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Modifications moléculaires et fonctionnelles au cours du vieillissement des poudres d'isolats de protéines solubles (WPI)

Eve-Anne Norwood

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Eve-Anne NORWOOD • 13 octobre 2016

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Science et Technologie du Lait et de l'Œuf (STLO)

Modifications moléculaires et fonctionnelles au cours du vieillissement des poudres d'isolats de protéines solubles (WPI)

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Doctoral College: Vie-Agro-Santé (VAS)

Presented by:

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Rennes, 13 October 2016

**Molecular and functional changes occurring during ageing
of whey protein isolate powders**

JURY

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“Ever tried.

Ever failed.

No matter.

Try again.

Fail again.

Fail better.”

Samuel Beckett (1906-1989)

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THESIS OUTPUTS

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Norwood EA, Le Floch Fouéré C, Schuck P, Croguennec T, & Jeantet R. (2016). Structural markers of the evolution of whey protein isolate powder during aging and effects on foaming properties. *Journal of Dairy Science*, 99(7), 5265–5272. doi:10.3168/jds.2015-10788.

Burgain J, El Zein R, Petit J, Norwood EA, Francius G, & Gaiani C. (2016). Local modification of whey protein isolate powder surface during high temperature storage. *Journal of Food Engineering*, 178, 39–46. doi:10.1016/j.jfoodeng.2016.01.005.

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Review

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Oral communications

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LIST OF ABBREVIATIONS

AFM: atomic force microscopy

AGE: advanced glycation end-products

α -LA: α -lactalbumin

Asp: aspartic acid

ATR: attenuated total reflection

AU: arbitrary units

a_w : water activity

β -CN: β -casein

β -LG: β -lactoglobulin

BI: browning index

BSA: bovine serum albumin

C_b : bulk concentration

CD: circular dichroism

D: diffusion coefficient

Δ : ellipsometric angle

DLS: dynamic light scattering

DM: dry matter

DTNB: 2,2'-Dinitro-5,5-dithiodibenzoate

DTT: dithiothreitol

ESCA: electron spectroscopy for chemical analysis

FD: foam density

FS: foam stability

FTIR: Fourier transform Infrared spectroscopy

γ : surface tension

Γ_0 : surface concentration at which π is different from zero

GPC-HPLC: gel permeation chromatography – HPLC

HPLC: high performance liquid chromatography

I(lact): intensity of the lactosylated protein

I(NAP): intensity of the non-aggregated protein

Lys: Lysine residue

mo: month

MPC: milk protein concentrate

MPI: milk protein isolate

M_w: molecular weight

NCN: non-casein nitrogen

NP: native protein

NPN: non-protein nitrogen

PC: principal component

PCA: principal component analysis

pI: isoelectric point

π: surface pressure

Ref: Control WPI powder

RP-HPLC: reverse-phase – HPLC

SAXS: small angle X-rays scattering

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: scanning electron microscopy

SMP: skim milk powder

t: time

T: temperature

TEM: transmission electron microscopy

θ: the amplitude of interactions at the air/water interface

θ_B: Brewster angle

List of abbreviations

TFA: trifluoroacetic acid

T_g : glass transition temperature

TN: total nitrogen

TOF – SIMS: time-of-flight secondary ion mass spectrometry

USD: US dollar

WMP: whole milk powder

WP: whey protein

WPC: whey protein concentrate

WPH: whey protein hydrolysate

WPI: whey protein isolate

XPS: X-Ray photoelectron spectroscopy

GENERAL INTRODUCTION

Milk and whey dehydration originally targeted to stabilize the surplus coming from the dairy industry to ensure storage. Long considered by-products, the resulting powders were mainly intended for animal feed and then generated only a low valuation of matter. Now milk and whey powders are mainly used as food ingredients and are produced at large scales. The global market for milk powders has indeed undergone two major transitions over the past four decades, first by integrating scientific and technological knowledge, and secondly due to the growing demand from the emerging markets. The use of separation techniques such as membrane filtration (*i.e.* microfiltration and ultrafiltration) allowed to access a new range of dry products with higher added value, including concentrates and isolates of specific milk protein fractions (Schuck 2002). By the production of these latter, the dairy sector lastly turned towards the nutritional control of these powders as well as their technological functionalities (texture properties, gelling, foaming and emulsifying).

If one emphasizes the significant share constituted by these dry functional products on the global market (uses representing about 150 000 tonnes equivalent protein in the sole European Union; FranceAgriMer 2012), the challenge represented by this market segment makes now these products a relevant axis of development for the industrial sector. This is particularly true in terms of how their conservation may affect their techno-functional properties. Moreover, the literature gathers a great number of studies on high protein dairy powders storage but they principally focused on protein concentrate powders or caseinate powders. A lack of knowledge remains on the ageing of whey protein isolate powders in terms of storage stability and functionality whereas the uses of this dry ingredient are expected to strongly increase in the next five years (Potier 2015). Thus, characterizing and measuring precisely the changes occurring during storage of whey protein isolate powders in order to better control their functionalities, resulted in a major research program conducted by the National Institute for Agricultural Research (INRA) in close interaction with the *Centre National Interprofessionnel de l'Economie laitière* (CNIEL).

The so called program *CODE POUDRE* aims to better understand the evolution of the functional properties undergone by high protein dairy powders over time and to link these changes with the environmental storage conditions. This includes:

- x Assessing the influence of both ageing and production conditions on rehydration properties and more generally on the functional properties

- x Identifying the mechanisms related to the structural changes in high protein dairy powders and the matter reorganization behind these changes. This characterization will be led at different scales of observation, which are the molecular, mesoscopic (particle), and macroscopic (powder) scales.
- x Finally, in terms of applied objectives, designing technological routes for increasing the high protein dairy powder stability and tools in order to define its storage conditions.

The CODE POUFRE program therefore aims to address the issues raised by the ageing of high protein dairy powders, which can be summed up by *“What conditions of processing and / or storage to adopt in order to perpetuate the functional properties of high protein dairy powders”*, relying on tools, methods and strategy reflected in previous programs.

CODE POUFRE is a 4-years program and is financially supported by the CNIEL. It includes two PhD studies, supported by the ANRT, and a post-doctoral study. These are coordinated by two INRA joint Research Units – the Science and Technology of Milk and Eggs, Agrocampus Ouest (STLO) and the Interface Processes and Hygiene of Materials (PIHM) - in partnership with the University of Lorraine (LIBio).

This present work is therefore one of the two PhD studies of the program and is focused on the structural and functional ageing of whey protein isolate powders. The underlying objective was to assess the ageing path of these powders to better control their changes occurring during storage. This work has been divided into 3 main parts dealing successively with the study of the structural markers of protein ageing (**Chapter 2**), the study of the impact of these structural changes on the heat-induced aggregation properties, the foaming properties and the interfacial properties of the powders (**Chapter 3**) and finally the study of technological levers limiting and/or minimizing the changes specific to WPI powders storage, ending with the integration of the new knowledge brought by this project (**Chapter 4** and **Chapter 5**). Ahead of these three parts, the scientific and economic contexts of the PhD project are presented in order to understand its originality, its goals and its objectives (**Chapter 1**).

CHAPTER 1. MILK MARKET AND IMPORTANCE OF DAIRY POWDERS STORAGE

This chapter aims to introduce the interest of dairy powders and their economic weight regarding the current global milk production. In addition to key statistics on milk production and dairy powders, a literature section will show that this way to store milk compounds creates significant end use issues for industries using these dry ingredients. In particular, this part lists exhaustively changes at molecular, mesoscopic and macroscopic scales that are responsible for changes in powder functional properties during preservation. This part has been submitted as a review in *Trends in Food Science and Technology* journal (Paper¹). Last and based on the concluding remarks of this review, the final section of this chapter will display the aims, the originality and the strategy of this PhD project. Indeed, understanding the changes occurring during high protein dairy powders' storage is essential to guarantee the preservation of their properties on long-term storage. This will help manufacturers to improve their competitiveness on a challenging market by reducing added value losses consecutive to uncontrolled changes in powder functionalities.

The aims of this chapter were to:

- x *provide an insight on the market of dairy powders and more specifically high protein dairy powders*
- x *provide highlights on the influence of storage conditions on high protein dairy powders ageing*
- x *define the PhD research area and display the research question*

¹ Submitted to *Trends in Food Science and Technology* as Norwood EA, Nasser S, Le Floch Fouéré C, Schuck P, Delaplace G, Croguennec T, Jeantet R. Storage of high protein dairy powders: changes in protein structures and functions. A review.

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PART 1. ECONOMIC CONTEXT

1.1.1. ECONOMIC POSITION AND INTEREST OF DAIRY POWDERS

The year 2014 was characterized by a significant 3% increase of overall milk production compared to 2013, which was estimated at around 802 million tonnes (m. t) all milk species included (**Figure 1**). This characterized the start of market turnover since this abundant milk delivery had to lead to further output, especially dairy powders.

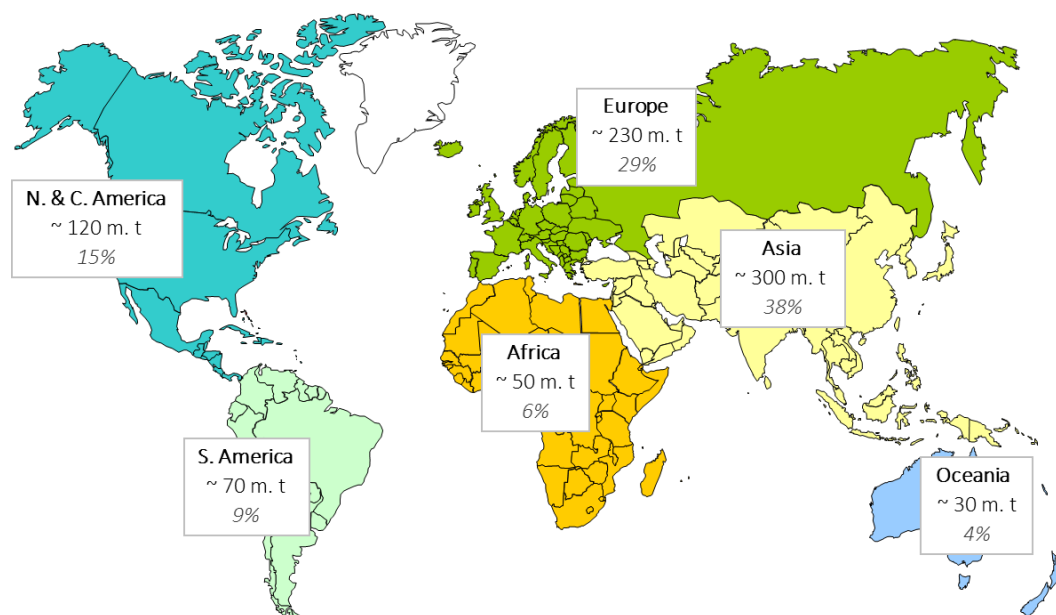


Figure 1: World milk production - all milk species (Pilet 2016)

Milk production is driven by three main world regions, being first Asia with 38% total milk followed by Europe and North & Central America with 29% and 15% total milk, respectively. As expected, the world trade is dominated by the exchanges between these regions and more precisely by North America, Western Europe and South East Asia, which represent alone 82% of the total milk production. Apart from these leader regions, South America, Africa and Oceania represent 9%, 6% and 4% of the total milk production, respectively.

However, all countries are not on the same basis of equality regarding milk production. A milk deficit compare to demand is observed in most countries, especially developing/emerging countries (**Figure 2**).

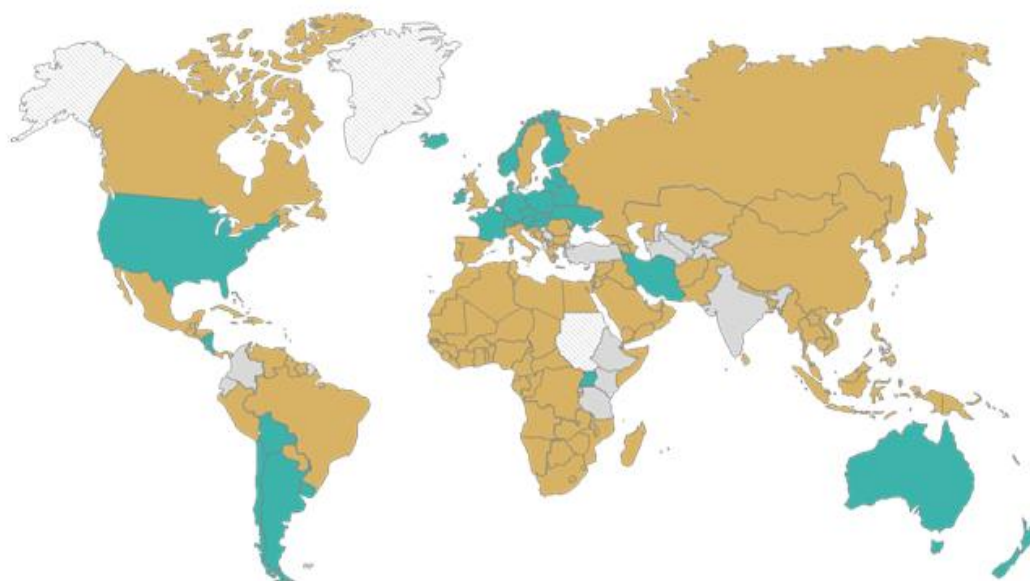


Figure 2: Dairy trade balances in 2013 composed of surplus countries with a positive trade balanced of more than 2% (blue), balanced countries (grey) and deficit countries with a negative trade balanced of less than 2% (yellow) (Pilet 2016)

The European Union (EU), Oceania, the United States (US) and some countries in South America (Chile, Argentine, Uruguay and Bolivia) have a surplus in milk production in relation to their domestic demand. Since mid-2013, the balance between the supply and the demand has shifted towards a bigger supply from the most producing countries (EU, New Zealand, US, Australia and Argentina) (Pilet 2016). This trends is emphasized in the EU with the abolition of the European milk quota in 2015. It is believed that French milk collection will increase of around 2% per year until 2020 (Maison de l’agriculture 2014).

The development of the international trade in the recent decades and the increased competition from Southeast Asian regions would suggest that today most regions heavily export and import to other parts of the world. However, as Asian countries are not self-sufficient with their milk production, a strong geographical trade polarization has been observed (**Figure 3**). Most of the global trade in milk is between rich countries. Here, the four parts of the world which constitute the main export areas are the EU and New Zealand with 25% of the exports share in the first place, the US and Australia in second place with between 5 and 15% of exports share and at last Argentina with less than 5%. These areas especially trade with each other and developed countries having milk deficit.

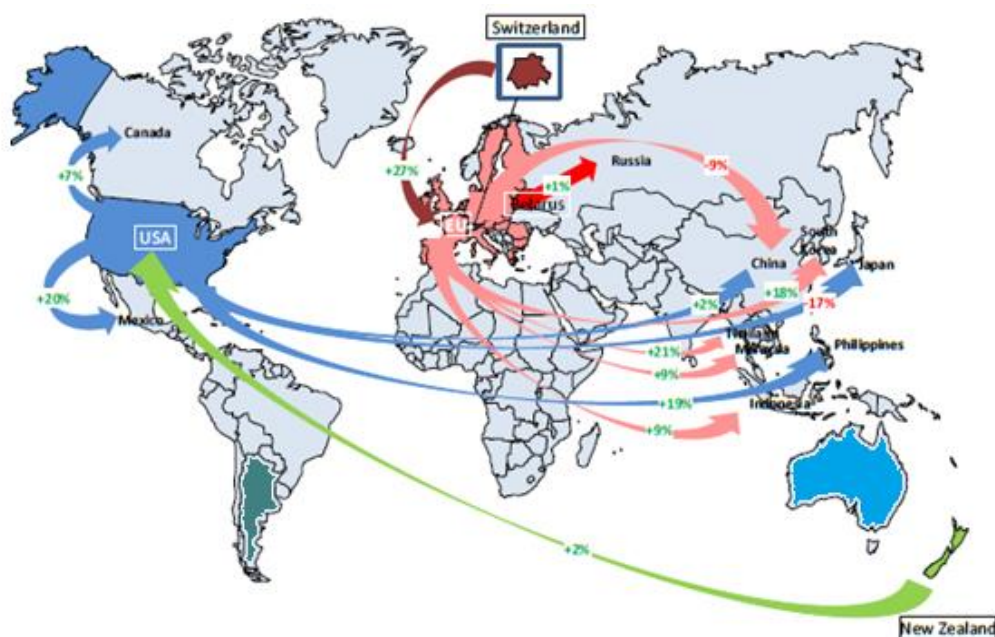


Figure 3: Top 5 export flows per exporter exceeding 10 000 tonnes. Development 2013/14 as indicated in % (IDF 2015)

As a consequence, the most producing countries had to find a way to preserve the milk production surplus and to export it in safe conditions. This has led to an increase in some types of dairy output and more specifically in dairy powders (**Table 1**). The global production of the main dairy powders showed an increase of about 20.6% between 2010 and 2014, which came up with an increase of about 29%, 20% and 10% for skim milk powder (SMP), whole milk powders (WMP) and whey powders, respectively. These increases are mainly due to the EU that increases its production of 47% and 15% in four years for SMP and whey powders, respectively, and to Oceania that increases its production of about 41% for WMP.

Table 1: Overall production of the main dairy powders between 2010 and 2014 (CNIEL 2016)

× 1000 tonnes	SMP		WMP		Whey powders	
	2010	2014	2010	2014	2010	2014
EU (28)	1078	1590	751	803	1939	2229
N. & C. America	900	1116	138	136	498	437
Asia	536	640	1000	1100	-	-
Oceania	566	653	1098	1551	61	52
S. America	130	154	705	844	29	64
Total	3210	4153	3692	4434	2527	2782

This evolution of dairy powders global production can be explain by the fact that powder processing is the major way to stabilize dairy products over long periods of time (up to 2 years), reduce the volume and consequently the cost of routing.

1.1.2. DRYING IN THE DAIRY INDUSTRY

The drying process is used in many fields such as pharmaceutical and food processing. Whatever the context, the aim is the same: *i.e.* improve stability and extend product shelf life, and reduce its transport and storage costs (Jeantet et al. 2011). The challenge is to keep the physical and functional properties of the dry material unchanged, for fresh powders as for long storage time.

Spray drying is the most commonly drying process used in the dairy industry for producing dairy powders and stabilizing dairy constituents. The spray drying method consists in obtaining a divided solid, through contact of a dispersed fluid sprayed into fine droplets with hot air (Schuck 2002). By varying the process parameters (spraying pressure and device, air flow rate and temperature, concentrate flow rate and dry matter), it is possible to control various properties of the finished product such as size, shape and porosity which will define its storage behaviour and eventually its functional properties.

1.1.3. COVETED HIGH PROTEIN DAIRY INGREDIENTS IN THE EUROPEAN UNION

DESCRIPTION OF HIGH PROTEIN DAIRY INGREDIENTS

Nearly 12% of milk collection is valued in a powder form. Until the eighties, the bulk of dairy powders gathered whole or skim milk, whey or buttermilk powders. Hardly better valued than the raw milk, these powders were used as market release products and carryovers ingredients in human food or in animal feed. Thanks to the advances in the technique of milk "cracking", the market for dry ingredients has grown exponentially (Schuck et al. 2013). Indeed, the emergence of filtration technologies made it possible for the dairy industry to design increasingly sophisticated preparation processes (**Figure 4**) in order to improve milk component valorisation and meet user needs in term of functional and nutritional properties (Schuck 2002).

This gave rise to the development of a wide variety of high added value milk protein concentrate (MPC) or whey proteins concentrates (WPC) and isolate powders (WPI), etc. called high protein dairy powders. In the last ten years, the nature of dairy powders has changed to more specific powders. Indeed, they have been developed as techno-functional ingredients according to their expected functional properties such as gelation, foaming and emulsification.

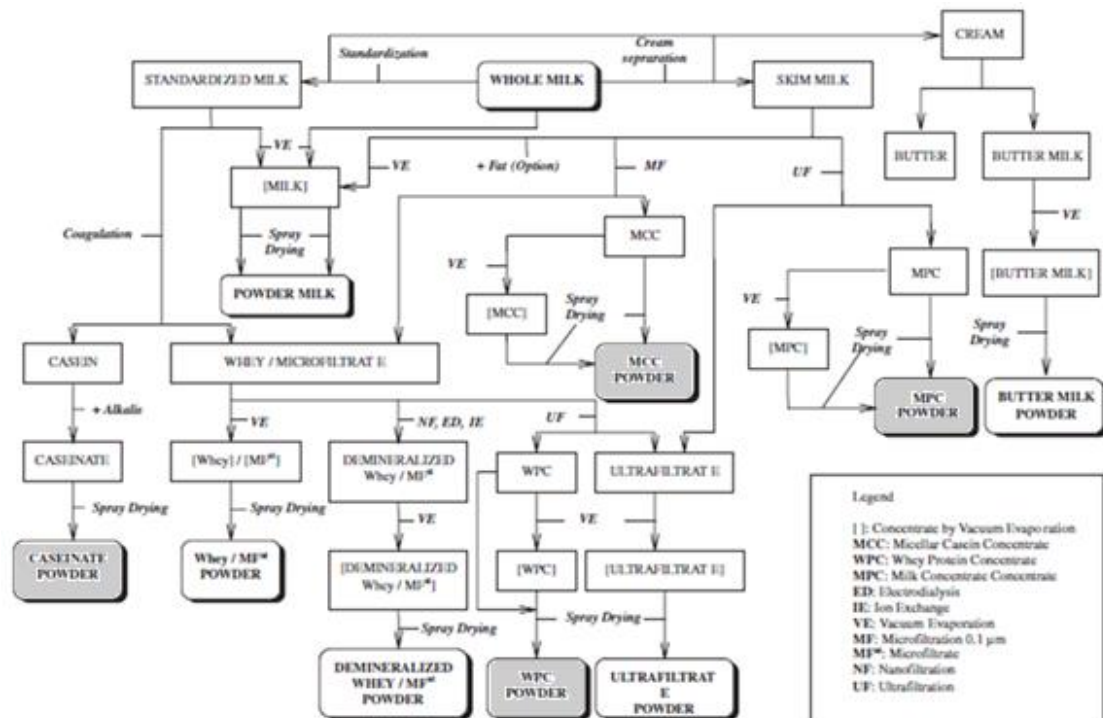


Figure 4: "Cracking" of milk (Schuck 2002)

Several levels of high added value products can be distinguish:

- x Concentrates, with a protein content < 90% of the dry matter (DM):
 - o Whey Protein Concentrate (WPC)
 - o Milk Protein Concentrate (MPC)
- x Isolates, which are highly purified protein concentrate with a protein content ≥ 90% of the DM:
 - o Whey Protein Isolate (WPI)
 - o Milk protein isolate (MPI)
 - o Whey protein hydrolysate (WPH)
- x Protein fractions:
 - o Micellar caseins
 - o Caseinates

Milk protein concentrate (MPC) powder obtained by ultrafiltration, skim milk powder or whole milk powder contain the milk proteins in the same ratio than in raw milk (80% caseins and 20% whey proteins) but with decreasing amount of protein and various amounts of lactose, mineral and fat (Schuck 2002). MPC may also be composed of 90:10 caseins / whey proteins if they are from microfiltration. Casein powders are obtained after separation of casein from whey proteins either by selective precipitation of casein at pH4.6 (casein, caseinate powders) or by microfiltration of milk

(micellar casein concentrate powders). Whey protein (WP) powders are obtained through the spray drying of whey protein fraction recovered either from milk microfiltrate, cheese whey or acid whey after casein removal.

High protein dairy powders represent a fast-growing market influenced by the thriving market of infant formulae and products with specific nutritional assets for the elderly, sportspeople and athletes. High protein dairy powders and infant formulae powders have thus become the driving force of investments in the dairy sector worldwide. In 2012, the uses of high protein dairy powders represented nearly 150 000 tonnes protein equivalent in the EU (FranceAgriMer 2012) and contributed significantly to the overall 8.4 billion USD of investments realised for dairy powders between 2012 and 2015 (Rouyer 2015). Nowadays, their properties – texturing, gelling, foaming or emulsifying – are well valued in the food industry. Besides, the pharmaceutical industry is also fond of dairy ingredients for skin moisturizing, sleep or appetite regulation, hypertension...

PRODUCTION VOLUME AND USES OF HIGH PROTEIN DAIRY INGREDIENTS

Casein and caseinates overall production in the EU reached approximately 125,000 tonnes with an availability for uses of 85,000 tonnes (FranceAgriMer 2012). WPC are used of up about 110,000 tonnes in protein equivalent in industry and the MPCs have an availability for uses of around 60 to 80,000 tonnes in the EU (FranceAgriMer 2012).

USES OF DAIRY POWDERS

Above the fact that there is a global increase in the dairy powder production, a change in the allocation of tonnage could be observed with a decrease in basic dried products towards more sophisticated ones (**Table 2**).

Table 2: Industrial uses in EU 27 between 2005 and 2010 (FranceAgriMer 2012)

	Industrial uses (%)	
	2005	2010
SMP	41	35
WMP	11	12
Whey powders	19	20
Caseins/caseinates	13	10
MPC	6	8
WPC	10	15

In the past decade, the substitution of milk powder, as SMP decreasing from 41% to 35% between 2005 and 2010, was essentially made with more concentrated dairy powders – high protein dairy powders – such as WPC and to a lesser extent through MPC. They are particularly found in animal feed, where the WPCs, whey permeate powder and vegetable proteins have come to replace the skim milk powder.

USES OF CASEIN AND CASEINATES

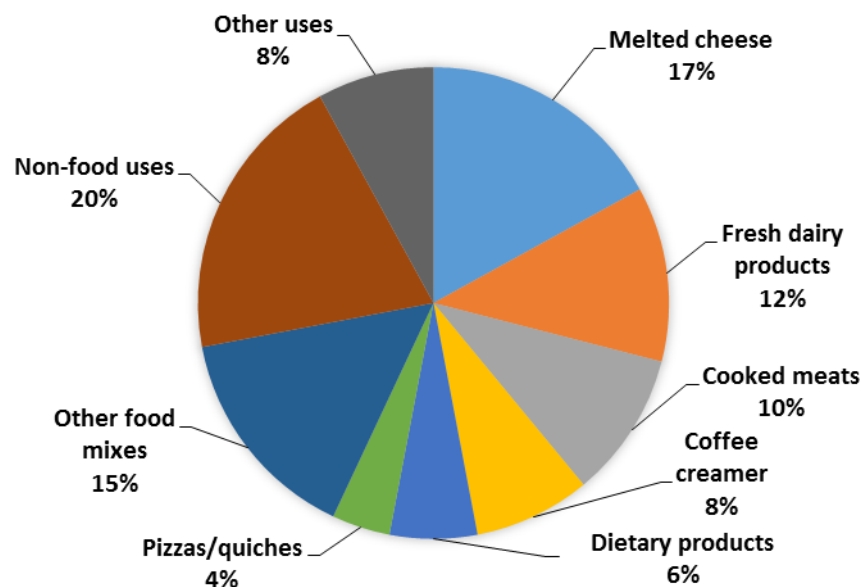


Figure 5: Identified uses of caseins and caseinates in the EU 27 in 2010 (FranceAgriMer 2012)

As shown in **Figure 5**, 72% of the caseins/caseinates are mainly used in the food industry for their texturizing properties (*e.g.* melted cheese, fresh dairy products), emulsifying properties (*e.g.* cooked meats) or their protein intake (*e.g.* dietary products). They are also involved in food mixes and ready to eat meals. Non-food uses include paint or glue and are of a significant use. These applications implies most often casein imported from third countries and of lower quality than that used in the food industry.

USES OF WHEY ISSUED PRODUCTS

Whey went from a status of waste to the status of by-product and co-product to finally become an ingredient itself. To illustrate this change of status in 2008, Smithers intitled its review “whey and whey proteins: from gutter to gold”. Now the trend is to extract the whey proteins from the whey in order to optimize its global value. This resulted in the development of WPC at various concentrations, WPI, WPH and even more fractionated pure protein fractions such as beta-lactoglobulin (β -LG), alpha-lactalbumin (α -LA), which are the two main whey proteins, lactoferrin, etc. In parallel, a rich in lactose - poor protein product is produced, leading to whey permeate powders.

Over the past decades, WPC and WPI have been part of a growing market owing to their specific functional properties, including both technological (*e.g.* gelling or foaming) and nutritional properties. The rapid expansion of some dietary products targeting specific populations (infant formulae, specific nutrition for elderly and sportspeople) has boosted their added value on the European and the US market. In Europe, this development concerns WPC35 (protein content of >35%) but also concentrate with higher protein content (>50%) and WPI. In the US, the increased production of high-value whey protein appears to be a real trend.

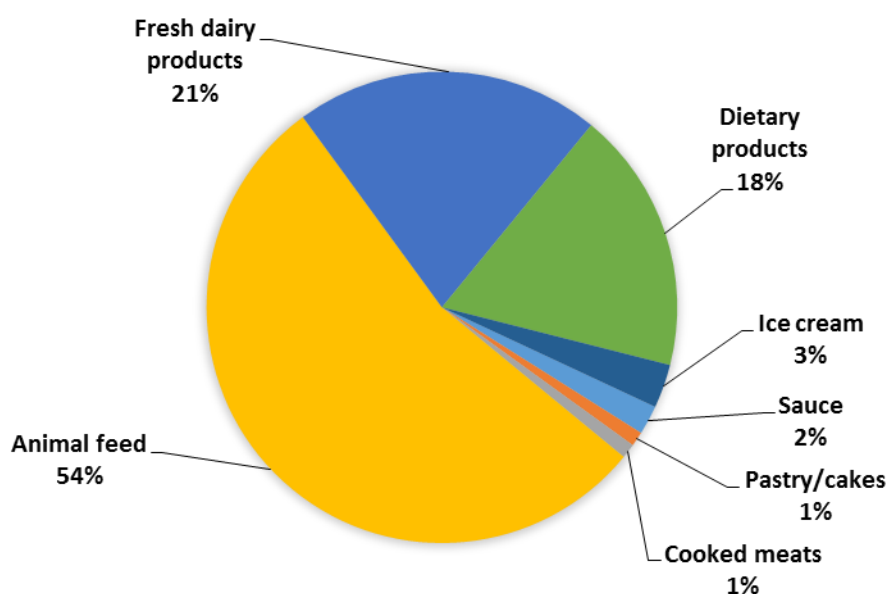


Figure 6: Distribution of WPC applications in the food sector in the EU 27 in 2010 (FranceAgriMer 2012)

The main WPC application sector is animal feed (calves breastfeeding), representing half of the uses of WPC35 (about 170,000 tonnes) (**Figure 6**). In second place, fresh dairy products and diet and health applications are found with the use of around 23,500 tonnes in protein equivalent. Uses of WPCs are more and more developed in the ice cream industry, pastry and sauces due to the society expectations leading to more natural food products (ideally without additives). Whey proteins promote foam formation, gelation, water uptake, emulsification of fat, and improve the melt behaviour of cheese. One of the advantages of WPC is to contain less lactose than the whey powders.

CONCLUSION

In recent years, the production and uses of WPC/MPC and WPI/MPI have developed primarily thanks to their specific functional properties. Indeed, it has been observed that conventional dairy ingredients like skim milk powders and whey powders were gradually replaced by MPC or WPC in food products (including animal feed) consumed in the EU.

It can be further noted that the present development of health and dietary foods should increase significantly the use of high protein dairy ingredients. The demand specifically targets the high protein dairy ingredients: caseinates, MPI, and even more especially the WPI powders.

Thereby controlling the quality of the high protein dairy powders during storage is of crucial importance in order to keep the end use properties of a fresh powder. Indeed, the literature showed that a poor control of storage could cause significant functional changes that may lead to crucial issues in the industrial uses.

PART 2. STORAGE OF HIGH PROTEIN DAIRY POWDERS

1.2.1. INTRODUCTION

Following a growing global demand and a world trade (without intra-EU trade) of 42 billion USD (Rouyer 2015), dairy companies increasingly convert milk and milk derivatives into powders to facilitate storage and transportation across the world. Indeed, drying keeps dairy products over long periods (0.5 to 2 years) and facilitate trade through exportation by lowering volumes from a 5 to 15 times factor, given that milk production and milk consumption zones do not perfectly match.

Considering dairy powders as key ingredients in the formulation of a wide variety of food products, the control of their functional and nutritional properties is crucial as it determines their value and uses. This is particularly the case for products such as those indicated above (infant formulae, products for elderly and sportspeople, etc.) because of their high added value. Consequently, dairy powder properties must be reproducible and remain constant during storage and delivery. However, studies indicate that dairy powders stored under unfavourable temperature and humidity conditions undergo physical (wettability, flowability, browning,...) (E. H.-J. Kim et al. 2009a; Morgan et al. 2005; Stapelfeldt et al. 1997) and chemical (lactose crystallization, Maillard reaction, changes in protein structure, fat oxidation ...) (Le, Holland, et al. 2013; Morgan et al. 2005; Thomas, Scher, Desobry-Banon, et al. 2004) changes with time. The type and kinetics of changes are dependent on the composition of the dairy powder and the storage conditions.

This review aims to report the physical and chemical changes that occur during storage of high protein dairy powders with a special focus on the modifications impacting protein structure and functionalities. The changes of other milk powders will be considered if needed to shed light on the changes occurring in high protein dairy powder.

1.2.2. POWDERS' STABILITY DURING STORAGE

Powders are considered stable as soon as the functional properties of the powder are unchanged after storage. On an industrial perspective, this state has to be extended as long as possible. Dairy powders are predominantly in an amorphous glassy state; therefore the mobility of the molecules in the matrix is highly limited and the powders are considered stable. Molecular mobility and powder stability is dependent on the glass transition temperature (T_g) which is specifically the property of an amorphous material (Bhandari and Howes 1999). When the temperature is above T_g , the amorphous glassy state (high viscosity) change to a rubbery state (low viscosity). This change in viscosity accelerates molecular mobility and then the displacement of reactants towards each other is initiated, which are at the source of physico-chemical and functional changes in the powders (Roos 2002). Powders are considered to be glassy food products which should remain physically, chemically and functionally unchanged over storage, but this is known not to be the case (Roudaut et al. 2004). Indeed, storage is not as well mastered as any other manufacturing steps and powders can be subjected to high temperatures (above T_g) for weeks during delivery (Leinberger 2006). This could affect the molecular mobility and thus induces functional and structural variabilities (**Figure 7**).

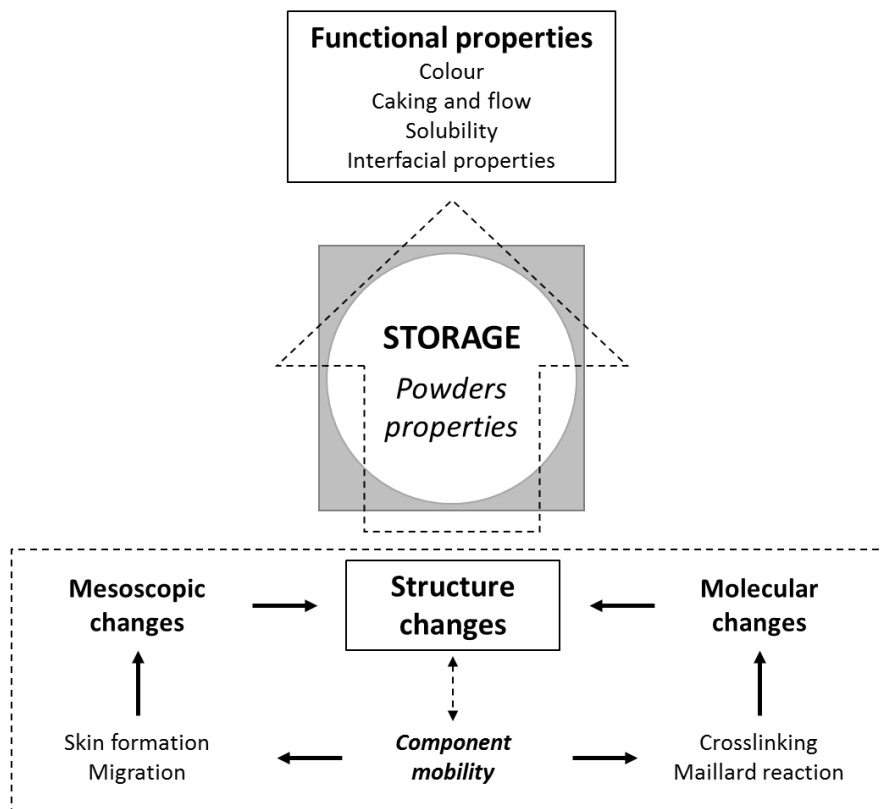


Figure 7: Properties of dairy powders upon storage

1.2.3. CHANGES IN FUNCTIONAL PROPERTIES UPON STORAGE

Ideally, dehydrated products must have similar properties to those of the original product after rehydration (Walstra et al. 2006). However dairy powders are complex systems and of varying composition (Schokker et al. 2011). During storage, some physico-chemical changes may occur depending on storage conditions, the powder composition and the physical state of the constituents. These can affect the physical and techno-functional properties including solubility, powders flowability and surfactant properties of proteins (Thomas, Scher, Desobry-Banon, et al. 2004).

PROPERTIES MODIFICATIONS IN THE DRY STATE

Colour properties. The colour measurements are often run in the L*a*b* space, where L* indicates lightness, and a* and b* are the chromaticity coordinates. Maskan (2001) proposed a formula combining the L*a*b* data to assess purity of the brown colour of food which was called "browning index" (BI):

$$BI = \frac{100 \times (x - 0.31)}{0.17} \quad (1)$$

With

$$x = \frac{a + 1.75 \times L}{5.645 \times L + a - 3.012 \times b} \quad (2)$$

This index is an indicator of Maillard reaction extent, as demonstrated by Martinez-Alvarenga et al. (2014). These authors followed the colour change of a WPI powders with a varying amine:carbonyl ratio, subjected to high temperature ($T \geq 50^\circ\text{C}$) and high humidity ($\geq 50\%$ relative humidity). They were able to establish that the powder increasingly browned as a function of heating time (**Figure 8**).



Figure 8: Colour development in samples treated under different experimental conditions. WPI means whey protein isolate and MD means maltodextrin. Numbers correspond to the reaction conditions: temperature ($^\circ\text{C}$)/ relative humidity (%)/ time (h)/ and molar ratio, respectively (Martinez-Alvarenga et al. 2014)

The same authors established a positive correlation between the disappearance of amino acids in the course of Maillard reaction and the appearance of brown colour (coefficient 0.743). Li-Chan (1983) also reported that, under accelerated storage conditions (37°C for 42 days and with a water activity (a_w) of 0.75), a dairy powder with a dry matter content of at least 35% browned. This browning was correlated to the decrease of residual lactose in the powder and also the decrease in lysine availability.

Caking and flow properties. The viscometer and the penetration test methods are widely used for characterization of caking and adhesion of dairy powders (Özkan et al. 2002).

During storage or transportation, dairy powders are subjected to temperature and a_w variations that affect powders caking and flow properties, depending on powder composition. Differences in caking and flow between WMP and SMP are explained by the particle surface composition. The surface of a WMP particle is mainly covered with a fine layer of fat, leading to a greater cohesion in the powder (Fitzpatrick et al. 2004; Özkan et al. 2002). Caking would primarily be due to the fact that fat forms non-covalent bonds in the powder (Özkan et al. 2002). It also appeared that the particle size is an important factor. The smaller the particles, the more the surface covered with a fat layer; thereby the cohesion of the powder increases which decreases its flow properties (Fitzpatrick et al. 2004). Amorphous lactose also influences flow properties, as it drives the moisture uptake of powder during storage. In fact, powders having a large amount of amorphous lactose are more susceptible to moisture uptake (hygroscopic powder). Water adsorption leads to a decrease of powder glass transition temperature, an increase of lactose crystallization kinetics and a release of water from lactose crystallization in the powder. This leads to the formation of bridges between particles responsible for powder caking phenomenon (Fitzpatrick, Barry, et al. 2007; Fitzpatrick et al. 2004; Fitzpatrick, Hodnett, et al. 2007). Therefore, SMP form more cohesive cakes than WMP because they are composed of a higher amount of amorphous lactose (49.5 - 52% against 36 - 38.5%) and this element is at the origin of caking (Özkan et al. 2002).

PROPERTIES MODIFICATION IN SOLUTION

Solubility properties. Solubility is a key step for powder rehydration, the latter being an essential property of dairy powders because most of them are rehydrated before use (Jeantet et al. 2010).

A well-known property change of micellar casein rich powders is the decrease of the rehydration kinetics and of the protein solubility with storage time, especially when stored above room temperature (Anema et al. 2006; Fyfe et al. 2011; Haque et al. 2010) and at high moisture content (>5%) (Fyfe et al. 2011; Haque et al. 2010), with whey proteins remaining soluble (Anema et al. 2006). By working on a MPC85 powder (protein content of >85%), Havea (2006) observed a reduction in the

powders solubility as a function of storage time because of the gradual formation of an insoluble material. The powders solubility decreased from 53% to 35% after two years storage at 20°C. However, unlike Havea (2006), Schokker et al. (2011) have demonstrated that, by modifying the non-micellar casein content in the powder, the rehydration process was not deteriorated by the presence of an insoluble material but because of a slowing down in the particles dispersion. These authors also showed that increasing the concentration of non-micellar caseins before drying reduced the loss of solubilisation after storage. To explain this result, they proposed two possible mechanisms. The first is based on the preferential adsorption of non-micellar caseins at the liquid-air interface during spray drying thereby reducing the proportion of casein micelles at the particle surface. As a consequence, the intensity of interactions between micelles is decreased. The second mechanism involves the limitation of micelle-micelle interactions through steric hindrance caused by the presence of non-micellar caseins. Gazi and Huppertz (2015) add that only micellar caseins become insoluble during storage, whereas non-micellar caseins remain soluble, strengthening the results of Schokker et al. (2011). Adding NaCl or chelating agents to micellar casein concentrate before spray-drying improve the rehydration properties of the powder (Schuck 2002). The release of caseins from the micellar casein structure was hypothesized to be responsible of the observed change in rehydration properties.

Foaming, emulsifying and interfacial properties. Whey proteins have good emulsifying and foaming abilities thanks to their amphiphilic nature, solubility and size. They are not as good as caseinate to stabilize emulsions but they are better foams stabilizers because they form a viscoelastic interface (Croguennec et al. 2008).

The Maillard reaction, which occurs in dairy powders ageing, seems to be one of the major cause of changes in the interfacial properties.

It appeared that surface tension of protein solutions was unaffected by Maillard reaction (Fechner et al. 2007; Hiller and Lorenzen 2010). However, all authors do not share these observations as for Baeza et al. (2005) who claimed that the Maillard reaction products increase the surface tension of protein solution. These differences could be due to the type of sugar used, not all have the same properties and the same ability to induce protein structural change after binding.

Regarding the foaming properties, foamability (surfactant ability to encapsulate air) is diminished by mixtures of milk protein/glucose and milk proteins/lactose due to the increase formation of neoformed Maillard reaction products (Hiller and Lorenzen 2010; Thomas, Scher, Desobry-Banon, et al. 2004). In addition, some authors noted an improvement in foamability (Martinez-Alvarenga et al. 2014), but it is probably due to the heat treatment in the dry state that induces protein denaturation

rather than the actual glycation (Báez et al. 2013). On the other hand, it has been shown that the previous mixtures improved foam stability (Hiller and Lorenzen 2010). These changes would be due to Maillard reaction products because they result in a steric protection against coalescence (Dunlap and Côté 2005), or because they are found in the interfacial film which would be thicker and more viscoelastic (Ganzevles et al. 2006; Wooster and Augustin 2007). For Báez et al. (2013), aggregates formed by Maillard reaction (β -LG + glucose) deteriorate foam stability by decreasing the rigidity and viscoelasticity of the interfacial film. The difference between these studies is in the foaming process, since the latter was using a bubbling apparatus whereas the former used an Aero-Latte stirrer, generating two different types of foam.

Changes in emulsifying properties, dependent on the type of milk powder, have also been highlighted. Hiller and Lorenzen (2010) observed a low emulsifying activity of sodium caseinate/lactose and glucose, whey proteins/glucose mixtures due to the formation of Maillard reaction products of high molecular weight causing a delay in the adsorption and in the deployment at the interface. Only mixtures of hydrolysed SMP and whey proteins with lactose have improved emulsifying activity. However, other authors have seen no difference, including Oliver et al. (2006) with sodium caseinate/glucose or lactose mixtures and French and Harper (2003) with a β -LG/lactose mixture.

Last, emulsion stability was also assessed. It increased for mixtures of milk proteins/glucose or lactose and decreased for mixtures of sodium caseinate or WPI/glucose or lactose. The decrease was explained by the fact that Maillard reaction products would cover an insufficient area of the interface and/or Maillard reaction products of high molecular weight would be left at the interface of several lipid droplets thereby promoting coalescence.

1.2.4. CHANGES IN STRUCTURAL PROPERTIES OF AGED POWDERS

Components in powders may evolve during storage; the kinetics of the changes is dependent on the composition of the powder and the storage conditions (Haque et al. 2012). Two levels of structural changes are commonly considered: the mesoscopic scale and the molecular scale, both being governed by molecular mobilities. These structural changes are assumed to be the sources of the modifications in the functional properties observed above.

The aim of this section is to understand the structures modifications during storage.

POWDER STABILITY AND COMPONENT MOBILITY

Variations in the component – or molecular – mobility including internal molecular motions and molecular migration or diffusion, are related to stability of dairy powders during storage (Fan and Roos

2016). Many milk powders are in an amorphous glassy state (Bhandari and Hartel 2005). Amorphous glassy systems are in an out-of-equilibrium, arrested state and as such are considered as stable as long as they are stored below T_g (Struik 1977). However, this condition is not always sufficient to ensure the physical and chemical stability of protein powders during long storage (Zylberman and Pilosof 2002). In terms of the free energy landscape, protein powders might still undergo a progressive relaxation process towards a more stable state. The change in powders occurring during the relaxation process is called physical aging (Schokker et al. 2011). The relaxation enthalpy is specific to the molecule and local including restricted long-range cooperative motions and internal molecular motions (not involving surroundings molecules) in the glass (Fan and Roos 2016) or it can also concern translational molecular motions of molecules or segments of molecules when the glassy material is heated to above its glass transition (Y. Liu et al. 2006). The level of physical aging is increasing above T_g and typically higher at high a_w , water acting as a plasticizer and promotes relaxation processes (Struik 1978). It is associated with macroscopic changes such as density, flowability and caking (Y. J. Kim et al. 2003) which are of practical importance as they play on the stability and shelf life of dairy powders.

Several studies have been carried out on the molecular mobility in milk powders during storage. Regarding to the study of Haque et al. (2012), increasing the moisture content initiates the relaxation of the proteins to a more thermodynamically stable state by molecular motions of protein segment. This has been verified for a MPC powder stored at $25 \pm 1^\circ\text{C}$ for 11 weeks under relatively high a_w conditions above or equal to 0.45. For a_w conditions below or equal to 0.23 molecular motions were restricted due to the lower amount of water molecules surrounding proteins. Molecular rearrangements would be the result of molecular motions in the relaxation processes and, with longer storage time, would lead to interactions between casein micelles responsible for the loss of solubility (Haque et al. 2012). During storage, molecular mobility can also be influenced by lactose crystallization, a process during which water is released into the reactive medium (Thomsen et al. 2005).

Rearrangements caused by the molecular mobility can therefore affect protein structures and thereby molecular interactions (Abdul-Fattah et al. 2007).

STRUCTURE MODIFICATIONS

MODIFICATIONS AT A MESOSCOPIC SCALE

Changes in surface, formation of skin. Several authors, as Anema et al. (2006), have shown that storage modulates powder functions through changes on the surface of powder particles which can be emphasise with time, temperature and relative humidity. As the particle surface is the first zone in

contact with water, any change at this point could be responsible for modification of rehydration properties (Fyfe et al. 2011).

Few changes of the particle surface of stored powders have been highlighted by Mimouni et al. (2010) using scanning electron microscopy (SEM) during powder rehydration. After 2 months storage at 20°C, a skin like layer was detected through the microstructure analysis of powder particles (**Figure 9**, A and B) while the particle surface of fresh MPC80 powder (protein content >80%) is smooth (Tamime et al. 2007) (**Figure 9**, C and D). This was explained by the increase of the particle density and a compaction of the casein micelles at the surface of the particle leading to short and direct inter-micellar bridges forming a new network. It is in accordance with Fyfe et al. (2011) who put forward that storage conditions of MPC powders, located between 25 and 40°C with relative humidity above 44% during 3 months, will increase the particle density with water uptake. Anema et al. (2006) mentioned that powders may undergo some degree of molecular rearrangements during long-term storage, such as protein crosslinking.

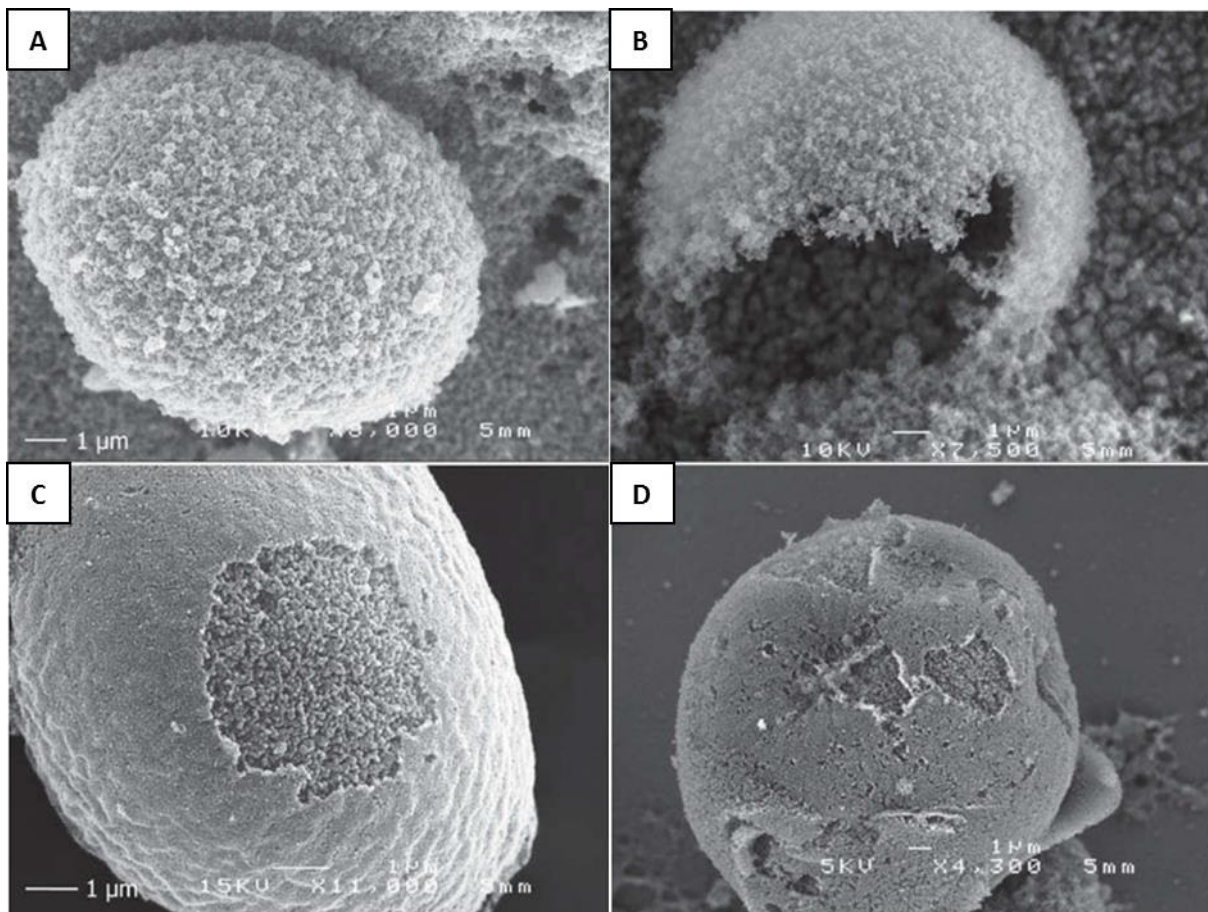


Figure 9: SEM images of particle surface of fresh powder after 10 and 80 min of rehydration (A, B respectively); of a 2 months stored powder at 20°C after 10 and 80 min of rehydration (C, D respectively) (Mimouni et al. 2010)

The dissolution of aged MPC powder particles is then much slower. During dissolution, the cross-linked micelles appeared to be the last portion of the particle to be dispersed which lead to a possible relation between the decrease in solubility of aged MPC powder and the skin formation from inter-micellar crosslinking.

Changes in surface, migration of lipids. Changes in the surface composition have also been observed during powder storage (E. H.-J. Kim et al. 2002; Nijdam and Langrish 2006). In order to monitor these modifications, X-Ray Photoelectron Spectroscopy (XPS), also known as Electron Spectroscopy for Chemical Analysis (ESCA), seems to be the ideal method to perform (Fyfe et al. 2011; Gaiani et al. 2006; E. H.-J. Kim et al. 2002). In fact, XPS gives information on elementary elements (C, O, and N percentages) within 10 nm of the particle surface. The method described by Fäldt et al. (1993) permitted to obtain fat, protein and lactose contents. Fyfe et al. (2011) observed that fat content at the particle surface of MPC powder decreased and the protein content increased after 30 days storage or more at 40°C and relative humidity between 44 and 84%. Beyond 90 days storage, the reduction of fat at the surface was set against the significant increase in lactose content. However, all researchers do not share the same point of view. For example E. H.-J. Kim et al. (2005) did not find significant changes in the surface composition of several milk powders after 6 months of storage at 20°C. On the other hand, Gaiani et al. (2009) shows that lipid content at the particle surface increases from 6 to 17% after 60 days of storage at 20°C or 50°C (with no effect of storage temperature) and the protein percentage at the surface was decreased from 94% to 83%. The difference between the first study and the latter might be attributed to the kind of powder as Gaiani et al. (2009) were working on micellar casein powder. Indeed, the fat content at the particle surface was around 30% for the first study against 1.5% for the latter. According to Fäldt (1995), an irregular and wrinkled surface is associated with the presence of fat thus supporting the hypothesis of a lipid release at the particle surface, which is also believed to be linked to the presence of pores (Baechler et al. 2005; Gaiani et al. 2009).

MODIFICATION AT A MOLECULAR SCALE

Changes in secondary and tertiary structures. Protein conformational modification is one of the major factors inducing instability of protein of MPC powder during processing and storage. Indeed, after denaturation, hydrophobic regions of proteins are exposed, favouring protein-protein interactions (Arrondo and Goñi 1999).

Several authors have used Fourier Transform Infrared spectroscopy (FTIR) to monitor changes in protein structure. Schokker et al. (2011) compared the protein structure of fresh and stored MPC85 powders during 54 days at 30°C and detected protein unfolding. Haque et al. (2010) highlighted the

importance of moisture content with an MPC powder stored 12 weeks at 25 and 45°C and at a_w from 0 to 0.85. The higher the moisture content, the higher the amount of α -helix decreases and of β -sheet increases.

Above, mesostructural analysis already demonstrated in MPC powder that casein micelles increasingly interact with storage time leading to a skin formation (Mimouni et al. 2010). However, spectral changes are too minor to enable direct correlation between loss of solubility and modification of the secondary structures of the caseins (Kher et al. 2007). The protein unfolding cannot be the only responsible for the loss of solubility, but it may be the initiation step leading to change in casein micelle structure, possibly involving intermolecular β -sheet formations, which at the end affect the solubility (Haque et al. 2010). The Small Angle X-rays Scattering (SAXS) profiles of fresh and stored MPC powders showed similar profiles showing that the internal organisation of casein micelles was not altered by storage (Schokker et al. 2011).

Concerning whey proteins, studies focused on dry heating of β -LG at 100 °C for 24 hours and controlled pH showed that secondary and tertiary structures evolve (Gulzar, Bouhallab, and Croguennec 2011). In particular, the authors observed that at acidic pH (2.5), the rigid environment of tryptophan is partially lost. Changes in the secondary structures of the proteins were detected at acidic pH and neutral pH. Other authors, such as Havea (2006), also showed changes in the structure of proteins with an increased exposure of hydrophobic residues by denaturation during storage.

Protein interactions and crosslinking. The insoluble material appearing upon reconstitution of stored micellar casein rich powders is thought to be the result of protein-protein interactions, which are mainly hydrophobic. It was observed for a storage in more severe conditions (50 days at 40°C) that caseins and the minor whey proteins would be involved in the formation of the insoluble material, while α -LA and β -LG remained soluble (Anema et al. 2006). Gazi and Huppertz (2015) believe that the insoluble fraction formed during storage of a MPC at 50°C is a combination of denatured whey proteins and casein micelles. It is also the case in the study of Havea (2006) who claimed that both soluble and insoluble covalent aggregates were mainly composed of K-casein and β -LG linked by disulphide bonds during storage of a MPC. Indeed, during storage of whey protein concentrate powders, whey proteins partially denature and a gradual polymerization was observed (Morr and Ha 1993). It is important to note that temperature and storage time have a positive effect on aggregation and this from 25°C (Morr and Ha 1993). But according to Havea (2006), these aggregates are also found in fresh MPC powder, which means they are not exclusively formed during storage. Casein interactions with each other also increase with storage time. These are responsible for the formation

of a very compact layer at the particle surface acting as a barrier to rehydration and thereby slowing the dissolution of powders (Anema et al. 2006; Mimouni et al. 2010).

After a dry heat treatment of WPI powder at 100°C for 24h at pH 2.5, pH 4.5 and pH 6.5, Gulzar, Bouhallab, Jeantet, et al. (2011) observed a decrease in the amount of native whey proteins in parallel with formation of aggregates whose size increases with pH. At acidic pH, aggregates are soluble and covalent bonds between proteins are only made of disulphide bridges while at higher pH, aggregates are partly soluble and covalently linked by disulphide bonds and other types of bonds that still have to be determined.

α-LA cyclization in WPI powder. Aside the formation of covalent aggregates, a significant proportion of the non-aggregated whey proteins during dry heating treatment was converted into non-native forms. Mass spectrometry analysis revealed a change in the molecular weight of the whey proteins attributed to a loss of one or two water molecules per proteins. The loss of water molecules was observed for 73% of non-aggregated α-LA and only 18% of non-aggregated β-LG molecules. For α-LA, water molecule loss was attributed to the formation of pyroglutamic acid from the N-terminal glutamic acid and to the formation of an internal cyclic imide at position Asp 64 (**Figure 10**).

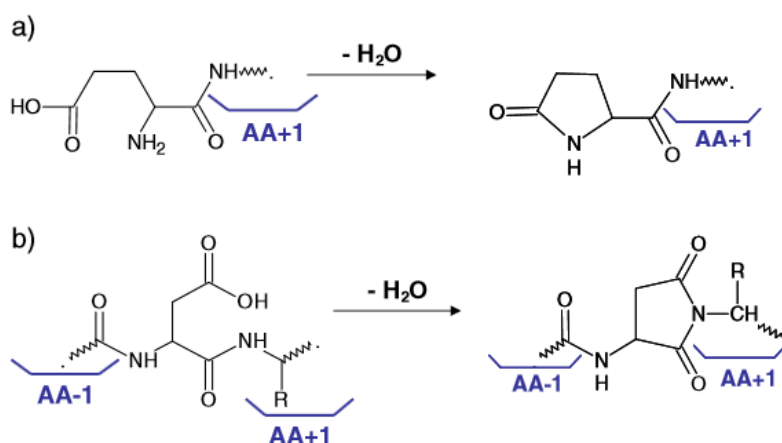


Figure 10: Chemical structures of (a) N-terminal pyroglutamic acid resulting from the cyclization of the N-terminal glutamic acid of α-LA, (b) a cyclic imide resulting from the cyclization of an internal aspartyl residue (Gulzar et al. 2013)

Maillard Reaction. Maillard reaction, or non-enzymatic browning, usually develops during processing and storage of milk protein powders. This reaction modifies protein structures and leads to Maillard reaction products with specific functional properties. Its control is difficult since it is composed of three steps involving a multitude of reactions whose kinetics are dependent on various intrinsic and

extrinsic factors (Martins et al. 2000). The first step is called "Early stage" and leads to lactosylation of an amine function basically of a protein. At this stage, the major consequence is the loss of available lysine (Van Boekel 1998). The second stage described as "Advanced stage" results in the formation of Advanced Glycated End products (AGE) initiating protein crosslinking. The third phase is the "Final stage" and leads to the formation of melanoidins which are molecules generated by polymerization reactions from the highly reactive AGE. Melanoidins are high molecular weight brown compounds (Van Boekel 1998).

The specificity of lactosylation sites is related to the exposure of amine residue at the protein surface and its chemical environment. Thus, a large number of combination of lactosylation sites would be expected for whey proteins in specific heat treatment conditions. β -LG and α -LA are composed of 15 and 12 lysines respectively and several authors who have worked on the β -LG agree on the lysines that are most involved in Maillard reaction in a powder state or in solution (Fogliano et al. 1998; Losito et al. 2010; Morgan et al. 1997; Morgan, Léonil, et al. 1998). Morgan, Bouhallab, et al. (1998) have identified every lysine which are involved in the lactosylation reaction of β -LG (**Table 3**). In particular, Lys 60, 83, 135, 141 and 8 have been reported to be particularly reactive in a powder state. Moreover, they observed by mass spectrometry a heterogeneity of populations of glycosylated β -LG by the number of lactose bound per protein, which can range up to 11 lactose molecules after 22h of reaction at 50°C and 65% relative humidity.

Table 3: Progressive reactivity of β -LG sites towards lactose in dry way glycation; protein/lactose ratio of 1:50 under 65% relative humidity at 50°C (Morgan, Bouhallab, et al. 1998)

Reaction time (h)	Glycated sites
0	Lys _{47,91}
2	NH ₂ -term, Lys _{15, 70, 100}
6	Lys _{60, 69, 75, 77, 83, 135, 138}
10	Lys _{8, 141}

Concerning SMP, Guyomarc'h et al. (2000) showed that the extent of protein lactosylation during storage were very slight or nil over 16 weeks at 20°C; at 37°C lactosylation rapidly increases over the first two weeks of storage then slows beyond; finally, lactosylation peaks were no longer detected after only 3.5 weeks at 52°C, the powder having a pronounced brown colour resulting from the Maillard reaction extent (Guyomarc'h et al. 2000; Van Boekel 1998).

The presence of lactose causes an increase in protein crosslinking in WPI powders, forming compounds of more than 100 kDa (Guyomarc'h et al. 2014; Le, Holland, et al. 2013). AGE are probably

involved in these interactions following lactosylation (Le, Holland, et al. 2013; Singh 1991). The greater the amount of lactose, the higher the level of crosslinking. Even if lactose is present in small quantities, whey proteins are modified through the formation of Maillard reaction products which then participated to the formation of large aggregates. The effect is more important on caseins, which become insoluble, than on whey proteins which remain soluble (Le, Holland, et al. 2013). It is noteworthy that lactosylation started during spray drying possibly due to the temperature applied as proteins in fresh milk are not lactosylated (Anema et al. 2006). In order to study this phenomenon, these authors stored a MPC powder during 10 days at 50°C. After 3 days of storage at 50°C, the level of lactosylated casein increased and species with two lactose groups attached were detected while 5 days later, lactosylation didn't evolve that much. As for whey proteins, Maillard reaction progress through interactions of lactose molecules on the lysines of caseins micelles which lead to further crosslinking affecting the powder solubility (Anema et al. 2006).

According to Hiller and Lorenzen (2010), the first step of Maillard reaction (lactosylation) does not lead to whey protein conformational changes. Indeed, the compact conformation (globular) of whey proteins would limit changes induced by the sugar binding. When the reaction progressed polymers of heterogeneous size are formed concomitantly to the disappearance of monomers present at first and the decrease in the soluble fraction of nitrogen. In this way, Guyomarc'h et al. (2014) reported a study on denaturation and aggregation of whey proteins in three types of powder – one commercial WPI (WPI_C) and two model WPI powders composed of pure proteins, one without added lactose (WPI_{M-L}) and the other one with lactose (WPI_{M+L}) in an equivalent amount as measured in WPI_C . It has been observed that the presence of lactose (WPI_{M+L}) has a dramatic effect on aggregates formation at pH 6.5, this suggests that some covalent intermolecular bonds formed in the WPI_C at pH 6.5 involved some degradation products of Maillard reaction. Conversely in WPI_{M+L} powders at acidic pH (pH 2.5), condensation reaction between lactose and whey proteins is a limiting step preventing further aggregation.

Finally, Hiller and Lorenzen (2010) reported that several studies suggested a loss of organised tertiary protein structures upon covalent binding of sugar molecules. Sugar binding induces a steric stress favouring protein unfolding by a decrease in intra/inter molecular interactions (Nacka et al. 1998; Wooster and Augustin 2007). The loss of tertiary structure was also observed upon temperature (Broersen et al. 2004) and humidity (Morgan et al. 1999) effects on proteins. For Báez et al. (2013), glycation did not result in changes in the secondary structure of proteins, but led to very slight changes in tertiary structure. This structural change come out as an exposure of tryptophan after partial protein unfolding as well as an appearance of a small amount of aggregates after glycation.

However, according to these authors, conformational changes are more due to heat treatment than protein glycation.

The Maillard reaction in powders is strongly dependent on the storage conditions: temperature, water activity (a_w) and time. Within these factors, temperature and a_w have the most significant impact in the extent of Maillard reaction (Martinez-Alvarenga et al. 2014; Pereyra Gonzales et al. 2010). The temperature influences the reaction kinetics (Cheriot 2007; Pereyra Gonzales et al. 2010; Thomsen et al. 2005) and in dairy powders the Maillard reaction proceeds maximally when a_w is between 0.50 and 0.80 (Martinez-Alvarenga et al. 2014; Thomas 2004). Above, the dilution of reactants and high water content lead to a reaction limitation because of the mass action law.

1.2.5. CONCLUSION

The shelf-life of a dairy powders clearly depends on its composition and storage conditions. Powders are relatively stable products compared to non-dehydrated products but they are still subjected to molecular mobility which is enhanced at temperature above the powder's T_g . The molecular mobility are responsible for mesoscopic structural changes (*e.g.* component migration) and for local molecular changes (*e.g.* protein cyclisation) and also for interaction reactions with other components in the medium (*e.g.* polymerization reactions, skin formation). These structural modifications are believed to be at the origin of the modification of the dairy powders' functions occurring during storage. Understanding the mechanisms of structural changes would help to avoid or at least minimize usability issues after long-term storage. Temperature, time and moisture content are crucial parameters to take into account in order to control the quality of powders. As such, optimum conditions should include storage at low temperature and low moisture content.

PART 3. ABOUT THE PHD PROJECT: AIMS AND STRATEGY

1.3.1. CONTEXT AND RESEARCH QUESTION

Regarding the economic weight of the high protein dairy powders worldwide, the control of their quality among storage or export is a major challenge in terms of compliance to consumers' requirements and dairy companies' competitiveness.

INDUSTRIAL CHALLENGE

This study tackles with a real industrial challenge since whey protein isolate powders are supposed to be extremely stable thanks to their high protein content and T_g . However, their functionality

sometimes does not match with the factory tests. Our aim is to identify the cause of these changes occurring during storage and delivery.

This PhD project is incorporated within the framework of the project CODE POUDRE, a research programme funded by the *Centre National Interprofessionnel de l'Economie Laitière* (CNIEL). This project aims to provide predictive tools of the high protein powder behaviour during their storage so as to adjust storage conditions according to the expected features or limiting their evolution among storage.

IMPORTANCE OF MONITORING THE DELIVERY AND STORAGE CONDITIONS

As shown by campaigns of temperature measurement inside containers during continental and intercontinental delivery, extreme temperatures – above 50°C – can be underwent by the powders and this repeatedly for some deliveries over 3 months (**Figure 11**) (Médecins Sans Frontières 2009). Similarly, Leinberger (2006) showed that more than 40% of shipments were experiencing temperature above 40°C (**Figure 12**). Deliveries also involve breakpoints (sometimes unexpected ones) and therefore powders can be stuck in temporary storage points that can last for several weeks. Leinberger study (2006) only shows the time of delivery but it does not take into account storage time of powders on their final destination, although they can be stored in non-insulated depot for up to 2 years before use.

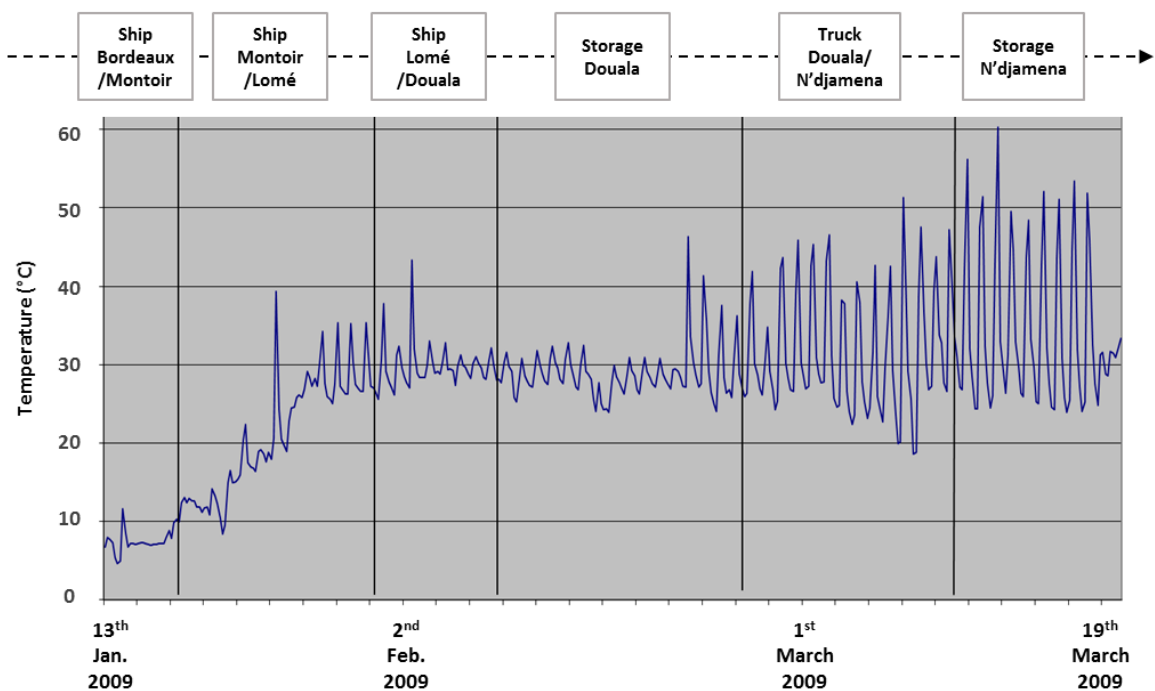


Figure 11: Temperature record during exportation: France toward Tchad (Médecins sans frontières 2009)

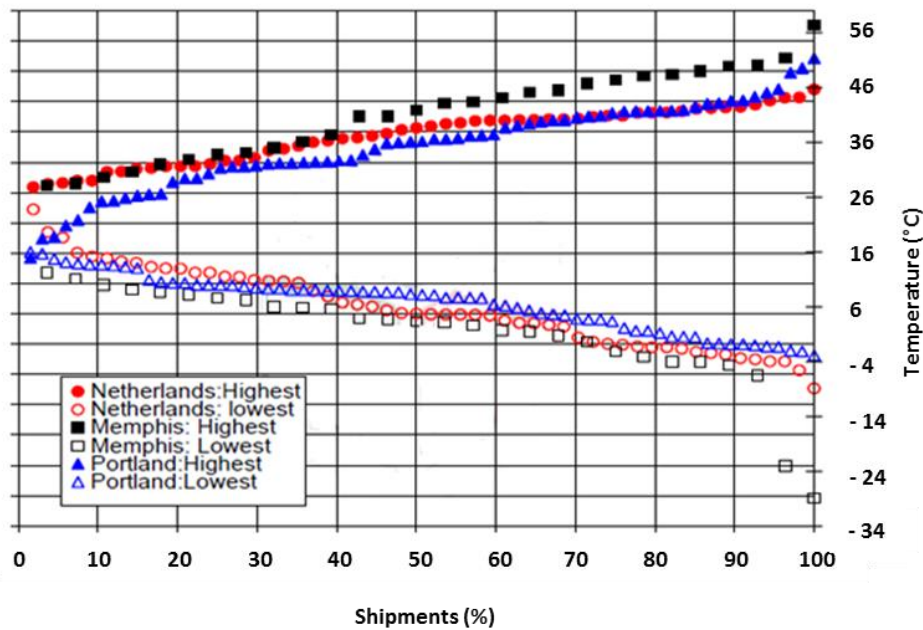


Figure 12: Temperatures reached by cumulative shipments (Leinberger 2006)

The literature review showed that these storage conditions involved changes in structural and functional properties of high protein dairy powders even at low storage temperature, thus making the definition of storage conditions a competitive key advantage on an industrial scale. Indeed, it will enable the increase in powders shelf-life and guarantee their functionality.

However, studies published to date were mainly conducted on MPC and native phosphocaseinate powders or on model systems of whey proteins. As a consequence, there is not much on WPI powders' storage in the literature even though this is a key ingredient for food industries. This comes to the *research question* of this PhD project:

'What are the structural markers of the evolution of whey protein isolate powders during storage and how they affect powders' functionalities after rehydration?'

Ultimately, monitoring of high protein dairy powders changes during storage will provide tools and predictive methods of structural ageing by identifying markers of ageing and qualifying sensors able to trace the storage history. These tools will make it possible to map the product evolution relative to the expected properties, and adapt storage to the type of product and/or the optimal duration of use of the route taken during export. Therefore, they are essential outputs to ensure the functionalities expression of these products.

1.3.2. OBJECTIVES AND STRATEGY OF THE PHD RESEARCH PROJECT

OBJECTIVES

Many studies have already been conducted on whey powders. However, few studies display the reactions occurring during storage of whey protein isolate (WPI) powders, the latter being constituted of at least 90% proteins (w/w) (Burin et al. 2000), of which essentially whey proteins including about 70% β -LG and 20% α -LA (McClements et al. 1993), and extremely low amount of minerals and lactose thanks to diafiltration before drying. Moreover, due to the specific properties and high added values of these high protein dairy powders, the knowledge of their behaviour during storage is a major concern for the dairy industry.

Ahead of this project, WPI ingredient users have been reporting changes in product functionality among ageing, involving browning and protein denaturation. At this stage, it was difficult to establish clear correlation between structural changes, changes in functionality and storage conditions, as these latter weren't controlled neither monitored. The general objective of this study was to fill this gap by conducting a systematic study on how the storage conditions of well-characterized WPI powders affect the structure-functionality relationships.

More precisely, the 3 main objectives of this PhD project were to:

- x Identify and quantify changes in protein structure and functional properties during ageing under controlled storage conditions
- x Identify the relevant structural markers of the evolution of WPI powders during storage affecting their functionalities and understand the contribution of protein modifications on powder functional changes
- x Validate these changes and define optimal storage conditions [initial state, duration t , temperature T] allowing to maintain the powders properties in the expected specifications, and conversely the extreme storage conditions above which these properties are irreversibly affected.

STRATEGY

Our study focused on the structural changes of whey protein isolate (WPI) powder stored under different conditions and their effects on the functional properties of the proteins once recombined in aqueous solution. In particular, this research aimed to investigate structural changes such as protein denaturation and aggregation in order to understand functional changes (foaming properties and heat-induced aggregation after recombination) that occurred in WPI powders after 15 months of

storage. Understanding these changes may represent a key point in the control of the properties of high protein dairy powders, providing a major competitive advantage for the dairy industry.

As mentioned earlier, it was of prime importance to control the initial state of the powder, particularly regarding protein denaturation limitation and exact environmental conditions underwent from the production stage. Therefore, all the WPI powders studied in this project were produced from fresh industrial WPI concentrate on the Bionov pilot workshop (Rennes, France).

The choice of the storage conditions derived principally from the ones currently used in the food industry. As powders are stored between 1 and 2 years, the storage time was of 15 months. Concerning the temperature, our aim was to choose temperatures allowing to sweep all the changes that can happen during storage and that are also relevant for the food industry. As such, 20°C, 40°C and 60°C were taken to lead this study. The ambient temperature is currently widely used to store all powders. 40°C appear to be a temperature midpoint during delivery (cf. 1.3.1., p28) so it appeared as a judicious temperature set. It was also interesting to go beyond, hence 60°C was taken as the highest temperature studied. It may seem to be a high storage temperature, however we have seen above (cf. 1.3.1., p27) that 60°C is an extreme condition but real for industrial exportations as it can be repeatedly reached during delivery. For longer storage time (>3 months), the treatment was pushed into more severe storage conditions in order to understand and/or validate the mechanisms of interaction between the various components in the powder (or solution) and thus to bring out the major players.

A 6-STAGES DEVELOPMENT

The project was conducted in 6 phases in which strategies were refined and validated during steering committees with the CNIEL (**Figure 13**).

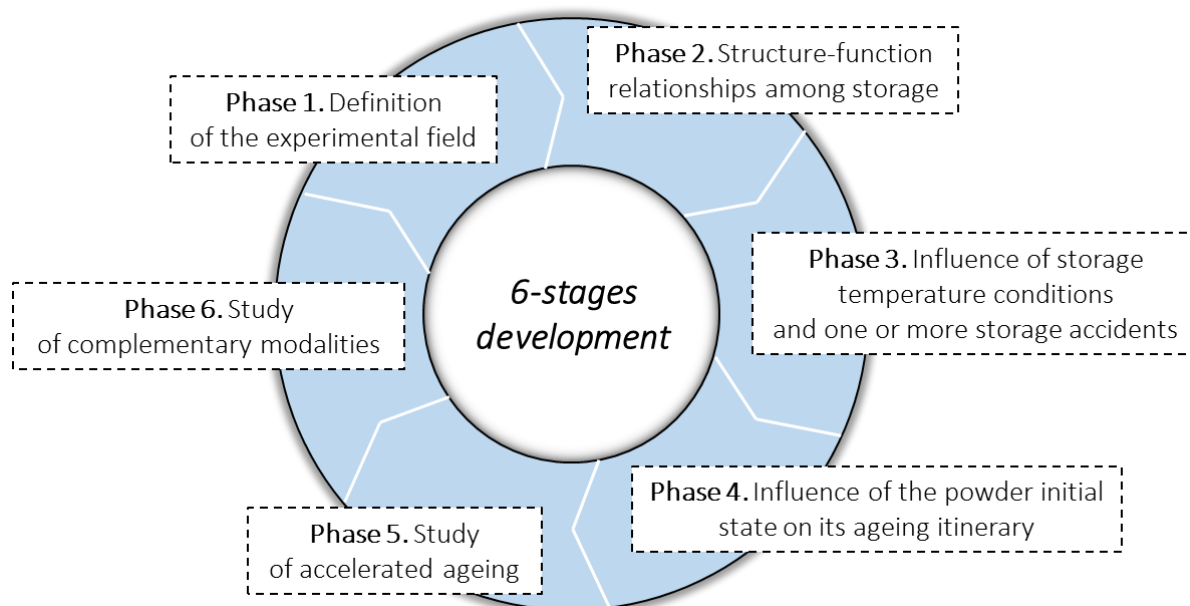


Figure 13: Organization of the PhD research project

The first step was conducted as a screening towards a better definition of the experimental field. The two following phases aimed to simultaneously quantify the kinetics of protein structural changes and functional properties under controlled storage conditions. The objective of these phases was to establish links between protein structural changes observed in the dry state and the modifications of functional properties both related to the storage conditions. These were orchestrated so as to answer the following question “*Are we in the presence of a catch-up phenomenon or different routes of ageing?*”. In addition, one or more storage accidents were mimed to quantify the impact of such an event on the powder ageing kinetics. The structural and functional parts of these phases are exposed in the second and the third chapters, respectively. Following this, the influence of the powder initial state on its ageing itinerary was studied. In the literature, it is known that the changes occurring during powders ageing are also inherent to the initial state of the powder (powder composition, drying parameters ...). The objective of this phase was to understand how the powder initial state in interaction with the storage conditions affects the structural and functional changes in the powder among storage. In particular and despite the very low lactose concentration, browning reported by WPI ingredients users suggested that the Maillard reaction still occurs in the powder and contributes to the ageing of the WPI powder. Therefore, this study, reported in the 4th chapter, aimed to precise the concentration threshold at which lactose still contributes to the ageing phenomenon. This chapter also gathers the study of complementary modalities including assessing the impact of the drying method. Finally, the general discussion (Chapter 5) provides correspondence between accelerated ageing conditions (*e.g.* high temperature) and actual kinetics considered in phases 2 and 3.

CHAPTER 2. STUDY OF THE STRUCTURAL CHANGES OCCURING IN THE WPI POWDER DURING STORAGE

In this chapter, the structural changes occurring during storage in the powder state are investigated in experimental conditions close to those on an industrial scale (time, temperature and water activity). It is highly important to identify the structural markers and understand the mechanisms involved in the ageing of WPI powders in order to control their end use properties. Six different storage conditions combining temperature and a_w control (20, 40 and 60°C; a_w 0.23 and 0.36) were applied over 15 months to WPI powders produced from fresh concentrate. These powders were characterised according to the mesoscopic and the molecular scales. The first was set to characterize changes on the particle surface and monitor the powder colour. The second involved protein denaturation, protein lactosylation and protein aggregation to better understand the role of proteins in the mechanisms of the storage-induced structural changes. It was revealed that the powders were relatively stable during storage at 20°C. On the other hand, powders presented hydrophobic patches and a loss in the amino acid content on the powder particles while significant levels of aggregation and protein modifications were observed at a molecular scale for storage at higher temperatures. A part of these results has been published in *Food And Bioprocess Technology* journal (Paper²), *Journal of Food Engineering* (Paper³) and *Journal of Dairy Science* journal (Paper⁴). WPI powders were also subjected to storage accidents, involving short exposure at high temperature. The purpose of this additional study was to assess their impact on changes occurring upon ageing.

The aims of this chapter were to:

- x *investigate powders' changes in the dry state under controlled storage conditions*
- x *specifically quantify the changes in protein structure*
- x *identify pertinent structural markers of WPI powders' ageing*

² Published as Norwood EA, Chevallier M, Le Floch Fouéré C, Schuck P, Jeantet R, Croguennec T. (2016). Heat-induced aggregation properties of whey proteins as affected by storage conditions of whey protein isolate powders. *Food and Bioprocess Technology*, 9(6), 993–1001. doi:10.1007/s11947-016-1686-1.

³ Published as Burgain J, El Zein R, Petit J, Norwood EA, Francius G, Gaiani C. (2016). Local modification of whey protein isolate powder surface during high temperature storage. *Journal of Food Engineering*, 178, 39–46. doi:10.1016/j.jfoodeng.2016.01.005.

⁴ Published as Norwood EA, Le Floch Fouéré C, Schuck P, Croguennec T, Jeantet R. (2016). Structural markers of the evolution of whey protein isolate powder during aging and effects on foaming properties. *Journal of Dairy Science*, 99(7), 5265–5272. doi:10.3168/jds.2015-10788.

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PART 1. INTRODUCTION

The drying process is widely applied at the industrial level with the main objective to increase the product shelf life and reduce transport costs. Ideally, dehydrated products should exhibit similar properties to those of the original product after reconstitution in water (Walstra et al. 2006). Thus, the main technological challenge in this field is to prevent unwanted changes that may occur during storage, and that could lead to a loss of techno-functional, sensory and/or nutritional characteristics of the rehydrated product (Walstra et al. 2006).

The preservation of powders properties depends on the processes implemented in the powders production (concentration processes, spray-drying parameters, etc.), their composition and physico-chemical characteristics, as well as their storage conditions (time, temperature, water activity). This set of parameters defines the powders sensitivity to different types of alteration, their end-use properties and the conditioning requirements with regard to their conservation ability.

As presented in Chapter 1, powders may be subjected to extreme conditions, such as temperatures as high as 57°C for weeks, during continental and intercontinental delivery (Leinberger 2006). Such conditions can lead to deterioration of powder properties and a reduction in their commercial value. The definition of storage conditions that increase powder shelf-life while guaranteeing their functionality is a competitive key advantage on an industrial scale.

Among the changes identified in the dry state, it can be found changes in protein structure including protein cyclisation or protein aggregation (Gulzar, Bouhallab, Jeantet, et al. 2011; Gulzar et al. 2013), the protein structure being the key of the expression of specific powders properties. Changes inherent to the particle surface have also been identified for high protein powders, in particular MPC80. This is an important issue in view of the powder uses, the surface being the first contact point with water when rehydrated (Fyfe et al. 2011); it is well known that the surface composition can affect the powders' functional properties, such as its rehydration, density and flow properties (Gaiani et al. 2006). Last, reactional changes have been exposed especially with the Maillard reaction, altering the powder visual appearance and which can lead to functional and/or nutritional issues. However, the literature is principally based on the study of protein concentrate powders and does not provide information on WPI powder stability upon ageing. This causes a research gap in the field of study of high protein dairy powder changes during storage.

Thus, in this study, two WPI powders were produced from fresh whey protein concentrate: the first one with an a_w close to industrial regular storage conditions (0.23), and a second one with a_w representative of a poor a_w control (0.36). These powders were then stored at representative storage

temperature conditions (from 4°C to 60°C). Then the evolution of powder particle and the protein structures were monitored up to 1 month and 15 months, respectively. The results will be mainly related to the powder of a_w 0.23 and only a part of the results for the powder of a_w 0.36 (mentioned in the text) will be exposed in the following sections.

PART 2. EXPERIMENTAL STRATEGY

2.2.1. PRODUCTION OF THE WPI POWDERS

WPI powder was obtained by spray drying a fresh whey protein concentrate originating from ultrafiltration and diafiltration of the milk microfiltrate. Provided by a dairy company based in Brittany, this concentrate had a dry matter of 27.8% (w/w) and contained 95% milk proteins. Spray drying was carried out to produce fine powders, using a three-stage spray drying pilot, Bionov (GEA, Niro Atomizer, Saint-Quentin-en-Yvelines, France). The flow was set at around 100 L.h⁻¹ and the inlet and outlet temperatures were fixed at 169°C and 66°C, respectively, in order to obtain a powder of a_w of 0.23, and at 146°C and 54.3°C in order to obtain a powder of a_w 0.36. After drying, WPI powders presented water content of 7.5% and 10.1% at a_w 0.23 and 0.36 respectively. Both were packed under air in 400 g airtight tins.

2.2.2. WPI POWDER STORAGE CONDITIONS

WPI powder tins were stored in heat chambers at 4 (control powder), 20, 40 and 60°C for periods of up to 15 months. After 0.5, 1, 3, 6, 9, 12 and 15 months of storage, 1 tin per temperature condition was opened in order to analyse the structural and functional properties of the powders.

2.2.3. POWDER COMPOSITION

MINERALS

Cations (calcium, magnesium, sodium, and potassium) and anions (inorganic phosphate, citrate and chloride) were measured using atomic absorption spectrometry (Varian 220FS spectrometer, Les Ulis, France) and ion chromatography (Dionex DX 500; Dionex, Voisin-le-Bretonneux, France) respectively (Gaucheron et al. 1996).

DRY MATTER

The dry matter (DM) was determined as described by Schuck et al. (2012). It was calculated by weight loss after drying 1 g of sample mixed with sand in a forced air oven at 105°C for 5 h.

LACTOSE

The amount of free lactose in the WPI powder was determined using a High Performance Liquid Chromatograph (HPLC) chain (Dionex, Germering, Germany) linked to a 6.5 mm diameter and 300 mm length column “house-packed” with an ion exchange resin Aminex A-6 (Biorad, St Louis Mo., USA). The oven was kept at 60°C and the elution flow was 0.4 mL.min⁻¹ using a 5 mM H₂SO₄ buffer. Free lactose content was detected through a differential refractometer (model RI 2031 plus, Jasco).

NITROGEN CONTENTS

The nitrogen contents of the initial powder were determined as described by Schuck et al. (2012). The total nitrogen (TN) content, non-casein nitrogen (NCN) content corresponding to the soluble fraction at pH 4.6, and non-protein nitrogen (NPN) content corresponding to the soluble fraction after their precipitation were determined by the Kjeldhal method. Nitrogen contents were converted into protein contents using 6.38 as multiplying factor.

2.2.4. POWDER SURFACE CHARACTERIZATION

SCANNING ELECTRON MICROSCOPY OBSERVATION

Scanning electron microscopy (SEM) was used to observe the morphology and surface structure of WPI powders. To this end, analysed powders were set on a double-sided adhesive tape and fixed to SEM stubs. Excess particles were removed by gentle tapping. Samples were then coated under partial vacuum with gold-palladium for 2 × 100 s (Polaron SC7640, Thermo VG Scientific, England). Finally, the samples were observed with a Stereoscan 240S/N (Léo, Rueil-Malmaison, France) operating at 15 kV.

X-RAY PHOTOELECTRON SPECTROSCOPY (XPS)

Elemental composition of WPI powder surface (up to 5 - 6 nm depth) was measured by X-ray Photoelectron Spectroscopy (XPS) (Fäldt et al. 1993). Spectra were obtained with a KRATOS Axis Ultra X-ray photoelectron spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromated Al K α X-ray ($h\nu = 1486.6$ eV) operated at 150 W. Spectra were collected at normal take-off angle (90°), and the analysis area was 700 × 300 μm^2 .

TOF-SIMS ANALYSIS

Measurements were performed in both positive and negative ion modes with TOF-SIMS 5 (IonTOF GmbH, Münster, Germany) in order to characterise the elemental and molecular composition of the sample surface. The analysis was performed using a bismuth liquid metal ion gun (LMIG, Bi₃⁺ ions, 25 keV). Images of WPI powder surface were acquired with a field of view of 200 × 200 μm^2

corresponding to 512×512 pixels, leading to a pixel size of $0.4 \mu\text{m}$. The fluence (also called primary ion dose density) was maintained to 1.0×10^{12} ions/cm² that is below the so-called static SIMS limit (Vickerman and Briggs 2001). The data acquisition and processing software was SurfaceLab 6.3 (ION-TOF GmbH, Münster, Germany).

ATOMIC FORCE MICROSCOPY

Gold-coated AFM tips (NPG-10, Bruker AXS, Palaiseau, France) were functionalized by immersing the tip for 15 h in a solution containing 2 mM 1-dodecanethiol ($\geq 98\%$, 471364 SIGMA). The process for tip functionalization consisted in the immobilization of thiol-based self-assembled monolayer on a gold-coated tip. This resulted in an oxidative addition of the thiol bond to the gold surface, concurrently with hydrogen removal. The functionalization of AFM tips with alkane-thiol monolayers is useful for mapping the spatial arrangement of hydrophobic chemical groups on powder surface by measuring adhesive forces. A glass substrate was coated with chromium through the use of a sputter coater (EMITECH, K575 Turbo, United Kingdom) and was further covered with a top gold layer. Gold-coated glass surfaces were immersed in a solution containing 1-dodecanethiol and rinsed with ethanol. The gold surfaces are used as references to control the correct functionalization of AFM tips (Alsteens et al. 2007). Interaction measurements were performed in liquid to avoid capillary forces. The spring constant of functionalized tips was determined using the thermal calibration method (Lévy and Maaloum 2002). The usual calibration process was employed to transform the experimental cantilever deflection curves as a function of the vertical scanner displacement Δz into force-distance curves (Figure 14).

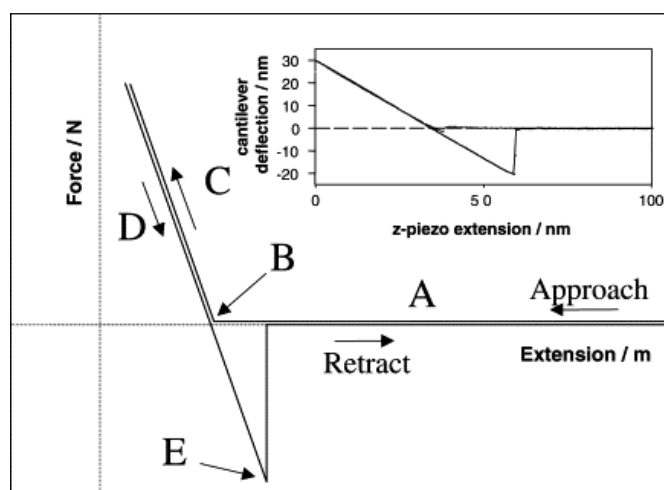


Figure 14: A force distance curve showing the approach and retract parts of the cycle: A, tip is a large distance from the surface and no interaction is measured; B, the tip contacts the surface; C, the cantilever is bent and a repulsive force (positive) is measured; D, the cantilever holder is retracted from the surface and an adhesive interaction (negative) between tip and surface is observed; E, the pull-off point (Smith et al. 2003)

In the contact region, the slope of the retraction deflection curve was used to convert the cantilever deflection into a force F using Hooke's law:

$$F = k_{exp} \times \Delta z \quad (3)$$

where k_{exp} is the experimentally determined spring constant.

2.2.5. CHEMICAL ANALYSIS

REVERSE PHASE CHROMATOGRAPHY

E-LG and D-LA were quantified in stored powders by reverse phase chromatography using a 300A 8 μ M 150x2.1 MM PLRP-S column (Polymer Laboratories, Amherst, MA, USA). The latter was connected to a HPLC made of a separation system Waters 2695, a double wavelength detector Waters 2487 and an acquisition and Empower data processing software (Milford, USA). The elution flow was 0.2 mL.min⁻¹ using a gradient of acetonitrile obtained by an appropriate combination of buffer solution A (trifluoroacetic acid, TFA, 0.1%) and buffer solution B (80% acetonitrile and 0.1% TFA). The column was first equilibrated with 35% buffer solution B and then linear gradients of buffer solution B moving from 35 to 44% in 3 min, from 44 to 48% in 11 min, from 48 to 53% in 12 min, and finally from 53 to 62% in 14 min were used to elute the proteins. Proteins were detected at 214 nm.

GEL PERMEATION CHROMATOGRAPHY

Native proteins (Gulzar et al. 2013) were quantified during powder storage after gel permeation chromatography (GPC) using a Yarra SEC 3000 column (Phenomenex, Le Pecq, France) connected to a high performance liquid chromatography (HPLC) apparatus comprising a Waters 2695 separation system, a Waters 2489 double wavelength detector and Empower (Milford, USA) acquisition and data processing software. The protein elution flow applied was 0.8 mL.min⁻¹ using a 0.05 M phosphate buffer at pH 7 containing 0.1 M NaCl. Proteins were detected at 214 nm.

SULFHYDRYL QUANTIFICATION

The thiol groups exposed on the protein surface were quantified using Ellman's method (Ellman 1959). Briefly, 100 μ L of a 10 g.L⁻¹ solution of rehydrated stored WPI powder were mixed with 900 μ L of Tris Glycine Buffer (50 mM, pH7.0) and 25 μ L of a 2,2'-Dinitro-5,5-dithiodibenzoate solution (8 mM) (DTNB, Merck, Darmstadt, Germany). After 2 h at room temperature, absorbance was measured at 412 nm and the accessible DTNB thiol groups were determined according to $H_{412nm} = 13,600 M^{-1}.cm^{-1}$.

CIRCULAR DICHROISM

Fresh and stored powders were diluted in 10 g.L⁻¹ solutions with milli-Q water and far-UV spectra were recorded at ambient temperature using the Synchrotron Radiation Circular Dichroism (SRCD) on DISCO beamline at SOLEIL synchrotron (Saint-Aubin, France). This technique is well explained by Miron et al. (2005).

MASS SPECTROMETRY

The percentages of lactosylated E-LG were determined by mass spectrometry after protein separation on reverse phase HPLC using a C4 VYDAC column (214TP5215, 150u2.1mm) (GRACE, Columbia, USA). The analysis was carried out with 15 µL of a 0.5% (w/w) solution reconstituted in milliQ water 2/3 diluted with a 0.212% buffer solution of TFA. The flow applied was 0.25 mL.min⁻¹ with an acetonitrile gradient. At the end of the column, a fraction of the eluate entered a QSTAR XL mass spectrometer (MDS SCIEX, Toronto, Ontario, Canada) at a flow rate of 75 µL.min⁻¹. Proteins were ionized with an Ion sprayer source before determining their mass with a TOF scanner (MDS SCIEX, Toronto, Ontario, Canada) previously calibrated with β-CN peptide from β-casein (193-209). The mass acquisition was carried out in the mass range of m/z 500 to 3,000 at 5 kV.

The percentage of lactosylation was determined using the following calculation:

$$\frac{I(lact)}{I(NAP) + I(lact)} * \%NP \quad (4)$$

With I(lact): intensity of the lactosylated protein

I(NAP): intensity of the non-aggregated protein

%NP: % native protein amount determined from reverse phase HPLC

SDS-PAGE ANALYSIS

SDS-PAGE was performed under reducing (with dithiothreitol (DTT)) and non-reducing conditions (without DTT) using a precast 4-20% gradient polyacrylamide tris/HCl gel (Biorad, St Louis Mo., USA) as described by Laemmli (1970). Solutions of 0.2% (w/w) were diluted with the denaturing buffer (0.5 M Tris-HCl pH 6.8; 20% glycerol; 10% SDS; 0.5% bromophenol blue). 15 µg of each samples was loaded in the well and was separated at 200 V for 30 min. Precision Plus Protein Standards unstained (10-250 kDa, Biorad, St Louis Mo., USA) was used for molecular weight calibration. Gels were stained with Bio-safe Coomassie Stain G250 (Biorad, St Louis Mo., USA) for an hour followed by a 20% ethanol solution. The gels were scanned by Image Scanner III (GE Healthcare, Paris, France).

2.2.6. PHYSICAL ANALYSIS

COLOUR

The browning index (BI) of powders was determined by measuring $L^*a^*b^*$ parameters (Konica Minolta Photo Imaging France SAS, Roissy, France) using the formula (Maskan 2001):

$$BI = \frac{100 * (x - 0.31)}{0.17} \quad (1)$$

with

$$x = \frac{a + 1.75 * L}{5.645 * L + a - 3.012 * b} \quad (2)$$

PART 3. CHANGES AT A MESOSCOPIC SCALE

2.3.1. FRESH POWDER COMPOSITION

The detailed composition of the fresh powder is visible in **Table 4**.

Table 4: Composition of the fresh powder

Dry matter (DM; w/w %)	DM	92.5
Nitrogen composition (% N × 6.38)	TN	88.81
	NPN	0.32
	NCN	84.66
Mineral composition (w/w %)	Na ⁺	0.100
	K ⁺	0.405
	Ca ²⁺	0.330
	Mg ²⁺	0.059
	Cl ⁻	0.003
	PO ₄ ³⁻	0.059
	Citrate	0.022
Carbohydrate (w/w %)	Lactose	1.28

Fresh WPI powder (t_0) of a_w 0.23 was characterized by 4.7% proteins that precipitated at pH 4.6. This fraction was considered to correspond to the proteins that had been denatured during spray drying or prior processing steps. As this study deals with the structural and functional evolution of proteins during storage, the contribution of these latter does not appear in the present results. Moreover, no structural or functional modifications were detected during 15 months' storage at 4°C. The powder

stored at 4°C was therefore taken as a control (Ref) and any difference from Ref properties were considered as structural and/or functional changes affected by storage conditions.

2.3.2. COLOUR OF STORED POWDERS

The colour of dairy powders constitutes an essential criterion for their end use properties and reflects overall changes occurring in powders because of the Maillard reaction, which is one of the major deteriorative evolution during storage of dehydrated dairy products. Indeed, dairy powders that incorporate amorphous lactose are susceptible to non-enzymatic browning through the Maillard reaction, because of melanoidins apparition. Moreover, Martinez-Alvarenga et al. (2014) have shown that powder browning was correlated with a loss of amino acids in the presence of sugar. The Maillard reaction development was therefore evaluated (**Figure 15**) and analysed through colour measurements, making it possible to quantify the change of the Browning Index (**Figure 16**).

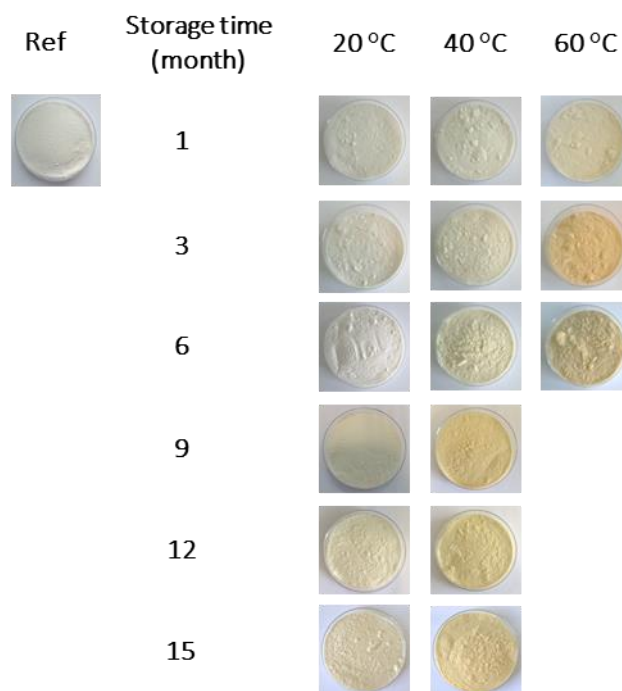


Figure 15: Images of powders after 15 months of storage at 4°C, after 1, 3, 6, 9 and 15 months of storage at 20°C, 40°C and after 1, 3, 6 months of storage at 60°C

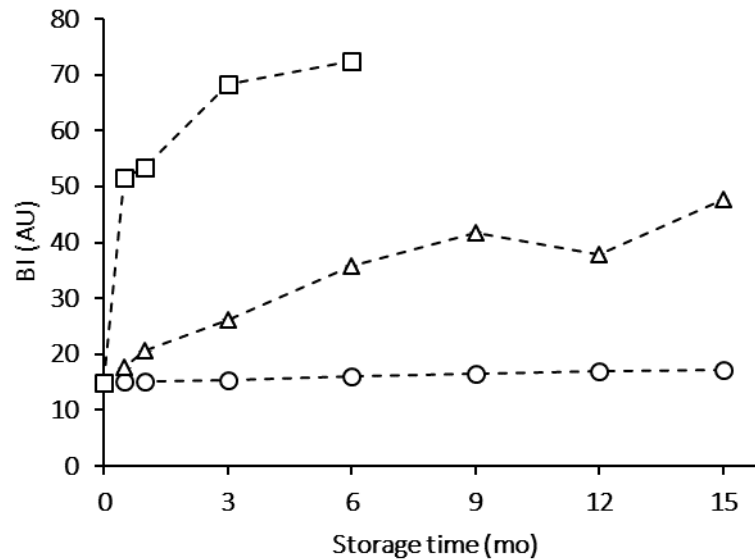


Figure 16: Evolution of Browning Index (Arbitrary Units, AU) for powders stored at 20°C (circle), 40°C (triangle) and 60°C (square) as a function of storage time, with experimental relative error < 0.7%

Powders developed colour changes with storage time and temperature (**Figure 15**). At t_0 , the browning index of WPI powder was 15, typical for a non-aged powder as shown in **Figure 16**. During storage at 20°C, changes in the browning index were very slight or nil over 15 months, whereas powders stored at temperature over 20°C browned from as early as 15 days during storage, with a sharper increase at 60°C. These results are in agreement with those of Stapelfeldt et al. (1997) and Burin et al. (2000) who showed that milk powder turned brown as a function of time and temperature during storage at $T \geq 25^\circ\text{C}$. Li-Chan (1983) reported that WPC35 browning was correlated with a decrease in the residual lactose as well as a decrease in lysine availability under accelerated storage conditions (37°C for 42 days and a_w of 0.75).

2.3.3. MORPHOLOGICAL STUDY

This part and the following one (cf. 2.3.4., p45-47) only tackle with the comparison of the evolutions of the particle surface of Ref powder and the powder stored one month at 60°C.

SEM analysis revealed powder particles with smooth surface and large dents (**Figure 17, A**), characteristics typically encountered in high protein dairy powders (Fyfe et al. 2011; Mistry 2002). After one month of storage at 60°C, the surface powder mainly remained smooth but cracks and broken structures were observed (**Figure 17, B**). This means that particle structure is affected by storage at high temperature.

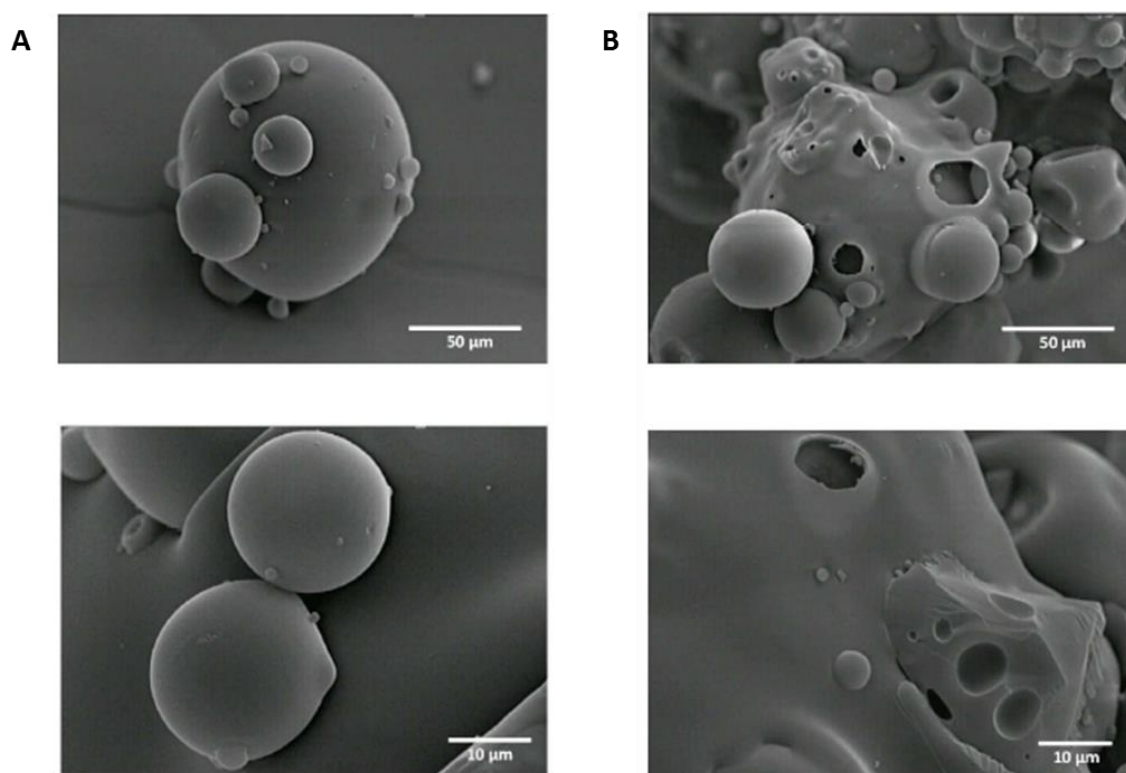


Figure 17: SEM images of A) Ref and B) aged powder. The bottom images are magnifications of their correspondent top ones

2.3.4. PARTICLE SURFACE CHEMISTRY

Adhesion force mapping with a hydrophobic AFM tip was used to probe the hydrophobic properties of the particle surface. Adhesion force histograms and maps are presented in **Figure 18**.

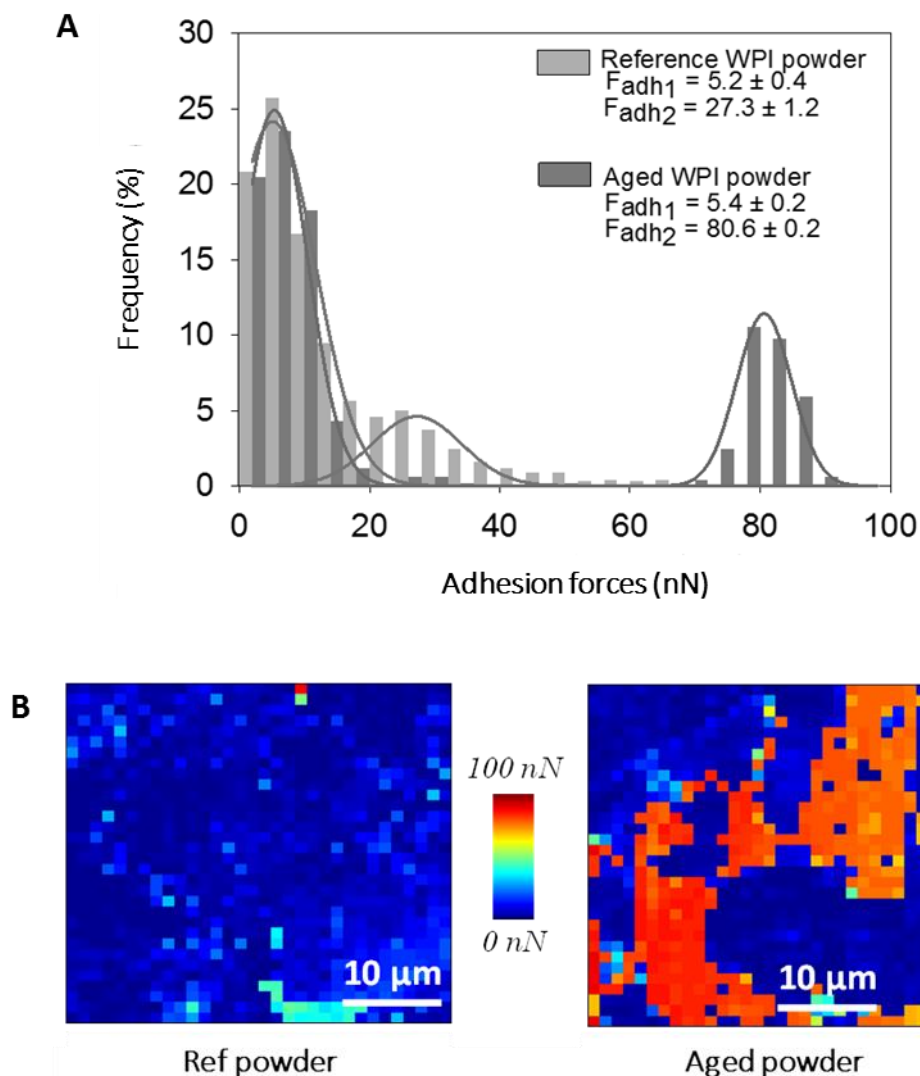


Figure 18: Evaluation of A) hydrophobic forces and representation of B) adhesion force maps for Ref and WPI powder stored 1 month at 60°C

Most of recorded curves showed two main areas of adhesion forces for each sample. The first area, of mean adhesion force of approximately 5 nN, is common to both Ref and aged powder. For Ref, the second area presented a mean adhesion force of 27.3 ± 1.2 nN whereas it was of 80.6 ± 0.2 nN for the aged powder. Interestingly, the adhesion maps of the aged WPI powder were heterogeneous and presented large hydrophobic patches. As these patches correlate with heterogeneous surface morphology, it is believed that a chemical modification on particle surface occurred upon storage at high temperature.

Many analysis methods can provide information on surface chemical composition; among them, XPS and ToF-SIMS stand out as the most relevant and most widely used techniques (Kingshott et al. 2011). In the present case, XPS analysis revealed no differences in surface composition between Ref and aged WPI powders (**Table 5**).

Table 5: XPS analysis of WPI powder (relative atomic percentage, %)

	Ref	Aged powder
O	17.00	17.38
N	14.01	14.08
Ca	0.14	0.13
C	68.39	67.93
S	0.32	0.37
P	0.14	0.12

A key advance for ToF-SIMS was the development of imaging combined with narrowly focused ion guns, which enables discriminating surface composition of smooth versus bump areas. During measurements, two regions of interest were analysed for each powder (**Figure 19**): the first one was on the smooth area and the second on the cracks previously described by SEM imaging. Results acquired in positive mode showed that the particle surface of powders stored at 60°C presented less amino acids than Ref (**Figure 19**).

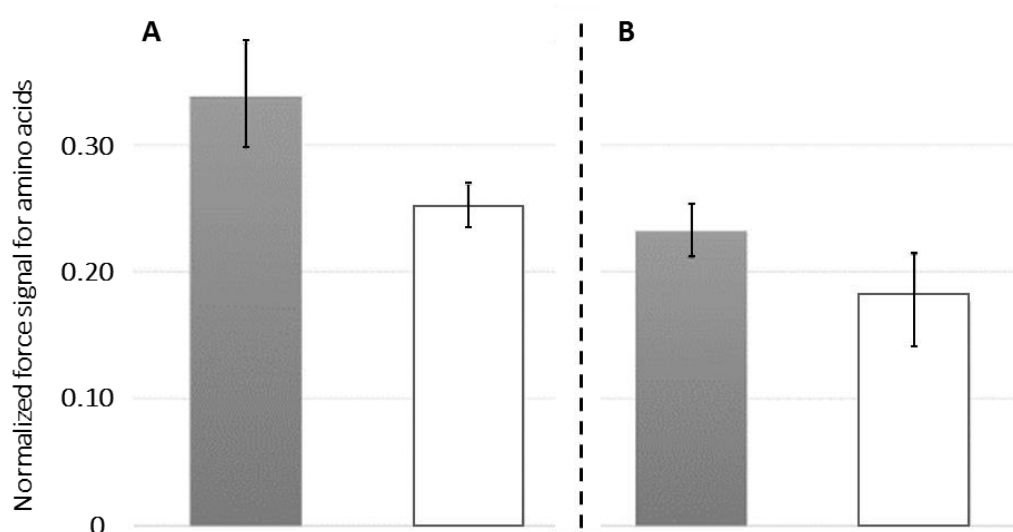


Figure 19: ToF-SIMS analysis of the smooth area (grey) and the cracks (white) for A) Ref and B) aged powder

In particular on Ref, the presence of amino acids was less detected on a crack area compared to the smooth area. The same observation was done for the aged powder. This means that changes in

surface morphology during storage is likely to be connected to surface chemical modification. To confirm these results, ToF-SIMS analysis were performed in negative mode. As expected, no lipids were found on the powder surface, as these latter are retained during the microfiltration step implemented for obtaining the WPI concentrate. This means that migration of lipids from the core to the surface was not responsible for the observed increase in surface hydrophobicity and decrease in amino acid content, even though it has been already described for skim milk powders (Gaiani et al. 2007). The changes in hydrophobicity and in amino acid content reported above are thus thought to be the result of the Maillard reaction. This reaction has been extensively described in the literature (Martins et al. 2000; Van Boekel 1998; Waller and Feather 1983) and is actually believed to occur in dairy powders ageing (Anema et al. 2006; Guyomarc'h et al. 2000). It leads to amino acid degradation (Patel et al. 2013), consistent with the decrease in amino acid surface content observed in ToF-SIMS experiments. Moreover, the final stage of the Maillard reaction consists in aldo condensation and aldehyde-amine polymerization, resulting in the production of heterocyclic nitrogen compounds (melanoidins) of brown colour, responsible for the evidenced colour change (**Figure 15**).

Fyfe et al. (2011) already described an increase in surface hydrophobicity for milk protein concentrate powders by evaluating particle adhesion to hydrophilic or hydrophobic materials. However, no differences in surface composition were evidenced by XPS in the current work. This can be explained by the fact that the XPS technique quantifies atoms and bonds on a relative large region. In the present study, it was demonstrated that modification on WPI particle surface might be at a very local stage. During one month storage of WPI powder at 60°C, powder surface principally underwent extremely local modifications.

From a structural point of view, the initially smooth surface presented cracks after storage and from a chemical point of view, a more hydrophobic material appeared on particle surface. According to the present work and literature, two main hypothesis can be issued: (1) The increase in hydrophobicity can originate from the unfolding of whey proteins resulting in an exposition of hydrophobic residues (Havea 2006), or (2) the increase in hydrophobicity and browning of the powder can result from the Maillard reaction, as heterocyclic nitrogen compounds, known to be hydrophobic and coloured, are produced (this second mechanism has clearly been evidenced by the multiscale approach of the current study). The alterations of powder surface during storage may result from many reactions involving all the powder components, among them, the 2 hypothesis highlighted previously. These latter are believed to be the main alteration mechanisms of WPI powders. So as to confirm the presence of these ageing alterations, a study at a molecular level was completed.

PART 4. CHANGES AT A MOLECULAR SCALE**2.4.1. PROTEIN DENATURATION OF STORED POWDERS**

In their native state, D-LA and E-LG are soluble at pH 4.6, and in reverse phase HPLC these proteins were eluted with a retention time of 20 min and 33-34 min, respectively (double peak corresponding to the two E-LG variants) with the gradients used in this study (**Figure 20, A**). Any reduction in the area of the native form peaks or any modification of their elution time was considered as a marker of non-native protein state and was attributed to denatured/aggregated and/or the lactosylated fractions of D-LA and E-LG during storage.

The denatured/aggregated proteins were quantified by difference with Ref using reverse phase chromatography after precipitation at pH 4.6. Powders stored at 20°C were stable for 15 months (**Figure 20, B**). A level of denaturation corresponding to 7% and approximately 45% of E-LG was observed for powders stored for 15 days at 40°C and 60°C, respectively. After 15 months' storage at 40°C, powders presented chromatograms with a shifted peak concerning approximately 38% of E-LG: the delayed elution time made it possible to consider the denatured proteins to be more hydrophobic (Gulzar et al. 2013). The level of denaturation was not quantifiable after 1 month at 60°C due to very low amount of remaining native proteins. As shown on **Figure 20, B** and in agreement with Stapelfeldt et al. (1997), the kinetics of powder property modifications were slightly faster at a_w 0.36 than those at a_w 0.23 for a storage temperature higher than 20°C. The difference between the two studied a_w was not that wide, making possible to state that there is a tolerance threshold on a_w of WPI powder between 0.2 and 0.36.

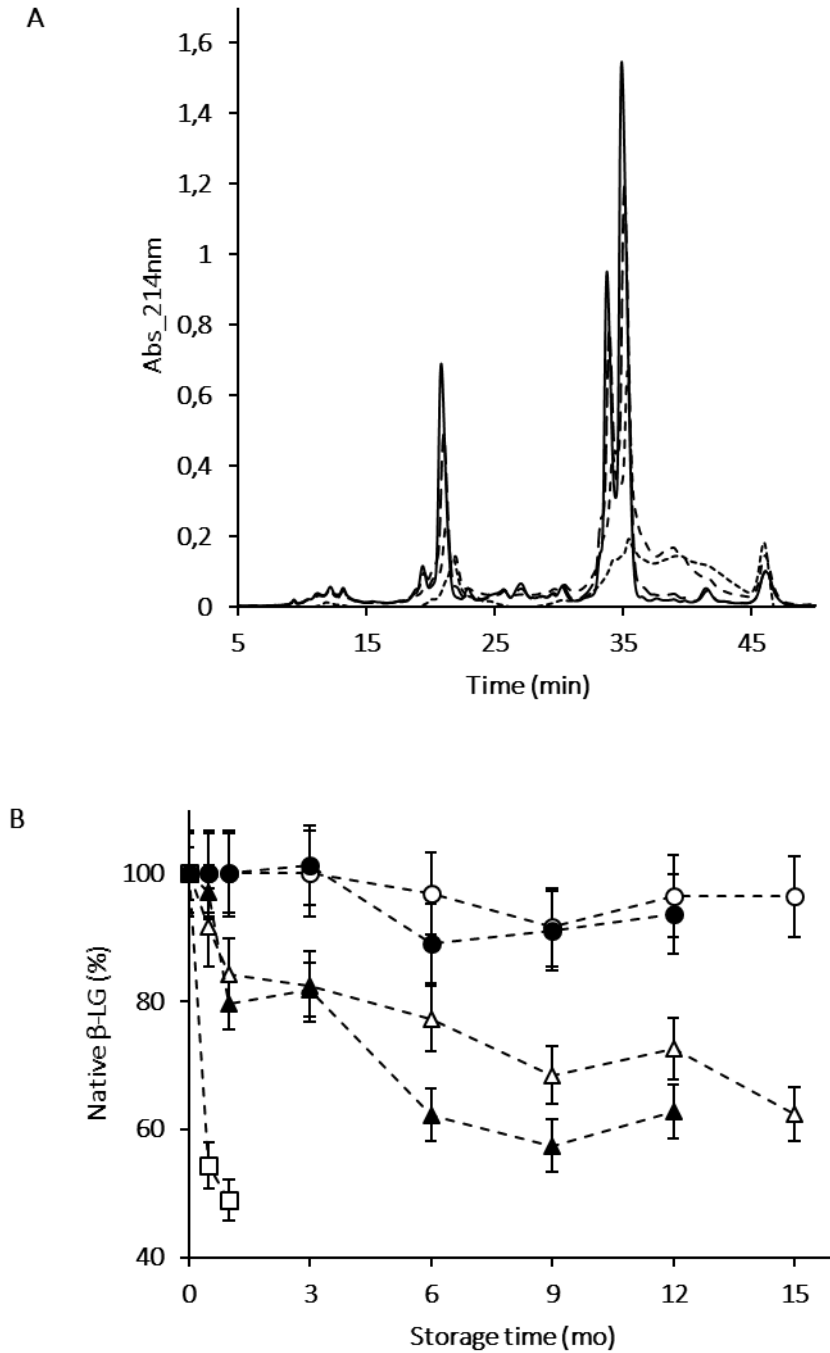


Figure 20: A) RP-HPLC profile of WPI powders at t_0 (solid line) and stored 15 months at 20°C (long-dashed line), at 40°C (dashed line) and stored 6 months at 60°C (dotted line). B) Influence of storage time and temperature on the amount of native β -LG at pH4.6 during storage à 20°C (circle), 40°C (triangle) and 60°C (square) for powders of a_w 0.23 (empty symbols) and a_w 0.36 (solid symbols)

Changes in protein conformation were monitored by quantification of free thiol groups accessible to the DTNB probe upon 12 months of storage (Figure 21).

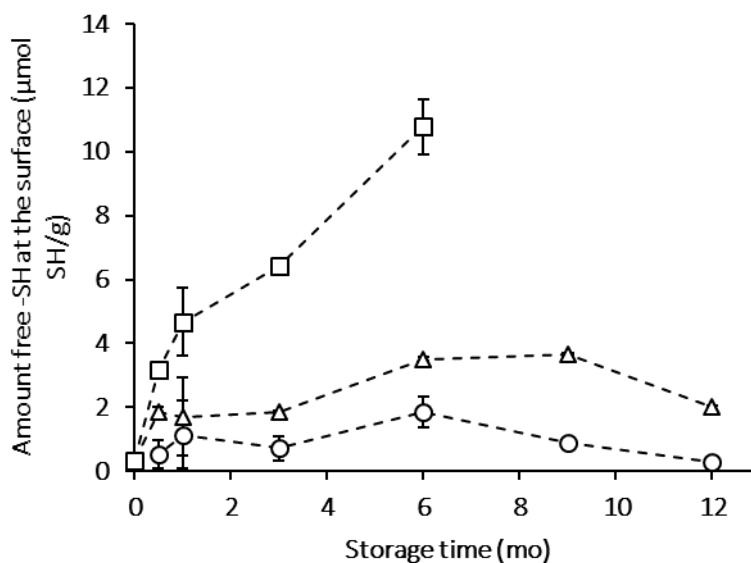


Figure 21: Influence of storage time on the amount of free thiol groups at the protein surface. Powders stored at 20°C (circle), at 40°C (triangle) and at 60°C (square)

At t_0 , the level of free thiol groups was $0.34 \pm 0.016 \mu\text{mol } -\text{SH/g}$ protein. Among the whey proteins, only E-LG and BSA have a free thiol group. It is possible to consider the concentration of BSA to be negligible compared to that of E-LG. On this basis, it can be estimated that only 1% of E-LG presented a free thiol group at t_0 , in agreement with the structural data indicating that the free thiol group of E-LG (Cys121) is buried in the protein structure (Sawyer et al. 1999). Exposure of free thiol groups increased during storage, reflecting an opening in protein structure (denaturation). For powders stored at 20°C for 12 months, the amount of free thiol was not significantly different from Ref. After 9 months' storage at 40°C, the level of free thiol was $3.66 \pm 0.11 \mu\text{mol } -\text{SH/g}$ protein. This level corresponded to 10% of E-LG that had a free thiol group accessible to the probe. A decrease was observed at around 12 months' storage. At 60°C, the level of free thiol groups reached $11 \pm 0.85 \mu\text{mol } -\text{SH/g}$ protein after 6 months' storage. In agreement with the previously reported changes in the denaturation level, this free thiol exposure might participate in exchange reactions with other protein disulphide bonds (Hoffmann and van Mil 1997; Roefs and De Kruif 1994; Visschers and de Jongh 2005).

2.4.2. PROTEIN LACTOSYLATION

Among the non-aggregated proteins that were detected by reverse phase chromatography at the characteristic retention time of E-LG and α -LA, some were lactosylated. The fraction of lactosylated E-LG (**Figure 22**), which could carry one or more lactose molecules per protein, was determined by mass

spectrometry. Only the results for β -LG are presented due to the difficulty of detecting lactosylation on α -LA with storage time.

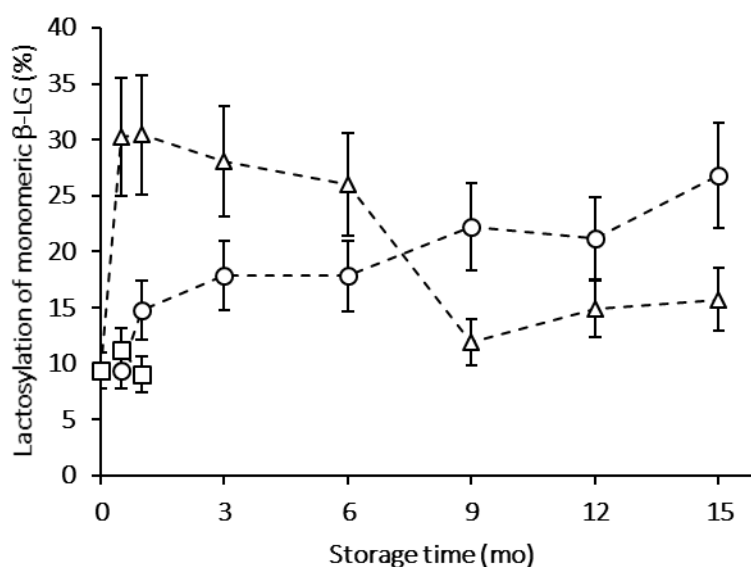


Figure 22: Evolution of lactosylation of β -LG for powders stored at 20°C (circle), 40°C (triangle) and 60°C (square) as a function of storage time

The fraction of lactosylated proteins was less than 10% of whey proteins at t_0 . This proportion regularly increased to reach 27% after 15 months' storage at 20°C. The lactosylation phenomenon evolved faster in powders stored at 40°C and 60°C. At 40°C, the level of lactosylated proteins reached 30% after 15 days storage, then remained stable for up to 3 months' storage before decreasing. Protein lactosylation constitutes therefore a marker of early change in the powder during storage. Lactosylation was rapidly undetectable in powders stored at 60°C. Indeed, over the first weeks of storage at 60°C, it was actually possible to quantify protein lactosylation; for longer storage time, lactosylated proteins were no longer quantifiable although still remaining detectable, as a result of the growing complexity of the signal. The decrease in proportion of lactosylated protein after 3 months' storage at 40°C and the rapidly undetectable lactosylation signal at 60°C corresponded to a high level of progression of denaturation (Figure 20, B).

The results of protein lactosylation have to be considered with caution. Indeed, these results are only estimated since ionization efficiency during mass spectrometry analysis could vary according to the level of lactosylation of the proteins. However, as the number of lactose molecules bound per protein was low (mainly one or two lactose molecules), the ionization efficiency between proteins was considered to remain stable.

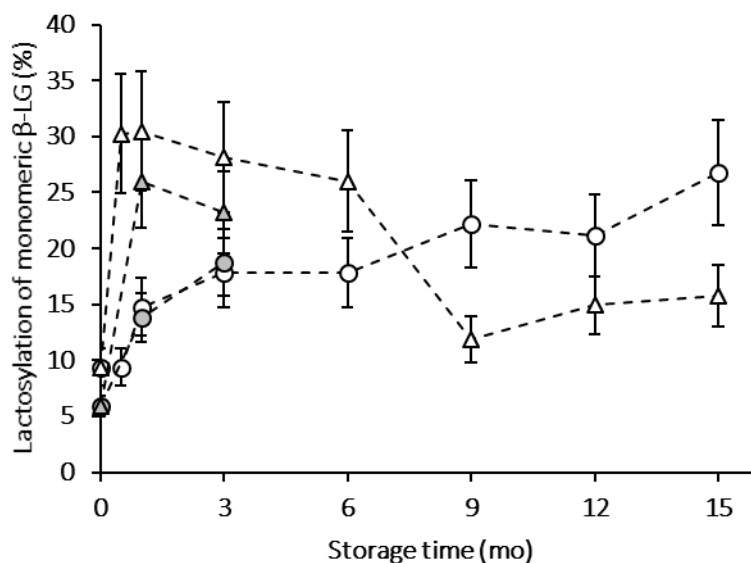


Figure 23: Comparison of the evolution of β -LG lactosylation for two different powder productions (production 1, white; production 2, grey), both stored at 20°C (circle) and 40°C (triangle) as a function of storage time

The study of the evolution of β -LG lactosylation was also repeated on another powder produced under the same conditions as the first one to look at the repeatability of these changes extended to different powder productions (**Figure 23**). The results showed that the level of lactosylation of the second production was superimposed to the first production for a storage at 20°C. At 40°C, the curve was in the standard deviation following the same path. These results confirm the evolution of this parameter for 3 months storage of a WPI powder.

2.4.3. CONFORMATIONAL STABILITY

The far-UV CD spectrum of fresh WPI powder (**Figure 24, A**) displayed extremes around 195, 210, and 218 nm comparable to previously reported spectra of β -LG (Broersen et al. 2004; Hirota-Nakaoka and Goto 1999). This result was consistent with our expectations since whey proteins are composed of around 70% of β -LG. A zero-crossing (around 200 nm) was retained upon 12 or 15 months' storage at 20 and 40°C, respectively (**Figure 24, A and B**), and the shape of the spectrum showed extremes around 195, 210, and 218 nm, similar to the fresh WPI powder. The difference in spectra intensity of stored WPI powders was attributed to the increasing rate of protein aggregation with longer storage time. As lactosylation seems to be the major protein modification during storage of WPI powders and as the shapes of the spectra showed only minor changes (mainly, same zero-crossing at 203 nm coupled with a difference in intensity), it can be concluded that the WPI powder was not significantly affected at any structural level by the lactosylation procedure. For samples stored under less severe conditions, these differences were even smaller.

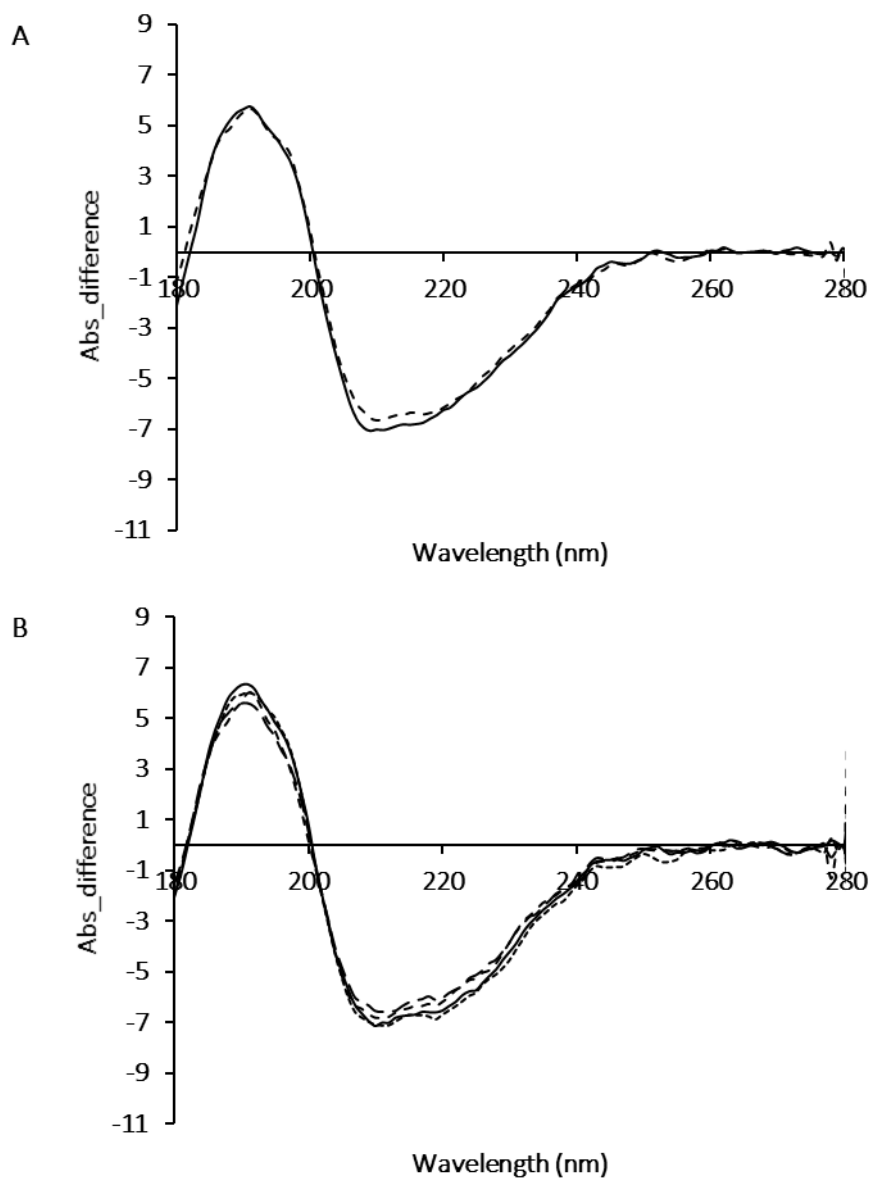


Figure 24: A) Far-UV CD spectra of WPI powders at t_0 (solid line) and stored at 20°C for 15 months (dashed line), and B) stored at 40°C for 3 months (dotted line), 6 months (dashed line) and 12 months (long-dashed line)

From these data it can be concluded that the binding of a lactose molecule to a protein does not apparently lead to a large loss of secondary structure if any.

2.4.4. AGGREGATION IN THE DRY STATE

QUANTIFICATION

Quantification of monomeric and aggregated forms of proteins upon storage has been determined using GPC-HPLC by difference of Ref and stored powders. Powders stored at 20°C were thus stable until 15 months (Figure 25).

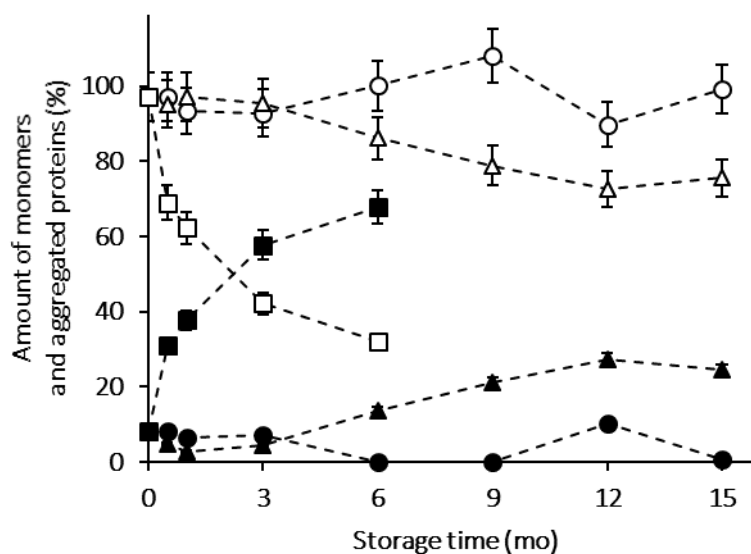


Figure 25: Amount of native (empty symbols) and aggregated (full symbols) proteins for powders stored at 20°C (circle), 40°C (triangle) and 60°C (square) as a function of storage time

Similar behaviour was observed for powders stored at 40°C for between 1 and 3 months; from this date, the fraction of native protein started to decrease, with a concomitant increase in aggregated proteins. After 15 months' storage at 40°C, powders presented around 25% aggregated protein. At 60°C, powders showed a high level of aggregation starting from the first 2 weeks of storage being of 31% aggregated proteins. This level continuously increased to reach 68% aggregated proteins after 6 months but no difference in solubility could be evidenced at this stage. Morr and Ha (1993) reported that storage of WPC powder resulted in partial denaturation and progressive polymerization. The amount of native protein therefore decreased from 60 to 33% during 7 days of storage at 80°C. Furthermore, one year storage at 25°C caused the polymerization of 18% of β -lg monomers. Havea (2006) reported that these proteins tended to unfold during storage at 40°C, making them more available to the formation of aggregates linked by disulphide bridges. Protein-protein interactions have been observed by other researchers (Anema et al. 2006; Mimouni et al. 2010; Zhou and Labuza 2007). This has also been reported for whey proteins dry heated in a powder state (Gulzar, Bouhallab, Jeantet, et al. 2011).

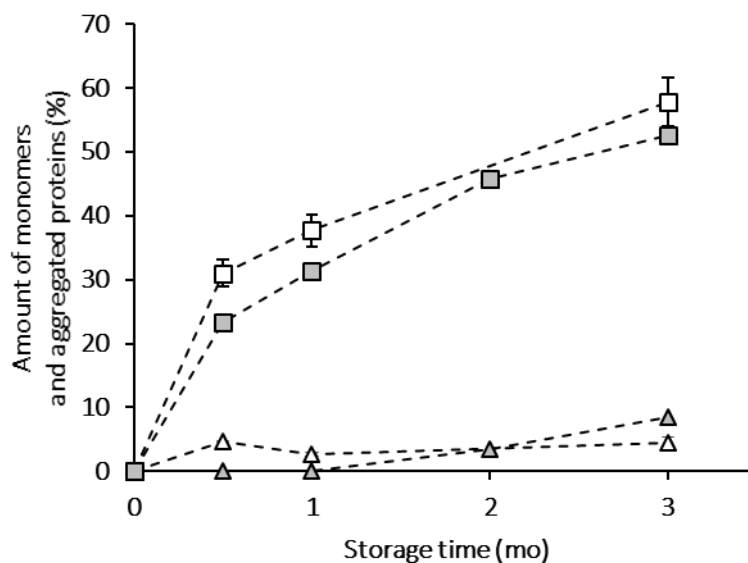


Figure 26: Comparison of the evolution of aggregated proteins amount for two different powder productions (production 1, white; production 2, grey), both stored at 40°C (triangle) and 60°C (square) as a function of storage time

As for β -LG lactosylation, the study of the amount of aggregated protein upon storage was repeated on another production (the same powder as for lactosylation) and this for the same purpose (**Figure 26**). The results showed that changes in the amount of aggregated proteins of the second production for storage at 40°C and 60°C are closely following their associated twins from the first production. These results confirm the evolution of this parameter during the first 3 months storage of a WPI powder.

CHARACTERISATION

SDS-PAGE analysis in the absence and presence of the reducing agent DTT was used to determine the type of intermolecular interactions in the formed aggregates.

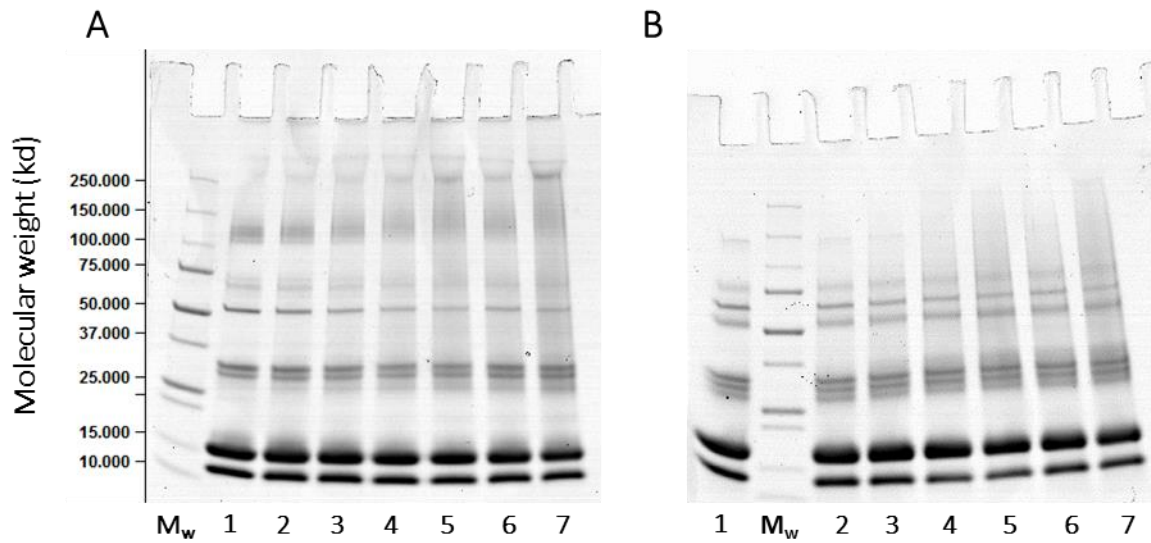


Figure 27: SDS-PAGE profile of fresh and stored powders at 40°C A) in the absence and B) presence of reducing agent DTT. M_w , low molecular weight markers; 1, Ref; 2, 1 month; 3, 3 months; 4, 6 months; 5, 9 months; 6, 12 months; 7, 15 months

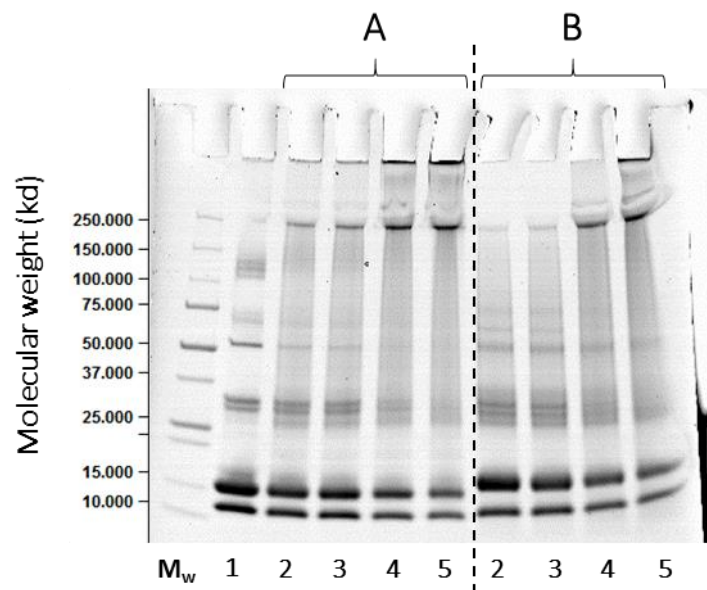


Figure 28: SDS-PAGE profile of fresh and stored powders at 60°C A) in the absence and B) presence of reducing agent DTT. M_w , low molecular weight markers; 1, Ref; 2, 15 days; 3, 1 month; 4, 3 months; 5, 6 months

In the absence of DTT (**Figure 27, A**), the fresh powder sample showed two major bands corresponding to monomers of α -LA and β -LG. At 40°C, the intensity of the bands corresponding to monomers of α -LA and β -LG decreased when increasing the storage time and the bands on top of the separating gel and in the wells, corresponding to large aggregates, intensified. Increasing the storage temperature at 60°C (**Figure 28**) accelerated the conversion of monomeric proteins into larger aggregates, in

agreement with size exclusion chromatography and reverse-phase chromatography. Moreover, these aggregates were covalently linked as SDS-PAGE would preserve this type of links while breaking non-covalent bonds.

To further investigate the type of covalent interactions, the samples were analysed by SDS-PAGE in the presence of the reducing agent, DTT (**Figure 27, B**). Under these conditions, the polymer bands observed in powders in the absence of DTT largely disappeared in profit of the monomeric forms, showing that a majority of the proteins in these aggregates were joined together by disulphide bonds. However, the recovery of the proteins in the monomeric band was incomplete, and darker smear was observed with storage time as significant amounts of polymers were still concentrated on top of the separating gel. This indicated that covalent bonds, other than disulphide bonds, also participated in protein cross-linking. Moreover the presence of smear showed that these aggregates were of heterogeneous size.

PART 5. DISCUSSION: MAILLARD REACTION IS THE MAIN REACTION OCCURRING DURING WPI POWDER STORAGE

During storage at 20°C, changes in the browning index were very slight or nil over 15 months, in accordance with the relatively small changes in protein lactosylation. At 40°C, powders browned to an intermediate level, BI values being in the 17.6 to 41.8 range. Several studies have reported powder colour changes with storage time and temperature ($\geq 50^\circ\text{C}$), suggesting participation of the Maillard reaction (Burin et al. 2000; Martinez-Alvarenga et al. 2014; Stapelfeldt et al. 1997). The Maillard reaction is based on the condensation of amino groups on reducing sugars, then followed by degradation of the condensation products and the formation of brown compounds. Due to diafiltration of the ultrafiltrate before spray drying, WPI powders consist of about 95% protein and a very small amount of lactose still remaining in an amorphous state (1.28% w/w corresponding to a lactose/protein molar ratio of 0.7) of which part is associated with proteins, as indicated by mass spectrometry analysis. Moreover, it is interesting to note that the level of lactosylation first increased for storage at 40°C up to 3 months when maximum lactosylation was reached with no change in the browning index. At this point, the latter started to increase continuously whereas the level of lactosylation decreased concomitantly and tended to be undetectable after 15 months' storage. The decrease in lactosylation after 3 months' storage also matched with a concomitant increase in protein aggregation. This suggests that, with storage time, lactosylated proteins are getting through the process of the Maillard reaction which ends up with the formation of aggregated proteins in the dry state (Guyomarc'h et al. 2000; Morgan et al. 1999).

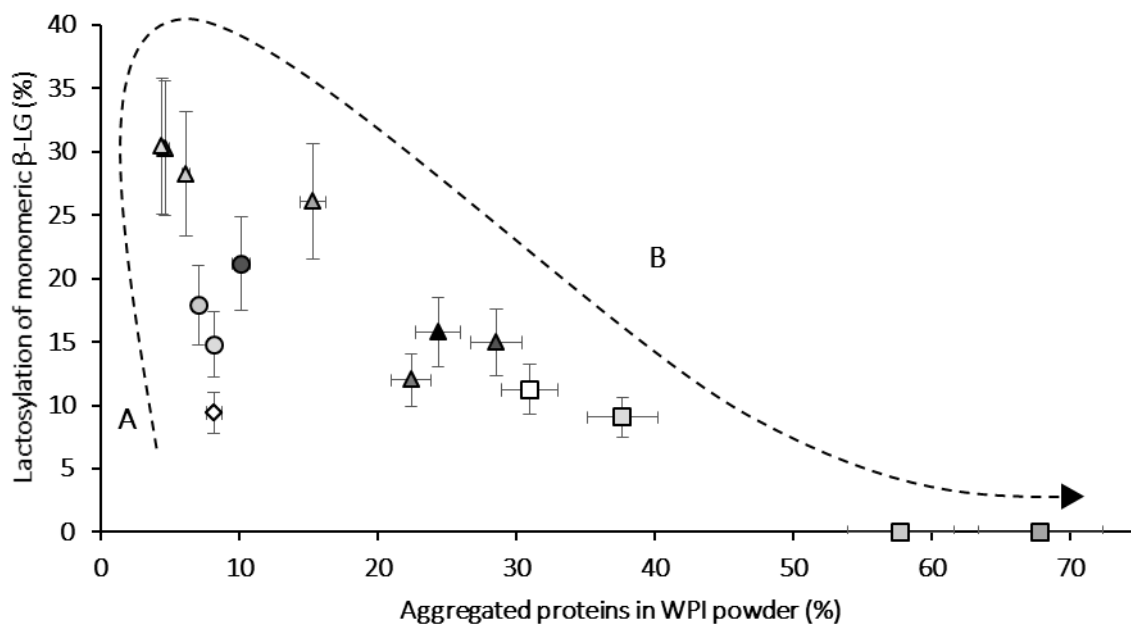


Figure 29: Correlation between lactosylation and aggregation for Ref (empty diamond) and powders stored at 20°C (circle), at 40°C (triangle) and at 60°C (square) from 15 days to 15 months (from white to black)

As shown in **Figure 29**, powders stored at 40°C for up to 15 months first presented a sharp increase in protein lactosylation (**Figure 29, A**), which then decreased towards protein aggregation (**Figure 29, B**). It is likely that free lactose molecules were in the process of binding to protein, while a fewer of these latter that were already bound were degraded into aggregates and tended to colour the powder from the first month of storage.

It can be hypothesized that proteins were extensively modified by lactosylation after 3 months' storage at 40°C, increasing the rate of the subsequent Maillard reaction and leading to the formation of new coloured compounds. These results are in agreement with previous studies showing that storage at high temperatures induces an increase in the amount of Maillard reaction products (Ipsen and Hansen 1988; Kieseker and Clarke 1984). SDS-PAGE showed that aggregates comprised disulphide bridges and also other covalent bonds. The latter are thought to be linked to the presence of lactose degradation products in the medium forming covalent protein crosslinks (Enomoto et al. 2007; Guyomarc'h et al. 2014; Le, Deeth, et al. 2013; Li et al. 2009).

Aggregation from crosslinking might also be due to heat-induced denaturation of proteins in view of the increase in free thiol groups exposed on the protein surface. In such a scheme, the storage temperature would act as a dry heat treatment which could affect the exposure of buried thiol groups with partial unfolding of β -LG, which initiates thiol or disulphide interchange reactions leading to protein aggregation (Busti et al. 2006; Hoffmann and van Mil 1997).

PART 6. ADDITIONAL RESULTS: INFLUENCE OF STORAGE ACCIDENTS**2.6.1. OBJECTIVES**

WPI powder may be subjected to non-constant environmental conditions during delivery, involving short exposure to high temperature. Such episodes are considered to be storage accidents. The purpose of this additional study was to assess whether the time dependence of changes occurring upon WPI powder ageing was according to a proportional scale or not.

2.6.2. EXPERIMENTAL STRATEGY

At first, we defined a reference time/temperature exposure representative of storage accident. Our choice fell on storage accidents of continuous 72 h rather than simulating combination of daily exposure (with a period of 12 h for example) at the critical temperature. Indeed, we first compared the evolution of the powder colour and the denaturation state of its protein (reverse phase HPLC) for a set made of 6 exposures at 60°C for 12 h, each separated by a 12 h lapse at room temperature, and for one exposure of 72 h at 60°C. We observed that the differences between powders were very low, although powders undergoing 72 h at 60°C were slightly browner than powders undergoing 6*12 h at 60°C. For the sake of simplification of the experimental strategy, the choice of a continuous period of 72 h was then adopted for defining one storage accident.

WPI powder was obtained as above using a three-stage spray drying pilot, Bionov (GEA, Niro Atomizer, Saint-Quentin-en-Yvelines, France). The flow was set at around 100 L.h⁻¹ and the inlet and outlet temperatures were fixed at 152°C and 58.9°C respectively. After drying, the WPI powder of a_w 0.28 and was packed under air in airtight tins of 400 g capacity.

6 powders were stored at 20°C for 4.5 months, 3 of them being subjected to 1, 2 or 3 storage accidents and the 3 others being subjected to 1 storage accident at 3 different times among the storage period (**Figure 30**).

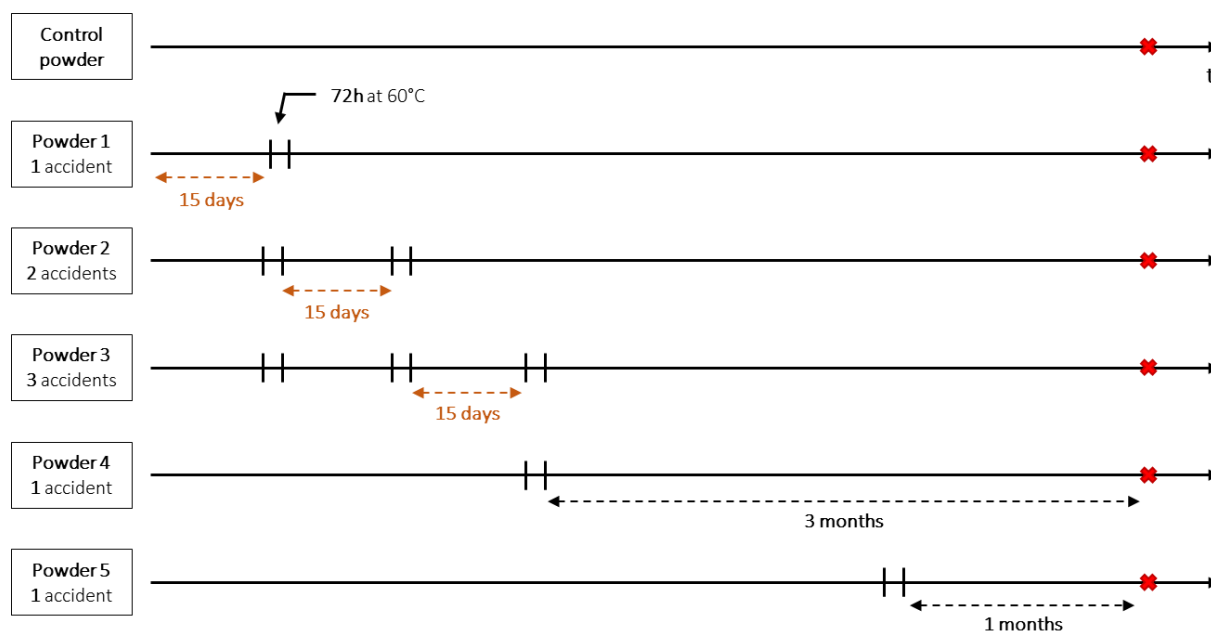


Figure 30: Diagram of the strategy adopted for the study of the influence of storage accidents on powders ageing

It was then possible to distinguish the impact of repeated storage accidents, from the time at which they occur: the comparison between powder 1, powder 4 and powder 5 gave an insight on the influence of the time at which the storage accident occurs; and the comparison between powder 1, powder 2 and powder 3 displayed the influence of the number of storage accidents.

2.6.3. RESULTS AND DISCUSSION

DATE OF THE STORAGE ACCIDENT

The results obtained showed that the rate of protein denaturation (**Figure 31, A**), the rate of monomeric and aggregated forms (**Figure 31, B**) and the Browning Index (**Figure 31, C**) were not significantly affected by the time at which the storage accident occurred. This indicated that the changes occurring between the storage accident at 20°C for 3 months were very low if measurable on this storage period (overall 4.5 months): consequently, the reactions induced by the storage accident should be considered as slowed/stopped during these relatively low temperature intervals.

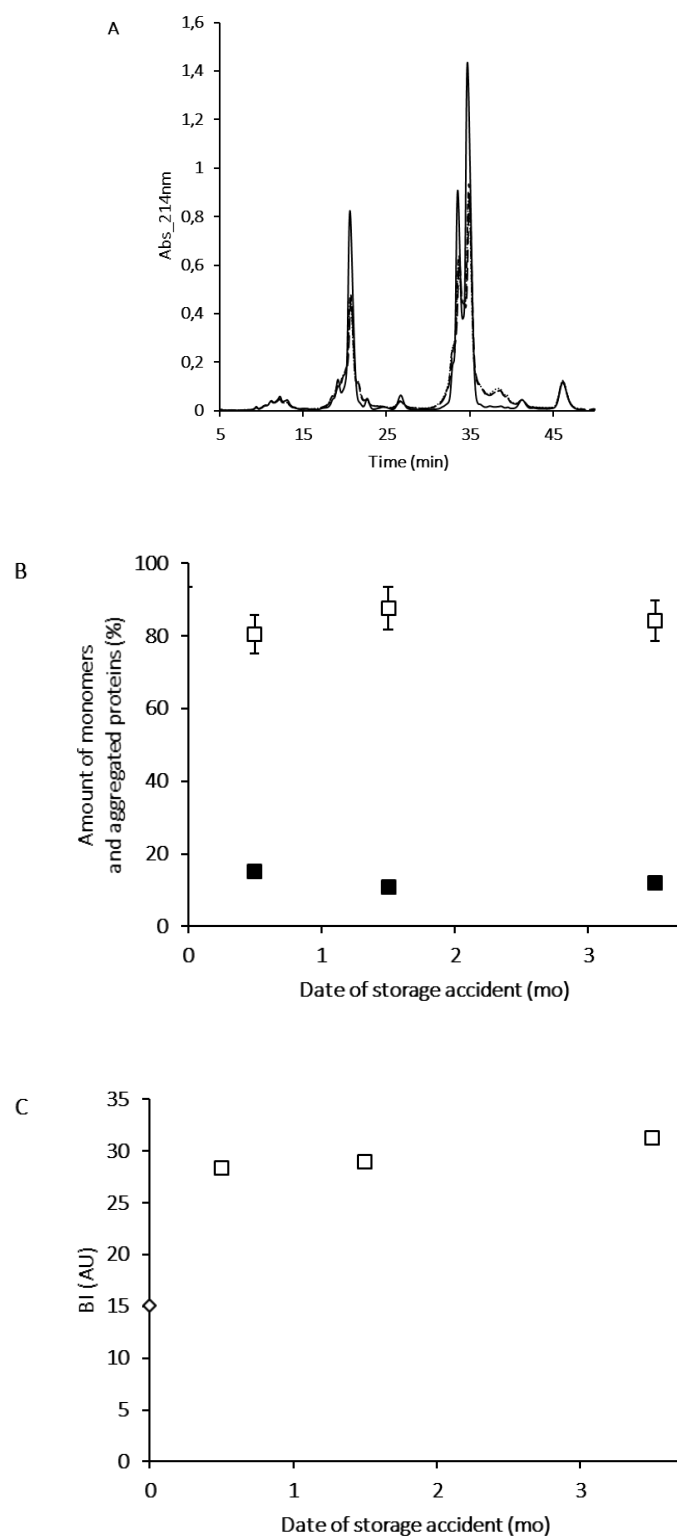


Figure 31: A) RP-Phase profile of WPI powders at pH 4.6 at t_0 (solid line) and powders with 1 storage accident after 2 weeks (long-dashed line), after 1.5 months (dashed line) and after 3.5 months (dotted line). B) Amount of monomers (empty square) and aggregated proteins (full square) and C) evolution of Browning Index for Ref powder (diamond) and powders with 1 storage accident (square) after 2 weeks, 1.5 months and 3.5 months, with experimental relative error $< 0.7\%$

NUMBER OF STORAGE ACCIDENTS

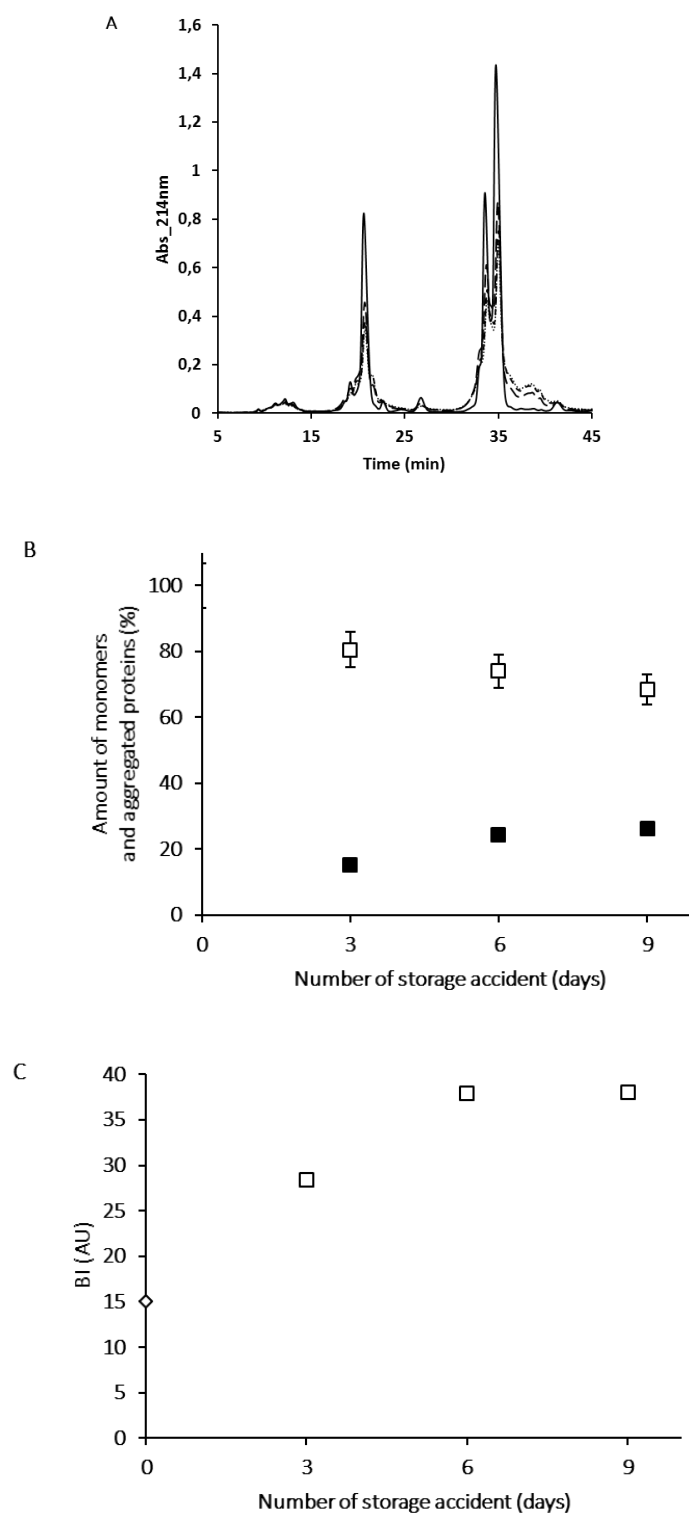


Figure 32: A) RP-Phase profile of WPI powders at pH 4.6 at t_0 (solid line) and powders with 1 storage accident (long-dashed line), 2 storage accidents (dashed line) and 3 storage accidents (dotted line); B) Amount of native (empty square) and aggregated (full square) proteins; and C) evolution of Browning Index for Ref powder (diamond) and powders with 1, 2 and 3 storage accidents (square), with experimental relative error < 0.7%

Our results showed that the more the number of storage accidents, the higher the rate of denaturation (**Figure 32, A**), the browning index (**Figure 32, C**) and the aggregation (**Figure 32, B**), and conversely the lower the monomer amount. Clearly, the structural evolutions – loss of monomeric forms through the formation of aggregated proteins – were cumulative among the number of storage accidents, given the linear shape of the curves of **Figure 32, B**.

This part of the study showed that several storage accidents can affect irreversibly the powder structural properties and this from the first storage accident. It is thus of high importance to control their storage conditions in the depot or in the course of delivery.

PART 7. CONCLUSIONS

In this chapter, we studied the influence of representative storage conditions on protein structural properties of WPI powders. The results showed that WPI powders stored at temperatures below or equal to 20°C were relatively stable from a structural point of view up to 15 months' storage with only a limited lactosylation increase. On the other hand, WPI powders particles presented hydrophobic patches and a decrease in amino acid content concomitant with a significant levels of aggregation and protein modifications. These involved first protein lactosylation and then degradation of Maillard reaction products resulting in a higher Browning Index compared to the reference, starting from the first two weeks of storage at 60°C. At 40°C, powders showed intermediate evolution.

Moreover, one to several storage accidents (72 h at 60°C) applied on powders stored at 20°C affected the structural properties of the WPI powder. The time at which the storage accidents occurred didn't influence the rate of modifications on 4.5 months; however, the number of storage accidents linearly affected the changes in protein structure and the Browning Index.

Although the WPI powder had been thoroughly diafiltered before spray drying, a very small amount of lactose still remains in the powder (1.28% w/w) to enable the development of the Maillard reaction. Indeed, two ageing steps depending on water activity, time and temperature have been highlighted in this study involving first protein lactosylation and then degradation of Maillard reaction products. This resulted in an increase in the aggregation rate in the dry state and a higher Browning Index compared to the reference.

The definition of storage conditions is obviously very important in order to keep the WPI powder structural properties upon ageing. However, how the storage-induced structural changes in the dry state affect the functional properties?

CHAPTER 3. STUDY OF CHANGES IN WHEY PROTEINS HEAT-INDUCED AGGREGATION, FOAMING AND INTERFACIAL PROPERTIES

The aim of this chapter is to identify which structural markers participate in the changes in protein functionality and to understand the mechanisms involved in these modifications. Functional properties of whey proteins are directly dependent on their structure, either native or in the form of protein aggregates, especially through their size, shape, surface properties, and also the type of bonds maintaining proteins together in the aggregates. In order to study the impact of whey proteins structural changes on their functional properties, WPI powders were characterized by three types of properties relevant to the industrials regarding their use: a) heat-induced aggregation, b) foaming and c) interfacial properties. It was demonstrated that even though protein lactosylation occurred in WPI powders stored at 20°C, the properties of these latter were not significantly different from the non-aged WPI powder. On the other hand, powders stored at 40°C, in which protein lactosylation and aggregation in the dry state occurred, showed a decrease in aggregate size after heat treatment at 5.8 d pH d 6.6 with concomitant changes in the aggregate shape. However, no changes in the foaming and interfacial properties among storage were observed. At 60°C, only foaming and interfacial properties have been studied and the changes in the dry state led to an improved foam stability in the most severe conditions although the diffusion coefficient of the proteins at the air/water interface decreased. A part of these results has been published in *Food And Bioprocess Technology* (Paper²) and in *Journal of Dairy Science* (Paper⁴).

The aims of that chapter were to:

- x *highlight the changes in functional properties caused by the structural changes in the dry state*
- x *identify the relevant structural markers affecting whey proteins functionality*
- x *understand their role in the functional properties modifications*

² Published as Norwood EA, Chevallier M, Le Floch Fouéré C, Schuck P, Jeantet R, Croguennec T. (2016). Heat-induced aggregation properties of whey proteins as affected by storage conditions of whey protein isolate powders. *Food and Bioprocess Technology*, 9(6), 993–1001. doi:10.1007/s11947-016-1686-1.

⁴ Published as Norwood EA, Le Floch Fouéré C, Schuck P, Croguennec T, Jeantet R. (2016). Structural markers of the evolution of whey protein isolate powder during aging and effects on foaming properties. *Journal of Dairy Science*, 99(7), 5265–5272. doi:10.3168/jds.2015-10788.

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PART 1. INTRODUCTION

As a reminder, WPI consists of protein fraction greater than or equal to 90% (w/w) (Burin et al. 2000), including about 70% β -lactoglobulin (β -LG) and 20% α -lactalbumin (α -LA) (McClements et al. 1993). Whey proteins in their native state have biological and functional properties that provide undeniable advantages in many food applications. They are recognized for their nutritional role, their solubility and their ability to form gels, emulsions and foams.

In the literature it has been shown that changes occurring in the dry state during storage affect powders functionality (Fyfe et al. 2011; Gaiani et al. 2009). However and from a scientific point of view, the main issue is to link structural modifications to powder's functional evolution in order to identify relevant markers giving information on early changes in properties.

On an industrial scale, heat treatment is an essential step since it modifies the structure of whey proteins, which is needed for some functional applications as a food ingredient. Depending on the severity of the treatment and the physicochemical environment of the proteins, structural changes occur, leading to aggregation that may be desired for structuring or texturing food (Nicolai 2015). Several types of whey protein aggregates that differ in size and morphology are used for specific functional properties such as the texture of dairy products (firmness, creaminess, etc.), as fat substitutes or agents in the stability of foams and emulsions (Guilmineau and Kulozik 2006), or for their stability in high protein drinks during long term storage (Sağlam et al. 2014).

Whey proteins also form stable viscoelastic layers at the air/water interface when they are adsorbed, which can lead to a resistant and cohesive interfacial network improving foam stability (Martin et al. 2002). Their interfacial properties allow the formation of a wide range of foamed products such as bread, meringues and ice creams. Moreover, proteins are often used as foaming agents in food since they contribute both to foam formation and foam stability; and controlling these properties is therefore of great interest for the food industry.

In order to study the impact of whey proteins structural modifications on their functional properties, powders were characterized through three types of relevant properties to the industrials: heat-induced aggregation, foaming and interfacial properties.

PART 2. EXPERIMENTAL STRATEGY**3.2.1. HEAT-INDUCED AGGREGATION****HEAT TREATMENT AFTER REHYDRATION**

Solutions of non-aged WPI powder (stored at 4°C – Ref) and WPI powders stored for 3, 6, 12 and 15 months dissolved in milli-Q water (4% w/w) were adjusted to pH 5.8, 6.2 and 6.6. Solutions were heated in a water bath at 80°C for 1 hour and then quenched in cold water. After heat treatment, 0.02% (w/w) sodium azide was added to each solution and the solutions were sampled for analysis. Samples which were analysed the same day were kept at room temperature (GPC, dynamic light scattering (DLS)) and those that were not analysed the same day (transmission electron microscopy (TEM)) were stored in a cold room.

HEAT-INDUCED AGGREGATION ANALYSIS

Particle size distribution of the heat-treated dispersions was determined by DLS using a Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK). Measurements were performed in triplicate at 20°C, measuring the backscatter at 173° over 120 s. Prior to measurement, samples were diluted 1:100 with milli-Q water. Average size diameters were reported. TEM was performed using negative staining. Samples were diluted to 160 mg.L⁻¹ protein and stained using a 2% uranyl acetate solution (Ayache et al. 2007). Samples were observed using a JEOL JEM 1400 transmission electron microscope (TEM, Croissy-sur-Seine, France) operating at 120 kV. Images were recorded with a GATAN Orius SC 1000 camera (Digital Micrograph software).

3.2.2. ANALYSIS OF FOAM PROPERTIES

Foaming properties were evaluated by beating 200 mL of 50 g.L⁻¹ WPI solution reconstituted in milliQ water at room temperature with a Hobart N50 whisk (Hobart, Peterborough, UK) at speed 3 (maximal speed) for 2 min. A foam core of 74 cm³ was removed and the initial foam weight was determined (time *t*_i). The foam core was placed in a glass funnel on a measuring tube previously tared and the exuded liquid was regularly measured for up to 60 min. The experiment was repeated in triplicate at room temperature.

The foam density (*FD*; g.cm⁻³) was evaluated according to:

$$FD = \frac{\text{Foam weight}}{\text{Foam volume}} \quad (5)$$

The foam stability (FS ; %) was calculated during drainage according to Howell and Taylor (1995):

$$FS = \frac{\text{Initial foam weight } (t_i) - \text{drained weight } (t)}{\text{Initial foam weight } (t_i)} * 100 \quad (6)$$

3.2.3. ANALYSIS OF THE INTERFACIAL PROPERTIES

Measurements of the ellipsometric angle value were carried out with an in-house automated ellipsometer in a “null ellipsometer” configuration (**Figure 33**) (Berge and Renault 1993; Azzam and Bashara 1977) and were repeated twice. A polarized He–Ne laser beam ($\lambda = 632.8 \text{ nm}$, Melles Griot, Glan-Thompson polarizer) was reflected on the liquid surface. The incidence angle was 53.12° , *i.e.* Brewster angle (θ_B) for the air/water interface. This particular angle is defined by (Lheveder et al. 2000):

$$\tan\theta_B = \frac{n_2}{n_1} \quad (7)$$

With n_1 : air index

n_2 : water index

After reflection on the liquid surface, the laser light passed through a $\lambda/4$ retardation plate, a Glan-Thompson analyser, and a photomultiplier. The analyser angle, multiplied by 2, yielded the value of the ellipsometric angle (Δ), *i.e.* the phase difference between parallel and perpendicular polarization of the reflected light. The laser beam probed the 1 mm^2 surface with a depth in the order of $1 \mu\text{m}$.

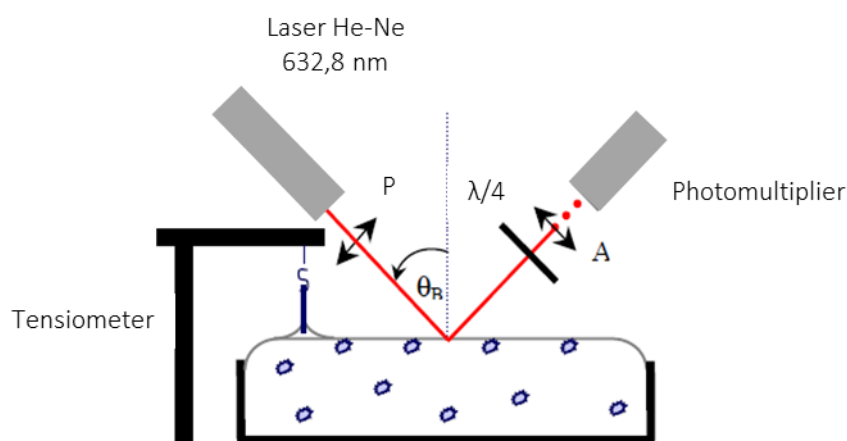


Figure 33: Diagram of the ellipsometric measurements

The surface pressure, π , was measured following the Wilhelmy plate method. The plate was a 10 mm \times 22 mm filter paper (Whatman, Velizy-Villacoublay, France) and was connected to a microelectronic feedback system (Nima PS4, Manchester, England). The surface pressure was recorded every 4 s with precision of $\pm 0.2 \text{ mN.m}^{-1}$. The measured surface pressure is defined by (Vargaftik et al. 1983):

$$\pi = \gamma_w - \gamma_f \quad (8)$$

With γ_w : surface tension of water, which is of 72.8 mN.m^{-1} at 20°C

γ_f : surface tension of the protein film

Initial values of the ellipsometric angle (Δ_0) and surface pressure (π_0) of milliQ water were recorded for at least half an hour to assure that the interface was clean. Experiments were performed only when obtaining a stable and minimal signal.

Fresh and stored powders were diluted in 3 mg.L^{-1} solutions with milli-Q water in order to study only the protein diffusion at the air/water interface and then values of the ellipsometric angle (Δ_t , $^\circ$) and surface pressure (π_t , mN.m^{-1}) for each solution were recorded for 3h at 20°C . Three parameters have been taken into account to characterize the interfacial properties of whey proteins of stored WPI powders: Γ_0 (mg.m^{-2}), the surface concentration at which π is different from zero; θ (mN.m.mg^{-1}), the amplitude of interactions at the air/water interface; and D ($\text{m}^2.\text{s}^{-1}$), the diffusion coefficient of the proteins at the air/water interface.

D was determined graphically according to De Feijter et al. (1978) and Ward and Tordai (1946) and is represented as the slope of the curve $d\Gamma/dt$ (**Figure 34**):

$$\Gamma = 0.2 * \Delta \quad (9)$$

$$\frac{d\Gamma}{dt} = C_b * \sqrt{\frac{D}{\pi * t}} \quad (10)$$

With C_b : protein concentration in the bulk ($\mu\text{g.mL}^{-1}$)

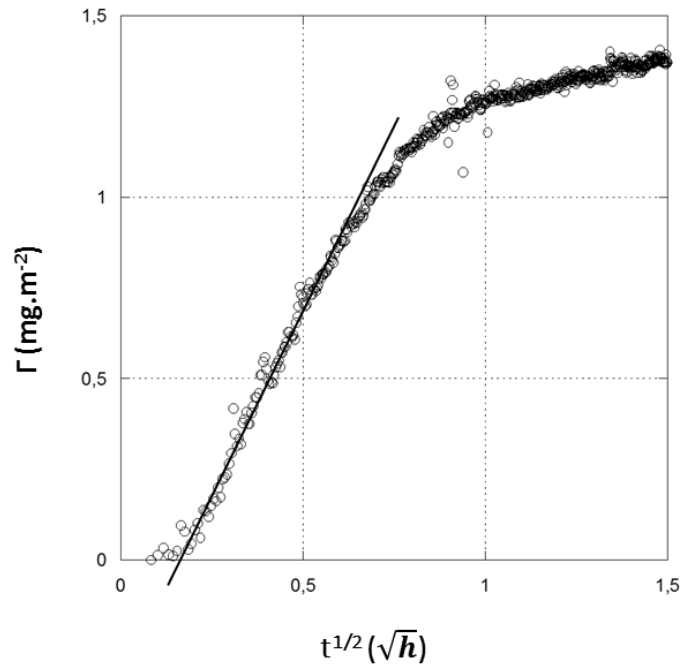


Figure 34: Graphic determination of the diffusion coefficient D

The two previous equations are true only for a diffusive regime. The parameters θ and Γ_0 were also determined graphically thanks to $\pi_t = \Gamma_t$ (**Figure 35**). θ is represented by the slope of the curve and Γ_0 the surface concentration at which π is different from zero.

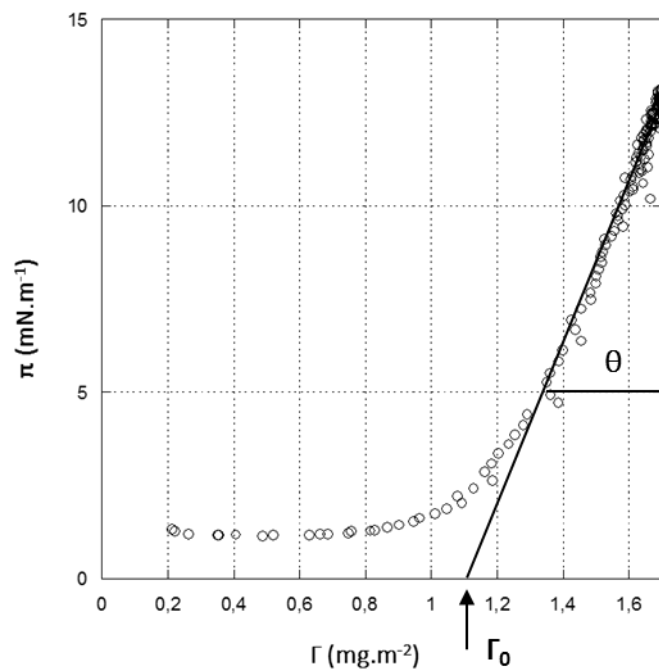


Figure 35: Graphic determination of Γ_0 , the surface concentration at which π is different from zero; and ϑ , the amplitude of interactions at the air/water interface

PART 3. HEAT-INDUCED AGGREGATION PROPERTIES OF WHEY PROTEINS AS AFFECTED BY STORAGE CONDITIONS OF WHEY PROTEIN ISOLATE POWDER

Whey proteins are widely used for their ability to form stable aggregates under controlled conditions (Donato et al. 2009), generally associated with the formation of intra- and inter-molecular interactions that occur during heating (Roefs and De Kruif 1994). It is known that heat-induced aggregation is very dependent on pH (Hoffmann and van Mil 1999), ionic strength and divalent cations (Ca^{2+}) content (Petit et al. 2016). Changes in techno-functional properties of stored WPI powders were analysed after reconstitution at pH ranging from 5.8 (just above their isoelectric point, pI) to 6.6 (regular pH of reconstitution in water).

3.3.1. STUDY OF THE HEAT-INDUCED AGGREGATES SIZE AND SHAPES

Heat-induced aggregation of reconstituted WPI powders was studied at pH 5.8, 6.2 and 6.6. Several studies have shown that heating generates microgels at pH 5.8, strands at pH 6.6 and a mixture of both at pH 6.2 (Nicolai et al. 2011; Nicolai and Durand 2013; Phan-Xuan et al. 2011).

Figure 36 shows the evolution of heat-induced aggregate size as a function of powder storage time and temperature after an hour of static heating of WPI solutions (4% w/w) adjusted to a given pH. The aggregate size of Ref was around 400 ± 24.96 nm, 170 ± 0.28 nm and 120 ± 2.75 nm at pH 5.8, 6.2 and 6.6, respectively. As expected, WPI powders stored at 20°C showed relative stability with regard to heat-induced aggregate size after 15 months of storage, whatever the pH (**Figure 36**, A). In fact, no significant variation was registered in DLS after repetition. However, storage at 40°C provided very different results. Heat-induced aggregate size decreased as a function of storage time regardless of pH (**Figure 36**, B); this decrease was significant from 3 months. At pH 5.8, 6.2 and 6.6, aggregate size decreased to 225 ± 19.23 nm, 130 ± 1.56 nm and 78 ± 1.16 nm after 12 months, respectively.

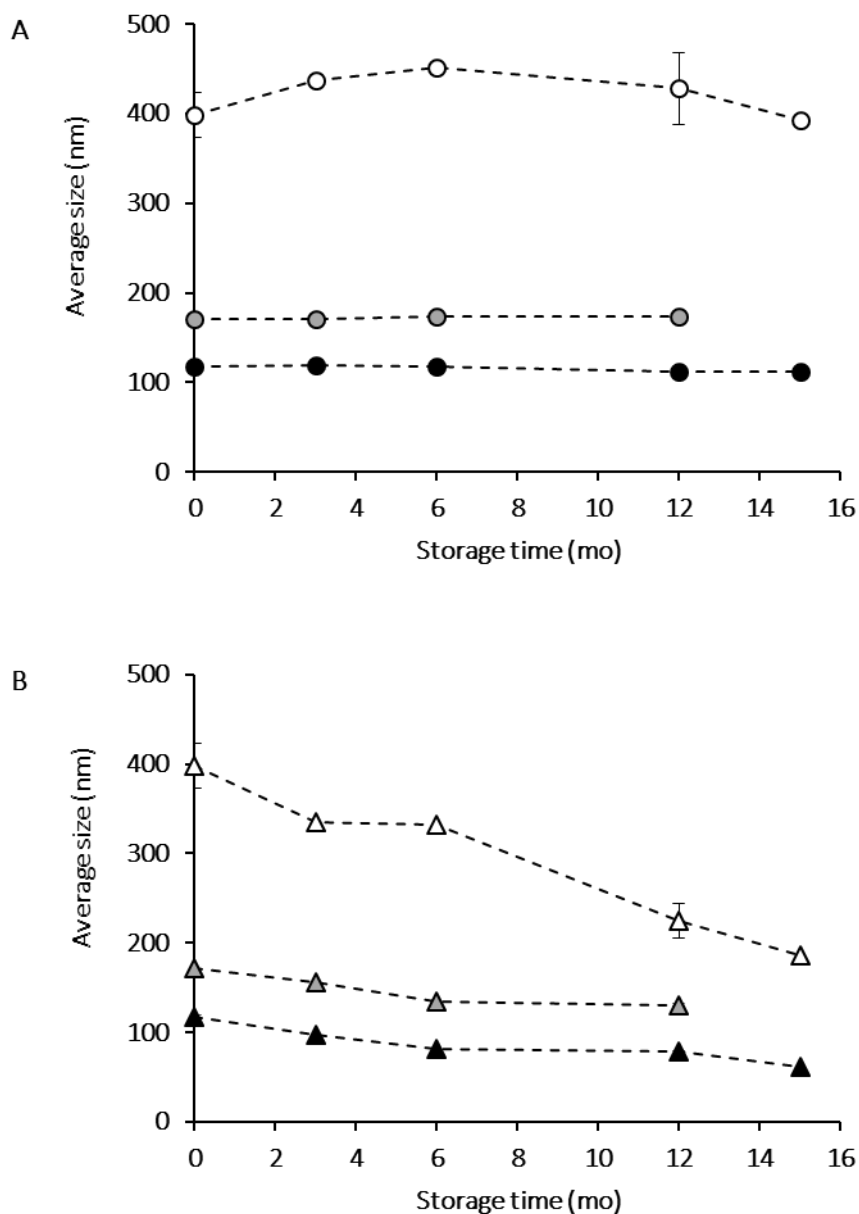


Figure 36: Average aggregate sizes as a function of storage time for powders stored at A) 20°C and B) 40°C after heat treatment at pH 5.8 (white symbols), pH 6.2 (grey symbols) and pH 6.6 (black symbols)

Heat-induced aggregates from WPI powders stored for 12 months at 40°C were observed by TEM (Figure 37). In the Ref samples, heat-induced aggregates consisted of microgels (spherical units) at pH 5.8 (Figure 37, A) and a mixture of smaller individual particles, mostly spherical and strand-like, at pH 6.2 (Figure 37, C) and 6.6 (Figure 37, E), respectively. Images of heat-induced aggregates using 12-months stored powder still showed spherical units although smaller than in Ref at pH 5.8 (Figure 37, B), mostly strands at pH 6.2 (Figure 37, D), and only strands at pH 6.6 (Figure 37, F). The morphological

features of Ref were not in agreement with the results reported by Phan-Xuan et al. (2011) for samples heat treated at pH 6.2 and 6.6. According to these authors, only strands could be formed at pH 6.6 and mostly strands at pH 6.2. The difference between our results and those of Phan-Xuan et al. (2011) might be related to the age of the powder as the shapes of the aggregates changed with storage time. It is also possible that differences in powder composition may affect the morphology of the aggregates. Phan-Xuan et al. (2013) indicated that small variations in amount of calcium could shift microgels to fine strands and vice-versa.

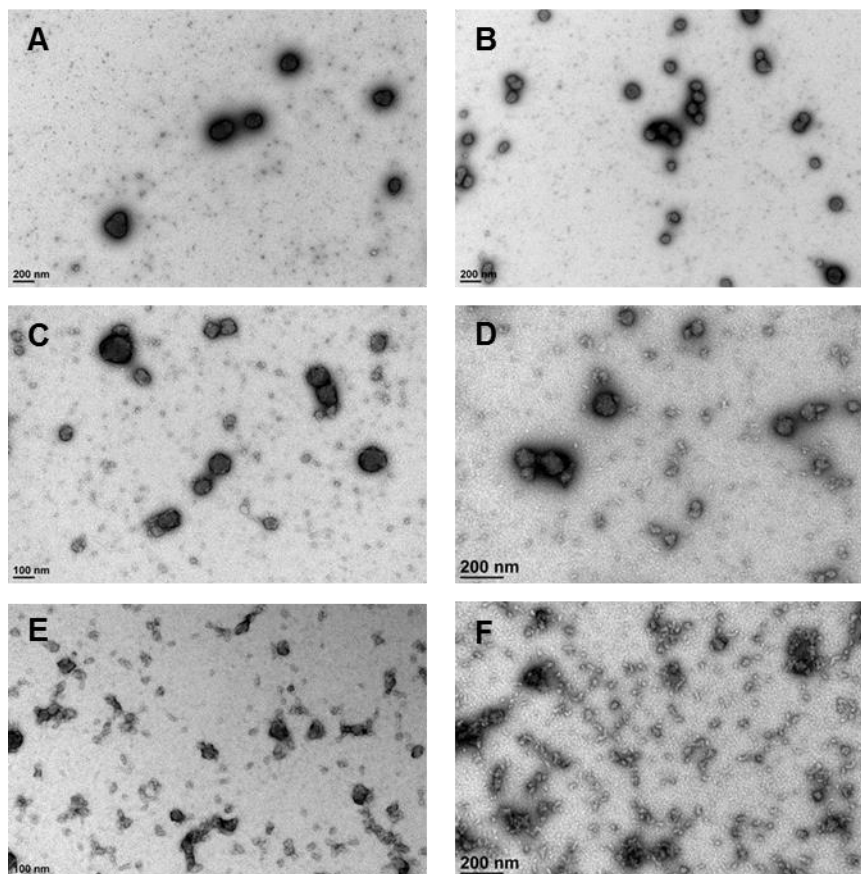


Figure 37: TEM images of negatively stained WPI Ref (A, C, E) and powder stored for 12 months at 40°C (B, D, F) when heat treated at pH 5.8 (A, B), pH 6.2 (C, D) and pH 6.6 (E, F)

3.3.2. ROLE OF THE STRUCTURAL MARKERS OF AGEING IN HEAT-INDUCED AGGREGATION

Upon heating, whey proteins shift to a reactive state. The protein structure is modified, free to unfold, exposing their hydrophobic core and free thiol groups so that they are able to interact with nearby molecules. First, covalent oligomers are formed linked by disulphide bridges (Croguennec et al. 2004; Surroca et al. 2002). Their structure is flexible and when the number of oligomers exceeds a critical level, they form relatively monodisperse structured aggregates (Nicolai et al. 2011; Schmitt et al. 2009). Their presence seems to be a condition for the formation of microgels (Da Silva Pinto et al.

2012; Schmitt et al. 2009), and according to Schmitt et al. (2010), small aggregates resulting from an association of oligomers are only a stage in the formation of final heat-induced aggregates. In fact, these small aggregates appear to be the source of new larger aggregates, first linked by hydrogen and hydrophobic bonds, and then stabilized by disulphide bridges. Other authors have hypothesized that the heat-induced aggregation originates from growth and nucleation processes (Aymard et al. 1996; Bromley et al. 2006; Phan-Xuan et al. 2011), the nuclei being the oligomers formed in the early stages of aggregation on which the denatured proteins aggregate (Phan-Xuan et al. 2013). Environmental conditions (pH, ionic strength, nature of salts) govern heat-induced aggregation as they determine the ability of proteins to unfold, change the net charge and thus the interaction between proteins which finally affect oligomers formation.

It was possible to see on **Figure 37** that all aggregates were not identical, depending on the solution pH when heat treated. At pH 5.8 microgel types were visible while at pH 6.2 and 6.6, mixtures of microgels and strands were observed. Phan-Xuan et al. (2014) highlighted the importance of the net charge density of native protein in the type of heat-induced aggregate formed, and therefore the key role of the solution pH during heat treatment (Hoffmann and van Mil 1999). Around pI, aggregates form a spherical structure (microgels) which is reinforced by new covalent bonds (Giroux et al. 2010), whereas when the pH increases, aggregate size decreases and strands are observed (Phan-Xuan et al. 2011). This can be explained by the repulsion generated by surface charges of denatured proteins, thereby limiting further aggregation. In our samples at $\text{pH} \geq 6.2$, a mixture of strands and microgels was present, in agreement with Hoffmann and van Mil (1999) and Phan-Xuan et al. (2011). In fact, heat-induced aggregation favours the formation of microgels before strands (Nicolai 2015) as the pH increases upon aggregation, leading to an increase in the protein net charge and then a shift from microgels to strands.

Structural findings confirmed that heat-induced aggregation was not significantly different from the Ref when the powders were stored at 20°C, although lactosylation was observed following 15 months of storage. When powders were stored at 40°C, the amount of native protein decreased in favour of aggregation in the dry state. According to Phan-Xuan et al. (2013), as oligomers are formed from native proteins, heat-induced aggregates are smaller because of the reduction in amount of native protein. A steady state is reached when the solution no longer contains native monomers. Our results were consistent with those of Jones et al. (2010) that explained that smaller heat-induced aggregates should probably be due to a reduction in available protein monomers. The literature also indicates that the reduction in aggregate size is due to the presence of sugar which modifies native proteins into AGE (Da Silva Pinto et al. 2012). In these conditions, proteins are found to be less flexible and thus lose

part of their ability to form aggregates and then inhibit protein aggregation (Lee et al. 2009). Indeed, in our study, proteins were partly lactosylated, without subsequently aggregation in a dry state during storage at 20°C, and no changes in heat-induced aggregation could be observed.

Moreover, heat-induced aggregates decreased in size at pH 6.2 and 6.6 but their shape moved from microgels to strands. The decrease in the amount of native protein after powder storage at 40°C could explain the change in aggregate morphology (Lee et al. 2009). The extensive binding of lactose molecule and Maillard reaction products on whey proteins (mainly on amino groups) stored at 40°C could also increase their net charge. Consequently, clustering of oligomers upon heating might rapidly reach a critical charge density, thus limiting aggregation, and strands would form preferentially.

To summarize, a mechanism for heat-induced aggregation of stored WPI powders under the experimental conditions tested is proposed in **Figure 38**.

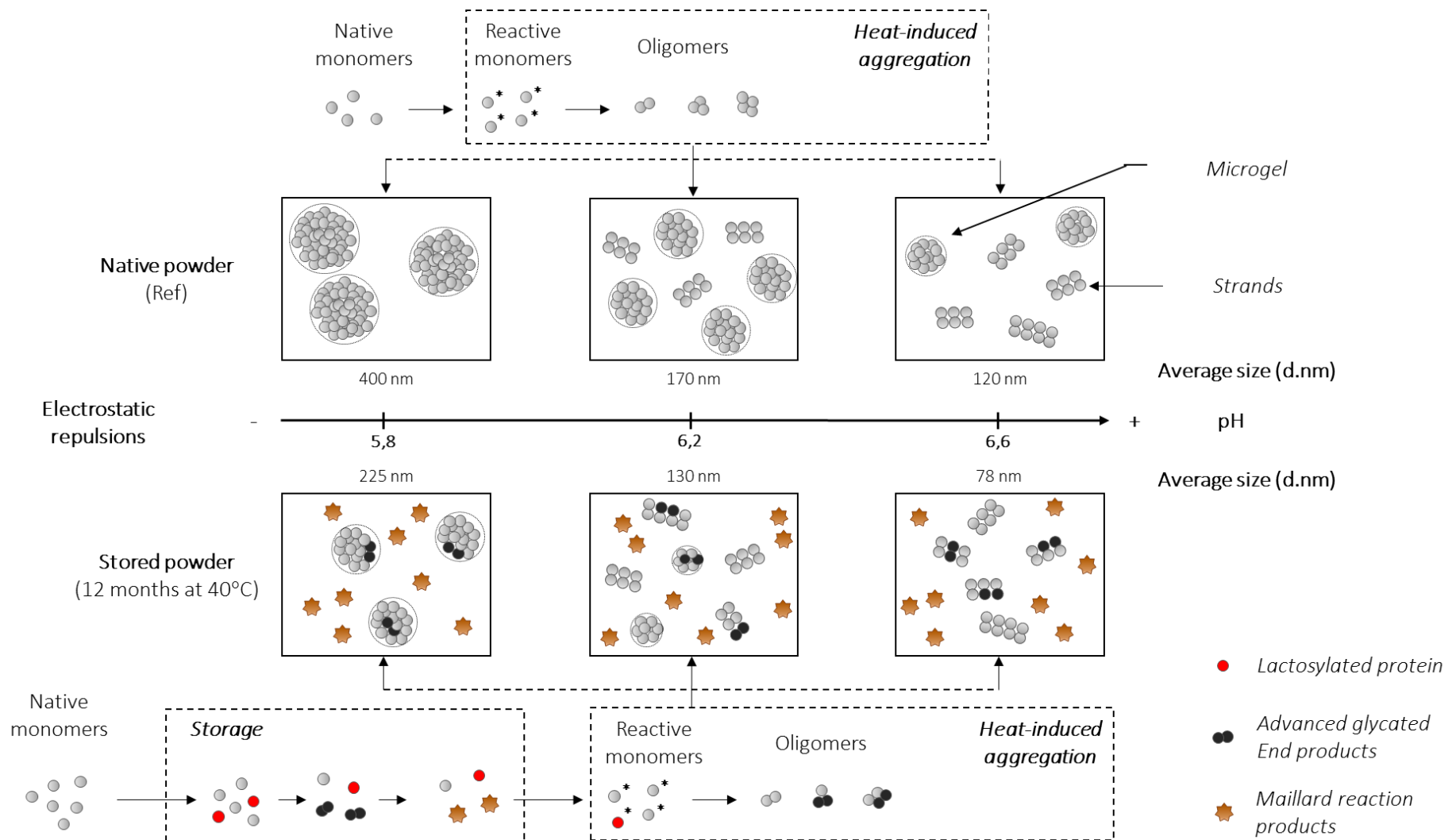


Figure 38: Proposed scheme for formation of heat-induced aggregates of native and stored powders as a function of pH

To put it in a nutshell, changes in heat-induced aggregation properties of whey proteins after storage are a consequence of structural changes occurring in the WPI powder in the dry state. Moreover, it can now be stated that lactosylated proteins are not directly responsible for the changes in aggregate size or shapes, but their degradation in AGE or Maillard aggregates is more likely to be involved in these modifications.

PART 4. LITTLE EFFECT ON THE FOAMING AND INTERFACIAL PROPERTIES

3.4.1. FOAM STABILITY

Foams are a dispersion of gas bubbles in a smaller volume of liquid with surface-active reagents, which increase the foam stability. In our case of study, the aqueous phase was composed of water, the gas was air and the surface-active molecules was represented by the proteins (modified or not). Foams are made of polyhedral gas bubbles with liquid films surrounding bubbles, which are called Plateau borders. The latter are formed by the meeting of three films and four of them join into a node. Liquid films and Plateau borders constitute a network for liquid drainage out of the system by gravity, and also by surface and capillary forces. Foams are thus thermodynamically unstable systems. Indeed, there are 3 major processes of destabilization of foams: film cohesion, drainage and disproportion (Ostwald ripening). One of them, drainage, was assessed over time through the change of FS (Equation n°6; **Figure 39**) and FD (Equation n°5; **Figure 40**). Only the results obtained after 12 months' storage at $T \leq 40^\circ\text{C}$ and 6 months at 60°C are presented.

The foam stability of powders stored at $T \leq 40^\circ\text{C}$ did not differ significantly from Ref powders. On the other hand, foams made from the powders stored at 60°C were denser and those of $a_w 0.36$ were slightly more stable ($p > 0.05$) than Ref powders after 40 min.

For powders stored at $T \leq 40^\circ\text{C}$, the amount of monomers did not appear to be limiting leading in no change in the foam stability. This results can also be explain by the presence of remaining caseins (**Table 4**), which are surface active components (Dickinson 2001) and may have participated to the foam stability.

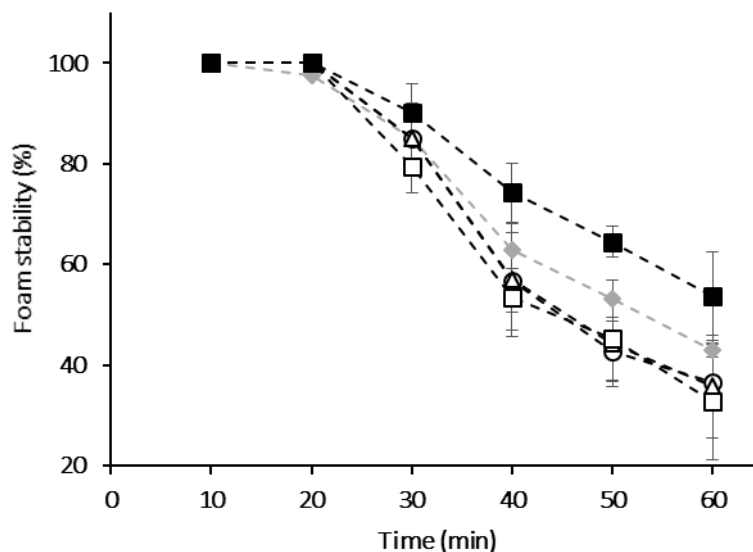


Figure 39: Effect of storage time and temperature on foam stability. Powders of a_w 0.23 at t_0 (full grey circle) and stored 12 months at 20°C (empty circle), at 40°C (empty triangle) and 6 months at 60°C (empty square). Powder of a_w 0.36 stored 6 months at 60°C (full square)

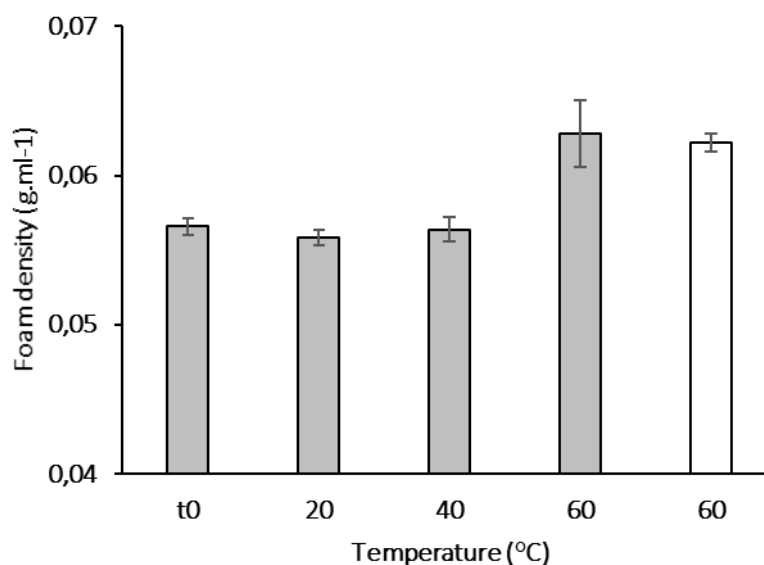


Figure 40: Effect of storage temperature on foam density. Powders of a_w 0.23 at t_0 and stored 12 months at 20°C, 40°C and 6 months at 60°C (grey bar). Powder of a_w 0.36 stored 6 months at 60°C (white bar)

The coexistence of aggregated proteins and non-aggregated proteins has been assessed to be a key parameter for foaming properties (Foegeding et al. 2006). In accordance with our results, the latter study showed that an increase in the relative percentage of whey protein polymers in the solution led to more stable foams by decreasing the drainage rate with a concomitant decrease in the air phase volume. Non-aggregated proteins contributed to the foam formation as they diffuse to the interface

faster, then unfold and adsorbed to form an aqueous film surrounding the air bubbles. These unfolded proteins at the interface can also serve as an anchor for aggregated proteins (Dimitrova et al. 2004). In our case, with more than 65% in the powder, the aggregates were likely to be forced to adsorption during foam threshing and participate in strengthening the viscoelasticity of the bubble interface. Moreover, these aggregated proteins contributed to the aqueous film stability by increasing its viscoelasticity and acting against the gravitational drainage and gas diffusion between bubbles (Davis and Foegeding 2004; Zhu and Damodaran 1994). Other authors have claimed that the increase in surface hydrophobicity could be considered as a decisive factor for the formation and stabilization of protein foams due to the rapid formation of viscoelastic films (Báez et al. 2013; Moro et al. 2011). The reverse phase HPLC results showed a decrease in the amount of native protein with storage time in favor of more hydrophobic denatured proteins. These new hydrophobic particles could adsorb irreversibly and form a solid shell stabilizing bubbles against disproportion (Kam and Rossen 1999).

Some of the aggregates which did not adsorb might provide additional stabilization mechanisms. They can become trapped in the film during foam formation and structure themselves into layers that are difficult to remove from the film (Sethumadhavan et al. 2001a, 2001b). The foam stability is then improved by repulsive forces which structure the aqueous film (Sethumadhavan et al. 2001a). This kind of aggregates is also subjected to crosslinking between the interfaces to form a gel-like network which prevents the film to become thinner, therefore preventing the interfacial layers of air bubbles to become closer and finally helping to retain the solution in the film (Rullier et al. 2008; Saint-Jalmes et al. 2005). Moreover, these aggregates can be located in the Plateau borders, playing a role of steric clutter impeding drainage.

It is assumed that shapes and sizes of aggregates are crucial to the stability of foams (Fameau and Salonen 2014; Rullier et al. 2010; Zhu and Damodaran 1994). However, the size of aggregates formed during storage in our study were too heterogeneous and their shapes couldn't be studied to confirm this hypothesis.

3.4.2. INTERFACIAL PROPERTIES

Because of their surface-active properties, whey proteins contribute to the formation of foam based food products and also improve their physical stability. They adsorb spontaneously from the aqueous solution to the air/water interface where their free energy is lower than in the unadsorbed state. Upon adsorption, proteins unfold and establish intermolecular interactions with other proteins at the interface or close to the interface leading to the formation of an interfacial film of proteins.

In this section, the influence of storage conditions on the interfacial properties of whey proteins considering three characteristic parameters of the adsorption, the diffusion coefficient D of the proteins at the air/water interface on the one hand, and the surface concentration Γ_0 at which the surface pressure π is different from zero and the amplitude of interactions θ at the air/water interface on the other hand.

ADSORPTION KINETICS

The characterization of the interfacial properties first consisted in determining the adsorption kinetics by ellipsometry (**Figure 41**).

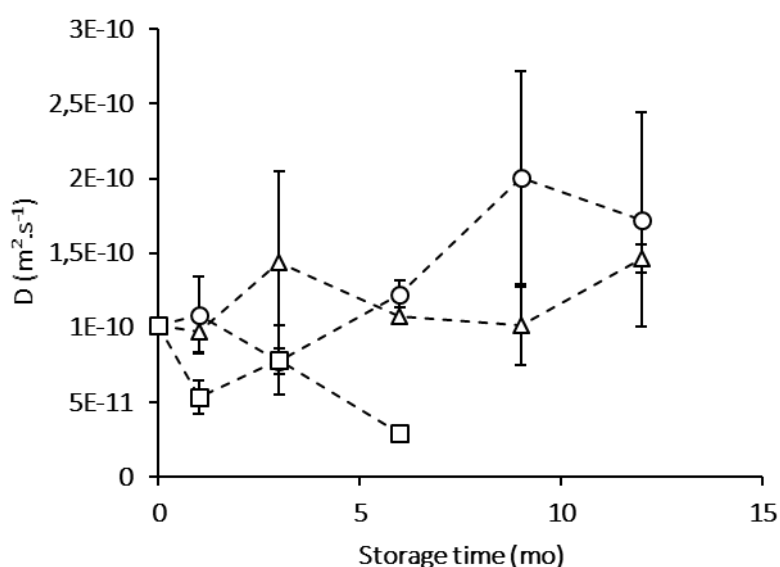


Figure 41: Diffusion coefficient (D , $m^2.s^{-1}$) of surface active molecules in $3 mg.L^{-1}$ solutions reconstituted with powders stored at $20^\circ C$ (circle), at $40^\circ C$ (triangle) and at $60^\circ C$ (square) as a function of storage time

The diffusion coefficient D of surface active molecules from fresh powder (Ref) was of $1.02 \times 10^{-10} m^2.s^{-1}$. As expected, D was comparable to the values found in the literature for proteins, namely ranging between 0.7 and $1.0 \times 10^{-10} m^2.s^{-1}$ (De Feijter et al. 1978). Indeed, the WPI powder consisting of at least 90% protein, it is likely to be the proteins who govern the adsorption at the air/water interface. At $20^\circ C$, D appears to increase with storage time of up to $2.00 \times 10^{-10} m^2.s^{-1}$ after 9 months of storage despite the broad standard deviation. Similar behaviour was observed for powders stored 3 months at $40^\circ C$, at around which time the diffusion coefficient reached $1.44 \times 10^{-10} m^2.s^{-1}$. However, it is worth noting that the recorded changes were not significant in terms of standard deviations at temperatures below or equal to $40^\circ C$. At $60^\circ C$, D seemed to decrease with storage time going from $5.36 \times 10^{-11} m^2.s^{-1}$ in the first month of storage to $2.94 \times 10^{-11} m^2.s^{-1}$ after 6 months.

The results obtained at 20 and 40°C can be explained by the level of lactosylation of the proteins being close to the maximum value measured (~30%). In the literature it has been reported that hydrophilic polysaccharides promote interfacial adsorption and dilatation of milk protein (Baeza et al. 2005; Hiller and Lorenzen 2010). Baeza et al. (2005) showed a cooperative behaviour between the hydrophilic polysaccharide and the protein leading to a significant increase of surface pressure of adsorbed films. At 60°C, the increase in molar mass of Maillard reaction products – aggregates formed in the dry state – is probably at the origin of the decrease in Γ_0 . Contrary to small protein aggregates, larger aggregates, coming from an extent of the Maillard reaction, weren't able to adsorb at the air/water interface in the given time of the experiment as demonstrated by Rullier et al. (2008, 2009).

ADSORPTION BEHAVIOUR

The surface concentration at which $\pi \neq 0$ (Γ_0) and the amplitude of interactions at the interface (θ) for different powder samples are given in **Figure 42** and **Figure 43**, respectively.

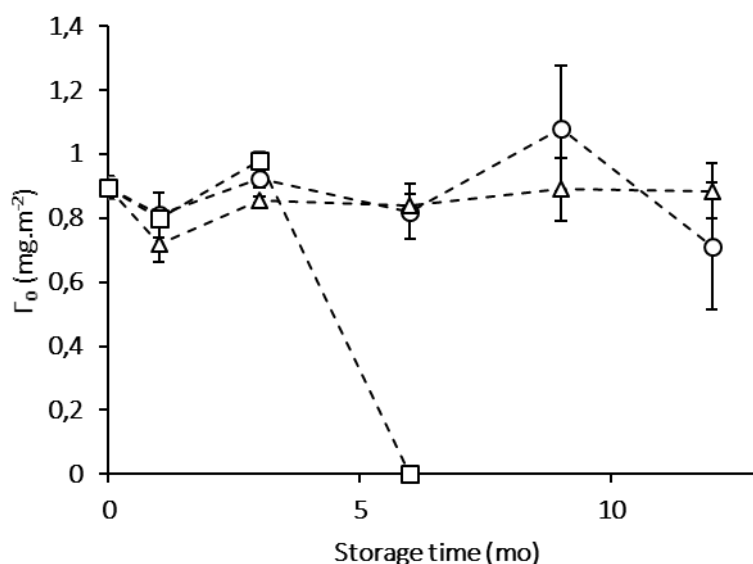


Figure 42: The surface concentration at which $\pi \neq 0$ (Γ_0 , mg.m^{-2}) of surface active molecules in 10^{-1} solutions reconstituted with powders stored at 20°C (circle), at 40°C (triangle) and at 60°C (square) as a function of storage time

For the fresh powder, Γ_0 was of 0.90 mg.m^{-2} . This value is close to those reported in the literature for protein and is between 1.04 and 1.17 mg.m^{-2} (Razumovsky and Damodaran 1999). At 20 and 40°C, the values of Γ_0 were not affected by storage time for a period of up to 12 months. By contrast, no measure of Γ_0 was possible in the time of adsorption experiments for powders stored 6 months at 60°C.

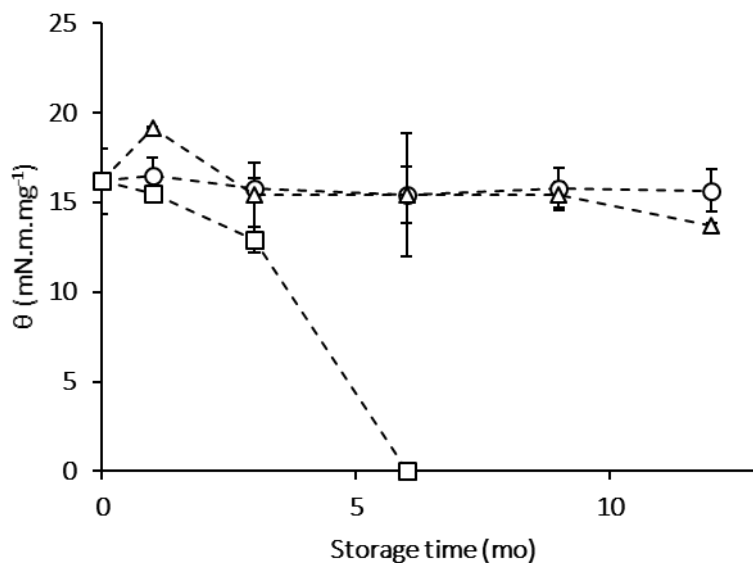


Figure 43: The amplitude of molecular interactions (θ , mN.m.mg^{-1}) of surface active molecules 5 mg.L^{-1} solutions reconstituted with powders stored (fresh), at 40°C (triangle) and at 60°C (square) as a function of storage time

The amplitude of molecular interactions (θ) at the interface between air and solution reconstituted from fresh powder was $16.18 \text{ mN.m.mg}^{-1}$, in agreement with those found in the literature for protein that are ranging from 16.1 to $19.9 \text{ mN.m.mg}^{-1}$ (Razumovsky and Damodaran 1999). This shows that the amplitude of the cohesive interactions was low in the early stages of adsorption for fresh powder. This happened to be similar for powders stored at 20 and 40°C , within the experimental error. At 60°C , the samples showed a decrease in θ after 3 months of storage which become non-measurable after 6 months storage.

Lactosylated proteins did not affect Γ_0 and θ as shown by the results of powders stored at 20°C for 12 months and at 40°C for a storage between 1 and 3 months. These results are consistent with Wierenga et al. (2009) who showed no difference in the interfacial properties of dry state glycosylated lysozyme. Moreover, powders stored for more than 3 months did not show any difference from the control sample while proteins had already started to aggregate in the dry state. The properties or the amount of aggregated proteins did not seem to be sufficient to modify Γ_0 and θ . However, results for powders stored 6 months at 60°C can be related to the previous paragraph showing no adsorption during the first times of adsorption. It is interesting to note that for shorter storage times, Γ_0 and θ were not affected or only slightly. With more than 65% aggregated proteins in the dry state, it looks like there were not enough proteins to adsorb at the air/water interface for powders stored 6 months

at 60°C, or that aggregated proteins were too slow to adsorb within the 3h experiments in accordance with the adsorption kinetics study.

To conclude and in combination with the results of the adsorption kinetics, there was no great change in the interfacial properties of WPI powders upon storage. As the literature reports that the interfacial properties can be modulated according to the degree of modification of the protein structures (Foegeding et al. 2002; Gauthier et al. 2001; Mackie et al. 1999), it can be concluded that the protein structure doesn't seem to be modified by lactosylation and that the extent of structural modifications, including protein denaturation and aggregation in the dry state, was too small to induce significant change in interfacial properties.

PART 5. ADDITIONAL RESULTS: EFFECT OF STORAGE ACCIDENT ON WHEY PROTEINS HEAT-INDUCED AGGREGATION PROPERTIES

3.5.1. EXPERIMENTAL STRATEGY

To study the effect of one or more storage accidents on heat-induced aggregation properties, the same powders as described in Chapter 2 (cf. 2.6.2., p59) were considered. Specifically, the experimentations were carried out on samples that differed in the number of storage accident. We have chosen not to determine the heat induced aggregation properties of the samples that differed in the moment at which the storage accident was set as they didn't show significant difference on the structural properties of whey proteins in the WPI powders (cf. 2.6.3., p60-63).

The study of heat induced aggregation properties was the same as described above (cf. 3.2.1., p68), but only considering reconstitution pH 5.8 and 6.6.

3.5.2. RESULTS AND DISCUSSION

It was seen in Chapter 2 that the number of storage accidents affected cumulatively structural changes, including aggregation and browning whose levels increased from the first storage accident. Moreover, it has been described above that the structural changes induced by storage had a significant impact on the heat-induced aggregation properties and especially through the products of degradation of lactosylated proteins (*e.g.* aggregates formed in the dry state).

Heat-induced aggregation of reconstituted WPI powders in milli-Q water was studied at pH 5.8 and 6.6 with the objective of studying the impact of storage accident on these properties.

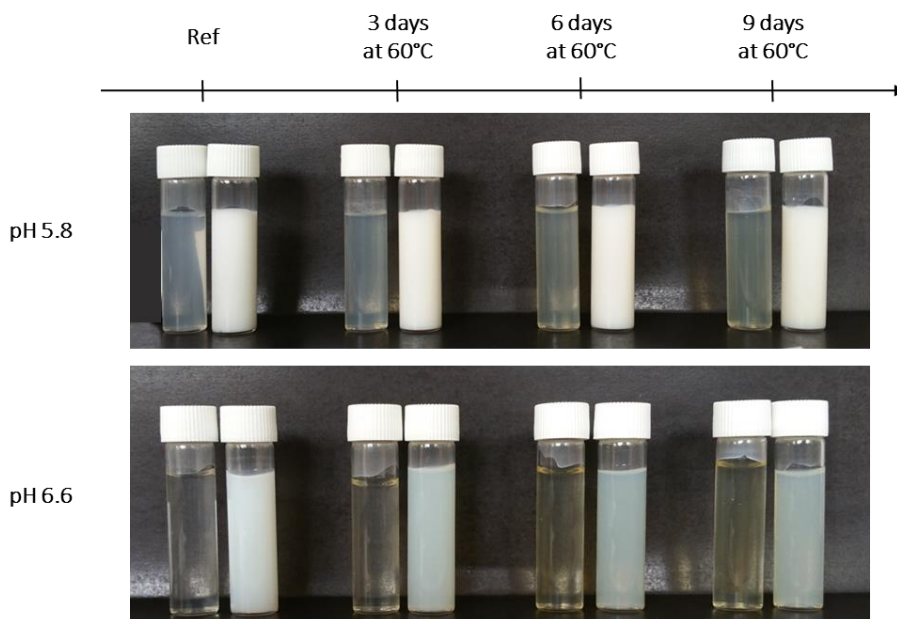


Figure 44: Pictures of samples arranged in pairs per conditions; unheated (left side) and heat treated (right side) solutions

Solutions reconstituted with fresh WPI powder (Ref) and WPI powders having undergone one to three storage accidents were analysed visually before and after heat treatment (**Figure 44**). In the Ref samples, the solution after heat treatment appeared to be milky and a little less turbid at pH 6.6 than pH 5.8. Heat treated solutions of powders subjected to storage accidents became less and less turbid supposing the heat-induced aggregates became smaller with the number of storage accident. In order to verify this hypothesis, the heat-induced aggregate size was studied through DLS.

Figure 45 shows the evolution of heat-induced aggregate size as a function of the number of storage accidents after one hour of static heating of WPI solutions (4% w/w) adjusted to a given pH. The aggregate size of Ref was around 385 nm and 128 nm at pH 5.8 and 6.6, respectively. The average size of heat-induced aggregate decreased as a function of the number of storage accident, regardless of pH. This decrease was significant from the first storage accident. At pH 5.8 and 6.6, size decreased to 186 nm and 77 nm after the third storage accident, respectively.

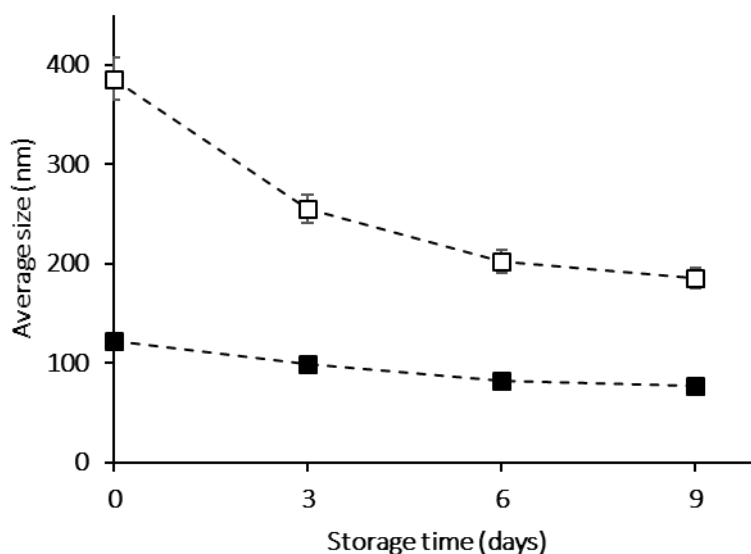


Figure 45: Average aggregate sizes as a function of the number of storage accidents at pH 5.8 (empty square) and pH 6.6 (full square)

A parallel can be drawn with the results and discussion presented in the paragraph 3.3.2. as the same phenomena were observed for powders subjected to a given number of storage accidents. The more the powder was subjected to storage accidents, the more the heat-induced aggregation properties were impacted. This involved storage-induced structural changes including an increase in the aggregation in the dry state with the number of storage accidents, leading to a reduction of the heat-induced aggregate size. Therefore, the scheme presented in **Figure 38** is also relevant for cumulative storage accident.

PART 6. CONCLUSIONS

In this study, changes in functional properties of WPI powders stored at representative storage conditions of the current modes of delivery were monitored. The results showed that WPI powders stored at temperatures below or equal to 20°C were relatively stable from a functional point of view for up to 15 months' storage even though the protein were lactosylated. On another hand, the results highlighted an evolution of heat-induced aggregation properties of WPI with moderate storage conditions (40°C) and storage accidents. Heat-induced aggregates appeared to decrease in size with storage time and also to change in their shapes. Regarding the foaming properties, a storage at 40°C did not induced significant changes in foam stability or the interfacial properties. At 60°C, the foaming stability was slightly improved for powders of a_w 0.36 after 6 months of storage.

The structural changes studied in the previous chapter could explain the variability of functional properties in WPI powder. At 40°C, the powder got browner and protein started to aggregate in the

dry state, but these evolutions did not impact the foaming properties whereas it was of a great influence on the heat-induced aggregation properties. Moreover, a high level of structural change in the WPI – storage at 60°C – resulted in a better foam stability.

Control of storage conditions is thus a critical step to ensure all the expected final properties of WPI powders. However, it is not as crucial as for other high protein powders (*e.g.* micellar casein), as only colour and heat induced aggregation properties were shown to be affected, whereas solubility, foaming and interfacial properties were not significantly changed. In order to preserve the entire native powder properties over a long period, one should recommend storage at temperatures below or equal to 20°C. The future perspective of this work will be to study and confirm the key role of lactose in the changes in protein structure and functional properties during storage of WPI powders.

CHAPTER 4. IS THERE A SOLUTION TO OVERCOME THESE STORAGE-RELATED ALTERATIONS?

In Chapter 2 and 3, we showed that even though WPI powders are made of at least 90% protein in a dry matter and assumed stable state, changes still occurred affecting the functional properties. The purpose of this chapter was to evaluate potential solutions to overcome the issues caused by the storage under moderate conditions. The presence of remaining lactose seemed to play a key role in WPI powder ageing. Thus, a part of this study aimed to validate the effect of lactose on the ageing-induced changes of WPI powders through a varying lactose content in WP powders from a very high to a very low content. This would eventually help us to evaluate the current stability of the WPI powders among storage and to determine the lactose content threshold at which the changes in WPI powders were minimized. In this scope, four powders differing in their lactose content were stored at 40°C and 60°C for up to 3 months and sampled periodically. Lactose was found to affect the structural and functional properties during powder ageing. In the presence of a higher lactose content, proteins were lactosylated more rapidly which were then aggregated in the dry state, causing a transition from soluble aggregate forms to insoluble ones. These results highlighted the great contribution of the Maillard reaction during ageing of WPI powders. Reducing lactose content in the WPI powders seemed to extend their stability among storage. A part of these has been published in *Journal of Food Engineering* (Paper⁵).

Moreover, the effect of particle size has been investigated, as the surface composition is known to be a parameter that strongly affects dairy powders properties. However, our results showed that it didn't modify the extent and kinetics of changes of WPI powders among storage. It can't therefore be proposed as a relevant tool to control WPI powder ageing.

The aims of this chapter were to:

- x study the effect of particle size on WPI powder's changes upon storage
- x validate the effect of lactose on the ageing-induced changes of WPI powders
- x define the lactose content threshold at which the WPI powders ageing is limited

⁵ Published as Norwood EA, Pezennec S, Burgain J, Briard-Bion V, Schuck P, Croguennec T, Jeantet R, Le Floch-Fouéré C. (2017). Crucial role of remaining lactose in whey protein isolate powders during storage. *Journal of Food Engineering*, 195, 206–216. doi:10.1016/j.jfoodeng.2016.10.010.

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PART 1. INTRODUCTION

Several researches have shown that storage conditions affect the structural and functional properties of dairy powder (Anema et al. 2006; Fitzpatrick, Barry, et al. 2007; Gazi and Huppertz 2015). However, the link between these two properties remains to date relatively indirect: namely, it would be inherent to the initial state of the powder, including composition and physical properties, and on reactions and migrations occurring upon storage. As an example, Schokker et al. (2011) demonstrated that increasing the concentration of non-micellar caseins in micellar caseins concentrate before drying reduced the solubility loss after storage. On the other hand, Gaiani et al. (2009) showed that native micellar caseins powder storage induced lipids release through pores onto the surface, which is believed to have repercussions on powder functional properties. Lipids were found to accumulate preferentially at the surface of the particle even when they were stored at 20°C. Lactose is also a component known to modify the properties of dairy powders during long-term storage (Guyomarc'h et al. 2000; Yazdanpanah and Langrish 2013). We have demonstrated in Chapter 2 that whey proteins were lactosylated among storage and that they seemed to engender aggregation in the dry state through the Maillard reaction. These changes were also supposed to affect whey proteins functionalities such as heat-induced aggregation, as shown in Chapter 3. Our results are in accordance with those of Guyomarc'h et al. (2000) who showed an increase in protein lactosylation during storage of a skim milk powder and dependant on the storage conditions. In addition, an insoluble material was observed upon reconstitution, probably due to largely modified proteins by advanced lactosylation. Moreover, the study of Fyfe et al. (2011) suggested that this lactose, which is located within the particle of a non-aged powder, would migrate to the particle surface of MPC80 up to twice the initial concentration after 90 days of storage at 25°C. This evolution has to be considered as the presence of lactose at the particle surface is known to strongly influence the agglomeration (Nijdam and Langrish 2006) or the wettability of milk powders (Gaiani et al. 2006). It is also believed that physical properties of the powder such as the particle size play a non-negligible role in powders ageing. Indeed, Fitzpatrick, Barry, et al. (2007) stated that surface composition of the powder particle is essential regarding the powder ageing capacities. The more the fat there is at the particle surface, the more the powder cakes. Ultimately, the smaller the particles, the larger the overall particle surface area: in such conditions, the changes in properties should be greater.

The present study is based on the storage of WP powders with a high protein content, namely WPC80 and WPI powders. Obtained from whey or milk microfiltration/ultrafiltration, and then spray dried, WPC80 and WPI powders are thus essentially composed of proteins (in the range of 80% and 95% of the dry matter, respectively), with a reduced lactose content and almost free from fat (Gulzar, Bouhallab, Jeantet, et al. 2011; Vignolles et al. 2007). The presence of the lactose, albeit at low

concentration (< 2%) in WPI powders, is believed to have a strong effect on the ingredient stability during storage thanks to our previous work (Norwood, Chevallier, et al. 2016; Norwood, Le Floch-Fouéré, et al. 2016). Due to its high content of proteins and its close-to-neutral pH, WPI powder gathers ideal physicochemical conditions for the occurrence of the Maillard reaction. Indeed, lactose, as a reducing sugar, reacts with free amino groups of proteins, peptides, and free amino acids and can thus negatively influence powder properties altering the powder quality (loss of lysine residue, development of a brown colour, protein cross-linking, ...) (Van Boekel 1998). The role of remaining lactose in WP powders will thus be studied so as to relate its presence to the changes observed in the previous chapters, and this with different lactose content.

Besides, the effect of the particle size is also considered in order to evaluate the ability of this parameter in better controlling storage induced changes in the WPI powder. In the literature, it is shown that powder particle surface plays an important role in powders' properties and ageing (Gaiani et al. 2010). Indeed, as the particle surface area per mass fraction unit increases as particle size decreases, it provides a greater interface area for reaction/interaction to take place, potentially resulting in more structural and functional changes. As a consequence, the local changes that occurred on the WPI powder surface observed previously (Paper⁵) would increase. Moreover, when particle size decreases, more contact points between particles are available for inter-particle interactions (Scholl and Schmidt 2014). Consequently, modulating the particle size of WPI powders may be an interesting tool to control their structural and functional changes during storage.

Therefore, it seems essential to study the influence of the initial composition and the physical properties of WPI powders, respectively and namely the lactose content and the particle size, on the powder physico-chemical changes and the ageing mechanisms at the molecular level.

PART 2. EXPERIMENTAL STRATEGY

All powders were obtained by spray drying a whey protein concentrate provided by a dairy company based in Brittany.

4.2.1. WPI POWDERS WITH DIFFERENT PARTICLE SIZE

Here, two complementary modalities assessing the impact of the drying method were studied (1. Particle size, 2. Production mode).

PRODUCTION

The non-granulated WPI powders with different particle size were all obtained from a protein concentrate (ultrafiltration / diafiltration of a skim milk microfiltrate) with a dry matter of 26.6%

(w/w). Three powders differing from their mean particle size (37, 62 and 74 μm for WPI_{B37}, WPI_{B62}, WPI_{B74}, respectively; **Table 6**) were obtained from a three-stage spray drying pilot, Bionov (GEA, Niro Atomizer, Saint-Quentin-en-Yvelines, France). In order to study the influence of the drying mode, a powder with a mean particle size of 37 μm (WPI_{M37}; **Table 6**) was obtained from a pilot-scale MOBILE MINOR™ spray-drier, MM-PSR type (GEA Niro A/S, Søborg, Denmark), equipped with a two fluid spray nozzle. The following parameters were set in order to vary the particle size of the produced powder (**Table 6**).

Table 6: Parameters set used to produce the four powders of different particle size

	WPI _{B37}	WPI _{B62}	WPI _{B74}	WPI _{M37}
Drying scale / pilot	Bionov	Bionov	Bionov	Minor
Nozzle	69/17	72/21	65/21	Orifice diameter: 0.80 mm
Pressure (bar)	280	140	70	-
Flow (L.h ⁻¹)	103	100	100	3.2
Inlet temperature (°C)	169	165	177	247
Outlet temperature (°C)	61.3	61	65	68.5
Mean particle size (d0.5; μm)	37	62	74	37

COMPOSITION

As the four different powders came from the same protein concentrate, their composition is very similar (**Table 7**).

Table 7: Composition of the four powders of different particle size

Dry matter (w/w %)	DM	≥ 93
Water activity	a _w	0.27
Nitrogen composition (% N × 6.38)	TN	≥ 88
	NPN	≥ 0.2
	NCN	≥ 74
Carbohydrates (w/w %)	Lactose	1.4

4.2.2. POWDERS WITH DIFFERENT LACTOSE CONTENT

PRODUCTION

The WP powders with different lactose content were all obtained from a protein concentrate (ultrafiltration / diafiltration of a skim milk microfiltrate) with a dry matter of ≥ 24% (w/w). Four

powders differing from their lactose content (16, 2.6, 1.2 and 0.1% for WPC, WPI^[+], WPI (control) and WPI^[-], respectively; **Table 8**) were obtained by spray drying. The WPI^[+] and WPI^[-] were obtained by diafiltration of the WPC and WPI liquid protein concentrates, respectively, in order to remove the lactose surplus. For this purpose, an ultrafiltration pilot (Carbosep TECH-SEP, Rhône-Poulenc, France) equipped with dual ceramic membranes (Tami Industries, Nyons, France) with a total membrane surface area of 13.3 m² and a molecular weight cut off of 10 kDa was used. The flow rate of the retentate was set at 40 L.h⁻¹. A diafiltration at 1.9 and 3 volumes was carried out on WPC and WPI liquid concentrate, respectively, with a salt solution to keep the soluble fraction of minerals constant. The composition of the salt solution was determined by quantifying the soluble fraction of minerals of a WPI (Control) reconstituted at the protein concentration of the retentate subjected to diafiltration. The WPI^[+] and WPI^[-] diafiltered retentates had a dry matter of 16.2 and 20.9% (w/w). After drying, the four powders were packed under air in airtight tins of 400 g capacity.

COMPOSITION

Table 8 provides the chemical composition of the four powders differing in their lactose content.

Table 8: Composition of the powders with different lactose content

		WPC	WPI ^[+]	WPI	WPI ^[-]
DM (w/w %)	DM	94.7	92.4	92.9	92.7
Water activity	a _w	0.33	0.36	0.25	0.27
Nitrogen composition (% N × 6.38)	TN	74.4	86.9	88.6	89.5
	NPN	0.85	0.28	0.19	0.21
	NCN	69.6	80.7	84.6	84.7
Mineral composition (w/w %)	Na ⁺	0.226	0.155	0.173	0.103
	K ⁺	0.818	0.508	0.270	0.179
	Ca ²⁺	0.297	0.260	0.414	0.389
	Mg ²⁺	0.063	0.045	0.055	0.05
	Cl ⁻	0.231	0.019	0.008	0.007
	PO ₄ ³⁻	0.31	0.127	0.025	0.05
	Citrate	0.491	0.119	0.033	0.034
Carbohydrates (w/w %)	Lactose	16.2	2.6	1.2	0.1

4.2.3. WP POWDERS STORAGE CONDITIONS

WP powder tins were stored in heat chambers at 4 (control powder), 40 and 60°C for periods of up to 3 months. After 1, 2 and 3 months of storage, 1 tin per temperature condition was opened in order to analyse the structural and functional properties of the powders.

4.2.4. CHEMICAL & PHYSICAL ANALYSIS

Chemical and physical analysis were the same as described in Chapter 2, Part 2. Additionally, the protein secondary structure in the dry state was monitored by FTIR measurements using a Bruker Tensor 27 instrument (Bruker Optics, Marne La Vallée, France). It was equipped with a mercury/cadmium telluride detector cooled with liquid nitrogen and used in the Attenuated Total Reflectance (ATR) mode using a single-reflection Ge crystal in a MIRacle accessory (Pike Technologies, Madison, WI, USA). Spectra were recorded in the region 850–4000 cm^{-1} at a 4 cm^{-1} resolution at room temperature taking the spectrum of blank diamond as the background. Spectra were corrected automatically for water vapour contributions using the dedicated routine of the OPUS 6.5 software (Bruker Optics, Marne La Vallée, France), and truncated to 1200–1900 cm^{-1} , which covers the typical amide bands, and normalised with the extended multiplicative scatter correction (Martens et al. 2003). Briefly, each spectrum is expressed as a linear combination of a reference spectrum and 'contaminant' spectra. The linear combination coefficients are computed by linear regression, and spectra are corrected for 'contaminations' weighed by the corresponding coefficients. The terms used in the regression were the mean spectrum (reference spectrum), the liquid water spectrum and linear and quadratic functions of the wave number ('contaminant' spectra). This procedure allows correcting each spectrum for variability in overall intensity, variability due to the liquid water signal (hence, to total protein concentration) and to the baseline changes.

A principal component analysis (PCA) was performed on spectra from fresh and stored WP powders. The general principles of this multivariate statistical technique have already been described in the literature (Abdi and Williams 2010; Jolliffe 2014; Wold et al. 1987). PCA is used to summarise a data set consisting in a numerical (continuous) *variables* \times *individuals* table. Based on the correlations between variables in the table, PCA computes an ordered, orthogonal set of 'new' variables, called principal components, which are linear combinations of the n original variables, in such a way that whatever m , $1 \leq m \leq n$, the scores of individuals on the first m principal components have the highest possible total variance, among all possible sets of linear combinations. In other words, PCA enables the projection of an n -dimensional data set onto a new p -dimensional coordinate system ($p < n$), while keeping the most part of the original 'information' content. The output of PCA therefore are

i) coefficients of the linear combination for every principal component; in the general case, correlations between the principal components and original variables are represented graphically on a correlation circle, and ii) the scores (or coordinates) of individuals in the new coordinate system, which are used to plot individuals similarity maps. In the case of spectral data, the original variables are the absorbances or intensities measured at each wavelength of wavenumber (variable i is the set of values measured for all spectra at the i -th wavelength). All the variables are expressed using the same unit, and differences in variance do have a physical meaning: scaling to unit variance is therefore not applied to spectral data. Being linear combinations of the original variables, principal components are homologous to spectra, and can be represented as such. Statistical analysis were done using the R package (R 2.9.2. Foundation for Statistical Computing) as an open source statistical computing environment.

4.2.5. HEAT-INDUCED AGGREGATION PROPERTIES

Heat treatment after rehydration and heat-induced aggregation analysis were the same as described in Chapter 3, Part 2. The heat treatment after rehydration was performed only at pH 6.6.

PART 3. STUDY OF THE EFFECT OF POWDERS PARTICLES SIZE UPON AGEING

4.3.1. DENATURATION STATE

Denaturation state analysis of WPI proteins after three months storage at 40°C and 60°C were studied using reverse phase HPLC and are shown in **Figure 46**, A and B, respectively.

The reduction in the area of the native form peaks represented in grey in **Figure 46**, A and B, and the modification of the elution time showed that after 3 months of storage non-native proteins are formed for all powders at both 40 and 60°C. However, no difference in the type of changes or in the ageing kinetics was observed between the four powders.

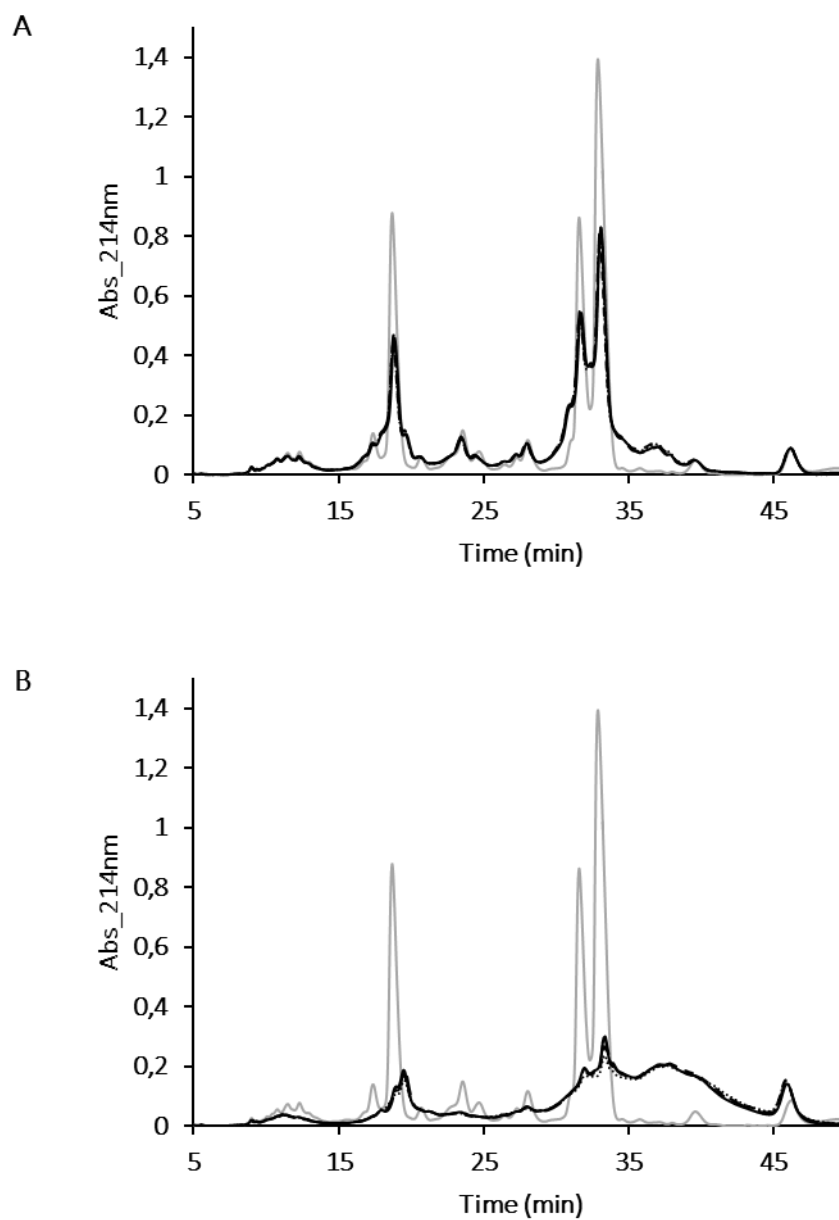


Figure 46: RP-Phase profiles of WPI powders at t_0 (grey solid line) and WPI_{B37} (black solid line), WPI_{B62} (black long-dashed line triangle), WPI_{B74} (black dashed line) and WPI_{M37} (black dotted line) stored 3 months at A) 40°C and at B) 60°C

4.3.2. PROTEIN LACTOSYLATION

Figure 47 shows the level of lactosylation of each powder during the first three months of storage.

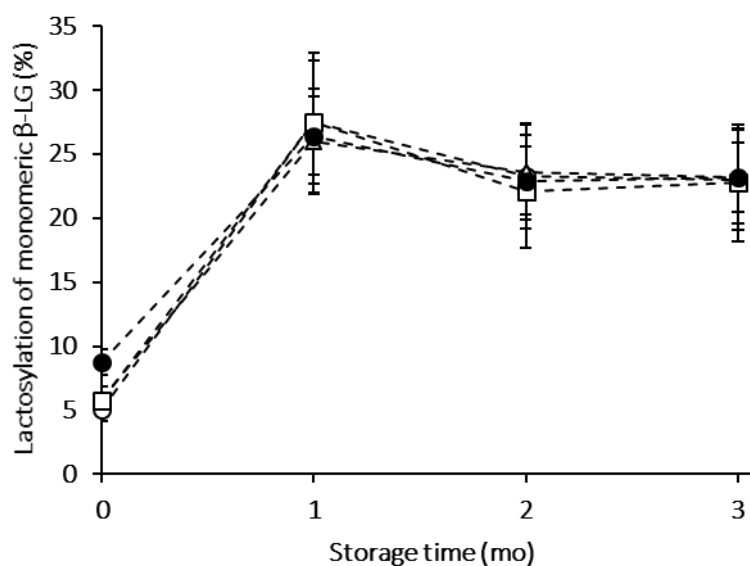


Figure 47: Evolution of β -LG lactosylation for WPI_{B37} (circle), WPI_{B62} (triangle), WPI_{B74} (square) and WPI_{M37} (solid circle) stored at 40°C as a function of storage time

The level of lactosylation reached values around 26.5% and 23% after 1 month and both 2 and 3 months respectively whatever the size of the particle. Consequently, all the points in **Figure 47** were superimposed or in the experimental error range, showing that the particle size did not have a significant effect regarding the level of lactosylation.

4.3.3. AGGREGATION IN THE DRY STATE

As in Chapter 2, the amount of aggregated proteins in the four powders stored at 40°C (**Figure 48, A**) and at 60°C (**Figure 48, B**) was determined using GPC-HPLC.

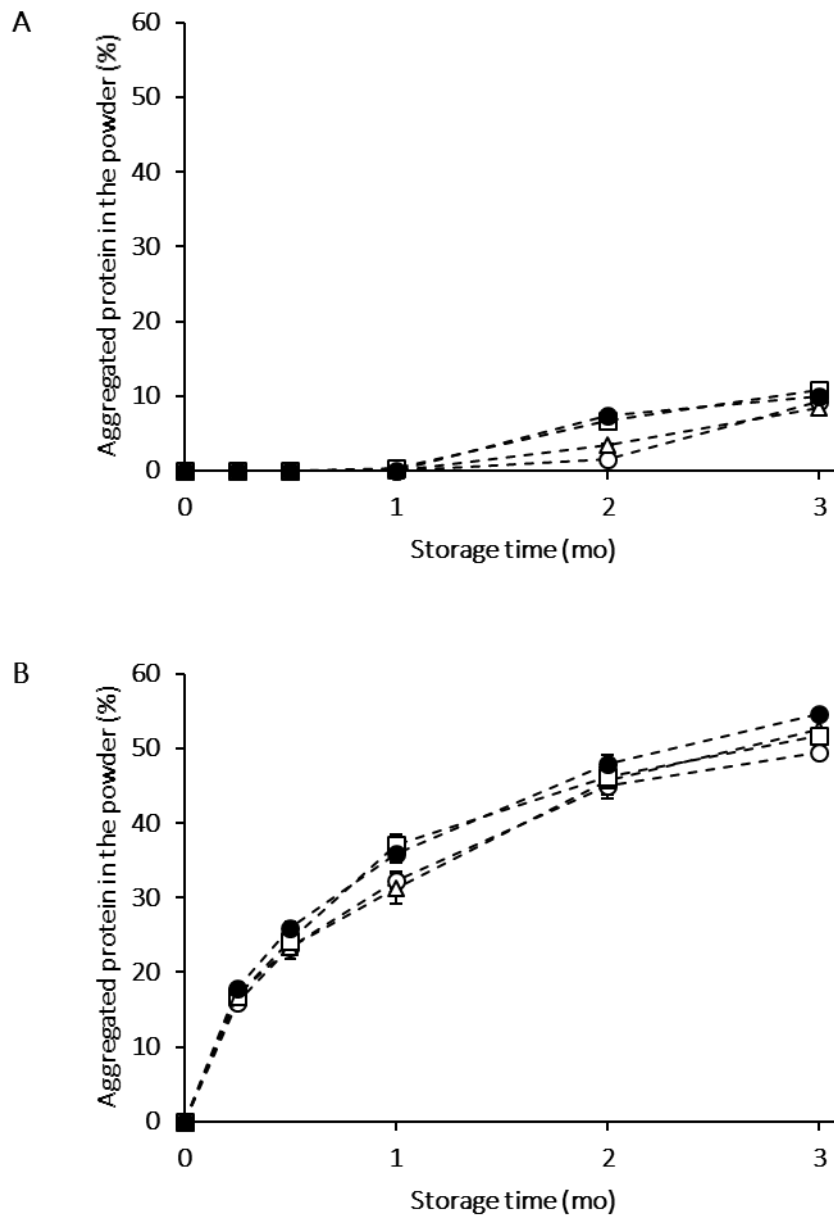


Figure 48: Amount of aggregated proteins for WPI_{B37} (circle), WPI_{B62} (triangle), WPI_{B74} (square) and WPI_{M37} (solid circle) stored at A) 40°C and B) 60°C as a function of storage time

The amount of aggregated proteins reached around 10% and 50% after 3 months storage at 40°C and 60°C, respectively, for all the powders. Thus, the results did not show significant changes between the four powders indicating that all the powders were ageing at the same rate regardless of the particle size.

4.3.4. HEAT-INDUCED AGGREGATION PROPERTIES

The aggregate sizes of all controls were around 395 ± 10 nm, 379 ± 39 nm, 378 ± 20 nm and 374 ± 23 nm for WPI_{B37}, WPI_{B62}, WPI_{B74} and WPI_{M37}, respectively. Heat-induced aggregation properties did not

differ from one powder to another after 2 months of storage. Heat-induced aggregate size decreased as a function of storage time regardless of the particle size. For WPI_{B37}, WPI_{B62}, WPI_{B74} and WPI_{M37} powders stored at 40°C, particle size decreased to 281 ± 7 nm, 258 ± 27 nm, 258 ± 14 nm and 254 ± 16 nm after 2 months, respectively. At 60°C, size decreased to 119 ± 3 nm, 129 ± 13 nm, 111 ± 6 and 108 ± 7 nm after 2 months for WPI_{B37}, WPI_{B62}, WPI_{B74} and WPI_{M37}, respectively.

The similar evolution of heat-induced aggregation properties for the four powders was not surprising. Indeed, all powders had very alike structural evolutions in terms of protein lactosylation and aggregation in the dry state, which are believed to play a significant role in the modifications of heat-induced aggregation properties.

4.3.5. DISCUSSION

As the study of Burgain et al. (2016) showed very interesting results by monitoring the changes on the particle surface, our first assumption was that changes were located on the particle surface making the particle size an important parameter to take into account. Indeed, particle size rules the extent of the reaction and/or interactions that may occur at the particle surface upon storage. However the present study refuted this hypothesis as no structural and no functional differences were observed for the four powders differing in their particle size upon storage. This could result of whether the Maillard reaction starting in the core of the particle and not on the particle surface or less likely of the component distribution within the particle.

Considering dairy powders particle composition, results differ from one powder type to another. Given the components properties (*e.g.* surface activity), their distribution into the particle occurs during the drying process resulting in heterogeneity between the particle surface concentration and the core concentration (Briggs 1994; (E. H.-J. Kim et al. 2002; Nijdam and Langrish 2006). Indeed, both protein and fat accumulate at the particle surface during spraying and drying, whereas lactose content is reported to be higher in the core of the particle (Nijdam and Langrish 2006). Proteins and moreover fat being surface active components adsorb preferentially to the droplet air / water interface during spray drying and thus appeared on the powder surface after drying (Fäldt 1995). Therefore, all dairy powders do not have the same component distribution in the particle due to variation in their bulk composition. As an example, powder particles of WMP and SMP have not the same surface composition: WMP is mainly covered with fat followed by lactose then proteins whereas SMP is mainly covered with proteins and a little lactose (Murrieta-Pazos et al. 2012). For high protein dairy powders, a different trend is observed as fat is only present at trace levels (micellar casein powders) or not present at all (whey protein isolate powders) due to skimming and filtration operations. Regarding

native micellar caseins, lipids and proteins are mainly located at the particle surface whereas lactose is located in the core of the particle for (Gaiani et al. 2010).

Our study is based on WPI powders, which is composed of at least 90% of proteins, small amounts of lactose and minerals and no fat. This results in a limited component segregation due to a low amount of lactose and minerals beside the whey proteins content. Thus protein should be located at a higher amount at the surface particle, and conversely lactose in the core of the particle. Our results showed that for a given bulk composition, lactosylation and changes occurring during ageing weren't affected by particle size and in turn didn't affect the overall particle surface area. This could be due to limiting lactose concentration at the particle surface to induce Maillard reaction. If so, the Maillard reaction would likely start in the core of the particle and then progress through a series of reactions toward the particle surface. This made it visible and quantifiable by ToF-SIMS afterwards (cf. 2.3.4., p46-47). In this view, the particle surface would not be the starting point of the Maillard reaction but only an indicator of its extent. This is in agreement with the same rates of structural and functional changes observed for the four powders differing in their particle size. Another hypothesis would be that the component distribution is more homogeneous in the particle due to concentrate high viscosity and spray drying conditions. It is known that high feed solid content and spray drying kinetics greatly affect the component distribution within the particle, this latter being reduced at high viscosity and high drying kinetics (E. H.-J. Kim et al. 2009b). The viscosity of our whey concentrate before drying was high thus decreasing the mobility of the components and resulting in a rather homogenous composition of our particle. This would lead to a relative homogenous spread of the Maillard reaction in the whole particle and as a consequence, explain why the four powders, although differing in their particle size, showed no difference upon ageing.

The results also showed that the WPI production mode (Minor vs Bionov) had no effect on powder ageing as WPI_{M37} and WPI_{B37} aged at the same rate. This means that there is no influence between the spray drying modes regarding the component distribution in the particle. This result is of interest for further powder research as spray drying at lower scale (Minor) is easier to implement.

PART 4. STUDY OF THE CRUCIAL ROLE OF REMAINING LACTOSE MOLECULES ON WHEY PROTEINS POWDER AGEING

4.4.1. COLOUR OF STORED POWDERS

The development of a brown colour is one of the characteristic changes of powders ageing caused by the Maillard reaction. According to a visual analysis (**Figure 49**), the colour development is more flagrant for powders stored at 60°C than at 40°C.

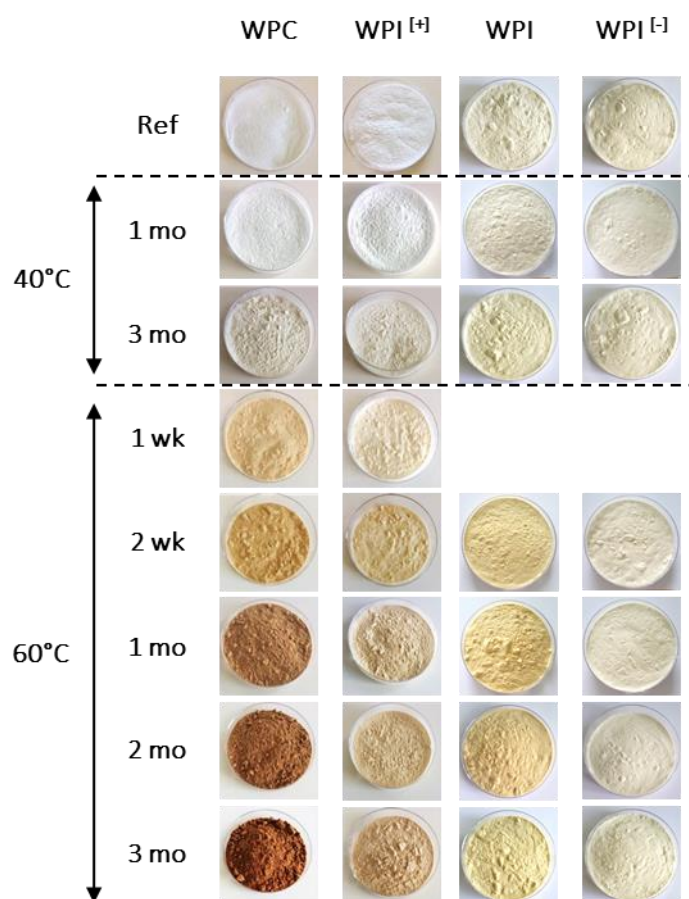


Figure 49: Images of Ref powder and WPC, WPI^[+], WPI and WPI^[-] stored for 1 and 3 months at 40°C; and stored for 1 week to 3 months at 60°C

No significant evolution was observed after 1 month at 40°C. After 3 months, WPC, WPI^[+] and WPI seemed to have a little more browning increase compared to WPI^[-]. For a storage at 60°C, the WPC powder developed a brown colour only after one week, which increased drastically among storage time reaching a dark brown colour. In the meantime, the WPC powder evolved in part as blocks, a result from caking possibly due to the progress of the Maillard reaction. This phenomenon was less obvious for the 3 other powders. WPI^[+] and WPI powders appeared yellower compared to the Ref during storage but not that different between each other. Finally, the colour of the WPI^[-] powder didn't seem to evolve at all among 3 months of storage.

In order to validate these observations, the Browning Index has been calculated for all powder samples stored at 40°C and 60°C (Figure 50, A and B, respectively).

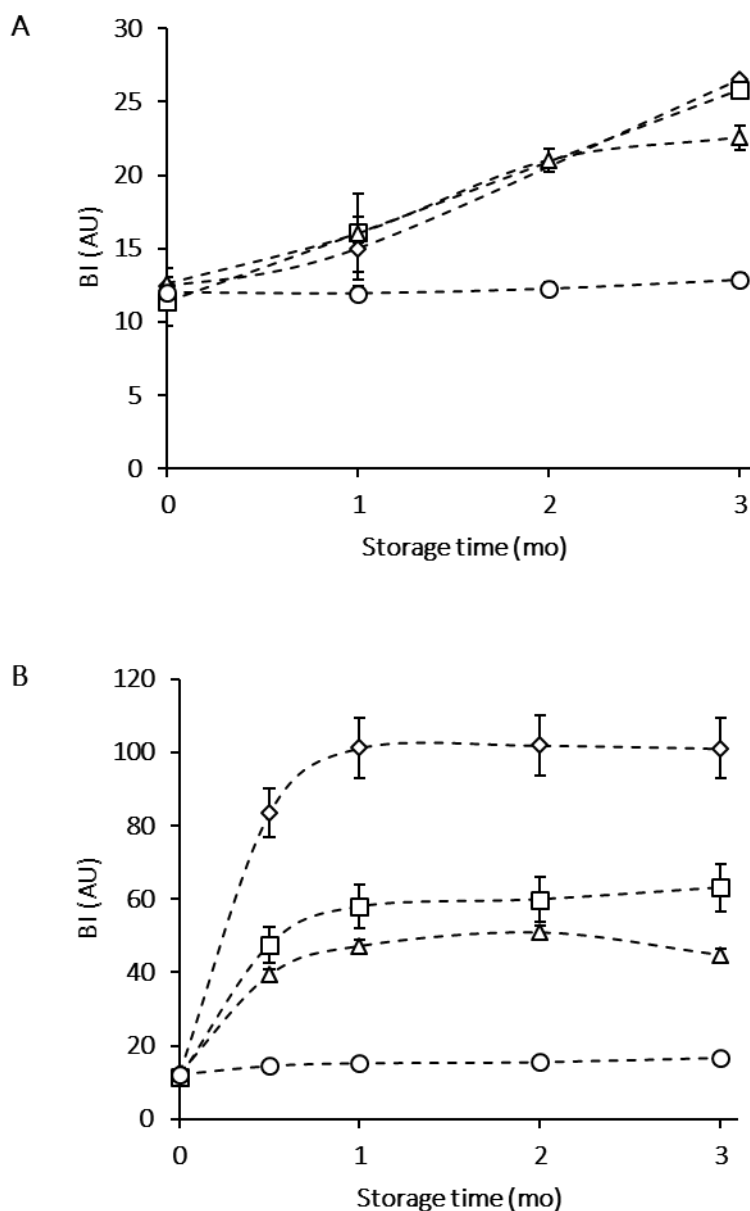


Figure 50: Evolution of Browning Index for WPC (diamond), WPI⁽⁺⁾ (square), WPI (triangle) and WPI⁽⁻⁾ (circle) powders stored at A) 40°C and B) 60°C as a function of storage time

The calculation of the Browning Index confirmed our previous observations. At 40°C (**Figure 50, A**), the BI of WPC, WPI⁽⁺⁾ and WPI powders evolved at a same rate reaching a value around 25 (AU) after 3 months of storage. At 60°C, the BI increased faster reaching different values for WPC, WPI⁽⁺⁾ and WPI powders (**Figure 50, B**). WPC powder had the higher BI followed by the WPI⁽⁺⁾ and WPI powders with values of 100, 63 and 45, respectively. The colour of the WPI⁽⁻⁾ powder was unchanged during the same storage period at 40°C and 60°C and remained stable around 16.8.

The storage temperature and lactose content are preponderant factors affecting powder colour. Indeed, the higher the storage temperature, the browner the powders. Furthermore, the lactose content has a significant effect as soon as the amount is as low as 1.2% in the powder.

4.4.2. DENATURATION PROFILES OF POWDERS WITH DIFFERENT LACTOSE CONTENT

RP-HPLC profiles of 3 months stored powders of WPC, WPI^[+], WPI and WPI^[-] are compared with the one of WPI powder at t_0 (Figure 51).

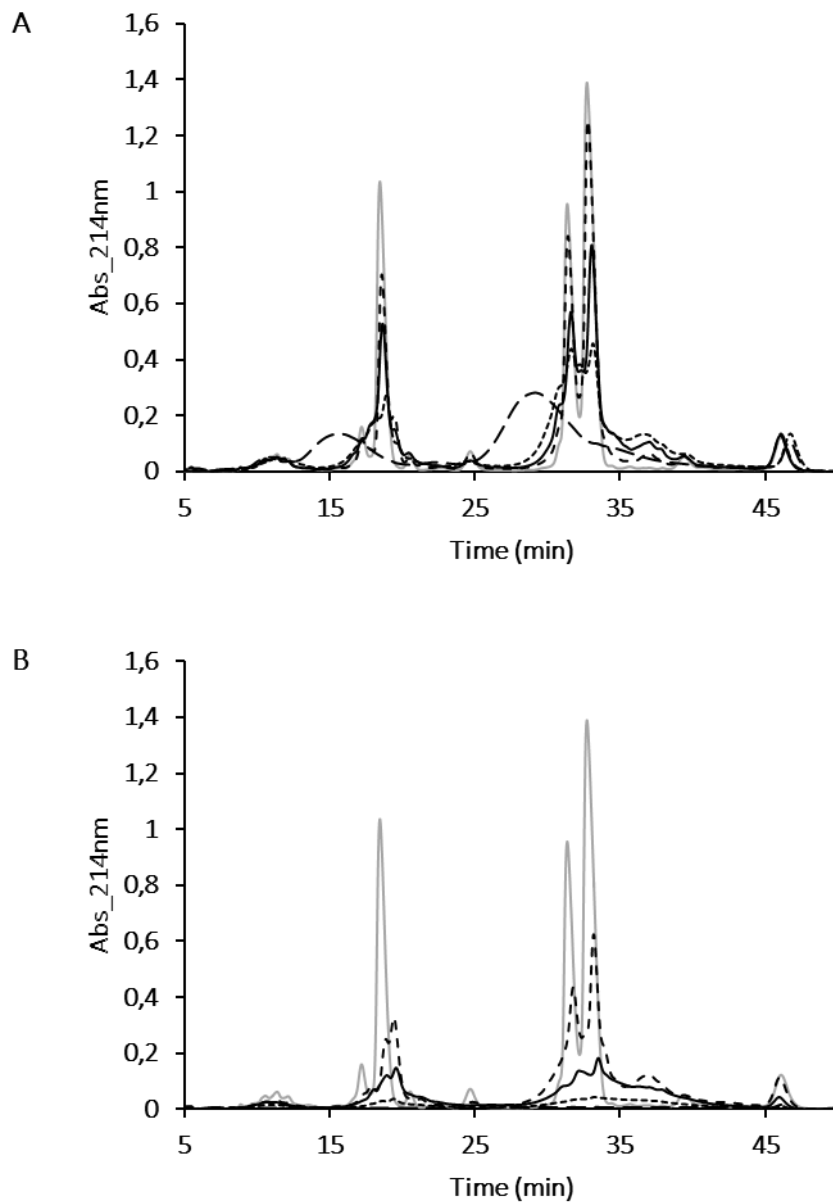


Figure 51: RP-HPLC profiles of WPI powders at t_0 (grey, solid line) and WPC (black, long-dashed line), WPI^[+] (black, dotted line), WPI (black, solid line) and WPI^[-] (black, dashed line) stored 3 months at A) 40°C and B) 60°C

At t_0 , the native proteins peaks of α -LA and the two variants β -LG B and A were eluted at 18 min, 31 and 32 minutes with the gradients used in this study, respectively. Any difference with the RP-HPLC profile of the WPI at t_0 is indicative of the denaturation and/or aggregation of the native proteins (α -LA and β -LG) during storage. RP-HPLC profiles of WPC, WPI^[+], WPI and WPI^[-] samples stored 3 months at 40°C showed that the reduction the native peaks forms was correlated to the lactose content in the powders (**Figure 51, A**). Furthermore, the presence of molecular species with shorter elution times in the RP-HPLC of WPC indicated the presence of more hydrophilic species. The 3 other powders only showed a shoulder, that could indicate that a lesser amount of hydrophilic species was formed. This increase in hydrophilicity is likely to be due to protein lactosylation during storage (Mulsow et al. 2008).

After powder storage at 60°C, the RP-HPLC profiles exhibit more drastic changes (**Figure 51, B**). After 3 months of storage at 60°C, the elution profile of WPC was completely flat meaning that the proteins did not go through the column. Indeed, during powders reconstitution in milli-Q water prior to the RP-HPLC analysis, an insoluble fraction was formed from two weeks of storage at 60°C. The RP-HPLC profile of WPI^[+] was very similar to the one of WPC. In contrast, the RP-HPLC profile of WPI^[-] still showed a large amount of native proteins after 3 months of storage at 60°C. However, it is worth noting that a shoulder appeared on the right hand side of α -LA peak (**Figure 51, B**). Similar modification has been reported to be typical of α -LA cyclisation by Gulzar et al. (2013). This was probably due to a lower competitiveness between reactions occurring in the powder with low lactose content among ageing resulting in a higher level of α -LA cyclisation. As expected, WPI has intermediate behaviour between WPI^[+] and WPI^[-].

In the presence of a higher lactose content, proteins were rapidly affected for storage at 40°C, including protein denaturation/aggregation and the formation of more hydrophilic forms. For more severe storage conditions (60°C), the rapid formation of an insoluble fraction was observed. This looks like coming from an advanced stage of the Maillard reaction.

4.4.3. PROTEIN LACTOSYLATION

The level of protein lactosylation was determined using liquid chromatography combined with mass spectrometry. The freshly prepared powders had already some lactosylated proteins that probably appeared during spray drying and prior processing steps. As indicated in Chapter 2, we focused on the lactosylation of β -IG as the detection of α -LA lactosylation became difficult with storage time.

The level of protein lactosylation in fresh powders was of 16.5%, 12.3%, 3.8% and 2.5% for WPC, WPI^[+], WPI and WPI^[-], respectively. The fractions of lactosylated E-LG among storage are shown in **Figure 52**.

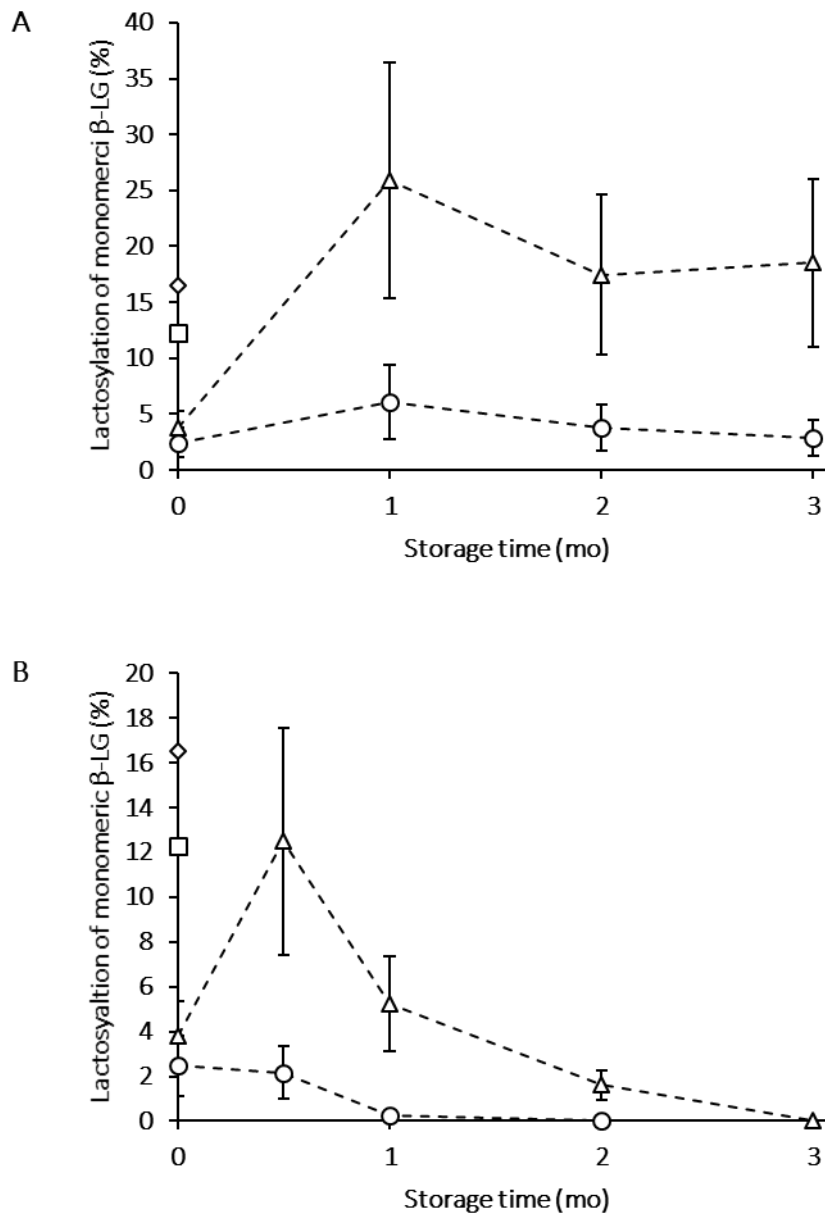


Figure 52: Evolution of lactosylation of β -LG (in % in regards to the native form) for WPI (triangle) and WPI^[-] (circle) powders stored at A) 40°C and B) 60°C as a function of storage time. The fraction of lactosylated β -LG in WPC and WPI^[+] powders at t_0 is also indicated

For the WPC and WPI^[+] samples, the level of protein lactosylation was undetectable after only a week of storage. This means that the Maillard reaction progressed rapidly in these powders. Regarding the

WPI powders stored at 40°C (**Figure 52, A**), a maximum fraction of lactosylated E-LG of 26% was reached after one month of storage and then remained stable in accordance with the results presented in Chapter 2. At 60°C, this fraction reached 12% after 15 days of storage and then decreased to no longer be detectable after 3 months of storage. A different evolution was observed for the WPI^[-] powder. The proportion of lactosylated proteins remained almost stable over 3 months of storage at 40°C (2 < 6%) while at 60°C, it rapidly decreased from the first month of storage. It can be assumed that lactose molecules reacted with proteins and due to their low amount in the powder, they rapidly became the limiting factor. The lactosylated proteins were then consumed resulting in a decrease in their proportion among storage at 60°C. Indeed, 0.1% w/w of lactose in the WPI^[-] corresponds to a lactose/protein molar ratio of 0.06. This means that a theoretical maximum of 6% of lactosylated proteins could be formed in this powder, which is in agreement with the results showed in **Figure 52**.

4.4.4. CONFORMATIONAL CHANGES IN PROTEIN STRUCTURE

Protein structures of the aged and non-aged WPC, WPI^[+], WPI and WPI^[-] powders were studied by FTIR and the spectra region between 1200 and 1900 cm⁻¹ was analysed by PCA. The reviewing of the latter region is relevant for this aim as it gathers the Amide I, II and III bands (Barth 2007; Dufour and Robert 2000). The Amide I band is located around 1650 cm⁻¹ and arises mainly from the C=O stretching vibration. The Amide II band is located around 1550 cm⁻¹ and comes from the combination of the NH in plane bend and the CN stretching vibration. The Amide III band, located around 1300 cm⁻¹, results mainly from the NH bending.

The PCA gathers 44 individuals differing in their lactose content (WPC, WPI^[+], WPI, WPI^[-]), their storage time and their storage temperature. The principal components are represented as spectral patterns. Besides, the factorial map gathers the individuals' projection following their similarities and the quality of their projection is defined by their size. The poorer the projection, the smaller the symbols will be.

According to FTIR analysis, proteins in the four powders showed only slight modifications of the secondary structure, as all spectra were more or less superimposed on each other (**Figure 53**).

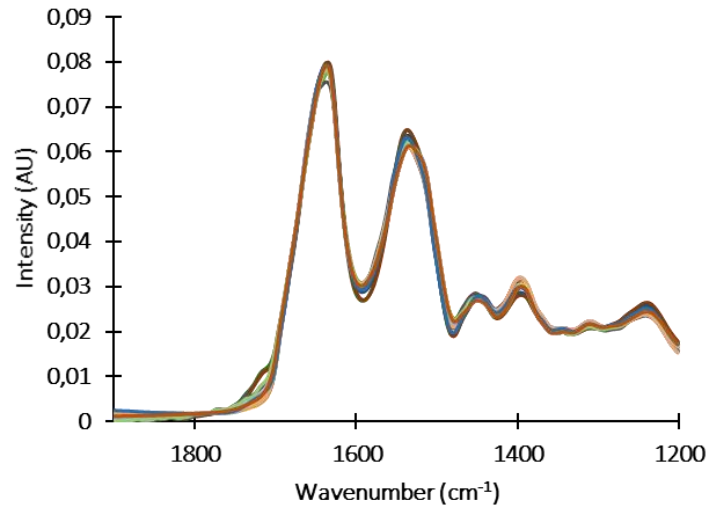


Figure 53: All spectra of the WPC, WPI^[+], WPI and WPI^[-] powders as Ref or stored at 40°C and 60°C up to 3 months

The factorial map gathers about 83% of the total inertia (**Figure 54**; projection of individuals), including PC1 that explains half of the total variability. This result is satisfying considering the number of individuals and variables.

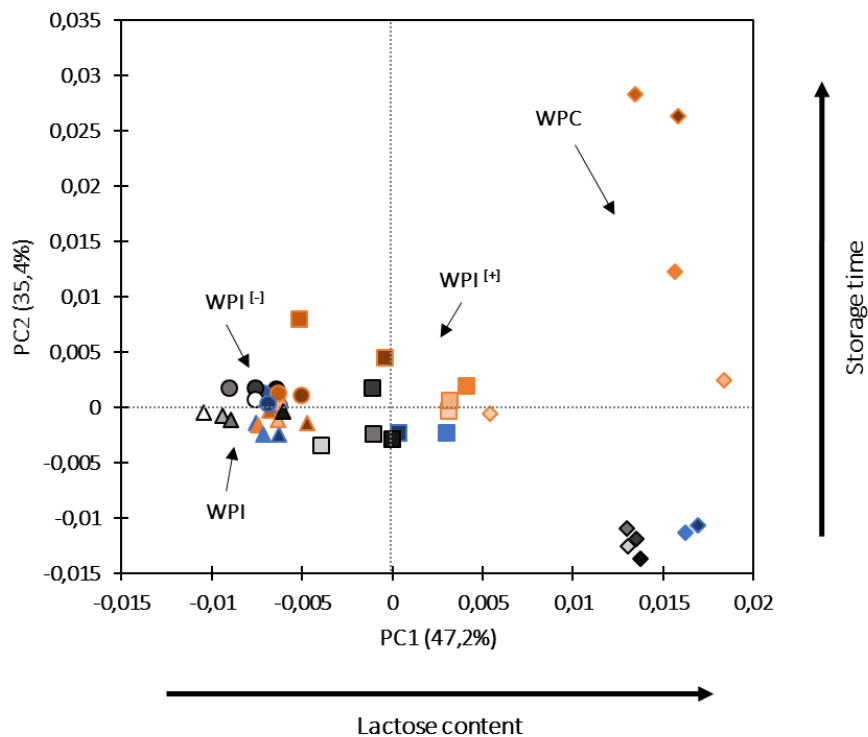


Figure 54: PC1–PC2 factorial map of the samples characterised by FTIR analysis in the region of amide I and II bands of WPC (diamond), WPI^[+] (square), WPI (triangle) and WPI^[-] (circle) powders for Ref (black symbols) and stored at 40°C (blue symbols) and 60°C (orange symbols); the darker the inner colour, the longer the storage time

Figure 54 shows the individuals' projection on the factorial map following their similarities. It includes the proportion of variance for PC1 and PC2 that are 47.2% and 35.4%, respectively, the individuals' projection quality and the scores of each samples on PC1 and PC2.

On the factorial map, the individuals' projection are satisfying. Moreover, it appears that the scores on PC1 seemed to increase as the lactose content increased. WPI^[1] and WPI had negative scores on PC1 whereas WPC had high positive scores. PC1 was thus independent of powders storage time. The differences in PC1 were not due to the contribution of free lactose in the powders as the IR signatures were out of the 1900 cm⁻¹ to 1200 cm⁻¹ wavenumbers range (W.-Q. Wang et al. 2013). The scores on PC2 seemed to increase with the increasing storage temperature and time.

Figure 55 shows the mean spectrum and the spectral patterns of PC1 and PC2. They indicate, by their intensity, the wavenumber at which the individuals differ. Spectral pattern of PC1 (or PC2) can be interpreted in two ways: i) peaks are correlated (negative peak at 1501 cm⁻¹ is correlated to the positive peak at 1547 cm⁻¹) and ii) for a positive peak on PC1 (or PC2), the higher the scores on PC1 (or PC2), the higher the peak intensity will be.

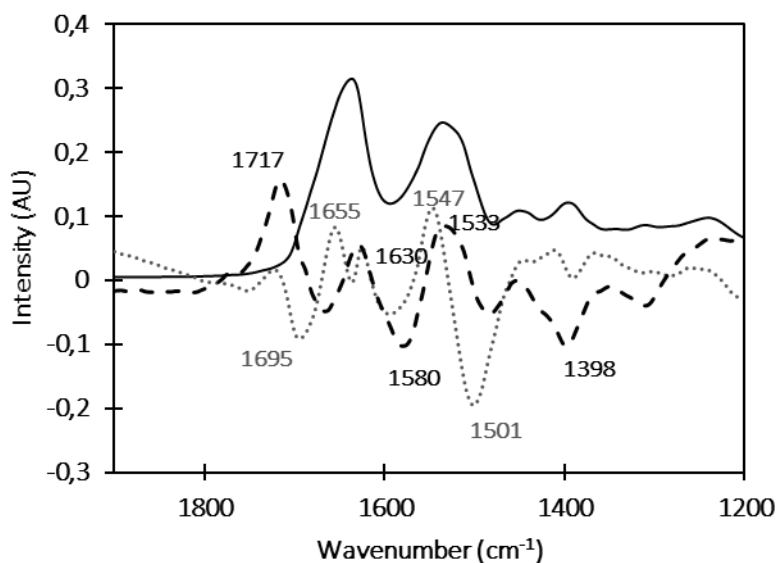


Figure 55: The mean spectrum (black) and the spectral pattern of PC1 (dotted line) and PC2 (dashed line) weighed by their standard deviations; some particular wavelengths on PC1 and PC2 are indicated on the curves

Regarding the spectral pattern of PC1, a positive peak at 1547 cm⁻¹ and a negative peak at 1501 cm⁻¹ reflect a great shift of the Amide II band towards higher frequencies for powders with a higher lactose content. This may be due to the presence of additional hydrogen bonding, which increased the bending vibrations frequency of H-C-N by producing an additional restoring force (Barth 2007).

Moreover, a positive peak was observed at 1655 cm^{-1} , which is attributed to a greater content of α -helices (Oboroceanu et al. 2010). This is in agreement with the shift previously mentioned in samples with high PC1 scores. A negative peak on PC1 at 1695 cm^{-1} was assigned to the protonation of the Arginine amino acid as reported by Barth (2007), reflecting a decrease in pH. The spectral pattern of PC2 was characterized by 3 positive peaks at 1717 , 1533 and 1630 cm^{-1} , and 2 negative peaks at 1580 and 1398 cm^{-1} . It is stated in the literature that the protonation of the carboxylate group results in a displacement of the absorption band around 1715 cm^{-1} corresponding to the stretching vibration of C=O (Dufour and Robert 2000) and a concomitant decrease of the bands 1580 cm^{-1} and 1402 cm^{-1} corresponding to the asymmetric and symmetric stretching vibration of the COO^- group, respectively (Dufour and Robert 2000; Barth 2007). Hence, the most discriminating positive peak on PC2 at 1717 cm^{-1} and the two negative peaks at 1580 cm^{-1} and 1398 cm^{-1} could be linked to the acidification of the medium due to Maillard reaction (Beck et al. 1990; Martins et al. 2000). Previous studies on whey protein denaturation assigned the 1630 cm^{-1} band to a loss of the secondary structure of the protein (Oboroceanu et al. 2010) and also to antiparallel β -sheets (Dufour and Robert 2000; Boye et al. 1996; Kher et al. 2007) that are strongly bonded (Kehoe et al. 2008). Moreover, the last positive peak of PC2 at 1533 cm^{-1} also refers to β -sheets (Dufour and Robert 2000). This means that the samples with higher PC2 scores were more aggregated.

In summary, the presence of lactose seems to have a stabilizing effect on the protein structure through a higher content of α -helices and β -sheets as PC1 was correlated with the bulk lactose content. From previous studies on the role of co-solute during drying or whey protein heat-induced aggregation, the lactose appeared to act as a hydrophilic protective agent toward whey proteins (Baier and McClements 2001; Chanasattru et al. 2007; Sun et al. 2011; Vignolles et al. 2009; W. Wang and Zhong 2014). The presence of sugar would cause protein molecules to fold more tightly with higher level of H-bonding (Baier and McClements 2001; Parsegian et al. 1995) in accordance with our results. On the other hand, a 3-order interaction between lactose content, storage time and temperature is suggested on PC2. The higher this interaction, the lower the pH and the higher the β -sheets content. This is associated to the extent of the Maillard reaction generating acid molecules (Beck et al. 1990; Martins et al. 2000) and reactive molecules for protein crosslinking (Norwood, Chevallier, et al. 2016).

However, the minor structural modifications of WPI^[1] seemed to be hidden by the WPC influence on the spectral PCA. Thus, it was chosen to perform another spectral PCA on the sole WPI and WPI^[1] powders so as to evaluate the effect of reducing the level of lactose on the 3-months ageing of WPI powders (Figure 56, Figure 57 and Figure 58).

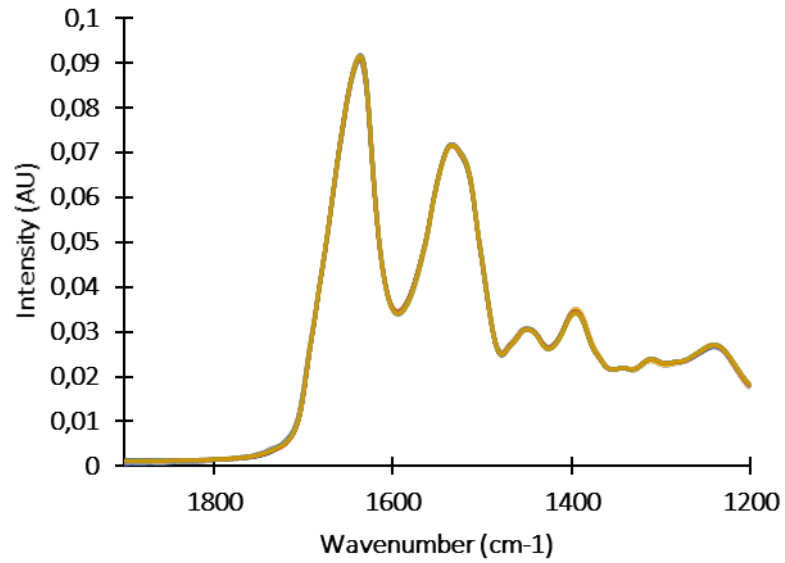


Figure 56: All spectra of the WPI and WPI^[L] powders as Ref or stored at 40°C and 60°C up to 3 months

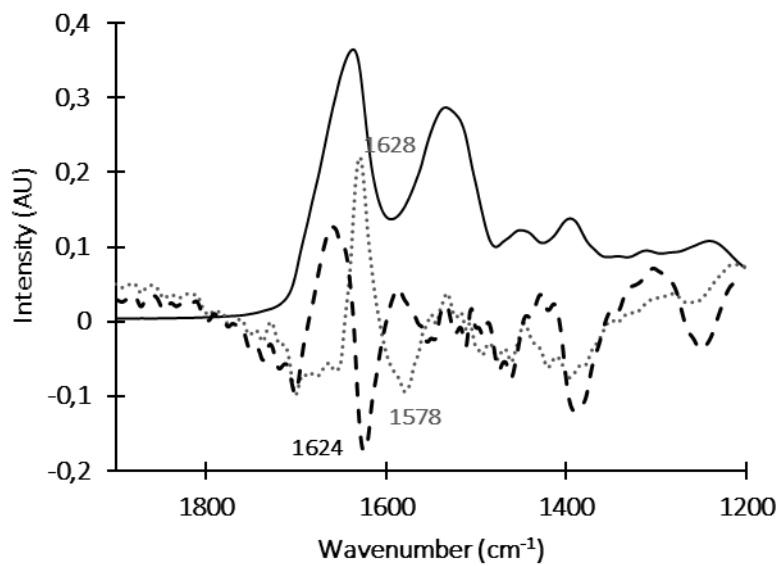


Figure 57: The mean spectrum (black) and the spectral pattern of PC1 (dotted line) and PC2 (dashed line) weighed by their standard deviations; some particular wavelengths on PC1 and PC2 are indicated on the curves

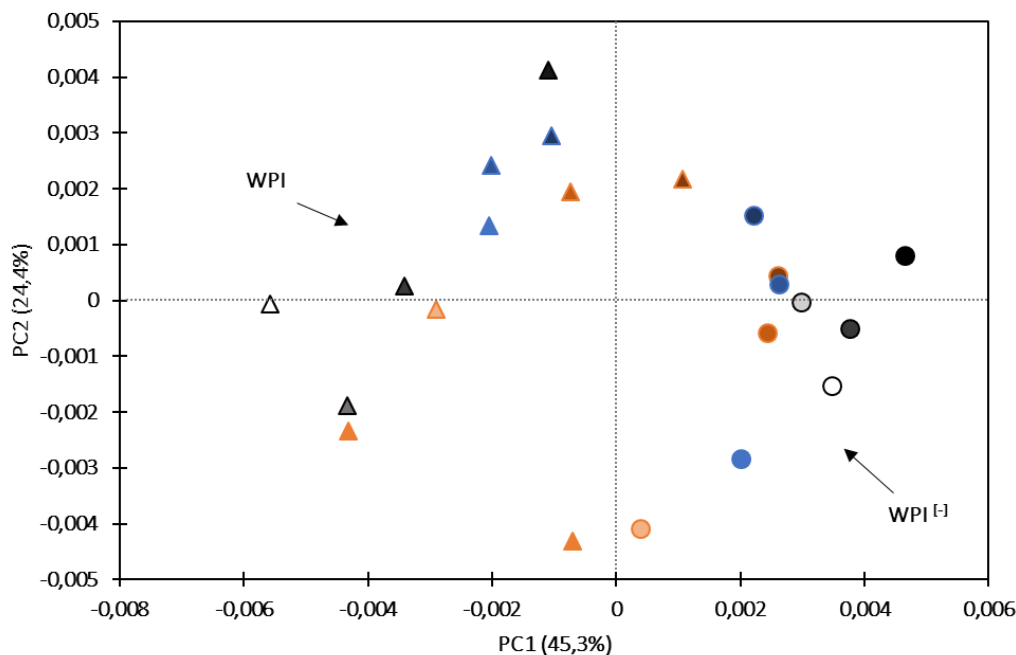


Figure 58: PC1–PC2 factorial map of the samples characterised by FTIR analysis in the region of amide I and II bands of WPI (triangle) and WPI^[-] (circle) powders for Ref (black symbols) and stored at 40°C (blue symbols) and 60°C (orange symbols); the darker the inner colour, the longer the storage time

The proportion of variance of PC1 and PC2 are 45.3% and 24.4%, respectively. From this analysis, it is worth noting that the differences between samples consisted in only very minor modifications of absorbance as all the spectra appeared superimposed (**Figure 56**). Graphically, the mixing up of all samples makes only PC1 relevant to discriminate WPI^[-] and WPI powders. On the factorial map (**Figure 58**), it appeared that the scores on PC1 slightly increased as the lactose content in the powders decreased. WPI^[-] and WPI had positive and negative scores, respectively. A positive peak was observed on PC1 at 1628 cm⁻¹ (**Figure 57**), meaning that high scores on PC1 corresponded to a greater amount of β -sheets. In the fresh WPI^[-] powder, it can be hypothesized that proteins are more aggregated than in the fresh WPI powder and this through intermolecular β -sheets even though it is located at higher frequencies than those found in the literature. This would only confirm that the higher the lactose content, the more stable the protein structures against aggregation. Regarding the samples distribution on the other principal components, it is hard to attribute the difference induced by the storage conditions even if the FTIR method still showed small changes. However, and as it has been said previously, the changes between WPI^[-] and WPI were very small, and FTIR might not be the most accurate method to investigate such tiny changes in protein structure (Desfougères, Jardin, et al. 2011).

4.4.5. AGGREGATION IN THE DRY STATE

QUANTIFICATION OF AGGREGATED PROTEINS

Figure 59 shows the evolution in the amount of aggregated proteins in the 4 powders during 3 months storage at 40°C and 60°C (**Figure 59**, A and B, respectively).

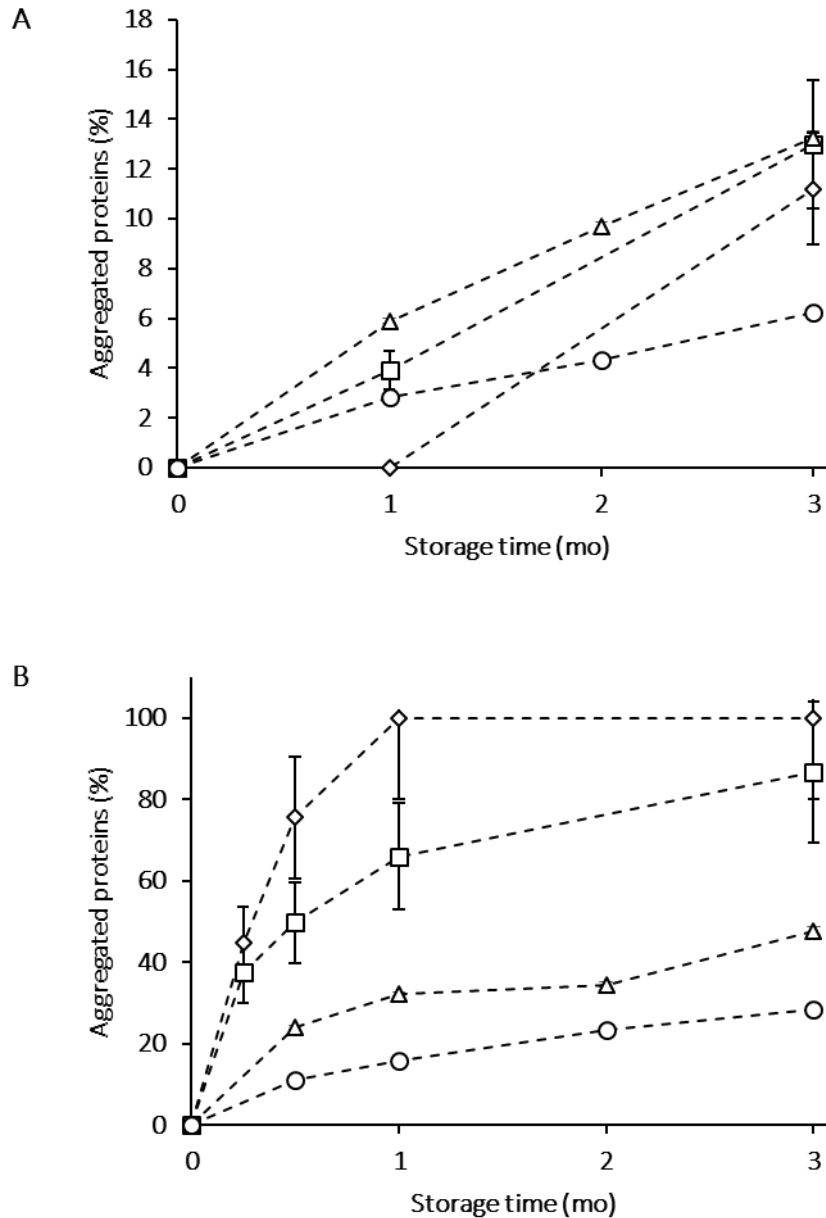


Figure 59: Amount of aggregated proteins for WPC (diamond), WPI^[+] (square), WPI (triangle) and WPI^[-] (circle) powders stored at A) 40°C and B) 60°C as a function of storage time

The gel permeation chromatography was used to determine the protein aggregation rates in the 4 powders as a function of storage time by difference with samples at t_0 . First, the results for powders

stored at 40°C showed that the process of aggregation was delayed in the WPC powder. This can be related to the protective effect of the high lactose content against whey proteins aggregation discussed in the previous paragraph.

The amount of aggregated proteins was not different for WPC, WPI^[+] and WPI after 3 months of storage at 40°C and reached 11 ± 2%, 13 ± 3% and 13 ± 1%, respectively, whereas it reached 6 ± 1% for the WPI^[-] powder. Considering a 3 months storage at 60°C, this amount increased with lactose content and reached 100 ± 20%, 87 ± 17%, 48 ± 1% and 29 ± 1% for WPC, WPI^[+], WPI and WPI^[-], respectively.

These results are in agreement with the findings of the RP-HPLC and FTIR analysis showing first a decrease in the amount of native proteins and a greater β -sheets content with higher storage time, temperature, and lactose content. Except WPI^[-], it is likely that the effect of lactose content starts to make a difference in the ageing of WP powders after 3 months of storage at 40°C. The former is indeed significantly different from the 3 others, whereas WPC, WPI^[+] and WPI are not significantly different from each other. At 60°C, apart from the WPC sample presenting an insoluble material, the other WPI samples (WPI^[+], WPI, WPI^[-]) were made of soluble aggregates. However, this study does not distinguish the soluble and insoluble aggregates. Therefore, both are included in the **Figure 59**. Nevertheless, the evolution of aggregated proteins is correlated with lactose content in WP powders. Lactose is playing a significant role on protein aggregation during powder ageing. The formation of the insoluble fraction in WPC is probably due to the Maillard induced protein polymerization (Le, Holland, et al. 2013; Serpen et al. 2007).

CHARACTERIZATION OF AGGREGATED PROTEINS

In order to observe possible differences in protein aggregation depending on storage conditions, SDS-PAGE were performed with the 4 WP powders stored for up to 3 months at 40°C and 60°C (**Figure 60**, **Figure 61** and **Figure 62**, **Figure 63**, respectively).

At 40°C, very slight or nil differences were observed regarding the aggregation state after 3 months of storage, which confirmed the results obtained above in GPC-HPLC. However, regarding the WPC powder after one month of storage, the bands of monomeric α -LA and β -LG (around 14 kDa and 18 kDa, respectively) seemed to evolve towards a higher molecular weight. This can be related to the observations of the RP-HPLC profiles (**Figure 51**), in which more hydrophilic species were supposed to be formed in place of native proteins, probably thanks to lactose molecule binding. These entities were not quantifiable in mass spectrometry, meaning that changes other than protein lactosylation also occurred in the dry state. The shift is only visible in the WPC as the lactose is in greater quantities

compared to the 3 other powders. In addition, mainly disulphide bound aggregates were also formed. It is interesting to note that these molecular species remain soluble among storage at 40°C, in accordance with the study of Hsu and Fennema (1989).

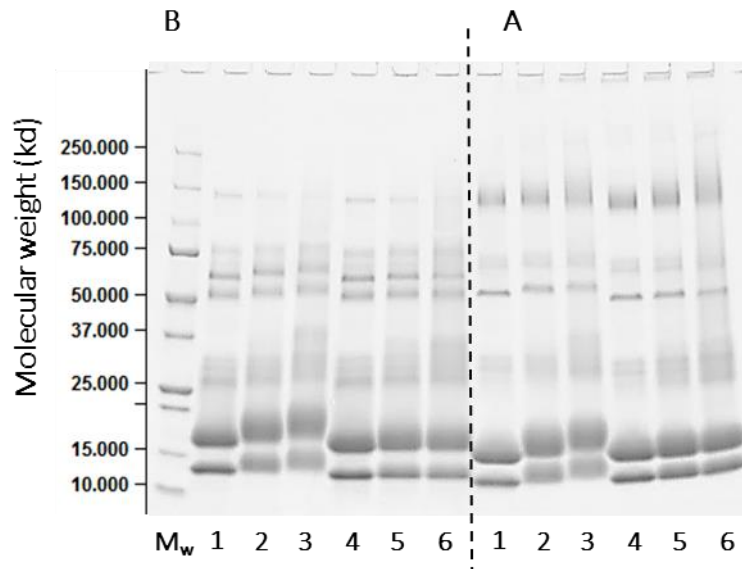


Figure 60: SDS–PAGE profile of fresh and stored WPC (1,2,3) and WPI^[4] (4,5,6) powders at 40°C A) in the absence and B) presence of reducing agent DTT. M_w, low molecular weight markers; 1, Ref; 2, 1 month; 3, 3 months; 4, Ref; 5, 1 month; 6, 3 months

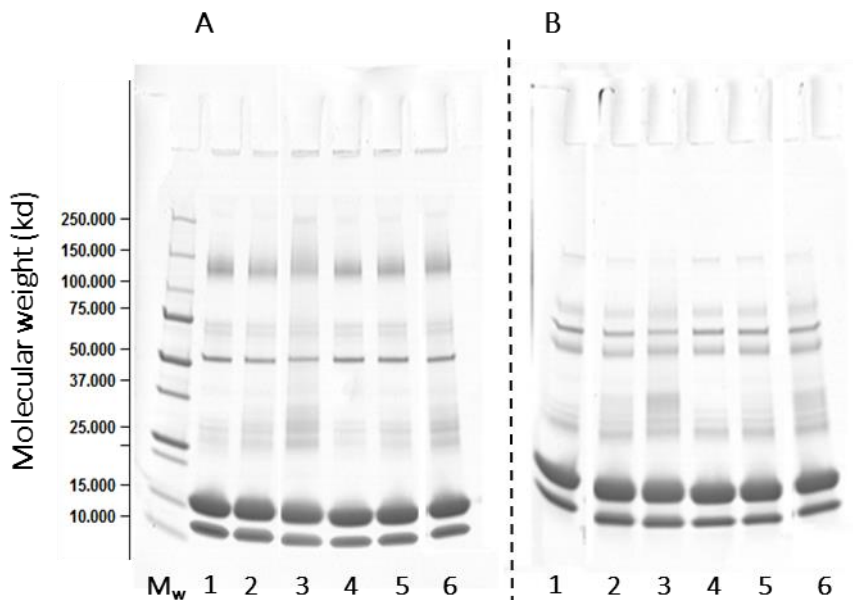


Figure 61: SDS–PAGE profile of fresh and stored WPI (1,2,3) and WPI^[4] (4,5,6) powders at 40°C A) in the absence and B) presence of reducing agent DTT. M_w, low molecular weight markers; 1, Ref; 2, 1 month; 3, 3 months; 4, Ref; 5, 1 month; 6, 3 months

At 60°C, long dark smears were observed on the gels from the first week of storage of the powders that were assigned to aggregates in accordance with the GPC-HPLC results. Moreover, they were mainly linked by covalent bonds. Disulphide bridges formed part of these, which were dissociated by DTT under reducing conditions. The other part was made of a different type of covalent bonding. During storage, the aggregates increased in size, as highlighted in the wells after two weeks storage for the WPC and much later for the WPI^[+], WPI and WPI^[-]. In addition, after one month of storage at 60°C, the aggregates of WPC became insoluble and are covalently bonded with a large number of bonds other than disulphide bridges. Lower lactose content in the powder delayed protein aggregation during powder ageing. These results are in agreement with those previously showed.

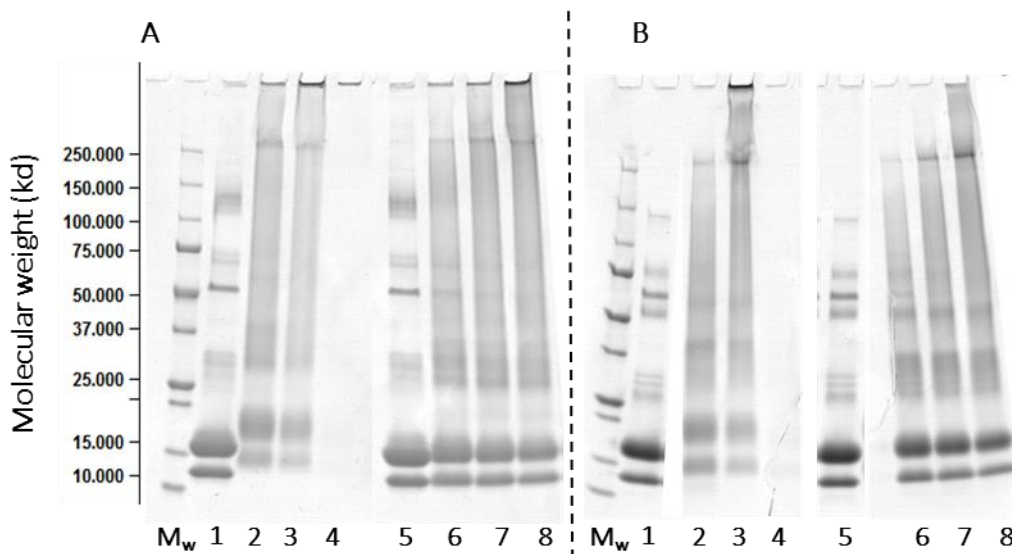


Figure 62: SDS-PAGE profile of fresh and stored WPC (1,2,3,4) and WPI^[+] (5,6,7,8) powders at 60°C A) in the absence and B) presence of reducing agent DTT. M_w, low molecular weight markers; 1, Ref; 2, 1 week; 3, 2 weeks; 4, 1 month; 5, Ref; 6, 1 week; 7, 2 weeks; 8, 1 month

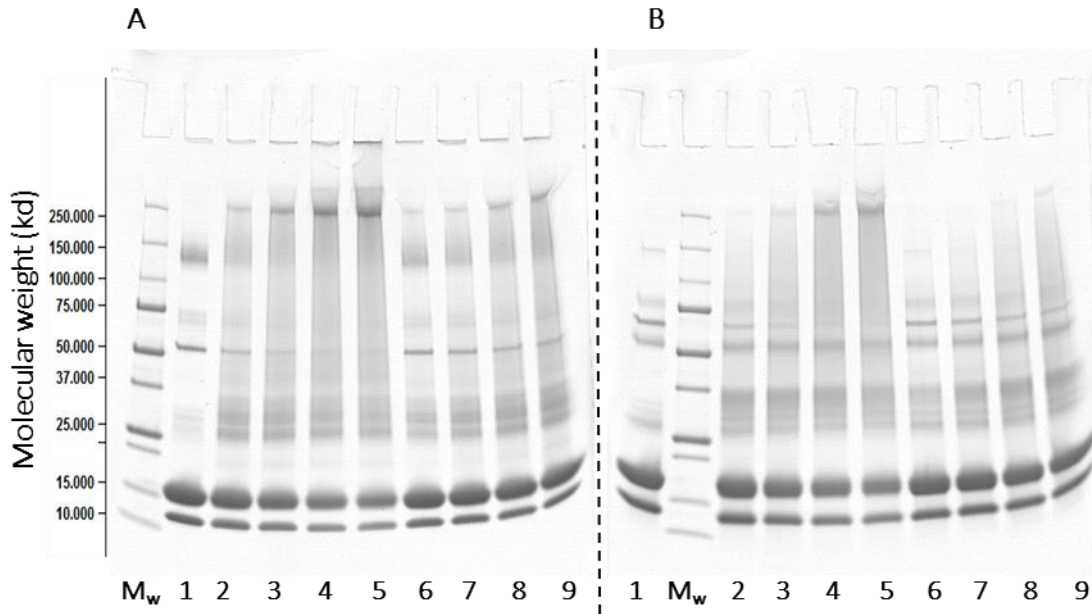


Figure 63: SDS-PAGE profile of fresh and stored WPI (1,2,3,4,5) and WPI^[-] (6,7,8,9) powders at 60°C A) in the absence and B) presence of reducing agent DTT. M_w , low molecular weight markers; 1, Ref; 2, 2 weeks; 3, 1 month; 4, 2 months; 5, 3 months; 6, 2 weeks; 7, 1 month; 8, 2 months; 9, 3 months

These results indicated that, during WPI powder ageing, the presence of lactose accelerated protein polymerization. Protein in the aggregates were linked by disulphide bridges and by other types of covalent bond. In presence of lactose in adequate amount (around 16%), these aggregates became insoluble and their kinetics of formation was function of the severity of the storage conditions and the lactose content in the powders.

4.4.6. HEAT-INDUCED AGGREGATION PROPERTIES

The heat-induced aggregation properties of reconstituted WPC, WPI^[+], WPI and WPI^[-] powders were studied at pH 6.6, according to the operating mode used in Chapter 3, Part 3. Reconstituted solutions of fresh WPC, WPI^[+], WPI and WPI^[-] powders (Ref) and of these same powders stored for up to 3 months were analysed visually before and after heat treatment at pH 6.6 (**Figure 64**).

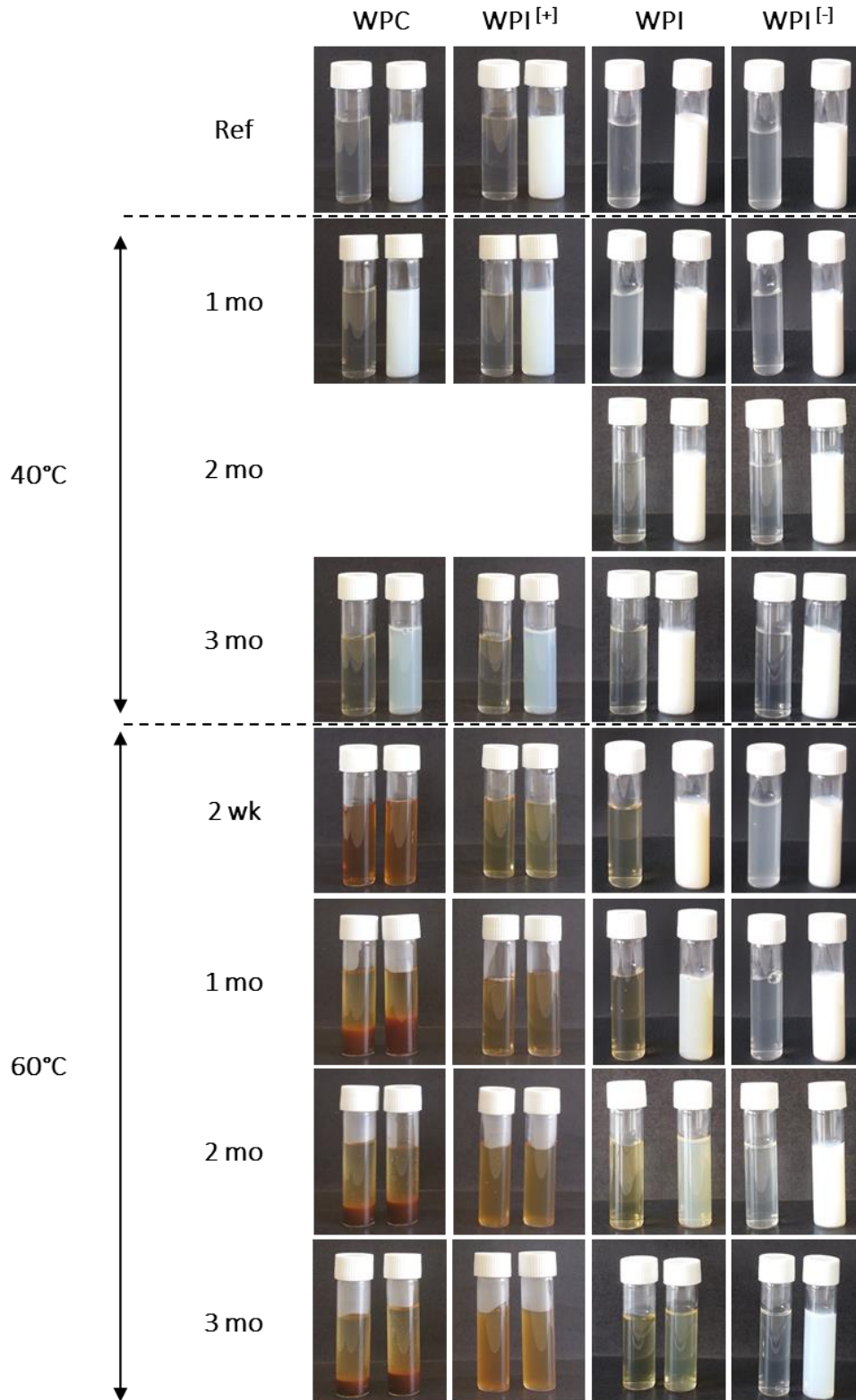


Figure 64: Pictures of samples arranged in pairs per conditions; unheated (left side) and heat treated (right side) solutions

The solutions made of the Ref samples were milky after heat treatment. They were a little less turbid for the WPC, WPI^[+] powders than for WPI, WPI^[-] powders. This difference was assigned to powder

composition as small molecules such as lactose and salts are known to affect whey protein heat-induced aggregation properties (Kulmyrzaev et al. 2000; Phan-Xuan et al. 2013, 2014; Schmitt et al. 2011; Vignolles et al. 2009; W. Wang and Zhong 2014). Heat treated solutions of stored WPC, WPI^[+] and WPI powders became less and less turbid with ageing time. The turbidity decreased much faster for the WPC powder, followed by WPI^[+] and WPI. In contrast, the solution of fresh and stored WPI^[-] did not seem to change at all. In order to go further in the characterization of heat-induced solutions, the size of heat-induced aggregates was studied through DLS.

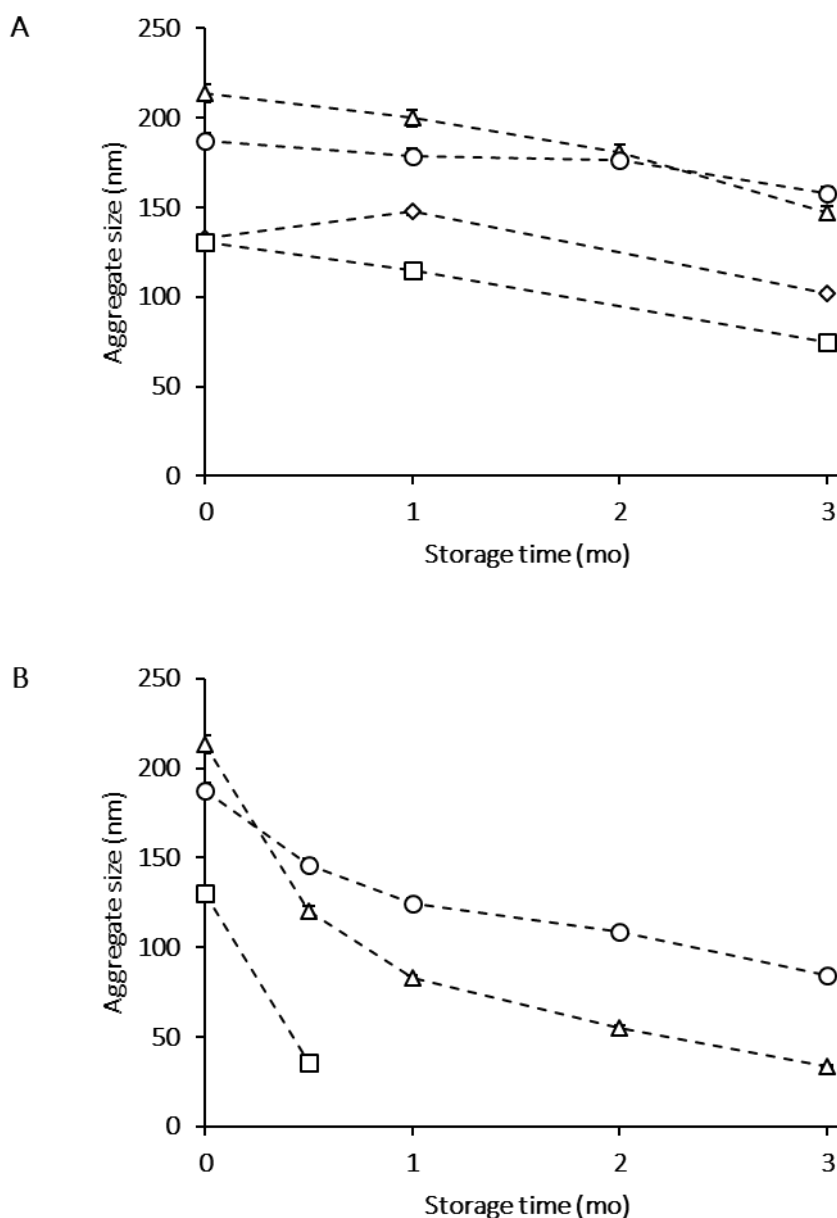


Figure 65: Average aggregate sizes as a function of storage time for WPC (diamond), WPI^[+] (square), WPI (triangle) and WPI^[-] (circle) powders stored at A) 40°C and B) 60°C after heat treatment at pH 6.6, with experimental relative error < 5%

Figure 65 shows the evolution of heat-induced aggregate size as a function of powder storage time and temperature of heat treated solutions of WPC, WPI^[+], WPI and WPI^[-] powders adjusted to pH 6.6. The aggregate size of Ref was around 133 ± 1.56 nm, 131 ± 3.05 nm, 215 ± 4.99 nm and 188 ± 4.19 nm for WPC, WPI^[+], WPI and WPI^[-], respectively. Whey powders stored at 40°C showed small decrease in the heat-induced aggregate size (**Figure 65, A**). However, a study on longer storage time at 40°C would be necessary in order to compare the kinetics of decrease in the heat-induced aggregate size even though the WPI^[-] powder seems to have the lowest one. Storage at 60°C provided larger differences between the 4 powders (**Figure 65, B**). For solutions of WPI and WPI^[-] powders, aggregate size decreased to 84 nm and 34 nm after 3 months, respectively. Moreover, the reduction of heat-induced aggregate size was lower for WPI^[-] than WPI, meaning that a reduction in the lactose content would prevent the decrease in the heat-induced aggregate size. Different results were obtained for solutions of WPC and WPI^[+] powders. The DLS analysis showed poly-dispersed aggregates for these two samples before and after heat treatment, which made difficult to measure the aggregate size. These results demonstrated that lactose content greatly impacted protein heat-induced aggregation properties.

4.4.7. DISCUSSION

From Chapter 2, it has been demonstrated that the level of lactosylated proteins increased from the first months of storage reaching a maximum, then decreased to an undetectable level with storage time. The decrease was concomitant with an increase in the level of aggregated proteins and powder browning. The hypothesis was that proteins were extensively modified by lactosylation. The degradation of the Maillard reaction products contributed to the formation of aggregated proteins in the dry state and the browning of the powder. Hence, varying the lactose content in whey powders would give a better understanding of the role played by lactosylated proteins on protein aggregation in the dry state, whether or not it is essential to the aggregation process, and if controlling its content is decisive for controlling powders ageing.

Based on the results obtained in this study, the lactose content was shown to have a significant effect on WP powder ageing. In the early stages of storage (up to 3 months storage at 40°C or during the first weeks at 60°C), the protein lactosylation was observed as the first step of powder ageing. For a lactose content of 1.2%, the same trend as in Chapter 2 was observed. For a content of 0.1%, the level of lactosylation did not increase during storage at 40°C and quickly decreased at 60°C without reaching 10% of lactosylated proteins. For higher lactose contents ($\geq 2.6\%$), the quantification of the level of lactosylation on non-aggregated proteins was not possible probably due to the rapid degradation of lactosylated proteins (Guyomarc'h et al. 2000; Van Boekel 1998). For identical storage conditions, this

difference in lactosylation rate may be found in the lactose/protein molar ratio, which varied from 12 to 0.06 for WPC and WPI^[1] powders, respectively. The Maillard reaction in powders with more than one lactose molecule per protein (WPC, WPI^[+]) proceeded very rapidly and in a much more extensive way than for the two other powders. By contrast, the reaction was more limited for lactose/protein molar ratio $\leq 0.7\%$ (WPI). For the WPI powder, over than 70% of the proteins can be lactosylated (if one protein binds no more than one molecule of lactose), but only a maximum of 30% was reached in this study. The most plausible explanation relates to the number of lactose bound per protein. Morgan, Bouhallab, et al. (1998) have distinguished several populations of glycosylated β -LG differing by the number of lactose bound per protein, which can range up to 11 lactose molecules. Thanks to the mass spectrometry analysis, it was observed that 2 to 3 lactose molecules were bound per protein. Consequently, the maximum level measured would approach half of the theoretical maximum, which corresponds to our results. Another explanation could involve the kinetics of the Maillard reaction. Immediately after lactosylation, bound lactose molecules are degraded. However, it seems less likely since no aggregation was observed before or at the time of the maximum protein lactosylation was reached, suggesting that the kinetics of protein lactosylation is faster than the kinetics of lactosylated protein aggregation. The disappearance of lactosylated proteins resulted in a parallel increased in protein aggregation from the first weeks of storage at 60°C. Furthermore, the higher the lactose content, the more the aggregation was boosted. This is the case of the WPC powder (16%) for which the extent of the Maillard reaction led to a high Browning Index and covalent aggregation of all proteins present in the medium. These results are in agreement with previous studies on WPC (M. N. Kim et al. 1981). With storage time, aggregates grew and were stabilized by more and more covalent bonding resistant to reducing agent, up to their insolubilization. On the other hand, a reduced lactose content ($\leq 0.1\%$) significantly limited the rate of protein aggregation and the browning of the whey powders during storage by reducing the level of lactosylated proteins. This shows that the ageing of WP powders was mostly due to the Maillard reaction and the step of lactosylation was essential for the aggregation as demonstrated in Chapter 2. This effect was much more pronounced on storage at high temperature.

The absence of lactose also allowed the observation of lactose-independent ageing mechanisms to take place in the powders. Studies on dry heating of purified whey proteins showed that the changes are not the same with and without lactose (Gulzar et al. 2013; Guyomarc'h et al. 2014). Dry heating in the absence of lactose caused changes in non-aggregated proteins. A high proportion of non-aggregated α -LA was converted into a non-native form corresponding either to the formation of a pyroglutamic acid or to the formation of an internal cyclic imide at position Asp₆₄ (Gulzar et al. 2013). This non-native form, more hydrophobic than the native form, seems to be observed on the

chromatographic profiles (**Figure 51, B**) at a content inversely proportional to the lactose content. These small structural changes can lead to a variation of functional properties, *e.g.* improved foaming and interfacial properties (Ibrahim et al. 1993). Desfougères et al. (2008) showed that the dry heating of pure hen egg white lysozyme resulted in a significant improvement in its foam ability. This is due to the formation of succinimidyl residues providing greater hydrophobicity of the protein and an increased flexibility promoting the adsorption and intermolecular interactions at the interface (Desfougères, Jardin, et al. 2011; Desfougères, Saint-Jalmes, et al. 2011). However, Zhou and Labuza (2007) showed that the absence of lactose in the WPI powder did not lead to greater structural changes compared to pure β -LG powder during storage, although the conditions applied (storage at 45°C for 2 weeks) appeared too soft to assess any differences between powders in views of our previous results. We can therefore conclude that the storage of a WPI powder with a lactose-reduced content may lead to the formation of new molecular species, the properties of which still have to be determined.

As already shown in Chapter 3, heat-induced aggregation is generally referred to a 2-stage mechanism. First proteins exposed their hydrophobic groups by unfolding. They assembled into oligomers to finally form larger aggregates thanks to hydrogen and hydrophobic interactions and subsequently, they stabilized through covalent disulphide bridges (Schmitt et al. 2010). Numerous studies displayed that protein glycation modified the process of protein heat-induced aggregation. One of them reported that glycation of WPI with lactose enabled to produce transparent dispersion after heating 7% (w/v) WPI solutions at 88°C for 2 minutes, whereas WPI control solution formed turbid solutions (G. Liu and Zhong 2013). The transparent solution was composed of particles smaller than 14 nm. Mulsow et al. (2008) also stated that covalent attachment of lactose to the whey proteins significantly improved the heat-stability of whey proteins. Three hypothesis have been formulated to explain the decrease of the aggregate size after heat treatment. The first, as stated in Chapter 3, would be that clustering of oligomers upon heating might rapidly reach a critical charge density, thus limiting aggregation, and smaller aggregates would form preferentially. However, Broersen et al. (2007) stated that the inhibition effect of glycosylation on heat-induced aggregation cannot be explain by this mechanism. Thereby, another mechanism has been proposed. The inhibition of aggregate growth would result from an increase in heat-induced aggregation kinetics. Indeed, glycosylation is believed to facilitate protein denaturation when heated at their denaturation temperature or above in the preliminary stage of aggregation (Broersen et al. 2007; Sun et al. 2011). Lastly, Mulsow et al. (2008) hypothesized that the increase in hydrophilicity resulting from lactose molecule binding to the protein may have weakened the hydrophobic interactions and thus hinder aggregation. This is partly in accordance with our results showing that heat-induced aggregates were smaller and smaller and

heat-treated solutions of whey proteins became more and more transparent when the lactose content of whey protein WP powders and the storage time and temperature increased.

Most of these studies stated that glycosylated proteins are responsible for these changes. By varying the lactose content in the WP powders, it was possible to demonstrate that heat induced-aggregation are affected by the Maillard reaction products resulting from the degradation of lactosylated proteins. Indeed, in chapter 2 we showed that the changes in heat-induced aggregation of whey proteins couldn't be explain by the sole protein lactosylation (results at 20°C). It is possible that the glycated protein powders considered in the previous stated works also contained some Maillard reaction products. For example, WPI dispersion exhibited a darkest colour in G. Liu and Zhong (2013) study and a higher furosine content in Mulsow et al. (2008) study. Moreover, Broersen et al. (2007) might have overestimated protein glycation using the OPA method, which refers to the blocking of free amine groups by reducing sugar. The quantification informs on protein glycation and its degradation products, but is not able to differentiate one from the other. This agrees with our results showing that the more lactose in the WP powder, the more degradation products are formed and thus the smaller the heat-induced aggregates. Indeed, heat-induced aggregation is almost completely prevented by ageing of powders due to the neo species. It is notable that the WPC powder stored two weeks at 60°C, corresponding to a high BI and aggregation rate in the dry state, seemed almost non-aggregated after heat treatment suggesting a non-participation of Maillard reaction products in the mechanism of heat-induced aggregation.

Therefore, controlling the lactose content in WPI powders is essential to control their properties. It determines the heat-induced aggregation rate of the proteins through the changes that occur in WPI powders during storage.

PART 5. CONCLUSIONS

In summary, this study provides important information regarding how to limit the storage-induced changes of WPI powders. Varying the particle size of the powder is not a relevant tool as it does not influence the powder ageing. Moreover, the drying mode (Bionov and Minor) does not seem to change the properties of the powder particle as it does not induce change either among storage. The powder composition, especially its lactose content is responsible for most of the storage-induced changes in WPI powders.

Varying the lactose content in WP powders gave useful details for further understanding of the changes occurring in whey protein isolate powder during storage. A WPC powder, widely used in food industry as an ingredient, turned out to be very unstable during storage. WPI powder is definitely

more stable among time than WPC. Although the presence of lactose stabilized the protein structure in a fresh powder, high lactose content greatly accelerated the kinetics of protein aggregation and the powder functional properties changes. On the contrary, the control of lactose content in a WPI powder is of major importance for its long-term preservation. In a WPI powder whose lactose content was greatly reduced (lactose/protein molar ratio <1), the Maillard reaction was not stopped but its occurrence was considerably limited. Increasing the diafiltration of lactose before spray drying would be an issue for food industrials so as to have a very stable WPI powder during storage. Moreover, at very low lactose content in WPI powder other reactions can occur due to the decreased competitiveness of the Maillard reaction and would affect the protein-ageing path by other aggregation mechanisms (Gulzar et al. 2013). They would eventually have an effect on the WPI powders properties after rehydration.

CHAPTER 5. KNOWLEDGE INTEGRATION

This chapter aims to clarify the ageing path of whey protein isolate powders when stored under specific conditions in order to provide industrial monitoring indicators and thus anticipate or predict the powder behaviour after different periods of storage and/or shipment. By graphically correlating the variables related to the changes of industrial interest, the objective was to evaluate the powder behaviour when stored, and more particularly the path they would follow for specific conditions of time and temperature. Several correspondences were found between the samples stored under different conditions. Finally, and in view of the previous results, a Principal Component Analysis (PCA) was performed in order to draw an ageing path of the whey protein isolate powder. This statistical approach helped in identifying a *catching up* behaviour from the changes obtained at lower temperatures to those at highest temperatures reflecting a clear and relevant ageing path of the WPI powder.

The aims of this chapter were to:

- x *highlight the ageing path of a whey protein isolate powder*
- x *suggest ageing indicators of WPI powders in order to predict and to better control WPI powder storage*

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PART 1. INTRODUCTION

The previous chapters have shown that the ageing of WPI powders is accompanied by changes in their functionalities depending on storage conditions (heat-induced aggregation, foam stability, colour). In parallel, global changes in protein structure were identified after powders rehydration. It has been shown that the presence of remaining lactose in the WPI powder was in major part responsible for these structural changes through the Maillard reaction causing the observed functional changes. Indeed, the Maillard reaction enhances protein interactions and aggregation, resulting in a better foam stability and a decrease in the heat-induced aggregate size (Norwood, Chevallier, et al. 2016; Norwood, Le Floch-Fouéré, et al. 2016).

Several authors showed that molecular mobility is favoured upon ageing (Fan and Roos 2016; Haque et al. 2012; Thomas, Scher, Desobry-Banon, et al. 2004), which is believed to be at the origin of the Maillard reaction (Thomas, Scher, and Desobry 2004). Evidences from the literature show that thermal history impacts molecular mobility in relation to the glass transition temperature (T_g). This implies fast dynamics (motions that involve small length scales), which is correlated to storage stability (Abdul-Fattah et al. 2007). Therefore, significant differences in overall ageing dynamics are observed according to the storage temperature conditions underwent by the powders. Namely, this is what seemed to happen in our study. The WPI powders were relatively stable during storage at 20°C. On the other hand, they presented significant levels of aggregation and protein modifications for storage at temperatures higher than 20°C. Storage temperature and relative humidity have been considered as the predominant factors involved, but our study made it possible to further demonstrate that time and milk components were also implied in these changes. The 3-order interaction – storage time and temperature, and powder composition – seems thus to determine the ageing kinetics of the WP powders (WPC and/or WPI). The more severe the storage conditions, the more pronounced the structural and functional changes in the WP powders.

However and to date, no relationship was established between the ageing-induced changes of stored WPI powders inherent to different and specific conditions of time and temperature. Two hypotheses have been proposed: 1. WPI powders undergo a *catching up* phenomenon or 2. WPI powders follow different paths according to the storage conditions. In the case of *catching up*, one model of ageing for all storage conditions is expected, which seems to already meet our previously stated results. The hypothesis of individual ageing paths is not completely excluded depending on the composition of WP powders.

In order to study which phenomenon occur in the WPI powders depending on the storage conditions, graphical correlation have been established between the most relevant structural markers of the

powder ageing. Moreover, a principal component analysis has been performed in order to draw the current ageing path of the WPI powder.

PART 2. STATISTICAL ANALYSIS

The complete data set was analysed using PCA, which has been described in Chapter 4 (cf. 4.2.4., p95). PCA was applied to the whole set of 11 variables including structural and functional experiments, which characterize 20 samples, using the Facto-MineR package of the R software (Lê et al. 2008). The variables were standardized (mean-centred and scaled to unit variance) by the software in order to give all of them the same weight on the analysis, regardless of the numerical magnitude and units of the original variables. The original variables used in PCA were *amount of monomers* and *aggregated proteins* in the WPI powders, *lactosylation level*, *thiols level*, *browning index*, ellipsometric variables (D , Γ_0 and ϑ), *air volume fraction*, *density* and *heat-induced aggregate size*. Hierarchical clustering on principal components was also performed onto the principal components and allows to evidence similarities between groups of individuals (Husson, Josse and Pagès 2010). Each of these groups, called clusters, has a characteristic inertia reflecting its homogeneity.

PART 3. AGEING PATH OF WHEY PROTEIN ISOLATE POWDERS

5.3.1. CORRESPONDENCE BETWEEN AGED SAMPLES

First, the possible relationships between the different ageing points were studied graphically by correlating variables of industrial interest, namely the browning index with either the aggregation level in the dry state, or the lactosylation level of the monomeric β -LG (**Figure 66** and **Figure 67**).

In **Figure 66**, several storage conditions come out to be equivalent by correlating the level of aggregation and the browning index. Indeed, the points corresponding to WPI powders stored 18 months at 20°C and 40°C were superimposed on those of powders stored 15 days at 40°C and 60°C, respectively. Furthermore, the storage accidents corresponding to 3, 6 and 9 days at 60°C were located in the following ranges: around 3 months, 6 to 9 months and around 12 months at 40°C, respectively. The results, including all storage temperatures and times, showed a positive correlation between the BI and the amount of aggregated proteins of 0.94. **Figure 67** shows the level of β -LG lactosylation according to the browning index. The evolutions of the samples stored at 20°C are more visible than in **Figure 66** making it possible to confirm the correspondences discussed above.

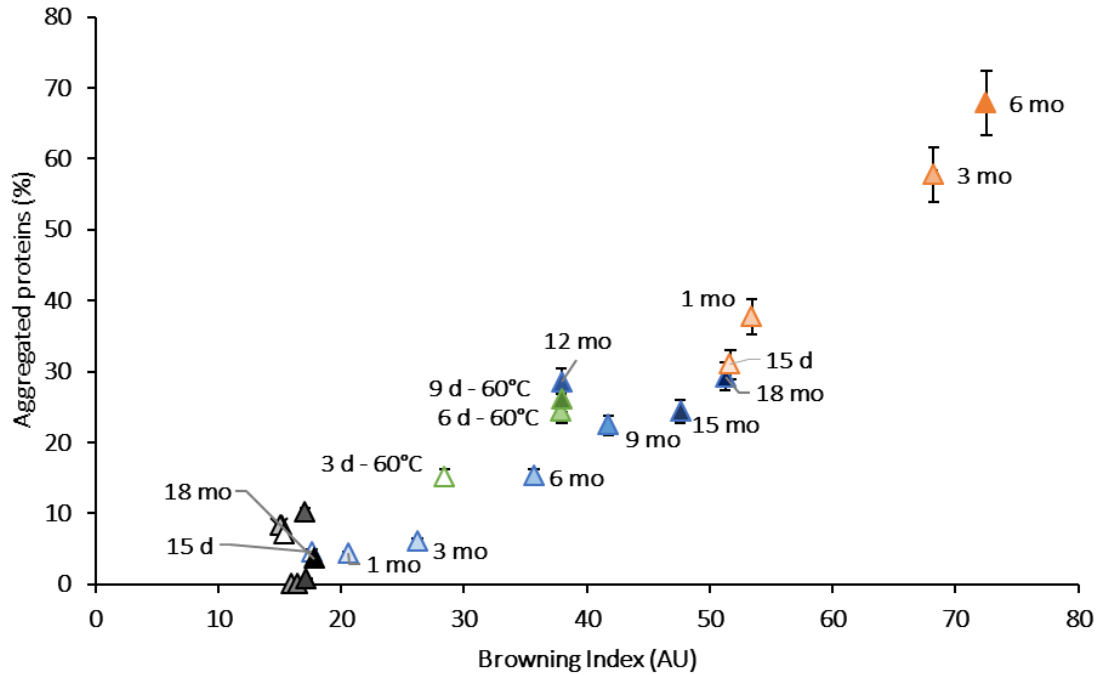


Figure 66: Correlation between the amount of aggregated proteins and the browning index for WPI powders stored at 20°C (black symbols), at 40°C (blue symbols) and at 60°C (orange symbols); and subjected to a number of storage accidents (green); the darker the inner colour, the longer the storage time

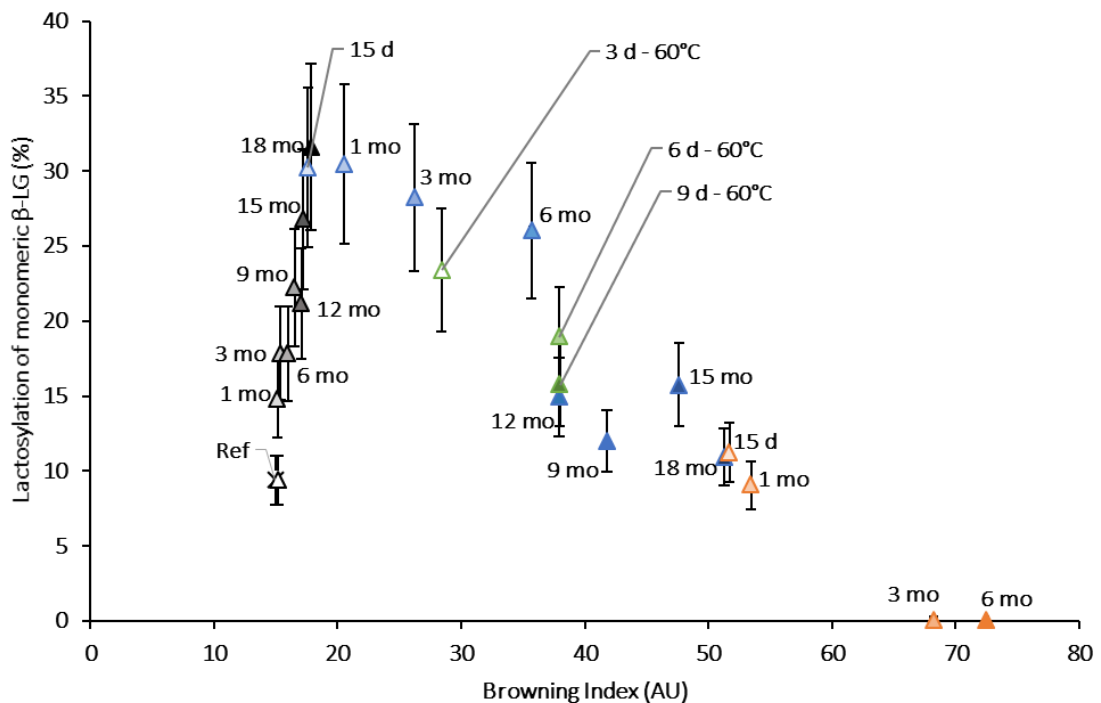


Figure 67: Correlation between the amount of lactosylated β -LG and the browning index for WPI powders stored at 20°C (black symbols), at 40°C (blue symbols) and at 60°C (orange symbols); and subjected to a number of storage accidents (green); the darker the inner colour, the longer the storage time

Despite the exploratory nature of this approach, patterns emerge and this in a clearer manner for most samples. In Chapter 2, we had already foreseen a shift in the time scale from the changes induced at low temperatures to those at higher temperatures (**Figure 29**). Samples stored at 20°C, showing only an increase in protein lactosylation during storage, seemed to move towards the samples stored for a shorter time at 40°C where the lactosylation level was high without displaying an initiation of protein aggregation. For longer storage time at 40°C, the latter seemed to catch up the samples stored for a short time at 60°C by a decrease in the level of lactosylation and an increase in the amount of aggregates in the dry state. However, no overlapping between the ageing paths of the similar WPI powders stored in different conditions could be highlighted, maybe due to not long enough storage times.

5.3.2. WPI AGEING PATH DETERMINED BY PCA

In order to characterize the samples, which are described by quantitative variables, a principal component analysis was performed. As a reminder, this statistical analysis allows to identify an individual profile or a group of individuals and illustrates the links between variables from characteristic individuals through the study of individuals' similarities with a global view of all variables.

The factorial map summarised about 84% of the total inertia (**Figure 68**, projection of variables; **Figure 69**, projection of individuals), including PC1 that explained almost $\frac{3}{4}$ of the total variability alone. This means that only 16% of initial information has been lost while projecting a 20-dimension space (individuals) or an 11-dimension space (variables) on the 2-dimensions of the factorial map. This can be considered as a very satisfactory representation considering the 20 active individuals and the 11 variables included in this analysis.

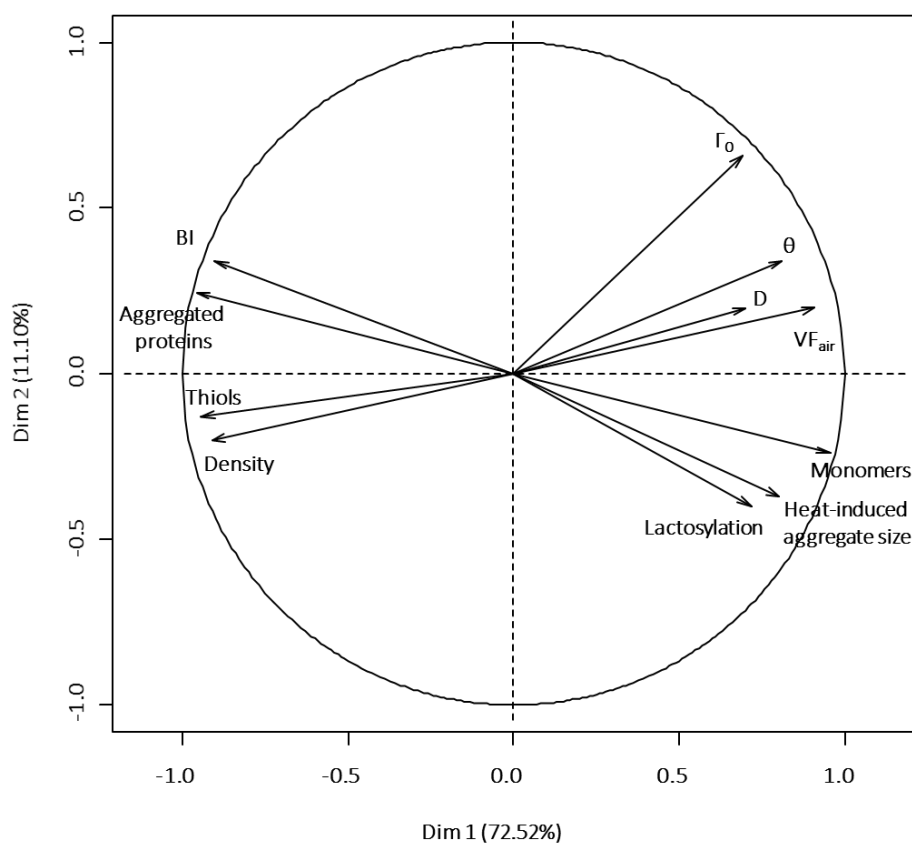


Figure 68: Correlation circle of the principal components analysis (PCA)

Figure 68 displays the correlation circle, which is a projection of the variables on the factorial map. Two ways of interpretation have to be considered in order to analyse the results: i) the angle between the variables projections in the circle shows the correlations between each other and alignment of the vectors results from high correlations between the variables, and ii) the projection quality is shown by the proximity of the vector to the circle (for example, *aggregated proteins* and *BI* have a very good projection and they are highly correlated). In our study, the variables were thus pretty well projected on the 2-D graphic. The first principal component (PC1) was positively and highly correlated with the amount of monomeric proteins and the air volume fraction in the foam (96% and 91%, respectively). It was also positively correlated to the heat-induced aggregate size, the protein lactosylation and the ellipsometric variables to a lesser extent (80%, 72% and ~70%, respectively). On the contrary, the aggregated proteins, the amount of free thiols groups, the foam density and the browning index were negatively correlated to PC1 (96%, 94%, 91% and 90%, respectively). It can thus be considered that this axis exposes the functional changes according to the protein structure. The second principal component (PC2) was positively correlated with one of the ellipsometric variables, namely Γ_0 (66%) representing the surface concentration at which π is different from zero. It can be interpreted as the moment when structural changes begin to impact the protein properties in solution or once at the

interface, either because they are lactosylated or aggregated. To summarise, the first and the second principal components tended to scatter individuals through their functions highly correlated with the protein structure and the protein properties, respectively.

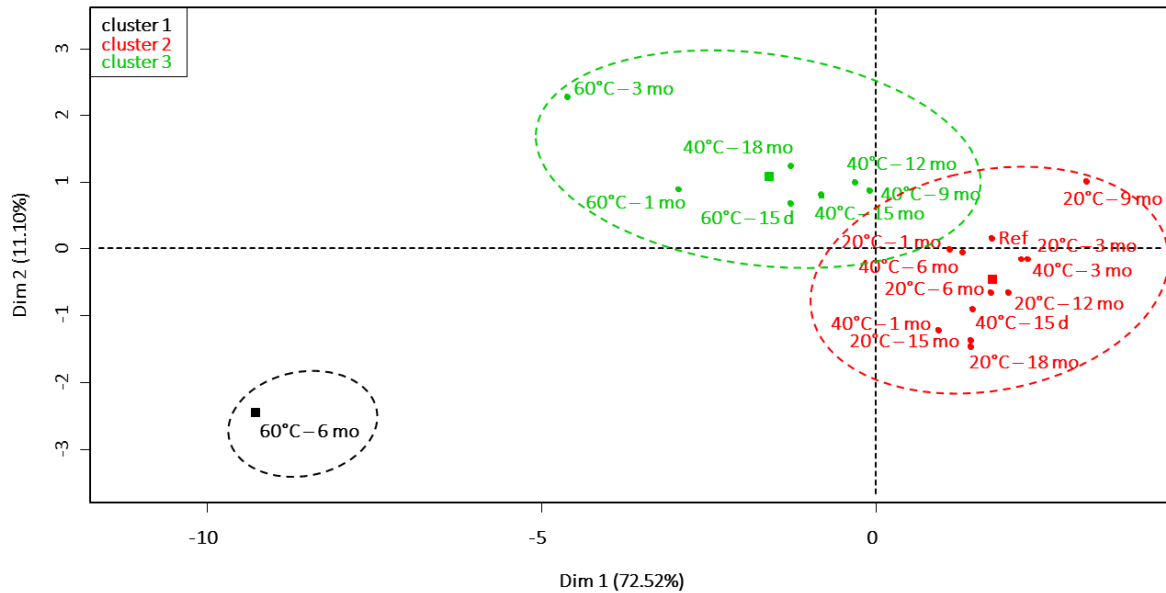


Figure 69: Graphic of individuals (dot) with hierarchical clustering on principal components; black, cluster 1; red, cluster 2; green, cluster 3; square, geometrical centre of clusters

The individuals' projections on the factorial map following their similarities are shown in **Figure 69**. The geometrical centres of the clusters formed by hierarchical clustering on principal components is also projected. Cluster 1 (in black) was clearly different from the other two clusters. It presented negative scores on PC1 and PC2. Considering **Figure 68**, it can be clearly interpreted as a “highly aged powder” cluster including protein aggregation, powder browning and great changes in the functional properties (foam and interfacial properties, and heat-induced aggregation properties). Cluster 2 (in red) was the largest cluster and is made of individuals that are not very different from Ref. Thus it was seen as the “Ref” cluster, the reference being the closest point to the cluster inertia. Cluster 3 (in green) contained all individuals with an intermediate rate of protein modification impacting only the heat-induced aggregation properties.

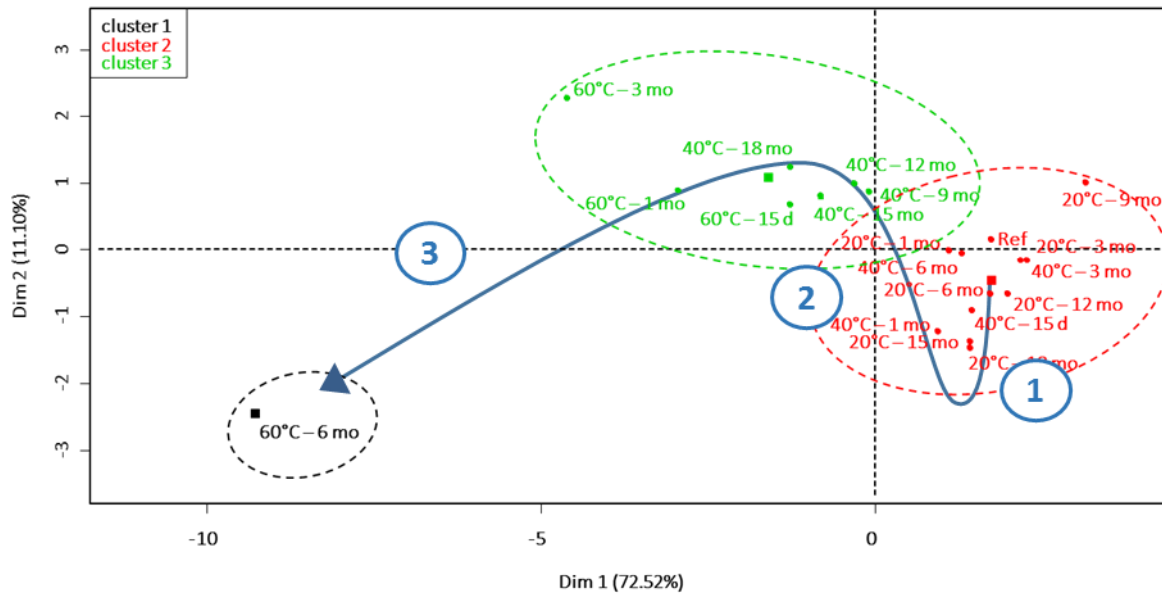


Figure 70: Drawing of the ageing path of WPI powder resulting from the PCA

Figure 70 shows the complexity of WPI powders ageing, which is composed of three interdependent stages. To date, this has never been clearly stated in the literature.

The first stage of WPI powder ageing is represented by the cluster 2 of the PCA (**Figure 70**, 1). It is characterized by the increase in protein lactosylation without affecting the aggregation state of the protein (which remains in a monomeric form) nor the functional properties. The second stage of ageing involves the consumption of lactosylated proteins previously formed feeding into aggregated products (**Figure 70**, 2). This phase is characterized by the presence of these two type of entities, the second having a significant impact on the heat-induced aggregation properties after rehydration. The third and final stage reflects the progress of the Maillard reaction with a total consumption of the lactosylated proteins, a high rate in aggregated proteins and a pronounced browning of the powder. Ultimately, these changes are accompanied by a change in foaming and interfacial properties, in addition to the heat-induced aggregation properties already modified under more moderate storage conditions. This stage is observed for very old WPI powders which have undergone high temperature episodes.

PART 4. BENEFICIAL CONTRIBUTIONS FOR INDUSTRIALS

It is now well known that high protein dairy powders represent a use-deferred strategy on a high-value market. Indeed, the market share allocated to these products has continuously increased in these recent years thanks to their change of status from a co-product to an ingredient itself and by

being involved in the production of many food and/or dairy products as infant formula. Securing specifications of high protein dairy powders proposed on the current market is therefore an issue of major importance for the French dairy industry. The advances provided by this PhD-project were needed to better understand and thus to better control the changes that occur during storage and/or transport of high protein dairy powders, considered as stable, but whose properties can significantly be changed depending on the conditions (time, temperature, a_w , etc.) to which they are subjected. According to the results of this chapter, keys are given for monitoring the evolution of WPI powders in order to better anticipate their use after storage.

Figure 66 and **Figure 67** allow to quickly identify the ageing path of a WPI powder by performing one or more test of accelerated ageing. The corresponding points would be placed on one of these 2 graphics. **Figure 67** is more appropriate for short storage time while **Figure 66** is for longer ones. This method can easily and rapidly predict the deterioration of the protein structure via the browning index and thus by analogy (PCA), the impact on the functional properties. A control of protein lactosylation and powder browning can actually considerably help to see what the WPI powders endured during storage or exportation. The sole measure of protein lactosylation would show that the powder is old while the measure of protein lactosylation accompanied by a browning would reflect one or more period of time at temperatures $\geq 40^\circ\text{C}$. However, it has to be noted that protein lactosylation can also come from pre-treatment of milk and powder manufacture preceding storage (milk or liquid concentrate heat-treatment, drying temperatures...). Thus, characterizing the average level of lactosylation of fresh WPI powder would be the first point to measure in order to run a good monitoring of its ageing.

From a more practical point of view for industrials, **Figure 66** and **Figure 67** allowed to establish correspondences, which are presented in **Table 9**. These are giving a quick estimation of accelerated ageing of WPI powders.

Table 9: Correspondences between samples stored in different conditions based on the Browning Index correlated with the amount of aggregated proteins in the powder and the level of lactosylation

20°C	18 mo	x	x	x	x
40°C	15 d	3 mo	6 – 9 mo	12 mo	18 mo
60°C	x	3 d	6 d	9 d	15 d

Moreover and by considering the Browning Index only, it was also possible to estimate the theoretical correspondences between the samples stored at 40°C and those stored at 60°C for times below 3 days. **Figure 71** shows the equation of the curve followed by the samples stored for times below 3

days at 60°C. Thanks to this quadratic equation, the correspondences between ageing at 40°C and 60°C presented in **Table 9** have been completed with theoretical correspondences below 3 days (**Table 10**).

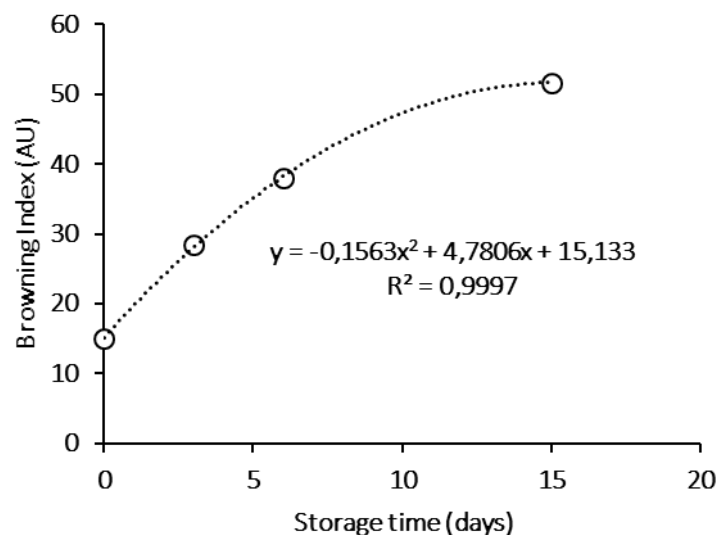


Figure 71: Determination of the curve equation considering the Browning Index of WPI powders stored at 60°C for up to 15 days

Table 10: Correspondences of the Browning Index at 40°C related to an accelerated storage at 60°C

Storage time at 40°C (days)	Storage time at 60°C (days)
0	0
15	0.5
30	1.2
60	1.3
90	2.5

These results allow to predict the WPI powder ageing by accelerated storage tests defined as several days at 60°C and these under identical conditions of a_w to those currently used by industrials (a_w of 0.23).

PART 5. CONCLUSIONS

This study presents a new approach to the drawing of WPI powder ageing regarding protein functional changes according to protein structure changes. A new draw describing WPI powders ageing was displayed. It takes into account the different phenomena occurring during WPI powders ageing such as protein lactosylation, protein aggregation, powder browning and powder functional properties. WPI

powders ageing follows a path of a *catching up* type depending on storage conditions, and started from low temperatures towards higher ones. The WPI powders present a 3-stage ageing, beginning with protein lactosylation, then protein aggregation and browning which finally end up by altering protein and thus powder functional properties. The colour remains thus a good indicator, easy to measure and reliable regarding structural or functional changes during storage of WPI powders. When the powder turns brown, the functional properties are affected. A very aged powder will be brown and its properties will be different from the original manufactured powder.

Our study provides new understanding by analysing more closely the specificity of the influence of the storage conditions on the ageing of WPI powders. Moreover, as WPI powders ageing is following a path of *catching up* type, it shows that temperature has an accelerating effect on structural and functional properties of whey proteins.

GENERAL CONCLUSION

This PhD project is a continuation of a major project undertaken by INRA in collaboration with the CNIEL which was initially focused on identifying methods for assessing the evolution of functional properties of proteins in connection with changes in their structures. This first step was needed to refine the strategy of this PhD project and ultimately optimize the times and conditions of storage of dairy powders in terms of maintaining their functionality during ageing. Indeed, one of the biggest challenges of this study was that it involved long storage times, up to 18 months. Hence previous work on qualifying methods was necessary. Another key element of our strategy was that the studied WPI powders were produced on the Bionov pilot workshop (Rennes, France) and therefore their initial state (t_0) was controlled, meaning WPI powders studied in this project were fresh powders – they have never been stored before.

This study focused on whey protein isolate powders, which are dry ingredients with high added value and whose structural and functional properties are strongly influenced by the storage conditions they are subjected to. WPI powders were stored under monitored storage conditions (20°C, 40°C and 60°C for up to 18 months) and the molecular changes (protein denaturation, protein lactosylation, protein aggregation) and functional changes (foaming and interfacial properties, heat-induced aggregation properties) were investigated in order to answer the 3 main objectives displayed earlier in Chapter 1:

1. Identify and quantify changes in protein structure and functional properties during ageing under controlled storage conditions

This research project has shown that the protein structural changes were minor and difficult to characterize, although the choice of working on a WPI powder reduces the complexity of the studied system because of its high protein content and its low lactose and minerals contents. This can be explained by storage temperatures and times much lower than those required for protein denaturation in the dry state (increased thermal resistance) and/or more likely to the fact that the Maillard reaction seems to be the main reaction taking place during WPI powders ageing. Indeed, the latter relates to the modification of most of the proteins in the powder and therefore, only a minor fraction of the proteins would be denatured. Protein changes would thus concern a change in the stability of side chains in interaction with lactose more than a change in their secondary structure. Bound lactose would favored protein aggregation in the dry state.

2. Identify the relevant structural markers of the evolution of WPI powders during storage affecting their functionality and understand their role in the properties modifications

It was found that lactosylated proteins did not affect the properties of WPI powders as their functional properties were not significantly different from the non-aged WPI powder. On the other hand, protein aggregation in the dry state led to a decrease in aggregate size after heat treatment at 5.8 d pH d 6.6 with concomitant changes in the heat-induced aggregate shape. Only a high amount of aggregated proteins ($\geq 65\%$), would eventually lead to an improved foam stability although the diffusion coefficient of the proteins at the air/water interface decreased. However, it is worth noting that protein lactosylation is one of the major critical step to protein aggregation in the dry state even if it does not interfere with the protein's functionality. Ultimately, the structural changes – in the protein state – induce a variability of functional properties in WPI powder after storage (**Figure 72**).

3. Validate these changes and define optimal storage conditions [initial state, duration t , temperature ϑ] allowing to maintain the powders properties in the expected specifications, and conversely the extreme storage conditions above which these properties are irreversibly affected

In this PhD project, we have shown that protein structural and functional changes during ageing of WPI powders depended on storage temperature, a key storage parameter, and the powder a_w . However, other parameters had a significant influence, namely the initial composition (contents in non-protein components, initial state / structure of these latter) of the powder. Playing on these, especially on the lactose content, will help to extend the shelf life of a WPI powder. From an industrial point of view, several levers have been provided to anticipate functional changes of whey proteins (level lactosylation, the powder color). Moreover, as the temperature appears as a key parameter in WPI powder ageing, storage at $\theta \leq 20^\circ\text{C}$ may help to extend the shelf life of the powder. Another solution would be the implementation of a further diafiltration. However, costs remain to be evaluated in terms of powder shelf life gains. Lastly, several days at 60°C are sufficient to cause irreversible structural modifications.

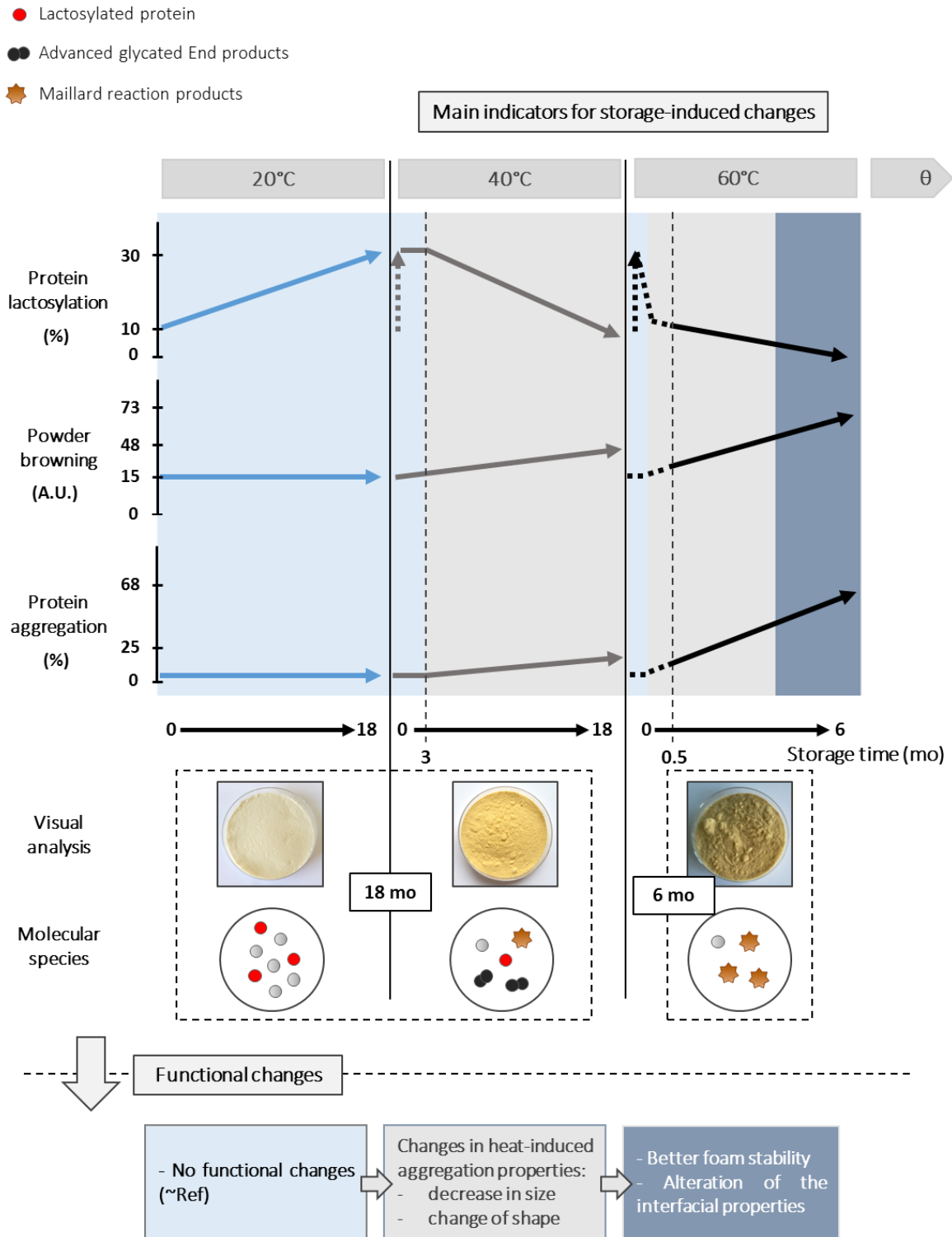


Figure 72: Highlights of the PhD project findings and conclusions

FURTHER OUTLOOK

This PhD project provided new and valuable conclusions considering the storage of whey protein powders; consequently this work brought us to new research questions exposed in **Figure 73**. The short-term continuation of this work lies in the understanding of the **other molecular interactions** that occur in the powder when stored in order to have a complete view of WPI powders ageing. Indeed, the powder being composed of more than 90% proteins, protein-protein interactions or protein modifications seem difficult to be avoided. Moreover, investigating the effect of **WPI powders composition** (e.g. level of storage-induced entities) on the **functional properties** would clear some grey areas as a part of our results on the foaming properties differ from those found in the literature. Lastly, long-term outlook would also focus on whey protein **powders composition** (e.g. variation of the initial composition), on the **process** as it greatly affects the powder quality and on the **nutritional & health aspects**, the Maillard reaction being already a hot topic in the nutrition area.

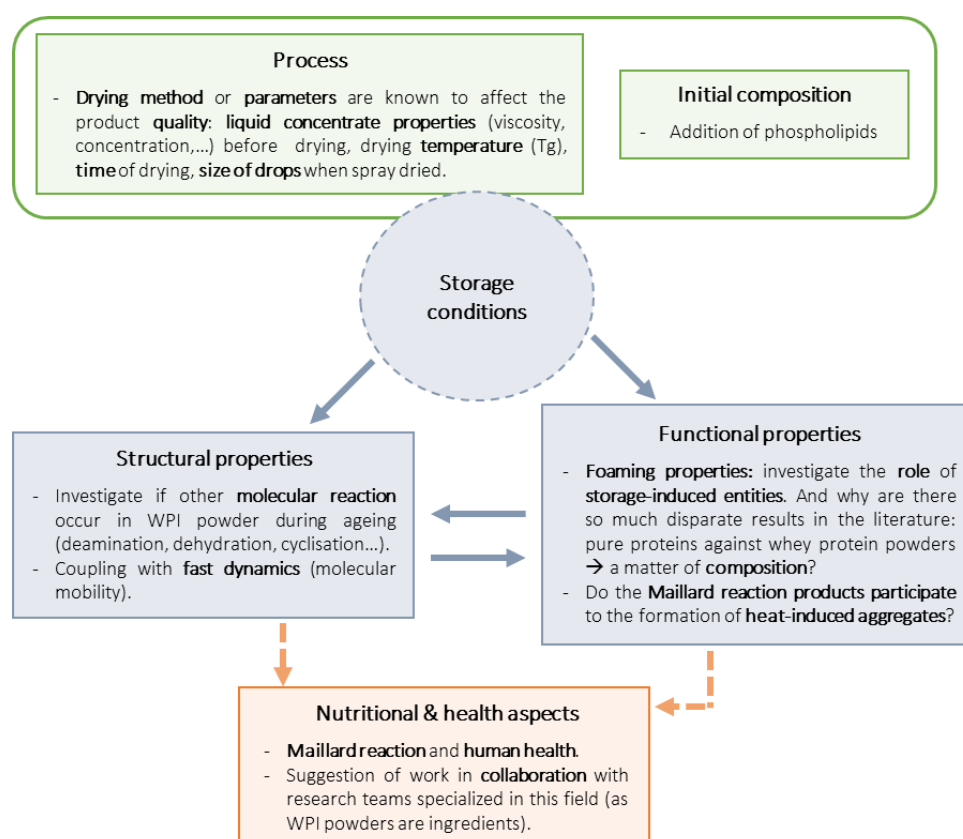


Figure 73: Diagram of the proposed future outlook of this PhD project with the short-term (blue) and long-term upstream (green) and downstream (orange) outlook

Short-term outlook – directly related to the PhD project

The study of interactions in the whey protein powder is not limited to the Maillard reaction, even if it seems to be mainly responsible for this powder kind ageing. Indeed, the characterization of aggregates formed in the powder during ageing has shown that the latter were formed by disulphide bridges and other covalent bonds possibly due to protein denaturation and Maillard reaction, respectively. However, the lowering of lactose content has restricted covalent aggregation without stopping it completely. One approach would be to investigate the different types of ageing-induced covalent bonds by mass spectrometry, including the monitoring of deamination, dehydration and/or protein cyclisation and to link these changes with aggregation in the dry state. A complementary approach would be the investigation of fast dynamics (Abdul-Fattah et al. 2007) in the solid state by light and inelastic neutron scattering. This kind of investigations would eventually lead to a better understanding of the formation of specific bonds by studying reaction competitiveness (interactions kinetics and forces) occurring during storage (Lerbret et al. 2012). This may be particularly interesting on powders with low lactose content or a model of whey protein powder made of pure whey proteins in an appropriate proportion.

Regarding the functional properties, further investigations still need to be done. On one hand, further study on the foaming properties would be necessary to elucidate such disparate results between our study and the literature. In the latter, it is found that dry-heated proteins make foam more stable (Desfougères et al. 2008) whereas in the case of our study, the results are not that obvious. Here, a matter of powder composition is suspected. Moreover, it would be interesting to further investigate the role of aggregates in the foam stability as they are of heterogeneous size (the literature often stated that the aggregate size is of major importance to stabilize foam). The idea would be to characterize the interface by its elasticity thanks to the interfacial dilatational rheometry or interfacial shear rheometry (Desfougères, Saint-Jalmes, et al. 2011), to determine the adsorbed molecules with foam film measurements (Rullier et al. 2009) or atomic force microscopy (AFM) coupled with imaging ellipsometry for more precise measurements (Ying et al. 2003), and finally to characterize the aqueous phase by rheological analysis. Another route for studying foam formation and stability, and for evaluating the role of every entities that were present in the solution at first has been initiated with the soft matter department located at the Institute of Physics – Rennes, more particularly with the Pr. A. Saint-Jalmes, and would need further investigations. On the other hand, even if our study made it possible to highlight that lactosylated proteins didn't interfere with the formation of heat-induced aggregates, the role of AGEs and the more advanced Maillard reaction products (*e.g.* melanoidins) still need to be considered. A proposed experiment to achieve this goal should be inspired from the one

given by Schmitt et al. (2011), who determined the content of native protein, soluble and insoluble aggregates, and heat-induced aggregates after heat treatment.

Long-term outlook – other focus of research

Process. Efforts should be made to better control the parameters of pre-treatments and especially spray drying as they define the powder properties. The powders characteristics are related to end-use properties of the powders and are known to affect their storage stability: water content and water activity for stability, size and size distribution for powder mixing and handling, bulk density and flowability for transportation, and wettability and dispersibility for instant properties (Aguilera and Lillford 2007; Huntington 2004).

Special attention should be accorded to:

- x the drying temperature and time as it can lead to different component initial state (eg, level of initial protein lactosylation)
- x the size of the droplet as it determines the particle size and the powder handling
- x and the liquid concentrate properties (composition, concentration, viscosity...)

Playing on these parameters will eventually modify the spray dried powder particle properties, namely the component distribution, the component native state and thus will affect the molecular mobility which is related to the interactions occurring during storage.

Powder composition. Whey protein powders are more and more used in infant formulas, beverages and health-care products. However, the initial composition of the powders involved in the formulation of these food products differs, leading very likely to another ageing path of the powders. The first interesting point to study would be the addition of purified phospholipid fractions in whey protein isolate powders and study the storage-induced changes as we did in our PhD project. Blending new components like lipids will engender new interactions and potentially lead to the production of new molecules upon storage, some of which having already been highlighted (Almansa et al. 2013; Angulo et al. 1998; Cesa et al. 2015). However, a possible two-way relationship might also be possible between the presence of lipids and the Maillard reaction products as we know that some of the latter have anti-oxidant properties. This hasn't been studied yet.

Nutritional & health aspects. The Maillard reaction has been observed in the stored whey protein powders. Beside the fact that this reaction produced odorous and brown compounds, which are desired by the food industry for appealing and palatability of foods, the nutritional and health aspects of these same compounds still has to be clearly determined: for example the melanoidins (brown

compounds) are given antioxidant (Cheriot 2007) and prebiotic properties (Tuohy et al. 2006). However, their effect on the intestinal microbiota remains uncertain to date. On the other hand, a nutritional loss is observed by the blocking of lysine residues, which results in an unavailability during digestion. Proteins can be further modified into other compounds whose digestion is still little known. In these are found AGEs that are particularly undesirable. They were originally discovered *in vivo* and involved in the complications of diabetes and renal failure (Basta 2004; Lalla et al. 2001; Uribarri et al. 2003). These compounds are found in food at varying levels. Their potentially deleterious health effects have led American researchers to rename them as glycotoxins. In summary, the scientific data on the biological effects (beneficial or deleterious) of AGEs and other Maillard products are insufficient to definitively conclude. A collaboration with a group of research specialised in this field could be considered in the future (*e.g.* Pr. F. Tessier, Université Lille-2/INSERM U995-LIRIC).

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RÉSUMÉ ABSTRACT

Modifications moléculaires et fonctionnelles au cours du vieillissement des poudres d'isolats de protéines solubles

Lors de la production des poudres protéiques laitières, des précautions sont prises pour assurer des fonctions technologiques optimales selon leurs utilisations. Cependant, des évolutions structurales et fonctionnelles surviennent lors du stockage. Ce projet vise ainsi à comprendre les mécanismes de modification qui interviennent lors du stockage des poudres d'isolat de protéines solubles du lait (WPI). Pour ce faire, des poudres de WPI ont été stockées en conditions contrôlées (15 mois à 20 °C, 40 °C et 60 °C), et leurs évolutions structurales et fonctionnelles après réhydratation ont été suivies expérimentalement à intervalles réguliers. Les résultats montrent que le vieillissement suit une trajectoire définie impliquant d'abord la lactosylation d'une partie des protéines, puis leur agrégation à l'état sec. De plus, cette trajectoire est caractérisée par un phénomène de rattrapage des modifications observées aux basses températures vers les plus hautes. L'impact des évolutions structurales sur les propriétés fonctionnelles est contrasté : les résultats montrent que les propriétés moussantes et interfaciales sont peu affectées, alors que les propriétés d'agrégation thermo-induites sont grandement modifiées. Pour minimiser ces modifications, deux pistes d'amélioration ont été suivies : 1. la granulométrie des poudres et 2. la teneur en lactose résiduel. La différence de granulométrie n'a pas eu d'influence sur le vieillissement alors que la diminution de la teneur en lactose a permis de limiter significativement l'étendue des modifications induites lors du stockage.

Mots-clés : poudre d'isolat de protéines solubles, poudre, stockage, structures protéiques, propriétés fonctionnelles

Molecular and functional changes occurring during ageing of whey protein isolate powders

During dairy powder manufacture, precautionary measures are taken to ensure optimal technological functionalities regarding their use requirements. However, changes in structural and functional properties appear during storage. This project aimed to understand the mechanisms of changes that occur during storage of whey protein isolate (WPI) powders. To do this, WPI powders were stored under controlled conditions (20 °C, 40 °C and 60 °C for 15 months), and their structural and functional changes after rehydration were experimentally monitored at regular intervals. The results showed that ageing follows a specific path involving first protein lactosylation and then their aggregation in the dry state. In addition, this path was characterized by a catching up behaviour from the changes obtained at lower temperatures to those at highest temperatures. The impact of these structural changes on the functional properties was mixed: the results showed that the foaming and interfacial properties were only slightly affected, while the heat-induced aggregation properties were greatly modified. To minimize these storage-induced changes, several areas for improvement were followed, playing on either the powder particle size, or on lactose content whose role appeared to be crucial for the development of the Maillard reaction. The study showed that the difference in powder particle size had no influence on their ageing path while lowering the lactose concentration allowed to significantly reduce the extent of the storage-induced changes.

Keywords: whey protein isolate powder, storage, protein structures, functional properties



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