colored over the storage time.

4.2. Powder Properties and High Protein Bars

Casein-based powders

Hardness of protein bars is extremely dependent on the type of protein used. The proteins used were divided into three groups: casein-based, whey-based and casein

hydrolysates. The casein-based are composed of NaCas, CaCas 1, acid casein, rennet casein, MCC80 and MPC90. As shown in Figure 22, for all the carbohydrate syrup concentration tested, acid and rennet casein were found to form the hardest bars, followed by the NaCas and CaCas 1. Acid and rennet caseins are considered insoluble-casein ingredients, as they are obtained by insolubilizing caseins. Although insoluble, acid and rennet casein are not inert, but they can interact and can hydrate by taking water. On the other hand, NaCas and CaCas are highly soluble in water (McSweeney & O'Mahony 2013). In general, it can be said that NaCas gave harder bars than CaCas 1. The difference in hardness for these two caseinates is caused by the presence of mono- or divalent cations. For monovalent caseinates as NaCas, the casein-casein interactions are dominated by electrostatic repulsions because the interactions between cation-polyanion are much weaker. This repulsion overcomes the hydrophobic association energy resulting in loose aggregates that are highly hydrated and exhibit high apparent viscosities in solution. In the case of divalent cations as CaCas, the inter- and intra- casein molecule bonds dominate causing a rearrangement of the casein in order to minimize the repulsion and form micelle-like particles with charged κ-caseins on the surface. This divalent caseinates are poorly hydrated and compact, giving origin to less viscous solutions than monovalent cations (Carr et al. 2003). Another fact that can be pointed out as a cause for the differences in texture is the presence or absence of whey proteins. These proteins are completely absent in caseinates and acid and rennet casein but they are part of MPC and MCC composition (Loveday et al. 2010). In general, the protein content in MPCs is composed by 80% caseins and 10% whey proteins. In the case of an MPC90, the 90% protein in the powder is in fact 72% caseins and 18% whey proteins. MCCs, on the other hand, are composed by ≥ 90% caseins and ≤ 10% whey proteins, that in the case of MCC80 (with 80% protein in its composition) totals 72% caseins and 8% whey proteins (McSweeney & O'Mahony 2013). Therefore, the softness of the bars made with MCC80 can be explained by lower protein content the powder material (90% in MCP90 versus 80% in MCC80)

Whey-based powders

The water activity of the dry powder material plays also an important role when differences in hardness of same powders types are compared. (Hogan et al. 2012) reported that differences in WPI-based bars could be largely related with differences between the a_w of powder and liquid phases, then other physical factors. In fact, it is well known that water migration occurs from the regions of high a_w to regions of low a_w and the discontinuities of water activity between bar ingredients (or micro-regions) are one of the major forces for water migration. From Figure 22 can be observed that a bar with WPI 3 in its composition had a harder texture than a bar made with WPI 1 or 2. The results are in line with the previous studies (Hogan et al. 2012), since WPI 3 had the lowest water activity value (a_w=0.241), followed by WPI 2 (a_w=0.242) and WPI 1 (a_w=0.477). These results show that minimizing the differences between the a_w of the different protein bar ingredients can improve bar stability and decrease moisture migration.

The main difference in using whey protein and casein-based ingredients is that the presence of whey proteins appears to plasticize the bar matrix.

Hydrolyzed powders

Figure 13 indicates that hydrolyzed proteins have a higher moisture sorption capacity than non-hydrolyzed proteins. During hydrolysis, peptides with different sizes are generated and their size will define the degree of hydrolysis, DH. A high DH is due to the presence of low molecular weight peptides. These small peptides have increased hygroscopicity due to the existence of more hydrophilic groups exposed and could further increase the plasticizing effect, leading to a decrease in the glass transition temperature, Tg. The Tg is defined as the transition of the physical state “glassy” to a “rubbery” state and it is time, composition and temperature dependent (Rao et al. 2016).

Solubility, EMC and viscosity

High protein bars are mostly composed of carbohydrate syrup and a protein powder material. Basically, the mixture of these two ingredients can produce two types of bars: a solution-like bar, where the protein is dissolved in the carbohydrate syrup and it forms a homogenous mixture; or a two-phase bar, where there is a carbohydrate phase which is absorbed into swollen protein powder particles. Figure 15 indicates that casein hydrolysates and whey protein isolates are soluble in a solution with 45 g carbohydrate syrup and 10 g protein powder. On other hand, it is not clear whether sodium caseinate is completely dissolved, or whether insoluble particles were still present in the solution. The same was confirmed by (Loveday et al. 2010) who found, by confocal microscopy, that WPI particles were almost completely dissolved in the bar matrix and bars made with calcium caseinate had some undissolved powder particles.

As previously mentioned, the water activity of protein bars is close to 0.65. Figure 44 shows the equilibrium moisture content at a_w=0.65 for protein bars prepared with different the protein ingredients. It was found that acid and rennet casein, as well as MCC80 are the powders that absorb the least water at a_w=0.65. In the other extreme are casein hydrolysates that absorb almost 0.3 g of water per g dry matter.

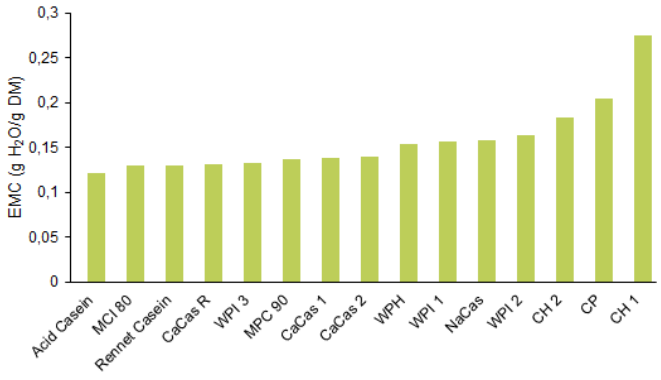

Figure 44 EMC at a_w of 0.65 for all the protein powders tested.

These protein ingredients can be divided into 5 categories: acid and rennet casein, casein micelles, caseinates, whey protein isolates and hydrolysates. The average EMC within these groups is shown in Figure 45.

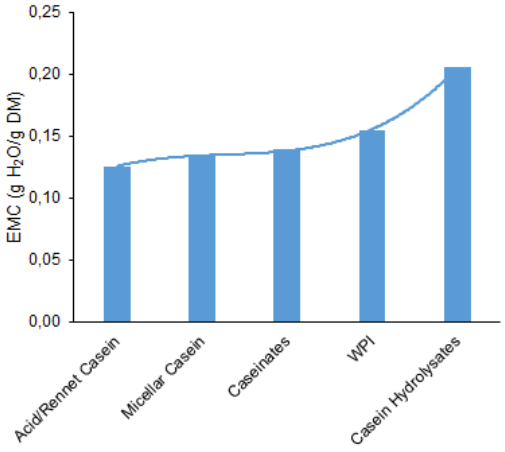

Figure 45 Average EMC at a_w of 0.65 for the 5 protein categories

The resulting texture of the bars can be explained by the solubility of the powder particles and the capacity to absorb water:

- casein hydrolysates have the highest water sorption capacity and are completely soluble. Bars made with these protein powders resulted in viscous solutions with a sticky texture;
- whey protein isolates are soluble, however they have a lower EMC at a_w of 0.65 than casein hydrolysates, resulting in concentrated solutions with a dough-like texture;
- caseinates-based bars contain soluble and insoluble particles and they have low water soprtion capacity, resulting in a hard bar with an appearance of something

between a concentrated solution and a particle gel. The same is valid for micellar caseins but they have a lower water sorption capacity than caseins;

- with insoluble particles and the lowest EMC are acid and rennet caseins that produced bars with a texture of a particle gel, dry and crumbly.

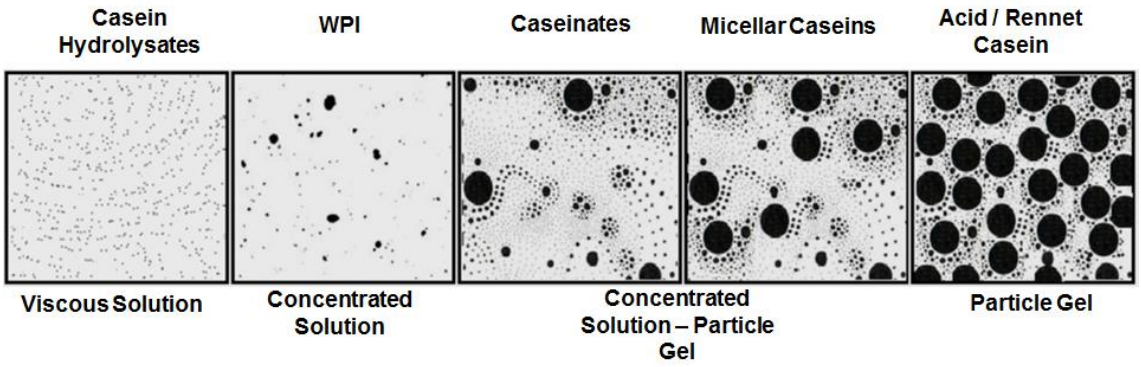

Figure 46 Illustration of the distribution of the protein powder particles within the bar matrix.

The bar matrix can be divided into two phases: a liquid phase composed by the carbohydrate syrup and glycerol and a powder phase composed by the powder particles. In the case of hydrolysates this phase separation is almost nonexistent because the powder is completely soluble in the liquid phase. However, other protein powders give origin to a two phase system. Micellar casein and caseinates-based powders are not completely soluble and so they will have some particles in the powder phase. Acid and rennet casein are completely insoluble, which means that the bar matrix will be composed by a liquid phase with no soluble particles and a powder phase with all the protein particles. The higher the fraction of protein particles that remained insoluble, the hard is the final texture of the bar. The expected results were that hardness would follow the order mentioned previously (hydrolysates < whey proteins < caseinates < micellar caseins < acid and rennet casein). Nevertheless, as can be seen in Figure 47, the average of the protein bars hardness with the proteins in each category revealed a switch between casein micelles and caseinates-based powders (hydrolysates < whey proteins < micellar caseins < caseinates < acid and rennet casein). Caseinates resulted in harder bars than micellar caseins due to the influence of the soluble particles in the bar. The NaCas soluble powder particles dissolved in the carbohydrate syrup have higher viscosity than an equivalent part of micellar casein soluble powder particles. Therefore, higher viscosity can be correlated with higher hardness.

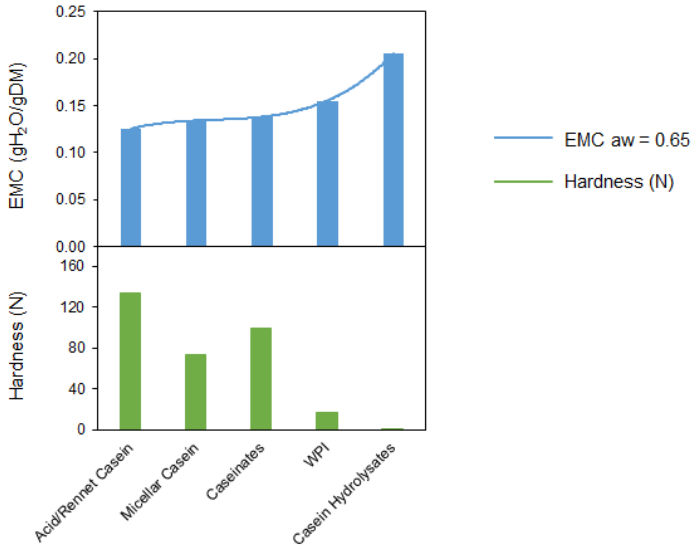

Figure 47 Comparison between the average EMC and hardness (N) of the 5 protein categories.

Effect of powder properties on the texture of protein bars

The same type of protein ingredient can have different powder properties and give origin to distinct protein bars depending on the manufacture process of the powder. Both CaCas 1 and CaCas 2 are spray dried powders and they therefore have a regular and spherical shape, with large central vacuoles or smaller vacuoles which are distributed throughout the interior of the particles. Although having the same method of drying, CaCas 1 and 2 were obtained from different suppliers. When used as the source of protein, they produced bars with different hardness over time. This occurs because the powders have different values for powder particle density, powder particle size and occluded air. The true density was calculated based on the composition of the powder and both CaCas 1 and 2 had almost the same value (Figure 6), as the main difference in powder composition was the moisture content. On other hand, powder particle density is dependent on the inherent characteristics of the powder, such as the amount of pores inside the particles (Bhandari et al. 2013) (occluded air) and had different values for the two spray dried CaCas: 0.80 g/cm^3 for CaCas 1 and 1.03 g/cm^3 for CaCas 2. This difference was reflected in the calculated content occluded air, that was found to be 54 g/cm^3 for CaCas 1 and 26 g/cm^3 for CaCas 2. Moreover, it was found that the powders had different particle sizes (74.9 µm for CaCas 1 and 61.7 µm for CaCas 2). The size of the particles would influence the appearance, reconstitution and flow characteristics of the powder, and is mostly depend on the spray drying conditions (atomization and viscosity of the concentrate) (Bhandari et al. 2013). Besides the powder properties it is important to take into account the differences in the initial water activity of the powders: 0.335 for CaCas 1 and 0.272 for CaCas 2. This lower value for CaCas 2 could be associated with a higher water migration from the carbohydrate phase to the protein phase. The influence on the final product is reflected in the values of hardness for the

bars made with the two CaCas. Over 30 days of storage, CaCas 1 had a hardness of 95.3 N, whereas for CaCas 2 the value measured was 114.3 N.

CaCas R was obtained from the same supplier as CaCas 1, but this powder was roller dried. Powders produced by roller drying have a compact structure and particles with irregular shape and very little occluded air (Bhandari et al. 2013). In fact, as can be seen in Figure 6, the calculated value of occluded air for CaCas R is much lower than the value for CaCas 1 and 2 (6 cm^3/100 g). This is reflected in the value of powder particle density, that is higher for the CaCas R. Furthermore, roller dried particles were found to be larger than spray dried particles, having a medium particle size of 118.9 µm. This powder produced softer bars than CaCas 1 and 2 at an early stage of storage, but over time the hardness reached the value of 95.4 N, which is very close to the value for CaCas 1. This increase in hardness for CaCas R can be explained by the differences in the initial water activity of the powder: CaCas R had an initial water activity of 0.217 and CaCas 1 of 0.335. The high value for CaCas 1 probably decreased the water migration within the bar due to the smaller difference in a_w of the ingredients used.

Volume of occluded air

The volume of occluded air in the powder particles can also be seen as the volume inside the powder particles that can be occupied by carbohydrate syrup. The higher the volume of occluded air, the less syrup will be free in the bar matrix. In the case of soluble particles, the dissolution of the particles can be favored by a high volume of occluded air, as the dissolution could also occur from the inside to outside of the particle. For insoluble particles, as acid and rennet casein, the resulting effect of the syrup in the volume of the occluded air will be only the decrease in the amount of syrup in the bar matrix. These insoluble particles could continue to suck up syrup, which result in harder bars. The amount of carbohydrate syrup that can actually fill the volume of occluded air was calculated based on the syrup density and the results are presented in Figure 48.

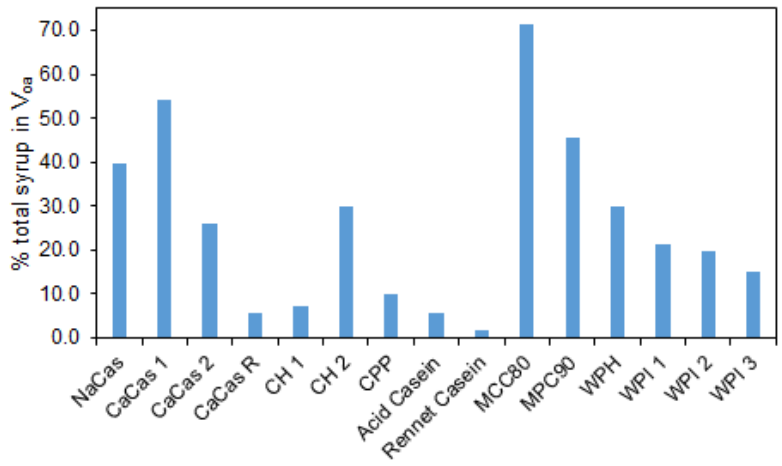

Figure 48 Percentage of the total syrup that corresponds to the V_{oa} for each protein powder.

Pre-equilibrated Powders

Pre-equilibration of powders in desiccators at a_w 0.65 resulted in softer bars than the non-equilibrated powders (Figure 34). The main reason for this result is the reduced difference in water activity between the ingredients that are in the protein bars formulation: the closer the material is to the osmotic equilibrium the less pronounced will be the rate of moisture transfer. Raising the water activity of the protein powder to 0.65 will reduce the competition between the powder and liquid phase for the moisture available, and so less water will migrate towards the powder particles. Although having a softening effect, pre-equilibrated powders did not eliminate the differences in texture between different protein types. NaCas continued to have more propensity to hardening than WPH and WPI 2.

- Both specific volume of occluded air and powder particle density should be considered, as a lower volume of occluded air and powder particle density are associated with harder bars;
- The difference in water activity between the ingredients of the protein bar should be minimized in order to avoid moisture migration

4.2.1. Fractionated Powders

As it was expected, all the fractions tested reveled an increase in hardness over time. The CaCas 1 powder material has a narrow range of particle sizes in its composition, which gave origin to fractions with very similar particle size. The same is not true for WPI 2, since the latter had broader particle size distribution, which made it possible to have fractions with distinct sizes. Therefore, for CaCas 1 no fraction specifically stood out from the others. All fractions of the same powder showed the same moisture sorption behavior, irrespective of the powder particle size. The sorption isotherms represented in Figure 18. However, the hydration of a powder material, and its ability to take up water is favored by the size of the particles due to the increased wettability, sinkability and dispersibility capacity with increasing particle size (Bhandari et al. 2013). Labuza & Hyman 1998 affirmed that smaller pore sizes will lower the moisture migration rate in foods as the water needs to pass through more tortuous pathways. Actually, in the case of WPI 2, F4 has hardened considerably when compared with the other fractions. This fraction has particles with a medium size of 327.8 µm, almost the twice of the size for the particle in fraction 3 (166.3 µm) which lead to an easily hydration of the particles and so, as a consequence, to bars with a higher value for hardness after the storage time. Another possible cause for these results is related with the atomization process of the powder. During drying the, the liquid is atomized into droplets of different sized. All droplets fall down the same drying tower, under the same conditions. The small droplets have less moisture and larger surface area and they will dry faster. The large droplets have more moisture and lower surface

area. Because water diffusion out of the droplet is slower in larger droplets, they stay wet for a longer time. This can lead to development of insolubility over time which can be related to the higher hardness of the bars with the largest particles.

Nonetheless, if the percentage of hardness over time was taken into account for each fraction, and not only the absolute value of hardness for the last measurement, for CaCas 1 F4 is also the fraction that had a higher increase in hardness over the 30 days (Table 10).

Table 10 Bar hardening represented as a percentage of hardness (N) over 30 days of storage

		Hardness day 30 - Hardness day 1 (N)	**% Hardness**
CaCas 1	Whole Powder	54.4	143
	F1	58.4	173
	F2	56.4	245
	F3	41.9	140
	F4	62.1	307
WPI 2	Whole Powder	10.2	707
	F1	13.5	973
	F2	13.8	1053
	F3	10.7	807
	F4	45.3	2827

There is no significant difference in the values of powder particle density and volume of occluded air within the fractions, which leads to the possibility of particle size be the principal property that is behind the hardness evolution of the bars.

4.2.2. Protein Blends

As mentioned before, proteins can have synergistic or antagonistic effects when mixed together. The results presented Figure 38 suggest that when mixing WPI 2 with WPH, the resulting bars have a hardness in each time point that is lower than the hardness for the single ingredients. It was also found that a mixture with a concentration of WPI 2 between 50 and 67% is the optimal concentration of proteins that result in the softest bars. The mechanism behind this behavior is not well known yet, but it can be associated with some effects on moisture sorption and also with the different sizes of the particles. Nevertheless, a possible cause for this effect is the concomitant better packing of protein particles. A good packing of protein particles is almost always favored by a blend of proteins due to the existence of a wide distribution of particle size. The inter-particular space, also called as void volume, and its fraction normally decrease with the presence of smaller particle size. The fine particles can occupy the interstitial space and pack together in bulk, which lead to a jamming effect. However, as can be seen in

Figure 49, above a certain concentration of fine particles, this effect is reversed (Bhandari et al. 2013).

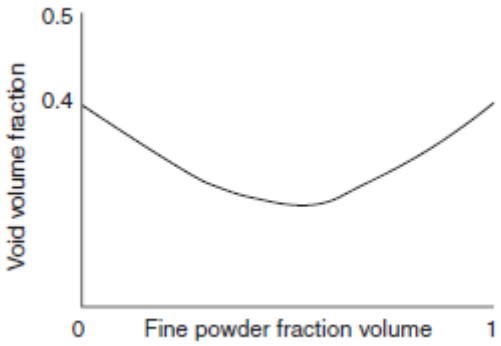

Figure 49 Void volume fraction as a function of the fine powder particle volume from (Bhandari et al. 2013)

The fine powder fraction volume has an ideal value between 0.5 and 0.6, which is concordant with the volume fraction of WPI 2 added to the mixture that was found to be the ideal ratio for WPI 2 and WPH.

When NaCas was mixed with CH 2 the same trend was observed for the first NaCas percentages: increasing the concentration of NaCas caused a decrease in hardness. However, percentages of NaCas higher than 33% lead to an increase of the hardness. For the first ratios, CH 2 was the dominant protein, due to the higher percentage in the mixture. When a half-half mixture was made, the effect of NaCas started to overcome the effect of CH 2. Hereupon, it was found that a bar made with 33% of NaCas and 77% of CH 2 produce the softest bars. However, the effect of the void volume in the packing of powder particles, and therefore in the resulting hardness, has only effect when particles remain insoluble. In the case of the NaCas and CH 2 mixture, the hydrolysate is completely soluble in the syrup and so it is not possible to mention void volume as the reason for the changes in hardness. When the two protein powders are mixed in the syrup there is a competition for the moisture available. In the first two ratios of NaCas and CH 2, the amount of NaCas powder particles was not sufficient to have any effect on texture and thus, the decrease in texture can be explained by the decrease in the CH 2 powder particles dissolved in the bar matrix. NaCas particles are mostly insoluble and their concentration in the first two ratios is not enough to allow particle interaction. Also, the effect of the swollen NaCas is not sufficient to have influence in the development of hardness. Nonetheless, when a blend of 50% NaCas and 50% of CH 2 was tested, the bars shown to develop a noticeable hardness. The amount of NaCas in the mixture allows particle-particle interactions and the syrup swelled by the particles increased their volume fraction (and decreased the water available to dissolve the hydrolysate), which resulted in harder bars. Increasing the concentrations of NaCas to 80% increased the effects mentioned above and gave origin to harder bars. In the case of WPI 2 and WPH, the same explanation is valid:

increasing the concentration of WPI 2 to about 50% will have no effect on hardness, and the texture of the bars is explained by the concomitant decrease in WPH in the formulation.

To test the veracity of this theory, a blend with NaCas and CPP was made, using the same ratios as before. The process of manufacturing the bars was changed: first, the CPP was added into the carbohydrate syrup and glycerol and mixed until all the powder was dissolved. After that, the NaCas was added and mixed for 30 s. The bars were stored at 37ºC and measurements were made at day 1, 4, 7 and 10. The texture results showed that the hardness behavior was the same seen previously for the other protein blends (see appendix B). However, the minimum that was reached for a 33% NaCas for the NaCas:CH 2 mixture was now reached for a 20% NaCas. The results can be caused by (1) the influence in texture given by CPP is not the same as CH 2, and (2) the dissolution of the particles in the syrup will minimize the competition for water and the effect of NaCas is noted earlier.

4.2.3. Coated Powders

The reactive groups present in proteins and that can absorb water are located at the exterior surface of the protein. Covering these reactive groups should decrease the interactions between the proteins and water (and also protein-protein interactions) and therefore, bars made with coated powders should have a softer texture than bars produced with non-coated powders. In effect, all the 10% coated powders had a lower moisture sorption, as can be seen in Figure 20. It was found that a higher content of the coating agent will cover more reactive sites and therefore less water will be taken from the liquid phase. Moreover, it was found that covering the powder with palm oil, lecithin or a mixture of both will result in softer bars. Nonetheless, there is no difference in using an amphiphilic molecule as lecithin or fat as palm oil. It can be said that lecithin gives softer bars according to Figure 40 , and a 10% lecithin coated powder made the softest bars, but the difference is not substantial.

When the carbohydrate syrup is mixed with the protein powder a suspension of powder particles is formed. However, this “equilibrium” state is not permanent. In fact, two reactions start almost immediately when mixing protein bar ingredients: (1) some powder particles, in this case NaCas particles, will dissolve in the liquid phase or (2) some of the carbohydrate syrup will get sucked up by the protein powder particles, that start to swell. These swollen particles can interact and form some aggregates that can be involved in the hardening process. When using palm oil, the softening effect is not so efficient compared to lecithin. It is known that lecithin is surface active and so it can act minimize the hardening in two ways: (1) by reducing the interactions between the powder particles or (2) by reducing the syrup that is taken by the particles. This amphiphilic molecule will be orientated with the polar (hydrophilic) domain to the syrup and with the non-polar (hydrophobic) domain to the powder particles and this distribution can reduce the interaction between particles, which lead to less aggregates formed. Also, the

attachment of these molecules on the surface of the protein particles can be more “permanent” that palm oil, which can lead to less syrup taken by protein particles.

When looking to the bars with the non-coated powders, the final hardness is practically the same as the ones with coated powders, although faster. Mixing the coating agent with the remain ingredients can have the same effect of using a powder that was coated previously. In the case of amphiphilic molecules as lecithin, when mixed with the other ingredients, the tendency will be to rearrange the position of the molecules with the hydrophobic part facing the powder particles and hydrophilic facing the carbohydrate/water syrup. This will have the same effect of using a powder that was previously coated. Loveday et al. 2010 studied the texture of protein bars consisting of glucose, protein powder, glycerol and cocoa butter. The results confirmed that some fat was probably distributed in the surface of the powder particles. When using palm oil, the fat molecules can migrate towards the protein particles and stayed located at their surface. This positioning of fat molecules will end up having the effect of using a coated powder.

4.2.4. Insoluble MPC90

As a result of heating, the casein micelle-based powders loose the layer κ-caseins that are located at the surface of the micelles. The κ-caseins collapse in the powder and form patches around the surface of the casein micelles.

When the powder is subjected to a higher water activity than its own some water is entering the system. This extra water allied with a high storage will confer some mobility to the proteins that rearrange in the powder particles through strong non-covalent interactions creating a thick and compact skin on the surface of the powder particles. This new rearrangement is very difficult to dissolve without high temperature and high shear.

In the case of protein bars, the MPC90 was subjected to a_w=0.4 and then stored at 60ºC and the resulting insoluble powder was used in the formulation of protein bars. Nevertheless, the process of making the bars does not involve high temperature or high shear and so the particles were not dissolved in the bar matrix.

As it can be seen in Figure 42, insoluble MPC90 (maintained at 60ºC or microwaved) produced harder bars than the MPC90 stored at 5ºC. Although insoluble, these powder particles can still be hydrated, swollen and interact with each other. The volume of the residual undissolved powder particles seems to be a key factor for the bar hardness. As it was mentioned previously, the fraction of powder particles is correlated with the hardness of the bars: a higher powder particle fraction is associated with a higher hardness. In the case of the microwaved MPC90, the hardness on the 15th day was almost the same as the soluble MPC90. This can be due to the non-homogeneity of the particles in the microwaved powder: some particles were affected by the heating and became insoluble, but others were not affected and remained soluble. These soluble particles contributed to the slightly lower value of hardness for

the microwaved MPC90. Moreover, the MPC90 that remained soluble formed the softest bars possibly due to the partial dissolution of powder particles into the syrup that formed a viscous liquid with a smaller fraction of (insoluble) powder particles.

5. Conclusions

5.1. Carbohydrates

- The concentration of the carbohydrate syrup appears to have no effect on bar hardening during storage;
- Using a reducing sugar, that participates in Maillard reaction, a non-reducing sugar or a polyol will produce bars with the same hardness behavior over storage time.

Maltose and sucrose bars had the approximately the same hardness development over time which indicates that Maillard reaction had no effect on bar hardening.

5.2. Effects of protein type

The differences in hardness were found to be primarily dependent on the protein type present in the bars.

- Acid and rennet casein will produce bars with a dry and crumbly texture, with the highest hardness over time;
- Using caseinates and micellar caseins will result in bars with a texture between a concentrated solution and a particle gel, dry and hard.
- WPI produced bars with a dough texture and with less hardening over time. Whey proteins underwent non-enzymatic browning (Maillard reaction);
- Using casein hydrolysates as protein ingredient resulted in viscous solutions, with a sticky texture.

Hardness of proteins bars showed to be highly dependent on the powder properties. When using the same powder type with different properties (powder particle density, occluded air, particle powder size) the resulting bars develop different hardness over time.

- Larger powder particles will produce harder bars due to the easiness of water migration through the space between the particles;
- Volume of occluded air can favor the dissolution of soluble powder particles but can also increase hardness by decreasing the amount of syrup in the bar matrix.

In terms of moisture content, a high EMC generally means that the protein powder will have more tendency to suck up water. However, the results showed that the powders with the highest EMC are also the one that are more soluble and produce the softest bars (hydrolysates and whey protein-based powders). Moreover, hardening of high protein bars appears to result

also from the competition of available moisture. Minimization of water activity differences between the ingredients (carbohydrate syrup and protein powder material) should provide an effective way to control and decrease bar hardness of time. Pre-equilibrated powders are an effective way to minimize the a_w difference between the protein bars ingredients, and so they result in softer bars than the original non-equilibrated powders. The pre-equilibration of the powders and the concomitant decrease in the difference between a_w of the different bar components represent an effective way to control textural deterioration and improve bar stability. Nonetheless, even controlling the moisture migration in multi-component food systems, different protein types will have different tendencies to harden. Although, minimizing the osmotic differential between different ingredients with high and low a_w will have no effect on the changes caused by the type of protein.

The fractionated powders confirmed the influence of the particle size in the bar hardness. Fractions with the largest particle sizes (F4) produced the hardest bars for both protein types tested (CaCas 1 and WPI 2). Larger particles will have more space between them, which allows the water to flow more easily in the inter-particles space.

From the bars prepared with powder blends it was observed that that protein powder blends with different particles sizes will produce softer bars. Actually, it was found that mix WPI 2 with WPH the resulting bars were softer than the bars made with the single ingredients. A powder with a wide range of particles size should give softer bars due to the better jamming/packing of protein particles. However, this effect is only important for insoluble particles. The effect of volume of occluded air should be taken into account, as it could be used as a softening agent in the case of soluble particles, and as a hardening agent in the case of insoluble particles (by decreasing the available syrup).

Coating a powder with palm oil, lecithin or a mixture of both did not have any softening effect on bars. In fact, the non-coated powders, where the coating agent was added as an ingredient in the bar formulation, developed the same hardness over time than the coated powders. This is related with the distribution of the coating agent in the bar matrix: the coating agent will be located at the surface of the powder particles and perform the same effect as coat the powder previously. Lecithin demonstrated a more permanent effect on softening the protein bars, however the mechanism behind this effect is not yet fully understood.

The insoluble MPC90 produced harder bars mostly due to the increase of powder particle fraction in the bar matrix, with the presence of swollen powder particles.

5.3. General Conclusions

Overall, it was found that a wide range of interactive physical and chemical factors can contribute to the hardening of HPB during storage, with protein type and powder particle size

and density being the major issues to consider. Some of the experiments revealed to be an effective way to control and improve bar stability, reducing hardening over time:

- Minimizing the osmotic differential between components with high and low a_w;
- Using a blend of proteins, rather than individual protein ingredients, e.g., using a mixture of intact protein and protein hydrolysate, or using a mixture of caseins and whey proteins;
- Using protein powders with a wide range of particles sizes;
- Adjusting the volume of occluded air that should be high for soluble particles and as low as possible for insoluble particles;
- Minimizing the powder fraction in the bar matrix.

This study reinforces that hardening is manly caused by the reorganization of protein particles, driven by osmotic pressure differences and their structural properties.

6. References

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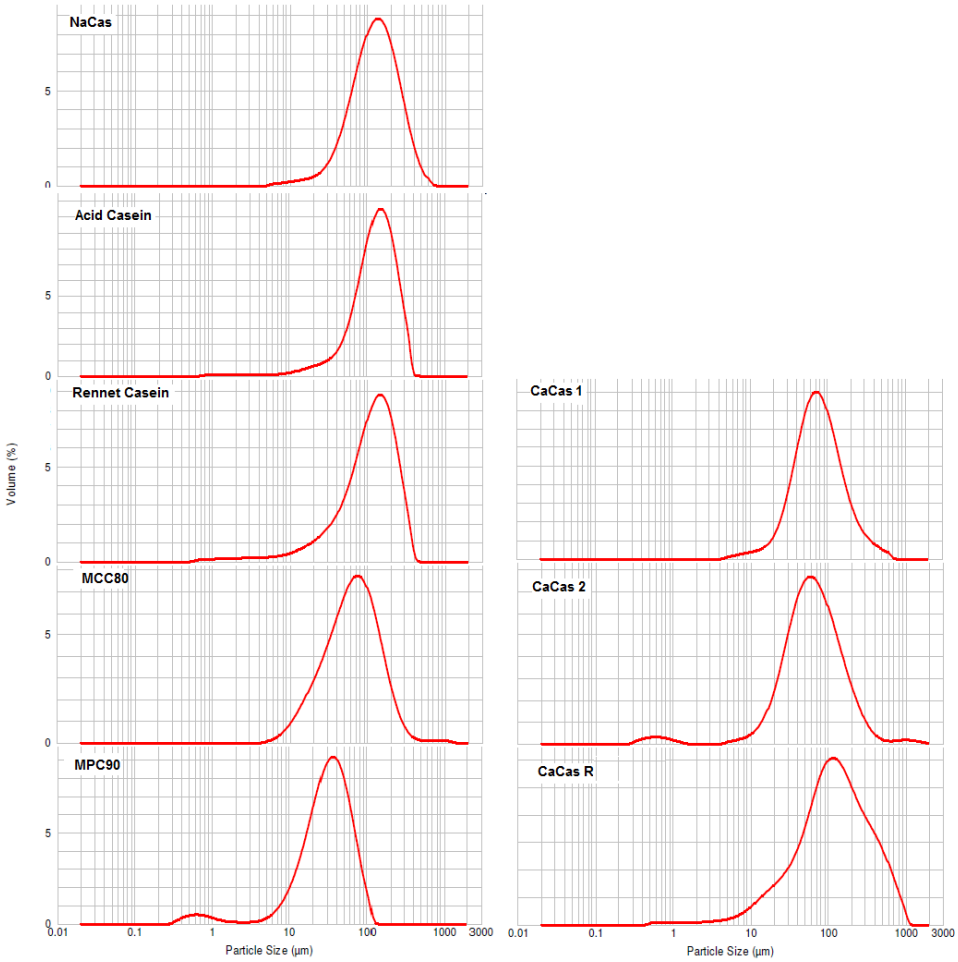
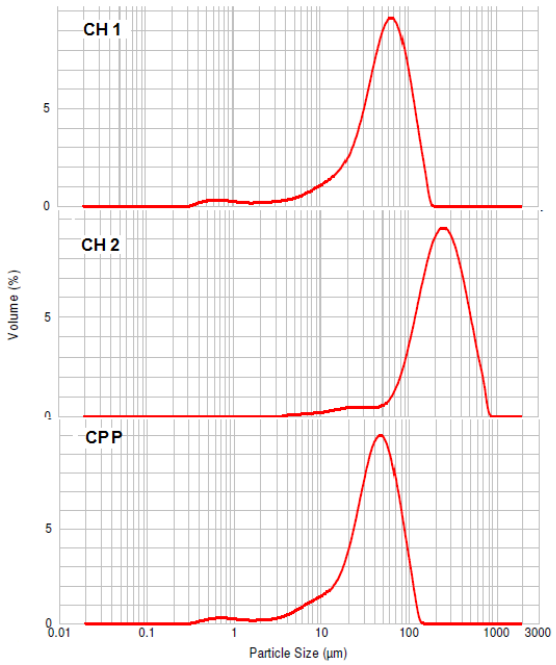
7. Appendix

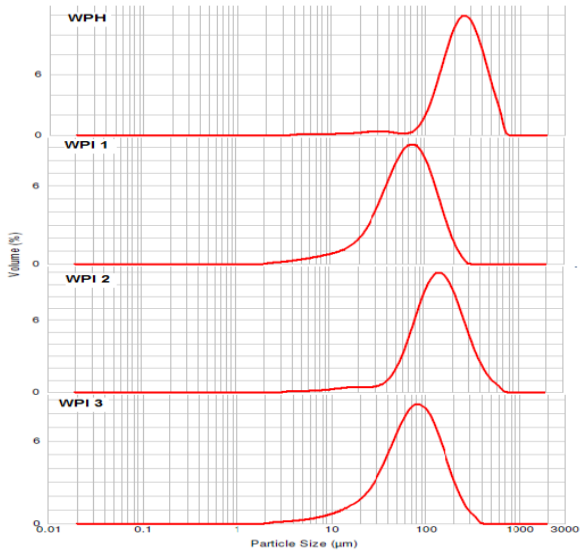
A. Powder particle size distribution



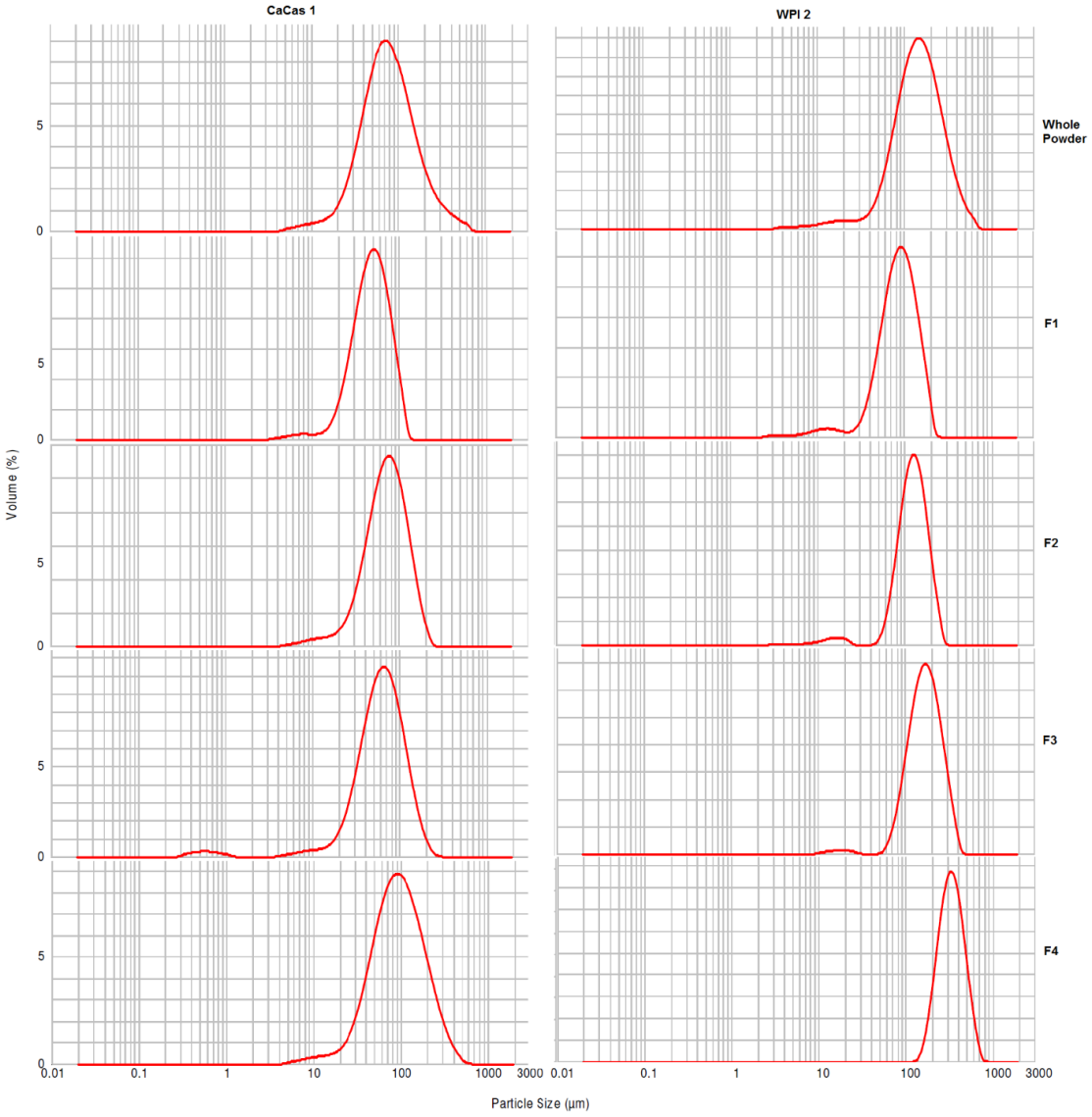
A 1 - Powder particle size distribution of the casein-based powders.



A 2 - Powder particle size distribution of the casein hydrolysate powders.



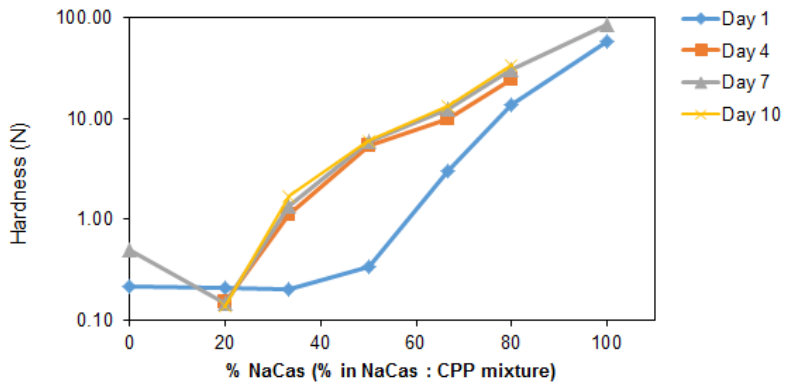
A 3 - Powder particle size distribution of the whey-based powders.



A 4 - Powder particle size distribution of the fractionated powders.

B. Protein blends – NaCas:CPP

		Hardness (N)			
	NaCas (% in NaCas : CPP)	Day 1	Day 4	Day 7	Day 10
NaCas:CPP	0	0.21		0.49	
	20	0.21	0.15	0.14	0.13
	33	0.20	1.11	1.36	1.68
	50	0.34	5.36	5.86	5.98
	67	2.98	9.74	12.19	13.33
	80	13.44	24.01	30.97	33.67
	100	58.99		86.83	



B 1 - Hardness (N) over 10 days of storage at 37ºC of the bars produced with the protein blends. The values of hardness are represented in a logarithmic scale.