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Par

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Evolution des propriétés structurales et fonctionnelles des poudres de PPCN en lien avec l'état initial et les conditions de vieillissement

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Soutenue le 19 mai 2017

Jury

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Liste des communications

Articles

Nasser S, Moreau A, Hédoux A, Jeantet R, Delaplace G. **Influence of storage conditions on the functional properties of micellar casein powder**. Soumis à *LWT-Food Science and Technology*, 2017

Nasser S, Hédoux A, Giuliani A, Le-Floch-Fouéré C, Santé-Lhoutellier V, de Waele I, Delaplace G. **Investigation of secondary structure evolution of micellar caseins powder upon ageing by FTIR and SRCD: Consequences on solubility**. Soumis à *Journal of the Science of Food and Agriculture;* 2017

Nasser S, Jeantet R, De-Sa-Peixoto P, Ronse G, Nuns N, Pourpoint F, Burgain J, Gaiani C, Hédoux A, Delaplace G. **Microstructure evolution of micellar casein powder upon ageing : Consequences on rehydration dynamics**. Accepté dans *Journal of Food Engineering*, 2017

Nasser S, Moreau A, Croguennec T, Tessier J F, Bray F, Rolando C, Hédoux A, Delaplace G . **Storage of micellar casein powder with and without lactose : Consequences on color, solubility and chemical modifications**. Soumis à *Journal of Agricultural and Food chemistry*, 2017

Revue

Norwood, E.-A., 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**. Soumis à *Food Engineers Review;* 2017

Communications par affiche

Nasser S, Moreau A, Hédoux A, Jeantet R, Delaplace G. **Evolutions of functions of Native Phospho Casein (NPC) powder during storage**, International Dairy Federation Word Dairy Summit, 20-24 september 2015, Vilnius, Lituanie**. Second best poster award**

Communication orale

Nasser S, Hédoux A, Giuliani A, Le-Floch-Fouéré C, Santé-Lhoutellier V, de Waele I, Delaplace G. **Evolution of secondary structure of milk powder during storage : Multivariate analysis FTIR and Circular Dichroism**, International Conference on Food Chemistry and Hydrocolloids, 8-10 august 2016, Toronto, Canada

Nasser S, Bray F, Rolando C, Delaplace G. **Ageing of powdered milk: comparison of bottom-up & top-down proteomic approaches**, Meeting réseau National de Spectrométrie de Masse FT-ICR à Haut Champ CNRS, 15 september 2016, Metz, France

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Abréviations

Français

2-DE : Electrophorèse bidimensionnelle

α-lb : Alpha lactalbumine

α_{s1}-caséine : Alpha caséine 1

α_{s2}-caséine : Alpha caséine 2

β-caséine : Béta caséine

β-lg : Béta-lactoglobuline

κ caséine : Kappa caséine

ACP : Analyse des composantes principales

CCP : Phosphates de calcium colloïdaux

CLSM : Chromatographie liquide couplée à un spectromètre de masse

CNIEL : Centre National Interprofessionnel de l'Economie Laitière

DCRS : Dichroïsme circulaire à rayonnement Synchrotron

DHA : Dehydroalanine

Electrophorèse SDS Page : Electrophorèse en gel de polyacrylamide contenant du dodécysulfate de sodium

FAO : Organisation des Nations Unies pour l'alimentation et l'agriculture *(Food and Agriculture Organization)*

HMF : Hydroxyméthylfurfural

Hr : Humidité relative

IB : Indice de brunissement

IDELE : Institut de l'élevage

IRTF : Infrarouge à transformée de Fourier (IRTF)

LAL : Lysinoalanine

MEB : Microscopie électronique à balayage

MPC : Concentré de protéines de lait

PPCN : Phosphocaséinates Natif

PRM : Produits de la réaction de Maillard

RM : Réaction de Maillard

RMN : Résonance magnétique nucléaire

ToF-SIMS : Spectrométrie de masse des ions secondaires à temps de vol *(Time-of-Flight Secondary Ion Mass Spectrometry)*

Tg : Transition vitreuse

UE : Union Européenne

USDA : Département de l'Agriculture des États-Unis *(United States Department of Agriculture)*

XPS : Spectrométrie photoélectronique à rayons X *(X-ray photoelectron spectrometry)*

WPI : Isolat de protéines sérique *(Whey protein isolate)*

Anglais

2-DE : Two dimensional gel electrophoresis

σ : Soluble material

α_{s1}-csn : Alpha casein 1

α_{s2}-csn : Alpha casein 2

β-csn : Beta casein

κ-csn : Kappa casein

ATR : Attenuated total reflection

BI : Browning index

DHA : Dehydroalanine

FDR : False discovery rate

FTIR : Fourier transform infrared

HMW : High molecular weight

HMF : Hydroxymethylfurfural

HPLC : High Performance Liquid Chromatography

LAL : Lysinoalanine

LCMS : Liquid Chromatography Mass spectrometry

LFQ : Label Free quantification

MC : Micellar casein

MPC : Milk protein concentrate

MR : Maillard Reaction

MRP : Maillard reaction products

MS : Mass spectrometry

NMR : Nuclear magnetic resonance

NR : Non reducing

PC : Principal components

PCA : Principal component analysis

PSD : Particle size distribution

R : Reducing

SEM : Scanning Electron Microscopy

SRCD : Synchrotron Radiation Circular Dichroism (SRCD)

Tricine SDS Page : Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis

Tof-SIMS : Time-of-Flight Secondary Ion Mass Spectrometry

UF : Ultrafiltration

XPS : X-ray photoelectron spectroscopy

Introduction générale

Aujourd'hui, plus de 800 millions de tonnes de lait sont produits annuellement dans le monde, dont 80 % concerne le lait de vache. Malheureusement, la production mondiale n'est pas uniforme par continent et par pays. Bien que certaines nations réalisent un excédent de production, d'autres présentent un déficit important (CNIEL, 2016). La transformation du lait en poudre a permis aux industriels, collectant le lait frais, de gérer leurs excédents en les stockant et/ou en les exportant.

Aujourd'hui, grâce aux avancées des opérations unitaires de séparation (filtration par membranes notamment) et de séchage (séchage par atomisation par exemple), des poudres de protéines de lait issues de différentes fractions du lait peuvent être obtenues. Ces poudres sont constituées de caséines, de protéines de lactosérum, de lactose, de matières grasses et de minéraux dans diverses proportions, selon les techniques séparatives utilisées en amont de l'opération de séchage par atomisation (Mistry, 2002). Ces poudres jouent un rôle fondamental dans la formulation de nombreux produits alimentaires et sont considérées comme des ingrédients fonctionnels à part entière, apportant des pouvoirs moussant, émulsifiant ou encore texturant (Singh et al., 2000).

Inévitablement, les poudres de protéines de lait ont besoin d'être réhydratées avant utilisation. C'est pourquoi, l'aptitude à la réhydratation est une de leur propriété d'usage majeure. Dans l'idéal, cette réhydratation se doit d'être rapide et complète (sans insolubles). Suivant la composition et les procédés de déshydratation subis, la cinétique de réhydratation des poudres peut s'avérer très longue (Bouvier et al., 2013; McKenna, 2000; Singh, 2007).

De plus, il a été démontré qu'au cours du stockage/transport, les propriétés de réhydratation des poudres de protéines de lait pouvaient être sévèrement altérées (en

partie à cause de la formation d'insolubles, au cours du vieillissement, difficiles à réhydrater), et ce d'autant plus que la teneur en caséine de la poudre est élevée (Anema et al., 2006; Havea, 2006; McKenna, 2000; Mimouni et al., 2010a; Schokker et al., 2011).

Comprendre les changements de structure au sein des grains de poudres à l'origine des évolutions des propriétés fonctionnelles des poudres de protéines de lait représente un enjeu important pour la filière des transformateurs du lait.

C'est dans ce contexte que le Centre National Interprofessionnel de l'Économie laitière (CNIEL) a lancé et financé un programme de recherche (dont l'acronyme a pour intitulé CODE POUDRE) impliquant 3 entités de recherche : l'UMR Science et la Technologie du Lait et des Œufs (SLTLO- UMR 1253) basée à Rennes, le laboratoire d'Ingénierie des Molécules (LiBio) de l'Université de Lorraine et l'UMR Unité Matériau et Transformation (UMET- UMR 8207) de l'université de Lille 1.

Les objectifs scientifiques de ce programme de recherche collectif CODE POUDRE ont été de décrypter les mécanismes impliqués dans la déstabilisation de deux poudres de protéines de lait au cours du stockage. Ces deux poudres (nommées WPI - Whey Protein Isolate et PPCN - PhosPhoCaséinate Natif) se différencient essentiellement par la nature et la composition de leurs assemblages protéiques. Les WPI sont composés principalement de protéines sériques et les PPCN essentiellement de caséines. Ces 2 poudres de protéines laitières ont toutes deux été obtenues après séchage par atomisation (sur la plateforme BIONOV, STLO, Rennes) et vieillies dans des conditions maitrisées.

Trois ressources humaines contractuelles (2 thèses et 1 Post doctorat) ont été recrutées pour réaliser le programme CODE POUDRE comme le montre la **Figure 1**.

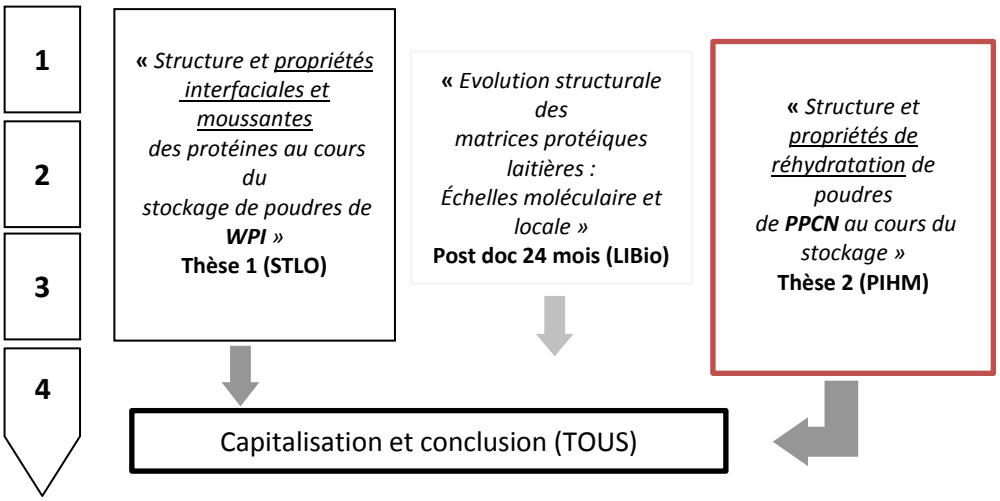


Figure 1: Articulation du programme CODE POUDRE

Le post-doctorat de Jenifer BURGAIN au LiBio a été consacré à du développement méthodologique transversal sur la caractérisation des évolutions structurales de matrices protéiques laitières lors du stockage.

Chacune des 2 thèses (celles d'Eve-Anne NORWOOD-STLO et de Sarah NASSER-UMET) a été centrée sur l'étude du vieillissement d'une poudre protéique de lait donnée (respectivement WPI et PPCN).

Les objectifs scientifiques communs de ces thèses étaient:

- d'évaluer l'influence couplée des conditions d'élaboration et de vieillissement des poudres sur certaines propriétés fonctionnelles ;

- d'identifier des marqueurs et méthodes d'analyses de modifications structurelles des poudres laitières (migration de composants, changement de conformation, réarrangements moléculaires..) susceptibles d'être à l'origine d'évolution de propriétés fonctionnelles.

Un des objectifs technologiques de ce programme CODE POUDRE est de mieux cerner des conditions de vieillissement pour lesquelles les propriétés fonctionnelles des poudres restent acceptables au regard de leur usage.

Le manuscrit de thèse présenté ici est consacré à l'étude de poudres de PPCN et les évolutions de structures/fonctions au cours du stockage. Ces poudres dont la

complexité structurale est unanimement reconnue, ont jusqu'alors été relativement peu étudiées et leurs comportements au vieillissement ont été très peu explorés. Ce projet de thèse s'est déroulé sur le site du CERTIA de l'UMR UMET, basé à Villeneuve d'Ascq et a impliqué deux équipes de l'UMR UMET : Processus aux Interfaces et Hygiène des Matériaux (PIHM) et Matériaux Moléculaire et Thérapeutiques (MMT). Les encadrants étaient Guillaume DELAPLACE, Directeur de recherche INRA et responsable de l'équipe PIHM et Alain HEDOUX, Professeur à l'Université de Lille 1 et responsable de l'équipe MMT.

De nombreuses collaborations ont été nouées au cours de ce projet de thèse avec des unités de recherche et plateformes (autres que celles précédemment citées) pour apporter une aide dans les caractérisations structurales multi-échelles de la poudre de PPCN et leurs expertises dans l'interprétation les données. On peut citer i) l'Unité de Catalyse et Chimie du Solide, (UCCS - UMR 8181) de l'Université de Lille 1 pour les analyses ToF-SIMS et RMN ii) la plateforme « Miniaturisation pour la Synthèse, l'Analyse et la Protéomique » (USR Lille1/CNRS n°3290) de l'Université de Lille 1 et l'UMR Lille Inflammation Research International Center, (LIRIC UMR 995) de l'Université de Lille 2 pour les analyses et interprétations de Spectrométrie de Masse iii) le Synchrotron SOLEIL de Gif-sur-Yvette pour les analyses de Dichroïsme Circulaire iv) le Laboratoire de Spectrochimie Infrarouge et Raman, (LASIR UMR 8516) de l'Université de Lille 1 et l'Unité «Qualité des Produits Animaux » (QuaPA,UR370) de Clermont-Ferrand-Theix, pour les analyses de spectroscopie Infrarouge et le traitement statistiques des données

Le manuscrit présenté est une thèse sur articles. Il a pour but d'être une contribution utile pour mieux cerner les évolutions de propriétés fonctionnelles et structurelles subies par les poudres de PPCN au cours du stockage, et de proposer des corrélations structure/fonction décrivant ces évolutions.

Le manuscrit de thèse est divisé en 3 parties :

La première partie est une bibliographie. Le contexte socio-économique laitier est abordé dans un premier temps. Dans un second temps, une présentation des poudres de protéines de lait, dont les PPCN, est proposée. Leurs itinéraires de productions et leurs applications en industrie, sont brièvement décrits. Enfin, les connaissances actuelles sur l'influence du stockage sur les poudres de protéines de lait sont présentées. Signalons que certains éléments de cette partie bibliographique ont été repris pour alimenter un article de revue en préparation sur les poudres de WPI et PPCN. Une version intermédiaire de ce manuscrit, prochainement soumis dans « Food Engineers Review» est proposée en Annexe 1.

La deuxième partie regroupe les résultats expérimentaux obtenus et interprétations proposées au cours de ce projet de thèse. Cette partie est divisée en 4 chapitres correspondant aux 4 articles soumis dans différents journaux à Comité de Lecture sur le temps du projet.

Le chapitre 1 présente les évolutions fonctionnelles de deux lots de poudres de PPCN lors d'un stockage. Premièrement, l'analyse des résultats expérimentaux examine la possibilité de corréler le temps de réhydratation et les trois indicateurs suivants: couleur, solubilité et temps de fragmentation. Dans un second temps, la possibilité d'appliquer des conditions de vieillissement accélérées couplées à un indicateur pour prévoir rapidement l'évolution de propriétés fonctionnelles de poudres de PPCN est évaluée. Enfin, les résultats obtenus pour des vieillissements réalisés à température élevée et à température ambiante ont été comparés afin d'identifier des correspondances temps-température. L'objectif de cet article, en évaluation dans *LWT - Food Science and Technology*, est d'identifier le comportement au vieillissement (couleur et aptitude à la réhydratation) de poudres de PPCN lors du stockage.

Les chapitres suivants sont consacrés à l'identification des mécanismes (migration de composants, évolution structure secondaire, réarrangements moléculaires..) pouvant être à l'origine des évolutions de propriétés fonctionnelles détaillées au chapitre 1.

Le chapitre 2 se focalise sur l'étude des évolutions de structures secondaires des PPCN lors d'un stockage. Pour cela, des méthodes de spectroscopie infrarouge à transformée de Fourrier et des techniques de dichroïsme circulaire ont été mises en œuvres. L'objectif est d'évaluer si un lien existe entre les évolutions de structures secondaires observées et l'altération des propriétés de réhydratation de la poudre de PPCN. Cet article est en évaluation dans *Journal of the Science of Food and Agriculture*.

Le chapitre 3 étudie l'évolution de la composition observée en surface des grains de poudres de PPCN lors du stockage. L'évolution de la structure de la surface des grains au cours de la réhydratation pour des poudres vieillies dans différentes conditions est également présentée. L'objectif de ce chapitre est d'illustrer clairement le lien entre ces évolutions de propriétés de surface des grains et l'augmentation du temps de réhydratation total. Cet article a été récemment accepté dans *Journal of Food Engineering*.

Enfin, le chapitre 4 étudie l'évolution des propriétés fonctionnelles et des liaisons chimiques de deux poudres de PPCN dont la teneur en lactose diffère. L'objectif est d'évaluer si le lactose est l'unique composé responsable de l'insolubilisation des poudres de PPCN lors d'un stockage et d'alimenter par cette étude expérimentale la réflexion sur les mécanismes d'insolubilisation. Cet article est en évaluation dans *Journal of Agricultural and Food chemistry*.

Afin de simplifier la lecture du manuscrit et d'éviter les redondances, nous avons fait le choix de ne pas exposer le matériel et méthode propre à chaque article dans une partie spécifique.

Enfin, la troisième et dernière partie du manuscrit est une conclusion générale, qui dresse un bilan des résultats et propose des perspectives de recherche future dans le domaine.

Partie 1. Etude bibliographique

Les deux premiers chapitres de cette étude bibliographique présentent le contexte économique et les perspectives actuelles du marché du lait et des poudres de protéines de lait. Le troisième chapitre de cette première partie est consacré à la présentation des poudres de PPCN étudiées lors de ce projet de thèse. Enfin, le dernier chapitre présente les altérations structurelles et fonctionnelles relevées sur des poudres de protéines de lait lors de leur stockage.

Chapitre 1. Contexte socio-économique du marché du lait

1.1. Accroissement de la production mondiale de lait de 1983 à 2025

Au cours des trois dernières décennies, la production mondiale de lait a augmenté de plus de 50 %, passant de 500 millions de tonnes (m.t) en 1983 à 769 m.t en 2013 (FAO, 2016). L'union européenne (UE) est le plus grand producteur de lait au monde, suivie par l'Inde, les États-Unis d'Amérique, la Chine, le Pakistan la Russie et le Brésil (**Figure 2**).

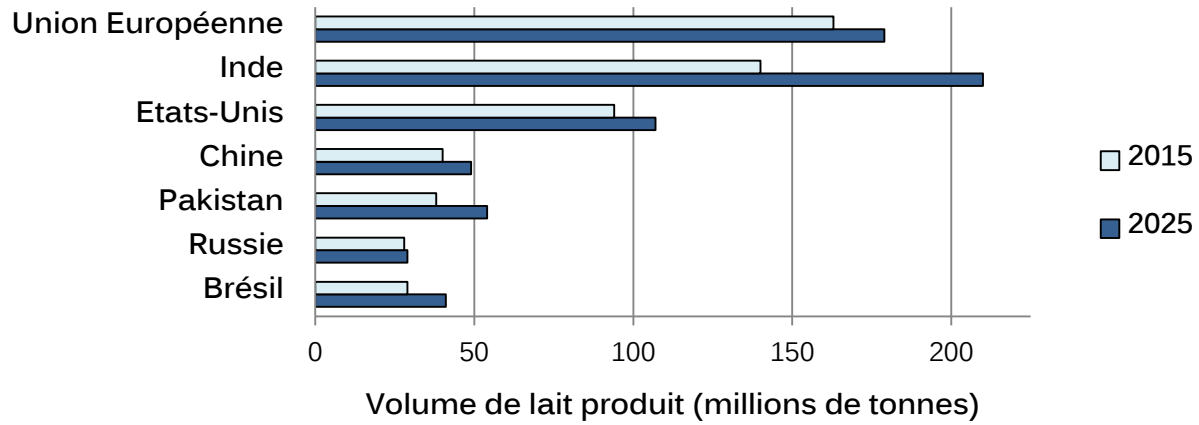


Figure 2: Evolution de la production de lait des principaux producteurs de lait entre 2013 et 2025 (OECD-FAO, 2016)

A long terme, la production mondiale de lait devrait continuer d'augmenter de 177 m.t entre 2013 et 2025, soit +23 %. Cependant, cette croissance (1.9 % par année) serait légèrement plus faible que les années précédentes (2.7 % par année). Les pays concernés par la croissance la plus importante seraient les pays en développement comme l'Inde et le Pakistan, poussés par le prix des aliments faibles et l'augmentation des troupeaux laitiers (OECD-FAO, 2016; Sharma et al., 2012).

En 2015, la France était le deuxième producteur en Europe et 7ème dans le monde avec 25.3 m.t. de lait produit par an (CNIEL, 2016), et une augmentation de 11 % est prévue d'ici 2020 (IDELE, 2016), entre autre expliquée par la suppression des quotas laitiers en 2015.

1.2. Surplus de production mondiale de lait : La nécessité de stocker

Depuis 2001, comme l'illustre la **Figure 3**, la production et la consommation mondiale de produits laitiers sont en hausse (IDELE, 2016).

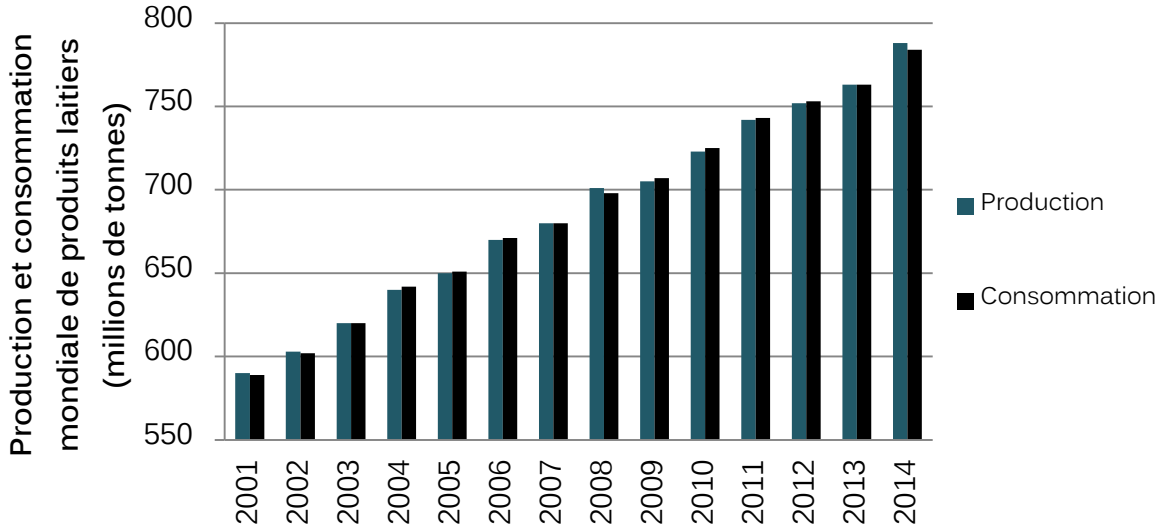


Figure 3 :Production et consommation mondiales de lait (IDELE, 2016)

L'augmentation des gammes de produits laitiers et l'occidentalisation des régimes alimentaires sont des facteurs clés expliquant l'évolution du marché des produits

laitiers dans le monde entier. En 2014, la baisse de la demande chinoise et l'embargo russe ont contribué à un inversement de la tendance, avec une production mondiale supérieure à la consommation. Pour faire face à cet excédent, les transformateurs européens et néozélandais ont dû augmenter leurs stocks de produits de report (poudre de lait, beurre, fromage) passant ainsi de 5 à 6 m.t. équivalent lait entre 2013 et 2014 (CNIEL, 2016).

1.3. Abondance ou carence en lait selon les pays : La nécessité d'exporter

Le ratio production/consommation diffère selon les pays. Il existe ainsi des pays excédentaires en lait, dont les principaux sont la Nouvelle-Zélande, les États-Unis d'Amérique, les pays de l'UE (dont la France), l'Australie et l'Argentine, comme le montre la **Figure 4**. En revanche des pays sont déficitaires comme la Chine, la Russie, le Mexique, l'Algérie et l'Indonésie.

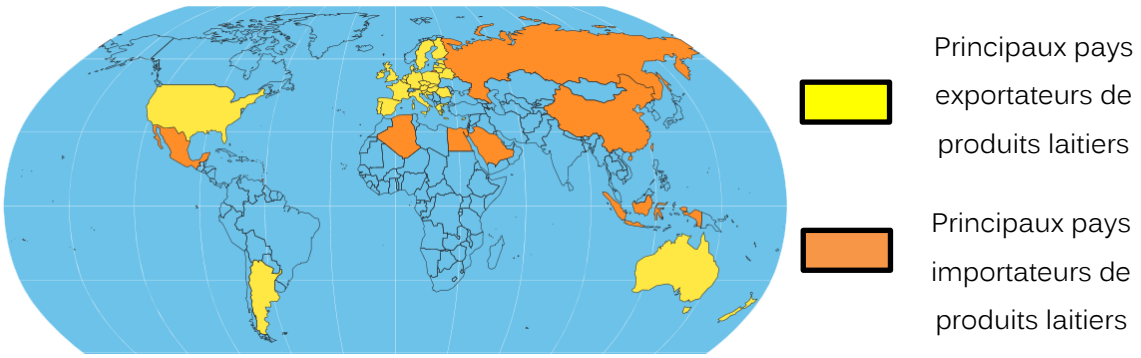


Figure 4 : Principaux pays exportateurs et importateurs de produits laitiers (FAO, 2015, 2016) .

En transformant le lait en poudre, les pays dont la production de lait est la plus élevée ont trouvé un moyen de préserver l'excédent de production de lait et peuvent envisager sereinement son exportation en limitant fortement les risques sanitaires.

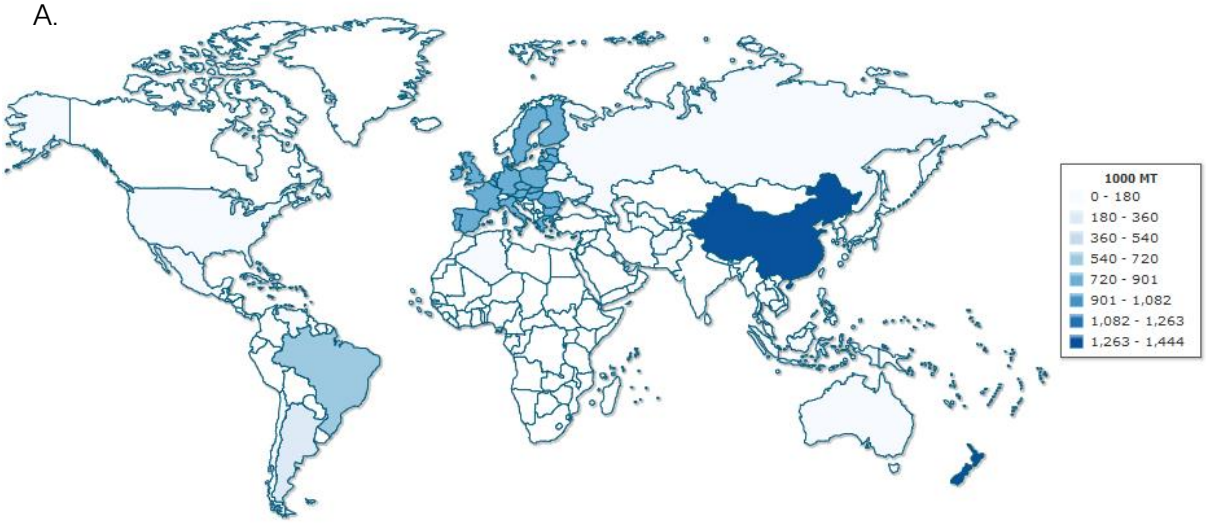
Les exportations et importations ont été encouragées par les politiques de libéralisation commerciale imposées par les pays industrialisés et émergents, et légitimées par les crises alimentaires et les problèmes nutritionnels des pays pauvres.

Les besoins d'exportation et de stockage du lait ont donc fait du séchage par atomisation, un procédé incontournable des industries laitières.

1.4. Production et exportation de la poudre de lait

En 2016, la chine (1400 m.t), la Nouvelle-Zélande (1350 m.t), l'union européenne (1200 m.t), le brésil (810 m.t) et l'argentine (630 m.t) sont respectivement les producteurs majeurs de poudre de lait (**Figure 5.A**). La Nouvelle-Zélande et les pays de l'UE ne consomment qu'une infime partie de leurs productions, tandis que les populations du Brésil et de la Chine consomment la quasi-totalité de leur production. De ce fait, le classement des pays exportant le plus de poudre de lait n'est pas une image fidèle du classement des pays producteurs. La Nouvelle-Zélande (1300 m.t), l'UE (1000 m.t) et l'Argentine (350 m.t) sont les 3 plus gros exportateurs, comme l'illustre la **Figure 5.B**.

A.



B.

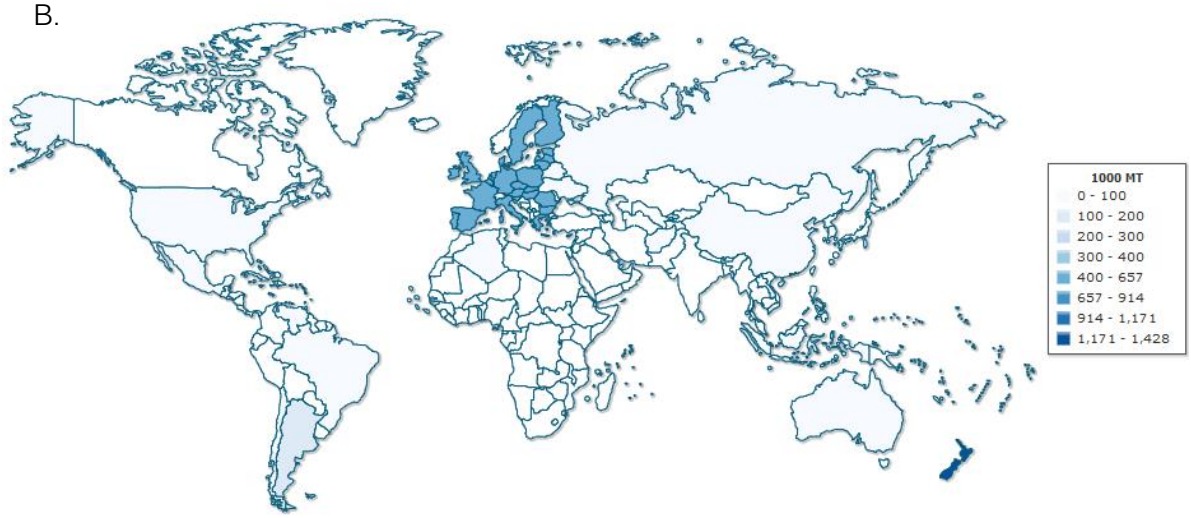


Figure 5.A (En haut): Production et B. (En bas) : Exportation de la poudre de lait en 2016 (USDA, 2016) .

Chapitre 2. Les poudres de protéines de lait : De leurs productions à leurs applications en industries

2.1. Séchage de concentrés laitiers : généralités et conséquences sur les poudres élaborées

L'objectif du séchage est d'améliorer la stabilité du lait frais mais également de faciliter son transport et son stockage (Jeantet et al., 2011). Le premier brevet sur le procédé de séchage du lait a été déposé par T.S. Grimwade en 1855 (Hunziker, 1920). Aujourd'hui, et depuis une trentaine d'année, le séchage par atomisation est le procédé

de séchage le plus couramment utilisé dans l'industrie laitière. Afin d'obtenir des particules fines de poudres (50 à 200 µm), trois étapes sont nécessaires : i) la pulvérisation du liquide pompé par la buse de l'atomiseur en fines gouttelettes ii) l'évaporation des gouttelettes d'eau par un air chaud et sec, au niveau de la chambre de séchage iii) l'entrainement de la poudre obtenue par un flux de chaleur jusqu'à un cyclone ou un filtre à manche afin de séparer l'air de la poudre (Refstrup, 2000; Schuck, 2002).

En faisant varier les paramètres du procédé tels que la pression ou la taille de la buse utilisée, il est possible d'obtenir différentes tailles, formes ou encore porosités de particules, et d'ainsi contrôler les propriétés du produit fini (Sadek et al., 2015).

Cependant, les paramètres du séchage, tels que la température de l'air dans laquelle le lait est pulvérisé, le degré de concentration et la température du concentré avant le séchage, la taille des gouttelettes de séchage et la température du mélange air / poudre sortant (température de l'air à la sortie), peuvent être responsables de la dénaturation des protéines et d'agrégation (Fang et al., 2012; Singh, 2007; Singh and Creamer, 1991). Ainsi, les modifications sur les ingrédients laitiers, le comportement au stockage et les propriétés fonctionnelles sont dépendants des conditions de séchage. Le défi est donc de conserver les propriétés physiques et fonctionnelles du concentré liquide à sécher.

2.2. Opérations unitaires mises en œuvre pour obtenir des poudres de protéines de lait aux propriétés désirées

Initialement, la transformation du lait en poudre avait pour unique objectif de stabiliser l'excédent provenant de l'industrie laitière, afin de permettre le stockage. Considérées comme sous-produits, les poudres étaient peu valorisées et étaient principalement destinées à l'alimentation animale. Aujourd'hui, les techniques de séchage ont évolué

et ont permis une meilleure valorisation des poudres. De plus, l'émergence des techniques de fractionnement et de séparation (ultrafiltration tangentielle, microfiltration, osmose inverse et nanofiltration) a permis à l'industrie laitière d'obtenir des poudres de protéines de lait présentant des propriétés fonctionnelles avancées, comme la gélification, des propriétés moussantes ou émulsifiantes (Isleten and Karagul-Yuceer, 2006; Schuck, 2002; Selomulya and Fang, 2013).

Des ratios spécifiques entre protéines (la caséine et le lactosérum individuellement ou le ratio caséine / lactosérum souhaité), lipides, glucides (lactose) et minéraux (y compris addition d'ion spécifique) peuvent ainsi être définis préalablement afin d'obtenir les propriétés fonctionnelles souhaitées. Ainsi, il est désormais possible de produire à grande échelle des ingrédients alimentaires (Snappe et al., 2010), en satisfaisant la plupart des exigences fonctionnelles et nutritionnelles des consommateurs. La **Figure 6** illustre, à titre d'exemple, quelques opérations de séparation réalisées en amont du séchage par atomisation, couramment appliquées à grande échelle, pour obtenir différents types de poudres de protéines de lait à partir de lait entier.

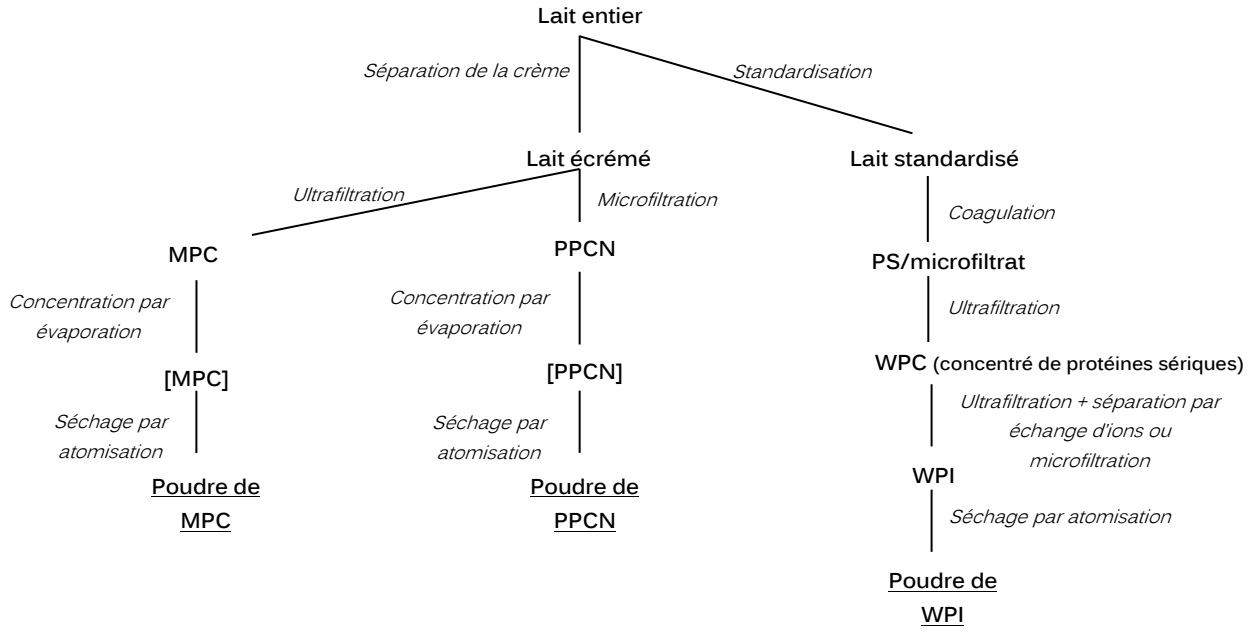


Figure 6 : Opérations nécessaires à l'obtention des poudres de MPC/PPCN/WPI .

La poudre de concentré de protéines de lait (MPC), est la poudre dont la production mondiale est la plus importante. La poudre de MPC contient entre 40 et 90 % de protéines et le ratio caséines / protéines sériques est quasiment identique au lait écrémé de départ. L'étape d'ultrafiltration du lait écrémé, permet d'obtenir un concentré de lait écrémé dont la teneur en lactose est réduit et la concentration en protéines totales augmentée (Schuck et al., 1998), tout en conservant la structure native du produit (Kelly, 2006; Schuck et al., 1998).

Les poudres d'isolat de protéines sériques (WPI) sont des poudres dont la concentration en protéines sériques est supérieure à 90 %.

Les poudres de phosphocaséinate natif (PPCN) sont des poudres dont la quasi-totalité des protéines sériques, du lactose et des minéraux du retentât est éliminée afin de concentrer les micelles de caséines (MC) (Schuck et al., 1994a).

Notons que les poudres de WPI sont les 2 poudres du programme CODE POUDRE

2.3. Secteur d'utilisation et propriétés fonctionnelles apportées par des poudres de protéines de lait

Les poudres de WPI et de PPCN sont utilisées pour différentes applications. Les principales applications sont listées dans le **Tableau 1** (Huppertz and Patel, 2013; Mistry, 2002)

Poudres de WPI	Poudres de PPCN
- Alimentation animale - Produit laitier frais - Produit diététique	- Usage non alimentaire (peinture, glue) - Fromage fondu - Produit laitier frais - Viande cuisine - Boisson chaude

Tableau 1. Principales applications des poudres de WPI et de PPCN

Les principales propriétés fonctionnelles apportées par les PPCN, les WPI et les MPC sont résumées **Tableau 2** (Sharma et al., 2012; Singh, 2002; Singh et al., 2000).

Poudres de PPCN	Poudre de WPI	Poudre de MPC
- Emulsifiant (Améliore apparence du produit final) - Moussant (Maintient propriétés moussantes + améliore visuel, goût et texture du produit final) - Viscosité (Améliore texture du produit final) - Stabilité à la chaleur (Permet aux protéines de résister aux traitements à haute température sans précipiter)	- Gélatineux (Améliore sensation en bouche + aide à obtenir texture crémeuse et lisse) - Moussant	- Solubilité (Bonne dispersion des poudres + empêche la sédimentation dans les boissons, les soupes et les sauces) - Stabilité à la chaleur - Coagulation présure (Temps de coagulation plus rapides et forces de gel plus élevées)

Tableau 2 : Propriétés fonctionnelles apportées par les poudres de WPI, de PPCN et MPC

Mentionnons qu'il a été établi par de nombreux auteurs que les propriétés fonctionnelles des protéines de lait variaient avec le pH, la température, la force ionique et la concentration de calcium et d'autres ions polyvalents, des sucres et des hydrocolloïdes (Augustin, 2000; Dalgleish and Law, 1989; Dewit, 1984; Luo et al., 2016; Post et al., 2012; Udabage et al., 2000).

Chapitre 3. Composition et structure des poudres de phosphocaseinate natif (PPCN)

Les poudres étudiées dans ce projet de thèse sont des PPCN. Cette poudre de protéines de lait est composée de 90 % de caséines et 10 % de protéines sériques. Nous présentons donc succinctement les connaissances existantes sur ces 2 familles de protéines.

3.1 Composition des caséines du lait et les modèles d'organisation micellaire

Les caséines sont composées de quatre protéines primaires, la caséine alpha 1 (α_{s1}-caséine) (40 %), la caséine béta (β-caséine) (35 %), la caséine kappa (κ-caséine) (12 %), la caséine alpha 2 (α_{s2}-caséine) (10 %) et la caséine gamma (3 à 7 %) (Payens and Vreeman, 1982). Les caséines présentent comme caractéristique essentielle le fait de précipiter à pH 4,6 (McMahon and Brown, 1984).

Le **tableau 3** présente la composition de ces 4 caséines.

Protéine	Acide aminés	Masse moléculaire (KDa)	Résidus de proline	Résidus de phosphosérine	Ponts disulfures
α_{s1}-caséine	199	23.164	17	8-9	0
α_{s2}-caséine	207	25.388	10	10-13	1
β-caséine	209	23.983	35	5	0
κ-caséine	169	19.038	20	1-2	1-2

Tableau 3 : Composition des caséines (Broyard and Gaucheron, 2015; Farrell et al., 2004)

95 % des caséines natives se trouvent à l'état naturel sous forme de structures associées et stabilisées, appelée micelles de caséine (MC) et d'une taille moyenne de 150 nm. Les micelles sont composées à 94 % de protéines et de 6 % de phosphates de calcium colloïdaux (CCP) et sont stabilisées par des liaisons hydrogènes (Gaucheron, 2005; Walstra, 1990). La présence élevée de proline (répartie de façon homogène le long des séquences de protéines), de résidus de phosphosérine et la faible présence de ponts disulfures, explique le peu de conformations sous forme d'hélices α ou de feuillets β des caséines.

La structure ouverte et flexible des caséines favorise l'exposition des régions hydrophobes des acides aminés, augmentant ainsi l'hydrophobie de surface (Griffiths, 2010). Cette conformation, résultant d'attractions hydrophobes et de répulsions électrostatiques, permet aux caséines de conserver leur stabilité face à la chaleur. Cependant certaines caséines sont sensibles à la teneur en calcium. En effet, augmenter la teneur de ce dernier peut entrainer la précipitation des caséines (Considine and Flanagan, 2009). α_{s2}-caséine est la caséine la plus sensible à la présence du calcium, cela s'explique par la présence importante de groupes phosphate, capables de se lier au calcium. Au contraire, κ-caséine, dont la teneur en groupes phosphate est la plus faible, n'est pas sensible à la teneur en calcium (Ginger and Grigor, 1999).

Les différences entre les caséines résultent ainsi principalement de leur structure primaire; via la répartition des zones hydrophiles et hydrophobes, du nombre de sites phosphorylés, de leur sensibilité au calcium et de leur capacité à former des ponts disulfures (Payens, 1966; Payens and Vreeman, 1982).

En dépit de la grande quantité de recherches menées et bien qu'il y ait un consensus entre les modèles sur l'emplacement en surface de la κ-caséine, il n'existe toujours pas de modèle définissant clairement la structure interne des MC. Plusieurs modèles ont été proposés reposant sur les propriétés et le comportement des caséines individuelles et des MC, les plus reconnus sont présentés ci-dessous.

- **Modèle structure ouverte**

Ce premier modèle, **Figure 7**, démontrait que les κ-caséines recouvraient la surface des micelles et que la composition interne était différente de la surface (Waugh, 1958).

Ce modèle primaire a été abandonné car il ne prenait pas en compte les CCP qui jouent un grand rôle dans la structure et la stabilité des micelles (de Kruif et al., 2012).

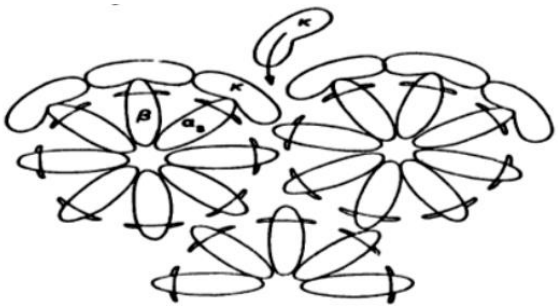


Figure 7 : Modèle de la micelle de caséine proposé par Waugh's, issu de Wong., (1988)

- **Modèle submicellaire**

D'après le modèle submicellaire, les α_{s1}-caséines, α_{s2}-caséines et β-caséines polymériseraient via des interactions hydrophobes et des liaisons hydrogènes pour former des submicelles. Les submicelles seraient reliées entre elles via des liaisons entre les α_{s1}-caséines, α_{s2}-caséines, β-caséines et les CCP. Enfin, les κ-caséines seraient réparties de façon hétérogène au niveau des couches extérieures de la submicelle.

Selon leur position dans la micelle, différents types de submicelles se distinguent. Les submicelles formées d' α_{s1}-caséine et de β-caséine reliées par des interactions hydrophobes sont localisées à l'intérieur de la micelle. Tandis que les submicelles constituées d' α_{s1}-caséine et de κ-caséine et de taille plus importante sont situées en surface.

La **Figure 8** illustre un modèle proposé par Schmidt (1982).

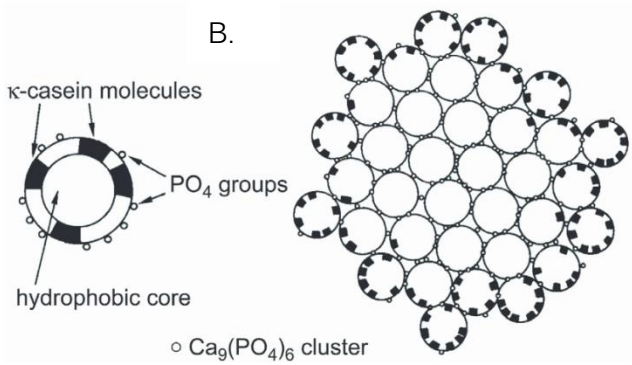


Figure 8. A. (A gauche) : Submicelle de caséine et B. (A droite) : Micelle de caséine composées de submicelles (Schmidt, 1982)

Le modèle submicellaire a été largement accepté et les rôles des CCP dans le maintien de la structure micellaire admis (de Kruif et al., 2012; Holt, 1992; Walstra and Jennes, 1984). Cependant d'autres modèles complémentaires ont été proposés.

- **Modèle de Holt**

Holt, (1992) décrit la micelle comme un réseau de gel minéralisé de protéines cimentées par les CCP, comme illustré à la **Figure 9**. Les clusters de phosphosérines réagissent avec les CCP afin de former des nanoclusters de 2-3 nm. En neutralisant les charges, les CCP permettent aux interactions hydrophobes de maintenir l'intégrité de la micelle.

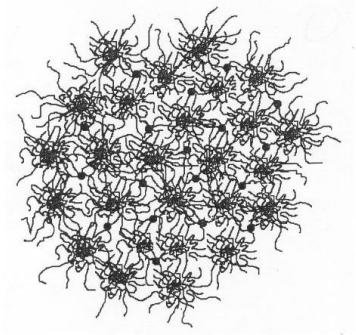


Figure 9 : Modèle de la micelle de caséine proposé par Holt., (1992).

Walstra., (1999) propose une version actualisée de ce modèle. Selon ce modèle, l'édifice micellaire serait constitué d'un assemblage de submicelles de 10 à 15 nm de diamètre.

Par la suite, un nouveau modèle de type « structure ouverte » a remis en cause la présence de submicelles : Le modèle de Horne (Horne, 1998).

- **Modèle de Horne**

Comme observé à la **Figure 10**, les chaines polypeptidiques seraient liées par les CCP. Les MC seraient liées entre elle via des interactions hydrophobes attractives (à l'origine de la formation des MC) et des interactions électrostatiques répulsives (limitant la polymérisation). l'α-caséine and la β-caséine interagiraient avec les CCP mais également entre eux via leurs sites hydrophobes. La κ-caséine ne possédant pas de site Ca^{2+} serait naturellement en bout de chaine, et constituerait la partie hydrophile exposée.

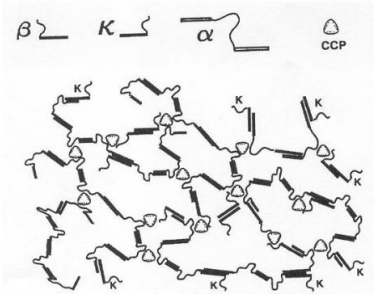


Figure 10 : Interactions au sein de la micelle de caséine, modèle proposé par Horne., (1998)

- **Modèle en éponge**

Un modèle plus récent suggère que la structure de la micelle de caséine suivrait un modèle « en éponge » séparé en 3 niveaux. Ce modèle est issu de l'étude de Bouchoux et al. (2010)(Cf. **Figure 11**), observant une séparation de la structure en différents niveaux hétérogènes. La structure est partagée entre régions denses et

« molles » lors de la compression d'une micelle de caséine suivie par diffusion des rayons X aux petits angles.

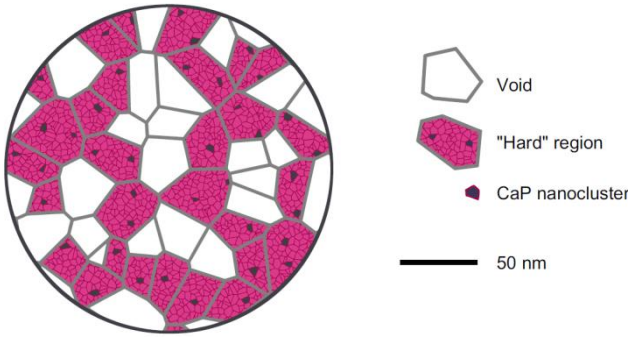


Figure 11 : Modèle de la micelle de caséine proposé par Bouchoux et al., (2010)

Bouchoux et al., (2010) décrivent les MC comme un assemblage de colloïdes, principalement reliés par des interactions hydrophobes. Ces interactions concernent, soit deux régions hydrophobes de différentes molécules de caséine, soit les nanoclusters de phosphate de calcium et les résidus phosphorylés des molécules de caséine. Les κ-caséines, ne pouvant se lier que par interactions hydrophobes, termineraient la croissance des micelles.

Pour conclure, la présence de κ-caséines en surface et le rôle des CCP dans le maintien de la micelle de caséine, sont des éléments de structure partagés par l'ensemble des récents modèles présentés dans cette partie.

3.2. Composition et structure des protéines sériques

Les protéines du lait restantes après précipitation isoélectrique des caséines, sont des protéines sériques. Elles sont principalement constituées de béta-lactoglobuline (β-lg) et d'alpha-lactalbumine (α-lb), mais contiennent également des immunoglobulines, du sérum albumine bovine, de la lactoferrine et des enzymes (lipases, protéases, etc.) (Fox and Brodkorb, 2008). Le **tableau 4** présente la composition de la β-lg et de l'α-lb.

Protéine	Acide aminés	Masse moléculaire (KDa)	Résidus de proline	Résidus de phosphosérine	Ponts disulfures
β-lg	162	18.277	8	0	2
α-lb	123	14.175	2	0	4

Tableau 4 : Composition de la β-lg et de l'α-lb (Broyard and Gaucheron, 2015; Farrell et al., 2004; Lucy et al., 2008)

Les protéines sériques ont une structure secondaire et tertiaire stable. Leur structure globulaire est stabilisée grâce à des ponts disulfures, des interactions hydrophobes, de van der Waal's et des liaisons hydrogènes (Singh and Havea, 2003).

Grâce à la présence de nombreux résidus hydrophiles à la surface de la structure globulaire et de nombreux ponts disulfures, les protéines sériques sont solubles (Dissanayake and Vasiljevic, 2009).

Au cours d'un traitement thermique, les protéines sériques se déplient et des ponts disulfures peuvent se former avec la κ-caséine (Donato and Guyomarc'H, 2009). Les protéines sériques sont donc des entités protéiques très importantes dans la détermination de la stabilité thermique des concentrés de protéines de lait (Havea et al., 2001) et donc des poudres de protéines de lait.

Les paragraphes suivant détaillent la composition et la structure des principales protéines sériques composant les WPI : la β-lg et l'α-lb.

-Béta-lactoglobuline

La β-lg est une protéine globulaire, représentant 40 % des protéines du lactosérum, extrêmement stable en milieu acide. La β-lg s'associe en dimères en solution à l'état natif (structure quaternaire). A pH acide les dimères se dissocient en monomères (Sakurai and Goto, 2002).

C'est une protéine de 18 kDa constituée de 162 résidus d'acides aminés, un groupe thiol libre et deux ponts disulfures. Son point isoélectrique est de 5,1 (Wong, 1988). La

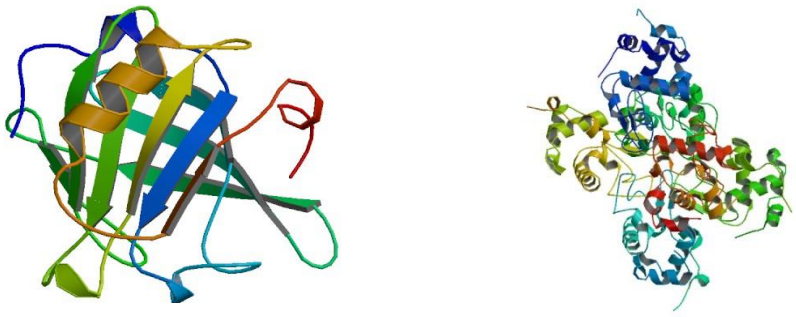
structure secondaire est constituée de 10 à 15 % de structure en hélice α, de 20 à 50% de feuillets β et de 30% de structure aléatoire.

Lors d'une dénaturation, la β-lg expose son groupe thiol libre et des résidus hydrophobes, ce qui conduit à la possibilité d'une variété de liaisons covalentes intermoléculaires et des associations hydrophobes, mais également des ponts disulfures avec les autres protéines sériques (Havea et al., 2001; Wijayanti et al., 2014).

-Alpha-lactalbumine

L'α-lb représentant 3 % des protéines du lactosérum. Elle comporte 123 acides aminés pour une masse de 14,3 kDa. Son point isoélectrique est de 4,8. Elle est majoritairement constituée d'hélice α et de quelques feuillets β, qui sont reliés par des ions calcium et 4 ponts disulfures (Permyakov and Berliner, 2000; Wijayanti et al., 2014; Wong, 1988). La protéine native est dénaturée vers 65 °C mais si le milieu est refroidi, il y a renaturation de 80 à 90 % de la protéine. Grâce à sa structure et la force des liaisons intermoléculaires, l'α-lb est la plus résistante des protéines sériques à la dénaturation thermique (Anema, 2014).

Les structures tridimensionnelles de la β-lg et de l'α-lb sont présentées à la **Figure 12**.



Béta-lactoglobuline Alpha-lactalbumine

Figure 12 : Structures tridimensionnelles de la β-lg (Brownlow et al., 1997) et de l'α-lb (Johnke and Petersen, 2012).

Chapitre 4. Influence du stockage sur les concentrés de poudres de lait.

4.1. Le stockage : une étape du cycle de vie des poudres longtemps sous étudié

Le séchage par atomisation est une opération unitaire utilisée depuis très longtemps pour augmenter la durée d'utilisation des produits laitiers (les périodes d'utilisation des poudres varient en général de 0,5 à 1 an après leur élaboration (Sharma et al., 2012).

Un autre avantage connu de l'élimination de l'eau est la réduction des volumes et masses des produits laitiers à transporter. Un facteur de réduction sur volume de 5 à 15 est en général apporté par l'opération de séchage par atomisation, ce qui permet de minimiser de façon très significative les coûts d'expédition.

Néanmoins, ce n'est que très récemment que les industriels se sont intéressés à identifier plus finement les altérations de propriétés caractéristiques des poudres lors du stockage, faisant face et anticipant une demande sociétale de plus en plus sévère dans ce domaine.

Ainsi des études scientifiques pionnières dans ce domaine datent uniquement des années 2000 (Anema et al., 2006; Burgain et al., 2016; Fyfe et al., 2011; Gaiani et al., 2007a; Gaiani et al., 2009; Gazi and Huppertz, 2015; Guyomarc'h et al., 2000; Haque et al., 2010; Haque et al., 2011, 2015; Haque et al., 2012; Kher et al., 2007; Le et al., 2011a; Le et al., 2012; Le et al., 2013; Mimouni et al., 2010a, b; Schokker et al., 2011; Thomas et al., 2004; Yazdanpanah and Langrish, 2013).

Les conditions de stockages étudiées ont majoritairement consisté à exposer la poudre à des températures (entre 4 et 50 °C) et humidités (entre 44 et 84 %) contrôlées pendant des durées plus ou moins longues (entre 10 jours et 10 mois) en régime permanent (ie les grandeurs physiques de température et d'humidité ont été maintenues constantes durant toute la durée du stockage).

Ces études, réalisées sur un ensemble de poudres aux compositions différentes, ont établi que des modifications structurelles (Burgain et al., 2016; Fyfe et al., 2011; Gaiani et al., 2009; Haque et al., 2010; Haque et al., 2012; Kher et al., 2007; Le et al., 2011a; Le et al., 2011b; Le et al., 2012; Mimouni et al., 2010a, b; Schokker et al., 2011) et fonctionnelles (Anema et al., 2006; Fyfe et al., 2011; Haque et al., 2012; Le et al., 2011b; Schokker et al., 2011; Thomas et al., 2004) évoluaient au cours du stockage. Comme attendu, il a été notifié que ces évolutions étaient d'autant plus marquées que les conditions de stockages étaient sévères.

Beaucoup de mécanismes décrivant les impacts structure/fonction restent cependant non élucidés : notamment les phénomènes à l'échelle moléculaire responsable de ces changements de propriétés fonctionnelles (migration de constituants, changement de conformation et réarrangements chimiques). Parallèlement, très peu de connaissances existent sur les marqueurs de vieillissement des poudres.

4.2. Conditions de température et humidité relative lors du stockage

Dans cette section, nous exposons les contraintes en températures et humidités relatives subies par les poudres de lait lors de leurs expéditions, ce qui permettra de mieux comprendre les conditions de stockages (températures et humidité) retenues pour les poudres de PPCN.

Sur la **Figure 13** apparait un relevé de températures réalisé lors d'une expédition de poudre laitière partant de la France jusqu'en Afrique (source confidentielle). Les données indiquent clairement que

i) l'acheminement des poudres est long et nécessite près de 3 mois

ii) des températures entre 45 et 50 °C peuvent être atteintes sur cette période

iii) la durée totale pour laquelle la température est au-dessus de 30°C est importante

iv) les accidents de stockage au-dessus de 45°C ne sont pas des événements isolés

On constate également que l'histoire en température est très éloignée d'une rampe idéale. En effet, l'histoire en température est une alternance jour/nuit influencée par l'ensoleillement et dépend fortement de l'enchainement des différents modes de transports (camion, stockage intermédiaire, bateau..).

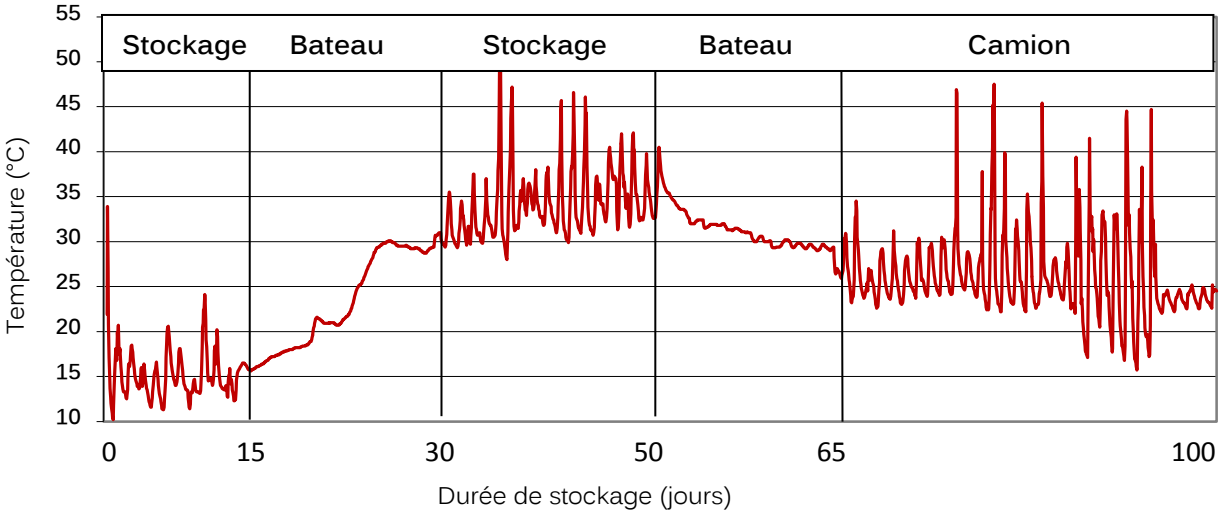


Figure 13 : Enregistrement des températures lors d'un transport de France en Afrique (Source confidentiel).

La **Figure 14**, issue d'une autre source et correspondant à un trajet différent, confirme que des températures extrêmes, proches de 55 °C sont couramment atteintes lors d'un transport inter-continent (Premtitikul, 2005). On observe également une variation de l'humidité relative entre 30 à 90 %.

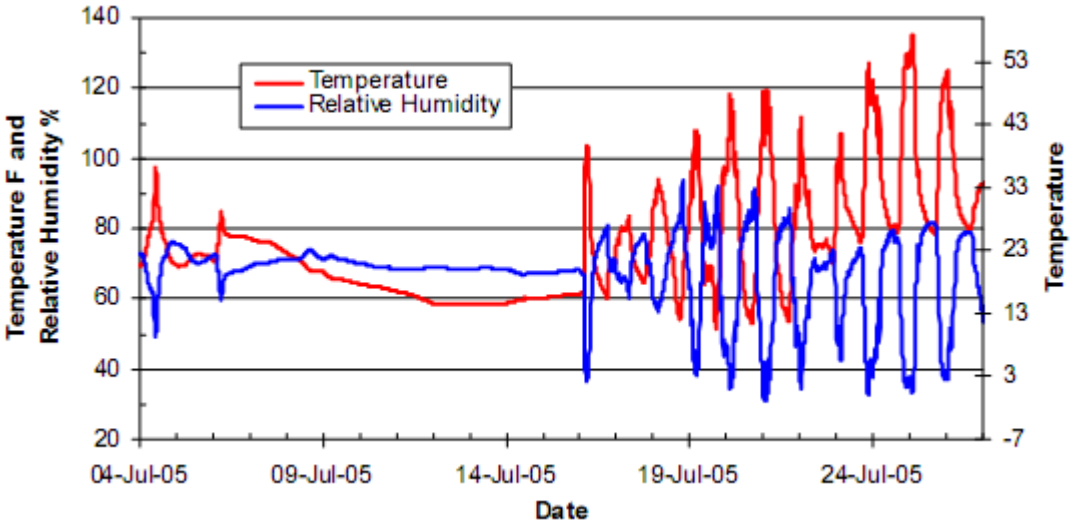


Figure 14 : Enregistrement des températures et humidités relatives lors d'un transport du Japon à Memphis.

4.3. Impact du stockage sur les propriétés caractéristiques des poudres de protéines de lait

Dans la section qui suit, nous discutons essentiellement des travaux qui répertorient des évolutions de couleur et d'aptitude à la réhydratation lors du stockage de poudres protéines de lait car ce sont ces propriétés caractéristiques qui seront suivies pour la poudre de PPCN étudiée.

- **Changement de couleur et brunissement**

La couleur de la poudre est une propriété essentielle pour le consommateur, gage de qualité, naturalité et authenticité. La couleur blanche initiale des poudres de lait est donc LA propriété indispensable à conserver pour les poudres. De plus, cette propriété est facilement accessible. De ce fait, la plupart des études scientifiques sur le vieillissement des poudres se sont intéressées à l'évolution de la couleur lors du stockage des poudres de protéines de lait, et ont notamment tenté d'éclaircir le lien existant entre le brunissement des poudres et la réaction de Maillard (RM) (Anema et al., 2006; Le et al., 2011a; Le et al., 2011b; Le et al., 2012; Le et al., 2013). Ces premières

études discutent, soit directement de l'évolution au cours du stockage des composantes du modèle colorimétrique choisi pour représenter les couleurs (par exemple RVB ou CIEL*a*b*), soit de l'évolution d'un indice global de brunissement recalculé à partir des composantes du modèle colorimétrique tel que défini par Maskan et al., (2001). Dans le modèle colorimétrique CIE L*a*b*, la composante a* représente les variations rouge-vert. La composante a* varie de 600 niveaux sur un axe vert (-300) → rouge (+299). La composante b* représente les variations jaune-bleu. La composante b* varie 600 niveaux sur un axe bleu (-300) → jaune (+299). La composante L* décrit la clarté (valeurs de gris) et va de 0 (Noir) à 100 (Blanc).

Maskan et al., (2001) ou encore Anese et al., (1999) ont démontré que le séchage d'aliments, avant stockage, pouvait engendrer une RM.

Par exemple, Maskan (2001) montrent clairement que son indice de brunissement calculé par l'équation (1) dépend très fortement du temps et de la température de l'air utilisé au séchage.

$$Indice\ de\ brunissement = \frac{[100(x-0{,}31)]}{0{,}17} \quad \text{with:} \quad x = \frac{(a+1{,}75L)}{(5{,}645L+a-3{,}012b)} \quad \text{(Eq).1}$$

Dans l'équation (1), le modèle colorimétrique CIE L*a*b* est utilisé.

La RM peut se poursuivre lors du stockage. Le et al., (2012) ont suivi l'évolution des composantes L* a* et b* durant un stockage à 30 °C d'une poudre de lait concentré en protéines à 80 % et d'une poudre de protéines sériques. Ils ont démontré une augmentation de l'indicateur b* au cours du stockage et donc un jaunissement de la poudre. Ces auteurs montrent que la température et le temps de stockage amplifient et accélèrent le brunissement de la poudre. Ces auteurs ont également établi une

corrélation entre l'augmentation d'hydroxyméthylfurfural (HMF), produit de la réaction de Maillard (RM), et l'apparition de couleur brune.

Beaucoup d'auteurs, abordant le brunissement, évoquent que le lactose est à l'origine du déclenchement de la RM (Anema et al., 2006; Le et al., 2011b; Morgan et al., 2005; van Boekel, 1998). Néanmoins le taux de lactose nécessaire à l'initiation de la RM n'est que très rarement discuté. Signalons qu'au-delà des évolutions de couleur, il est souvent mentionné que les réarrangements avec le lactose sont à l'origine de la formation d'insolubles. Des études suggèrent ainsi que les nouveaux composés formés lors de la RM comme le glyoxal, methylglyoxal ou3-deoxyglucosone peuvent être responsables des liaisons entre caséines lors du stockage (Le et al., 2011b; Le et al., 2013). Mais là encore, le lien entre lactose et insoluble est plus pressenti que réellement établi.

- **Diminution de la solubilité et de la capacité de réhydratation**

L'aptitude à la réhydratation des poudres est une propriété d'usage vitale et il est essentiel que cette propriété soit conservée lors du stockage. En effet, une dissolution incomplète engendre une perte en protéine mais aussi un risque de ne pas atteindre la fonctionnalité (pouvoir texturant, moussant,...) visée du dérivé laitier au terme de la reconstitution de la poudre (Crowley et al., 2015). Malheureusement, il a été observé par de nombreux auteurs que les propriétés de réhydratation étaient modifiées de façon significative au cours du stockage (Anema et al., 2006; Gazi and Huppertz, 2015; Haque et al., 2011; Mimouni et al., 2010b; Schokker et al., 2011) en particulier sous conditions sévères (stockage à haute température et humidité élevée).

Selon Havea et al., (2006), l'augmentation du taux d'insolubles serait due principalement aux interactions entre protéines hydrophobes. Anema et al., (2006) ont pour leur part établi que les insolubles étaient principalement constitués de caséines mais contenaient également des protéines sériques.

Plus récemment, Schokker et al. (2011) ont suggéré que le ralentissement des cinétiques de réhydratation était majoritairement dû aux difficultés croissantes rencontrées pour individualiser les MC des poudres vieillies.

Enfin, Gazi et Huppertz et al., (2015) ont démontré que seules les caséines micellaires voient leurs aptitudes à la réhydratation réduites au cours du stockage, alors que les caséines non micellaires restaient non altérées par le stockage. Cela a permis de confirmer les résultats de Schokker et al., (2011) qui suggéraient que l'augmentation de la concentration en caséines non micellaires avant le séchage pouvait réduire la perte de solubilisation lors du stockage. Pour augmenter la teneur en caséines non micellaires, Schokker et al., (2011) ont proposé l'ajout de caséinates de sodium avant le séchage du concentré.

Plusieurs études ont également cherché à agir en amont sur les paramètres du procédé (filtration, séchage par atomisation)(Schuck et al., 1994a). Schuck et al., (1994a) ont tenté de jouer sur les conditions de température d'entrée de séchage pour améliorer la solubilité des poudres. D'autres auteurs ont cherché à adapter la formulation initiale du retentât pour réduire la formation du taux d'insolubles constaté sur les poudres reconstituées après le stockage (Carr et al., 2002). Ainsi Carr et al., (2002) ont démontré que le remplacement des ions calcium par des ions sodium améliorait la solubilité des poudres de lait. De la même manière, l'addition de sels monovalents au retentât ultrafiltré avant l'étape d'évaporation/séchage a été suggérée afin d'augmenter la solubilité à froid.

4.4. Les mécanismes impliqués dans l'évolution des caractéristiques des poudres de protéines de lait au cours du stockage

Les mécanismes à l'échelle moléculaire gouvernant l'altération des propriétés des poudres au vieillissement ont été jusqu'ici très peu étudiés ou partiellement abordés.

Dans la section qui suit, nous discutons quelques études qui traitent de ce verrou scientifique et les mécanismes, souvent mis en avant, pour expliquer la déstabilisation au stockage des poudres de lait.

- **Mobilité moléculaire des protéines**

Les produits amorphes vitreux sont reconnus comme stables lorsqu'ils sont stockés sous leur température de transition vitreuse (Tg) (Struik, 1977). Seulement, lors d'un stockage prolongé, cet état amorphe vitreux, qui caractérise les poudres de lait (Bhandari and Hartel, 2005), ne peut pas toujours assurer une stabilité physique et chimique (Zylberman and Pilosof, 2002). Dans ce cas, des mobilités moléculaires peuvent engendrer des changements physico chimiques et fonctionnels importants (Roos, 2002), comme un réarrangement structural, des interactions entre micelles et une perte de solubilité. Ainsi Schokker et al.,(2011) concluent que même si la poudre de lait est stockée sous sa Tg, la mobilité des caséines est assez élevée pour permettre des interactions entre caséines.

Haque et al.,(2012) ont comparé les mouvements moléculaires par calorimétrie différentielle à balayage de deux MPC stockées à 25 ± 1 °C pendant 11 semaines. Ils ont observé que les mouvements moléculaires d'une poudre à teneur en eau réduite étaient moins importants et l'expliquent par la réduction de la teneur eau autour des protéines. Ils démontrent que l'augmentation de la teneur en eau de la poudre induit une relaxation de la protéine. En effet, l'eau est reconnue pour agir comme un

plastifiant, ce qui signifie qu'elle augmente la mobilité des chaînes et donc favorise la relaxation (Struik, 1978). En présence d'eau, la protéine cherche à atteindre un état thermodynamique plus stable, via l'augmentation des mouvements moléculaires de la protéine. Enfin, Thomsen et al.,(2005) ont démontré que pendant le stockage, l'eau libérée dans le milieu réactionnel lors de la cristallisation du lactose pouvait conduire à une augmentation la mobilité moléculaire.

- **Migration de composés lipidiques vers la surface des grains**

Plusieurs auteurs ont étudié les changements dans la composition de surface de poudres de protéines de lait lors du stockage (Gaiani et al., 2009; Kim et al., 2005). La spectroscopie photoélectronique à rayon X a été jusqu'ici la technique la plus utilisée pour suivre ces modifications (Fyfe et al., 2011; Gaiani et al., 2009; Kim et al., 2002). Cependant tous ne partagent pas les mêmes conclusions.

Gaiani et al., (2009) ont démontré que la teneur en lipides à la surface des particules de poudres de PPCN augmentait de 6 à 17% après 60 jours de stockage à 20 °C ou 50 °C et que la teneur en protéines à la surface était réduite de 94% à 83%.

Après 6 mois de stockage à 20 °C, Kim et al.,(2005) n'ont pas détecté de changements significatifs dans la composition en surface de plusieurs poudres de protéines lait.

La différence de résultat entre les auteurs pourrait être attribuée au type de poudre. Gaiani et al., (2009) ont analysé des poudres de PPCN dont la teneur en matière grasse à la surface des particules est chiffrée à 1,5% contre 30% pour les poudres analysées par Kim et al.,(2005).

- **Evolution des conformations et des structures secondaires**

Kher et al., (2007) ont suggéré que les modifications de conformations au cours du stockage pouvaient être à l'origine d'une perte de solubilité des poudres de protéines de lait. En effet, en étant dénaturées lors du stockage, les protéines exposeraient plus

facilement leurs régions hydrophobes à la surface, favorisant ainsi les structures en feuillets β et les interactions entre protéines (Anema, 2014; Arrondo and Goni, 1999; Singh and Latham, 1993). Dickinson and Parkinson., (2004) ont aussi avancé que l'augmentation des interactions entre protéines et l'agrégation affectaient la stabilité de la protéine.

Kher et al., (2007) ont étudié une poudre de protéines de lait par analyse spectroscopie infrarouge à transformée de Fourier (IRTF) et ont constaté un changement d'intensité de la bande à 1631 cm^{-1} (associée aux feuillets β), alors que la bande dans la région 1660-1650 cm^{-1} (associée aux hélices α) n'évoluait pas de façon significative.

Haque et al.,(2010) ont complété cette étude en étudiant la bande Amide I d'une poudre de protéines de lait par FTIR pendant un stockage à 24 et 45 °C. Une diminution d'hélice α et une augmentation des feuillets β ont été observées après 10 semaines à 24 °C. Le stockage à 45 °C a entraîné une diminution des structures en hélice α plus importante. Ils ont également souligné l'importance de la teneur en eau de la poudre via l'étude d'une poudre de protéines de lait stockée 12 semaines à 25 et 45 °C avec une teneur en eau comprise entre 0 à 0,85. Ils ont démontré que plus la teneur en eau était élevée, plus l'évolution des structures secondaires était importante.

Même si les modifications spectrales obtenues par ces auteurs suggèrent qu'il est tout à fait plausible qu'une évolution de la structure secondaire puisse entraîner une perte de solubilité irréversible, il n'a été possible pour aucun d'entre eux de proposer une corrélation directe entre la perte de la solubilité et l'évolution de la structure secondaire de la caséine. Signalons également que ce travail est rendu compliqué car il est connu que les structures secondaires des caséines sont très peu nombreuses.

- **Réactions chimiques et réarrangements moléculaires dans le volume des grains**

Des réactions chimiques impliquant des protéines peuvent également expliquer l'augmentation des interactions entre protéines au cours du stockage.

Anema et al.,(2006) ont montré par spectrométrie de masse que la caséine des poudres protéines de lait pouvait être lactosylée au cours d'un stockage. La lactosylation est la fixation du groupe aldéhyde du lactose avec le groupe ε-amino de la lysine, elle constitue la première étape de la RM (van Boekel, 1998). Cette liaison conduit à la formation de lactosyl-lysine (Abd El-Salam, 2014) et des composés sensibles à l'agrégation. C'est pourquoi, Le et al., (2011b) ont suggéré que la RM pouvait causer une perte de solubilité.

Le et al., (2012) ont couplé l'électrophorèse bidimensionnelle (2-DE) et la spectrométrie de masse pour observer les modifications chimiques d'une poudre de protéines de lait lors du stockage. Ils démontrent une lactosylation des protéines lors du séchage et du stockage. Ils observent également des désamidations (réaction chimique au cours de laquelle une molécule perd un groupement amide) au cours du stockage.

Le et al.,(2012) ont également tenté d'observer par 2-DE l'évolution d'autres modifications chimiques, dont la phosphorylation, lors du stockage d'une poudre de protéines de lait. La déphosphorylation est la perte du groupe phosphate des sérines par β-élimination, entrainant la formation de DeHydroAlanine (DHA) (DeGnore and Qin, 1998). La DHA est très réactive avec les acides aminés de la caséine, comme la lysine, cystéine ou histidine, et peut former des composés comme la LysinoALanine (LAL), la lanthionine ou l'histidinoalanine (Pellegrino et al., 2011). Cependant, cette étude n'a pas permis de détecter et d'identifier des peptides phosphorylés (Le et al., 2012). Le et al., (2013) ont réalisé une dephosphorylation d'α_{s1}-caséine avant un traitement thermique

en présence de lactose. Ils ont conclu que la dephosphorylation n'influençait pas l'aggrégation entre l' α_{s1}-caséine et le lactose lors d'un traitement thermique.

Signalons que les travaux de Schokker et al., (2011) n'ont pas permis de détecter d'évolution de la structure interne, par diffusion des rayons X aux petits angles, lors du stockage de poudre de protéines de lait.

- **Réarrangement moléculaire en surface des particules et formation d'une peau**

Quelques études sur le vieillissement des poudres de lait ont prouvé qu'une évolution des propriétés de surface des particules s'opérait lors du stockage (Burgain et al., 2016; Fyfe et al., 2011; Mimouni et al., 2010a, b).

Mimouni et al.,(2010a) ont observé par microscopie électronique à balayage (MEB) qu'une croûte se formait à la périphérie des poudre de PPCN et que cette couche restait nettement visible au début du processus de réhydratation. Ils observent que 2 mois de stockage à 20 °C sont suffisants pour détecter cette peau en surface. Ils suggèrent que cette peau est issue d'un compactage des MC à la surface de la particule, conduisant à des ponts et des liaisons inter-micellaires et un ralentissement du processus de réhydratation. Mimouni et al., (2010b) ont montré que la présence de la peau à la surface des particules primaires retardait considérablement la réhydratation de la poudre, évoquant un transfert inhibé de l'eau et / ou de la dispersion ralentie des MC liées entre elles. Ces études corroborent aussi les travaux pionniers d'Anema et al., (2006) suggérant que des réarrangements moléculaires, impliquant des liaisons entre MC, se produisent au cours de stockage. Fyfe et al.,(2011) ont plus tard confirmé la présence d'une couche mince en surface des particules, composée de MC agrégées, lors d'un stockage.

L'évolution de cette peau en surface et de ses propriétés mécaniques au cours du temps a récemment été confirmée par les travaux de Burgain et al., (2016) par microscope à force atomique.

Synthèse bibliographique et objectifs de la thèse

En résumé, il a été démontré que la production et l'exportation des poudres de lait étaient en perpétuelle hausse depuis une dizaine d'années et que la France était un des principaux acteurs de ce marché aux enjeux importants.

Il a également été établi que les poudres de lait subissaient des variations importantes de température et d'humidité sur des périodes de plus de 3 mois lors des exportations.

Il a été évoqué que les travaux scientifiques traitant de l'influence des conditions de stockage sur l'évolution des caractéristiques des poudres de protéines de lait étaient peu nombreux et récents. De ce fait, très peu de données quantitatives (évolution des propriétés fonctionnelles et sensorielles versus conditions de stockage) sont à notre disposition. De même, il existe peu de connaissances sur les évolutions structurelles des poudres au stockage et les marqueurs de vieillissement des poudres. Cet état de l'art ne permet pas par exemple d'être en capacité de prédire la perte d'aptitude à la réhydratation des poudres à base de caséines lors d'un stockage.

Des lacunes existent également sur les correspondances temps-température entre les vieillissements à différentes températures. Par exemple, aucune correspondance temporelle entre des vieillissements de poudres à températures élevées et ambiantes n'a été proposée. Enfin, l'influence exacte d'une montée ponctuelle en température de quelques heures des poudres au-dessus de 40 °C (désignée dans ce manuscrit par accident de stockage) et leurs effets cumulatifs dans le processus de vieillissement des poudres n'est pas connue.

A noter que le comportement de poudres à forte teneur en caséine comme les poudres de PPCN, ingrédients fonctionnels à haute valeur ajoutée et occupant aujourd'hui un rôle fondamental dans les formulations alimentaires, n'a été que rarement étudié.

Les études pionnières sur le vieillissement des poudres au stockage ont néanmoins établies que les variations de températures et d'humidité durant le stockage sont à l'origine d'altérations des propriétés fonctionnelles et sensorielles (couleur) des poudres de protéines de lait. Il a notamment été démontré par différents auteurs que des augmentations du temps de réhydratation, de l'insolubilité et du brunissement se produisaient lors du stockage de poudre de protéines de lait. Ces évolutions de propriétés fonctionnelles et sensorielles semblent d'autant plus marquées que les poudres ont une teneur en caséine élevée.

Les études mécanistiques sur le vieillissement des poudres de lait suggèrent que la migration de certains constituants, les changements de conformation des protéines, des lactosylations peuvent conduire à l'augmentation des interactions protéiques entre micelles et densifier la peau en périphérie du grain, ce qui altèrerait la solubilisation des poudres.

Des études supplémentaires sont néanmoins encore requises pour :

i) préciser les mécanismes et les espèces impliqués (ex. lactose) dans les réarrangements à l'origine de la déstabilisation des protéines et la perte de fonctionnalités des poudres de PPCN.

ii) être en capacité de proposer une corrélation claire entre les évolutions structurelles et fonctionnelles en fonction des conditions de stockage appliquées aux poudres.

L'objectif général de cette étude est donc de combler ces lacunes en menant une étude systématique des évolutions fonctionnelles et structurelles des poudres de PPCN au cours du stockage. Afin de répondre à une réalité (au niveau de l'histoire en température au stockage), les poudres de PPCN ont été conservées sous plusieurs températures (4 °C, 20 °C, 40 °C et 60 °C pendant 12 mois) sous humidité contrôlée. Les changements structuraux (structure secondaire, composition de surface, caractérisation des liaisons entre protéines, modifications chimiques), fonctionnels et

sensoriels (temps de réhydratation, de fragmentation, de relaxation, solubilité, couleur) de poudres de PPCN avec ou sans lactose ont été observés.

Les actions de recherche réalisées dans cette thèse sont une contribution pour répondre aux 6 objectifs opérationnels énoncés ci-dessous :

1. Identifier et quantifier les changements de propriétés fonctionnelles d'une poudre de PPCN au cours d'un vieillissement contrôlé [Partie 2 – chapitre 1].

2. Proposer des tests et/ou des méthodes permettant de prédire le temps de réhydratation [Partie 2 – chapitre 1].

3. Proposer des correspondances entre vieillissement à température élevée et température ambiante et déterminer l'influence d'un ou plusieurs accidents de stockage sur les propriétés de réhydratation des poudres de PPCN [Partie 2 – chapitre 1].

4. Identifier les évolutions de structure secondaire et modifications chimiques, pouvant être des marqueurs de la déstabilisation des protéines [Partie 2 – chapitre 2-3-4]

5. Etablir des relations entre ces marqueurs et la perte de fonctionnalité des poudres de PPCN [Partie 2 – chapitre 2-3-4].

6. Elucider le rôle du lactose dans les mécanismes de modifications chimiques et les évolutions des propriétés fonctionnelles et sensorielles des poudres de PPCN [Partie 2 – chapitre 4]

Références partie 1

Abd El-Salam, M. H. 2014. Application of proteomics to the areas of milk production, processing and quality control - A review. International Journal of Dairy Technology **67**:153-166.

Anema, S. G. 2014. The Whey Proteins in Milk: Thermal Denaturation, Physical Interactions, and Effects on the Functional Properties of Milk. Pages 269-318 *in* H. Singh, M. Boland, and A. Thompson, editors. Milk Proteins: from Expression to Food, 2nd Edition. Elsevier Academic Press Inc, San Diego.

Anema, S. G., D. N. Pinder, R. J. Hunter, and Y. Hemar. 2006. Effects of storage temperature on the solubility of milk protein concentrate (MPC85). Food Hydrocolloids **20**:386-393.

Anese, M., M. C. Nicoli, R. Massini, and C. R. Lerici. 1999. Effects of drying processing on the Maillard reaction in pasta. Food Research International **32**:193-199.

Arrondo, J. L. R., and F. M. Goni. 1999. Structure and dynamics of membrane proteins as studied by infrared spectroscopy. Progress in Biophysics & Molecular Biology **72**:367-405.

Augustin, M. A. 2000. Mineral salts and their effect on milk functionality. Australian Journal of Dairy Technology **55**:61-64.

Bhandari, B. R., and R. W. Hartel. 2005. Phase transitions during food powder production and powder stability. Pages 261-292 *in* C. Onwulata, editor. Encapsulated and Powdered Foods.

Bouchoux, A., G. Gesan-Guiziou, J. Perez, and B. Cabane. 2010. How to Squeeze a Sponge Casein Micelles under Osmotic Stress, a SAXS Study. Biophysical Journal **99**:3754-3762.

Bouvier, J.-M., M. Collado, D. Gardiner, M. Scott, and P. Schuck. 2013. Physical and rehydration properties of milk protein concentrates: comparison of spray-dried and extrusion-porosified powders. Dairy Science & Technology **93**:387-399.

Brownlow, S., J. H. M. Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polikarpov, A. C. T. North, and L. Sawyer. 1997. Bovine beta-lactoglobulin at 1.8 angstrom resolution - Still an enigmatic lipocalin. Structure **5**:481-495.

Broyard, C., and F. Gaucheron. 2015. Modifications of structures and functions of caseins: a scientific and technological challenge. Dairy Science & Technology **95**:831-862.

Burgain, J., J. Scher, J. Petit, G. Francius, and C. Gaiani. 2016. Links between particle surface hardening and rehydration impairment during micellar casein powder storage. Food hydrocolloids **61**:277-285.

Carr, A., G. Bhaskar, and S. Ram. 2002. Monovalent salt enhances solubility of milk protein concentrate. WO02/096208.

CNIEL. 2016. L'économie laitière en chiffres, édition 2016. http://www.maison-du-lait.com/chiffres-cles/des-donnees-reference.

Considine, T., and J. Flanagan. 2009. Interaction between milk proteins and micronutrients. Milk Proteins: from Expression to Food:377-407.

Crowley, S. V., B. Desautel, I. Gazi, A. L. Kelly, T. Huppertz, and J. A. O'Mahony. 2015. Rehydration characteristics of milk protein concentrate powders. Journal of Food Engineering **149**:105-113.

Dalgleish, D. G., and A. J. R. Law. 1989. pH-induced dissociation of bovine casein micelles. 2. Mineral solubilization and its relation to casein release. Journal of Dairy Research **56**:727-735.

de Kruif, C. G., T. Huppertz, V. S. Urban, and A. V. Petukhov. 2012. Casein micelles and their internal structure. Advances in Colloid and Interface Science **171**:36-52.

DeGnore, J. P., and J. Qin. 1998. Fragmentation of phosphopeptides in an ion trap mass spectrometer. Journal of the American Society for Mass Spectrometry **9**:1175-1188.

Dewit, J. N. 1984. Functional properties of whey proteins in food systems. Netherlands Milk and Dairy Journal **38**:71-89.

Dickinson, E., and E. L. Parkinson. 2004. Heat-induced aggregation of milk protein-stabilized emulsions: sensitivity to processing and composition. International Dairy Journal **14**:635-645.

Dissanayake, M., and T. Vasiljevic. 2009. Functional properties of whey proteins affected by heat treatment and hydrodynamic high-pressure shearing. Journal of Dairy Science **92**:1387-1397.

Donato, L., and F. Guyomarc'H. 2009. Formation and properties of the whey protein/kappa-casein complexes in heated skim milk - A review. Dairy Science & Technology **89**:3-29.

Fang, Y., S. Rogers, C. Selomulya, and X. D. Chen. 2012. Functionality of milk protein concentrate: Effect of spray drying temperature. Biochemical Engineering Journal **62**:101-105.

FAO. 2015. Milk and milk products. http://www.fao.org/fileadmin/templates/est/COMM_MARKETS_MONITORING/Dairy/Documents/FO_OCT_2015_-_Dairy2.pdf.

FAO. 2016. Lait et produits laitiers; http://www.fao.org/3/a-BO101f.pdf.

Farrell, H. M., R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, K. F. Ng-Kwai-Hang, and H. E. Swaisgood. 2004. Nomenclature of the proteins of cows' milk - Sixth revision. Journal of Dairy Science **87**:1641-1674.

Fox, P. F., and A. Brodkorb. 2008. The casein micelle: Historical aspects, current concepts and significance. International Dairy Journal **18**:677-684.

Fyfe, K. N., O. Kravchuk, T. Le, H. C. Deeth, A. V. Nguyen, and B. Bhandari. 2011. Storage induced changes to high protein powders: influence on surface properties and solubility. Journal of the Science of Food and Agriculture **91**:2566-2575.

Gaiani, C., J. Scher, J. J. Ehrhardt, M. Linder, P. Schuck, S. Desobry, and S. Banon. 2007. Relationships between dairy powder surface composition and wetting properties during storage: Importance of residual lipids. Journal of Agricultural and Food Chemistry **55**:6561-6567.

Gaiani, C., P. Schuck, J. Scher, J. J. Ehrhardt, E. Arab-Tehrany, M. Jacquot, and S. Banon. 2009. Native phosphocaseinate powder during storage: Lipids released onto the surface. Journal of Food Engineering **94**:130-134.

Gaucheron, F. 2005. The minerals of milk. Pages 473-483. Reprod. Nutr. Dev.

Gazi, I., and T. Huppertz. 2015. Influence of protein content and storage conditions on the solubility of caseins and whey proteins in milk protein concentrates. International Dairy Journal **46**:22-30.

Ginger, M. R., and M. R. Grigor. 1999. Comparative aspects of milk caseins. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology **124**:133-145.

Griffiths, M. W. 2010. Milk biochemistry. Pages 3-26 *in* M. W. Griffiths, editor. Improving the Safety and Quality of Milk, Volume 1: Milk Production and Processing. Woodhead Publ Ltd, Cambridge.

Guyomarc'h, F., F. Warin, D. D. Muir, and J. Leaver. 2000. Lactosylation of milk proteins during the manufacture and storage of skim milk powders. International Dairy Journal **10**:863-872.

Haque, E., B. R. Bhandari, M. J. Gidley, H. C. Deeth, S. M. Moller, and A. K. Whittaker. 2010. Protein Conformational Modifications and Kinetics of Water-Protein Interactions in Milk Protein Concentrate Powder upon Aging: Effect on Solubility. Journal of Agricultural and Food Chemistry **58**:7748-7755.

Haque, E., B. R. Bhandari, M. J. Gidley, H. C. Deeth, and A. K. Whittaker. 2011. Ageing-induced solubility loss in milk protein concentrate powder: effect of protein conformational modifications and interactions with water. Journal of the Science of Food and Agriculture **91**:2576-2581.

Haque, E., B. R. Bhandari, M. J. Gidley, H. C. Deeth, and A. K. Whittaker. 2015. Change in molecular structure and dynamics of protein in milk protein concentrate powder upon ageing by solid-state carbon NMR. Food Hydrocolloids **44**:66-70.

Haque, E., A. K. Whittaker, M. J. Gidley, H. C. Deeth, K. Fibrianto, and B. R. Bhandari. 2012. Kinetics of enthalpy relaxation of milk protein concentrate powder upon ageing and its effect on solubility. Food Chemistry **134**:1368-1373.

Havea, P. 2006. Protein interactions in milk protein concentrate powders. International Dairy Journal **16**:415-422.

Havea, P., H. Singh, and L. K. Creamer. 2001. Characterization of heat-induced aggregates of beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin in a whey protein concentrate environment. Journal of Dairy Research **68**:483-497.

Holt, C. 1992. STRUCTURE AND STABILITY OF BOVINE CASEIN MICELLES. Advances in Protein Chemistry **43**:63-151.

Horne, D. S. 1998. Casein interactions: Casting light on the black boxes, the structure in dairy products. International Dairy Journal **8**:171-177.

Hunziker, O. F. 1920. Condensed Milk and Milk Powder. Page 277.

Huppertz, T., and H. Patel. 2013. Advances in Milk Protein Ingredients. Innovation in Healthy and Functional Foods:363-385.

IDELE. 2016. Situation et devenir des marchés des produits laitiers-Forces et faiblesses de la France laitière.

Isleten, M., and Y. Karagul-Yuceer. 2006. Effects of dried dairy ingredients on physical and sensory properties of nonfat yogurt. Journal of Dairy Science **89**:2865-2872.

Jeantet, R., G. Brulé, and G. Delaplace. 2011. Génie des procédés appliqué à l'industrie laitière. Lavoisier.

Johnke, M., and T. E. Petersen. 2012. The Alpha-Lactalbumin/Oleic Acid Complex and Its Cytotoxic Activity. Milk Protein:119-144.

Kelly, P. M. 2006. Innovation in milk powder technology. International Journal of Dairy Technology **59**:70-75.

Kher, A., P. Udabage, I. McKinnon, D. McNaughton, and M. A. Augustin. 2007. FTIR investigation of spray-dried milk protein concentrate powders. Vibrational Spectroscopy **44**:375-381.

Kim, E. H. J., X. D. Chen, and D. Pearce. 2002. Surface characterization of four industrial spray-dried dairy powders in relation to chemical composition, structure and wetting property. Colloids and Surfaces B-Biointerfaces **26**:197-212.

Kim, E. H. J., X. D. Chen, and D. Pearce. 2005. Melting characteristics of fat present on the surface of industrial spray-dried dairy powders. Colloids and Surfaces B-Biointerfaces **42**:1-8.

Le, T. T., B. Bhandari, and H. C. Deeth. 2011a. Chemical and Physical Changes in Milk Protein Concentrate (MPC80) Powder during Storage. Journal of Agricultural and Food Chemistry **59**:5465-5473.

Le, T. T., B. Bhandari, J. W. Holland, and H. C. Deeth. 2011b. Maillard Reaction and Protein Cross-Linking in Relation to the Solubility of Milk Powders. Journal of Agricultural and Food Chemistry **59**:12473-12479.

Le, T. T., H. C. Deeth, B. Bhandari, P. F. Alewood, and J. W. Holland. 2012. A proteomic approach to detect lactosylation and other chemical changes in stored milk protein concentrate. Food Chemistry **132**:655-662.

Le, T. T., J. W. Holland, B. Bhandari, P. F. Alewood, and H. C. Deeth. 2013. Direct evidence for the role of Maillard reaction products in protein cross-linking in milk powder during storage. International Dairy Journal **31**:83-91.

Lucy, C. A., A. M. MacDonald, and M. D. Gulcev. 2008. Non-covalent capillary coatings for protein separations in capillary electrophoresis. Journal of Chromatography A **1184**:81-105.

Luo, X., T. Vasiljevic, and L. Ramchandran. 2016. Effect of adjusted pH prior to ultrafiltration of skim milk on membrane performance and physical functionality of milk protein concentrate. Journal of Dairy Science **99**:1083-1094.

Maskan, M. 2001. Kinetics of colour change of kiwifruits during hot air and microwave drying. Journal of Food Engineering **48**:169-175.

McKenna, A. B. 2000. Effect of processing and storage on reconstitution properties of whole milk and ultrafiltered skim milk powders. Massey University, New Zealand.

McMahon, D. J., and R. J. Brown. 1984. Composition, structure, and integrity of casein micelles - a review. Journal of Dairy Science **67**:499-512.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari. 2010a. Investigation of the microstructure of milk protein concentrate powders during rehydration: Alterations during storage. Journal of Dairy Science **93**:463-472.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari. 2010b. Rehydration of high-protein-containing dairy powder: Slow- and fast-dissolving components and storage effects. Dairy Science & Technology **90**:335-344.

Mistry, V. V. 2002. Manufacture and application of high milk protein powder. Lait **82**:515-522.

Morgan, F., C. A. Nouzille, R. Baechler, G. Vuataz, and A. Raemy. 2005. Lactose crystallisation and early Maillard reaction in skim milk powder and whey protein concentrates. Lait **85**:315-323.

OECD-FAO. 2016. The OECD-FAO Agricultural Outlook 2016-2025 reports; http://www.fao.org/3/a-i5778e.pdf.

Payens, T. A. J. 1966. Association of caseins and their possible relation to structure of casein micelle. Journal of Dairy Science **49**:1317-&.

Payens, T. A. J., and H. J. Vreeman. 1982. Casein Micelles and Micelles of **κ**- and β-Casein. Springer US.

Pellegrino, L., S. Cattaneo, and I. De Noni. 2011. Effects of processing on protein quality of milk and milk products. Encyclopedia of dairy sciences, San Diego, USA.

Permyakov, E. A., and L. J. Berliner. 2000. alpha-Lactalbumin: structure and function. Febs Letters **473**:269-274.

Post, A. E., B. Arnold, J. Weiss, and J. Hinrichs. 2012. Effect of temperature and pH on the solubility of caseins: Environmental influences on the dissociation of alpha(S)- and beta-casein. Journal of Dairy Science **95**:1603-1616.

Premtitikul, P. 2005. Ocean container temperature and humidity study. Inter dry moisture control.

Refstrup, E. 2000. Evaporation and drying technology developments. International Journal of Dairy Technology **53**:163-167.

Roos, Y. H. 2002. Importance of glass transition and water activity to spray drying and stability of dairy powders. Lait **82**:475-484.

Sadek, C., P. Schuck, Y. Fallourd, N. Pradeau, C. Le Floch-Fouere, and R. Jeantet. 2015. Drying of a single droplet to investigate process-structure-function relationships: a review. Dairy Science & Technology **95**:771-794.

Schmidt, D. G. 1982. Association of caseins and casein micelle structure. Developments in Dairy Chemistry.

Schokker, E. P., J. S. Church, J. P. Mata, E. P. Gilbert, A. Puvanenthiran, and P. Udabage. 2011. Reconstitution properties of micellar casein powder: Effects of composition and storage. International Dairy Journal **21**:877-886.

Schuck, M. Piot, S. Méjean, Y. Le Graet, J. Fauquant, G. Brulé, and J. L. Maubois. 1994. Déshydratation par atomisation de phosphocaséinate natif obtenu par microfiltration sur membrane. Le lait **74**:375-388.

Schuck, P. 2002. Spray drying of dairy products: state of the art. Pages 375-382. Elsevier.

Schuck, P., M. Roignant, G. Brule, A. Davenel, M. H. Famelart, and J. L. Maubois. 1998. Simulation of water transfer in spray drying. Drying Technology **16**:1371-1393.

Selomulya, C., and Y. Fang. 2013. Food powder rehydration. Pages 379-408 *in* B. Bhandari, N. Bansal, M. Zhang, and P. Schuck, editors. Handbook of Food Powders: Processes and Properties. Woodhead Publ Ltd, Cambridge.

Sharma, A., A. H. Jana, and R. S. Chavan. 2012. Functionality of Milk Powders and Milk-Based Powders for End Use Applications-A Review. Comprehensive Reviews in Food Science and Food Safety **11**:518-528.

Singh, H. 2002. Milk Proteins : Functional properties. Encyclopedia of Dairy Science:1976-1982.

Singh, H. 2007. Interactions of milk proteins during the manufacture of milk powders. Lait **87**:413-423.

Singh, H., and L. K. Creamer. 1991. Denaturation, aggregation and heat'stability of milk protein during the manufacture of skim milk powder. Journal of Dairy Research **58**:269-283.

Singh, H., and P. Havea. 2003. Thermal denaturation, aggregation and gelation of whey proteins. In Advanced Dairy Chemistry.

Singh, H., and J. M. Latham. 1993. Heat Stability of Milk: Aggregation and Dissociation of Protein at Ultra-high Temperatures. International Dairy Journal **3**:225-237.

Singh, H., A. Ye, and P. Havea. 2000. Milk protein interactions and functionality of dairy ingredients. Australian Journal of Dairy Technology **55**:71-77.

Snappe , J. J., A. Lepoudere, and N. Sredzinski. 2010. Protéines laitières. Techniques de l'ingénieur.

Struik, L. C. E. 1977. Physical aging in plastic and other glassy materials. Polymer Engineering and Science **17**:165-173.

Struik, L. C. E. 1978. Orientation effects and cooling stresses in amorphous polymers. Polymer Engineering and Science **18**:799-811.

Thomas, M. E. C., J. Scher, S. Desobry-Banon, and S. Desobry. 2004. Milk powders ageing: Effect on physical and functional properties. Critical Reviews in Food Science and Nutrition **44**:297-322.

Thomsen, M. K., L. Lauridsen, L. H. Skibsted, and J. Risbo. 2005. Temperature effect on lactose crystallization, Maillard reactions, and lipid oxidation in whole milk powder. Journal of Agricultural and Food Chemistry **53**:7082-7090.

Udabage, P., I. R. McKinnon, and M. A. Augustin. 2000. Mineral and casein equilibria in milk: effects of added salts and calcium-chelating agents. Journal of Dairy Research **67**:361-370.

USDA. 2016. Dairy, Dry Whole Milk Powder Production by Country in 1000 MT - Map. http://www.indexmundi.com/agriculture/?commodity=powdered-whole-milk&graph=production&display=map.

van Boekel, M. 1998. Effect of heating on Maillard reactions in milk. Food Chemistry **62**:403-414.

Walstra, P. 1990. On the stability of casein micelles. Journal of Dairy Science **73**:1965-1979.

Walstra, P. 1999. Casein sub-micelles: do they exist? International Dairy Journal **9**:189-192.

Walstra, P., and R. Jennes. 1984. Dairy chemistry and Physics. New-york.

Waugh, D. 1958. The interaction of αs, β- and κ-caseins in micelle formation. Faraday Society Discuss **25 :186-92**.

Wijayanti, H. B., N. Bansal, and H. C. Deeth. 2014. Stability of Whey Proteins during Thermal Processing: A Review. Comprehensive Reviews in Food Science and Food Safety **13**:1235-1251.

Wong. 1988. Fundamental of Dairy Chemistry. Pages 481-492. Fundamental of Dairy Chemistry, New-york.

Yazdanpanah, N., and T. A. G. Langrish. 2013. Comparative study of deteriorative changes in the ageing of milk powder. Journal of Food Engineering **114**:14-21.

Zylberman, V., and A. M. R. Pilosof. 2002. Relationship between the glass transition, molecular structure and functional stability of hydrolyzed soy proteins. Amorphous Food and Pharmaceutical Systems:158-168.

Partie 2. Résultats

Chapitre 1. Etude de l'influence du stockage sur les propriétés fonctionnelles des poudres de PPCN

L'aptitude à la réhydratation et la couleur des poudres de PPCN sont deux propriétés fonctionnelles et sensorielles essentielles qui doivent être maintenues tout au long du processus de stockage. Réhydrater des poudres de PPCN n'est pas une opération unitaire aussi aisée qu'il n'y paraît. En effet, suivant la composition, l'individualisation des MC jusqu'à la dissolution complète de certaines poudres de PPCN, peut nécessiter jusqu'à une dizaine d'heures à température ambiante.

De plus, il est reconnu que des conditions de stockage sévères (température, humidité et temps d'exposition élevés) des poudres peuvent contribuer à augmenter significativement l'insolubilité des poudres et les temps de réhydratation nécessaires.

Malheureusement, les relations exactes entre conditions de stockage d'une poudre de PPCN et changements de propriétés fonctionnelles et sensorielles, comme la solubilité, les temps caractéristiques du processus de réhydratation et la couleur ne sont pas établies. Les industriels soucieux de maîtriser leurs procédés de reconstitution et les propriétés finales du produit peuvent faire face à des difficultés lors de l'élaboration de dérivés laitiers à base de caséines.

Dans ce contexte, l'objectif de ce chapitre est d'identifier le comportement au vieillissement (couleur et aptitude à la réhydratation) de poudres de PPCN lors du stockage.

Pour cela, deux poudres de PPCN aux caractéristiques différentes ont été utilisées et des vieillissements contrôlés ont été appliqués (à 4, 20, 40 et 60 °C jusqu'à 12 mois). L'évolution de l'indice de brunissement a été mesurée par un chromamètre. Deux temps caractéristiques du processus de réhydratation, le temps de fragmentation

et le temps de réhydratation total, ont été suivis par différentes techniques de granulométrie. Enfin une mesure de la solubilité a également été réalisée.

L'analyse des résultats obtenus a consisté dans un premier temps à examiner la possibilité de corréler le temps de réhydratation et les trois indicateurs suivants : indice de brunissement, solubilité et temps de fragmentation. Ceci afin de proposer des méthodes et/ou des tests permettant de prédire rapidement le temps de réhydratation total, qui est la grandeur physique d'intérêt et la plus chronophage à déterminer.

Puis, les résultats obtenus pour des vieillissements réalisés à température élevée et à température ambiante ont été comparés afin d'identifier des correspondances temps-température.

Ce chapitre montre tout d'abord que les temps de réhydratation caractéristiques (temps de réhydratation total et de fragmentation) augmentent avec le temps et la température de stockage, pour les deux poudres de PPCN. Il en est de même pour l'indice de brunissement alors que la solubilité suit une tendance opposée et décroit avec la sévérité du stockage. Ces résultats sont conformes à ceux attendus et de la littérature.

L'évolution du temps de réhydratation en fonction de chaque indicateur (temps de fragmentation, indice de brunissement et solubilité) ont permis de tracer des courbes de vieillissement (obtenues par ajustement) pour chaque poudre et les équations de corrélations pour chaque courbe de vieillissement ont été données.

Chaque courbe de vieillissement regroupe l'ensemble des couples temps / température de stockage réalisés. Ceci montre clairement que des similitudes de comportement au vieillissement existent entre des échantillons conservés à température modérée et ceux stockés à température élevée.

En revanche, il est prouvé que l'allure de la courbe de vieillissement est indépendante des conditions de stockage mais dépendante du vécu de la poudre

avant son stockage (composition initiale du concentré et paramètres lors du séchage par atomisation).

Cet ensemble de résultat nous a permis d'établir que l'analyse de 3 échantillons de poudres (2 échantillons stockés à température élevée et 1 obtenu sur la poudre avant stockage) suffisent à approximer la courbe de vieillissement de référence de la poudre étudiée et de rapidement identifier la trajectoire au vieillissement. Des couples [temps-température] d'intérêts sont proposés pour établir la courbe de référence approximée des poudres de PPCN étudiées.

Enfin, une méthode empirique a été proposée pour prédire le temps de réhydratation nécessaire d'une poudre de PPCN ayant subi un vieillissement inconnu. Cette méthode est basée sur l'utilisation de la courbe de vieillissement approximée (qui peut être facilement déterminée au préalable – confère le paragraphe ci-dessus) et la mesure expérimentale de l'indicateur de la courbe de vieillissement pour l'échantillon de poudre à caractériser.

La méthode empirique proposée a été testée et validée en comparant les mesures expérimentales et prédites du temps de réhydratation des deux lots de poudres de PPCN. De plus, une série indépendante d'échantillons vieillis a été utilisée. Cette série indépendante d'échantillons a subi une ou plusieurs montées ponctuelles en température (accident de stockage) au lieu d'être vieillie à température constante tout au long du stockage. La bonne adéquation des prédictions avec les mesures expérimentales permet de valider la méthode.

Influence of storage conditions on the functional properties of micellar casein powder

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Abstract

Controlled ageing conditions have been applied to two micellar casein (MC) powders and the consequent impact on their rehydration capacity and colorimetric evolution (measured by browning index) has been reported. Two characteristic times (fragmentation and total rehydration time) and solubility have been experimentally determinated to evaluate the evolution of rehydration capacity with controlled ageing conditions (storage duration and temperature).

For the two MC powders tested, it was shown that the two characteristic times and the browning index increased with storage duration and temperature applied during ageing, whereas solubility decreased. For each MC powder studied, it was shown i) that there is a correlation (ageing curves) between the rehydration time (the target variable) and the indicator parameters (fragmentation time, browning index and solubility) and (ii) that the shape of each ageing curve is independent of the ageing conditions (ageing at ambient temperature or accelerated ageing at higher temperatures) but dependent on the MC powder studied (initial composition of the concentrate and spray drying parameters).

These results clearly suggest i) the possibility to obtain reference ageing curves for each indicator, linking total rehydration time and the following indicators: fragmentation time, browning index and solubility ii) the possibility to identify several ageing similarities between severe and moderate storage conditions iii) the feasibility of applying accelerated

ageing conditions to rapidly establish and identify the shape of the reference ageing curves for a given MC powder, and iv) the possibility of predicting the rehydration time of the MC powder studied using reference ageing curves (approximated or not) through the measurement of one indicator (fragmentation time, browning index and solubility). This predicting ability of the proposed approach has been ascertained by comparing experimental and predicted values of rehydration time for aged samples having undergone storage accidents.

Abbreviations

MPC: milk protein concentrate; MC: micellar casein; P1: powder 1; P2: powder 2

1. Introduction

Milk protein concentrate (MPC) powders are produced from skimmed milk by spray drying after ultrafiltration (Haque et al., 2010). These dairy-derived powders have a high protein content, with the dry matter content ranging from 40 to 90 % (De Castro-Morel and Harper, 2002). The casein to whey protein ratio of MPC is identical to that of skimmed milk.

Due to their high nutritional value and favourable functional properties (e.g. foaming, emulsifying, gelling, etc.), MPC powders are incorporated as an ingredient into a wide range of applications in downstream food industries (Selomulya and Fang, 2013). For example, they can be used to standardise the protein content of milk and are used as ingredients in many food applications including raising the protein content of cheese and yoghurt.

For most applications, a prior dissolution of MPC powder in water is mandatory in order to allow the powder to fully express its functional properties (Gaiani et al., 2007a) Consequently, rehydration and solubility are important end-use properties for MPC powders and require accurate assessment.

Unfortunately, several studies have reported that MPC powders are characterised by poor rehydration properties (Gaiani et al., 2007b; Richard et al., 2013; Richard et al., 2012b; Schuck et al., 2007). This is even truer for micellar casein (MC) powder, whether freshly prepared or stored. MC powder is a high-protein dairy powder obtained from milk microfiltration retentate. The objective is to eliminate almost all of the whey proteins, lactose and minerals (Schuck et al., 1994a) and to concentrate the casein micelles while preserving their native structure. Due to the enriched micellar casein content, MC powder is an attractive material for the food industry, as it can enhance the structure, texture and consistency of various foodstuffs (Paracha, 2011; Singh, 2002). For powder enriched in micellar casein, it was i) shown that the total rehydration process required more than 3 hours at 25 °C under stirring at 900 rpm, and ii) observed that despite the aforementioned treatment, some undissolved material remained, which is generally referred to as the 'insoluble fraction' (Richard et al., 2013).

The impact of storage on the properties of MPC has been extensively studied (Anema et al., 2006; Fang et al., 2011; Gazi and Huppertz, 2015; Haque et al., 2015; Haque et al., 2012; Havea, 2006; Hunter et al., 2011; Jimenezflores and Kosikowski, 1986; Le et al., 2011a; Marella et al., 2015; Mimouni et al., 2010a, c; Sikand et al., 2016; Udabage et al., 2012), with particular focus on the gradual loss of solubility during storage (Anema et al., 2006; Fyfe et al., 2011; Haque et al., 2012; Mimouni et al., 2010c). These studies have shown that the solubility and rehydration properties of MPC were negatively impacted by severe ageing conditions (temperature, humidity and storage time). Poor reconstitution properties, and hence poor functionality of high-micellar casein powders can prevent them from achieving full market potential (De Castro-Morel and Harper, 2002). Thus, various studies have investigated ways of preventing insolubility of a powder, either by attempting to adjust adequate process parameters during filtration and spray drying operations (namely the drying

inlet temperature conditions for example (Schuck et al., 1994a), or by adapting the initial retentate formulation. It was found that adding monovalent salts to the ultrafiltered retentate prior to drying may improve solubility while increasing the calcium/total mineral ratio decreases the solubility of MPC (Carr et al., 2002).

At present, protein destabilisation due to conformational changes (Fyfe et al., 2011; Haque et al., 2010; Haque et al., 2011, 2015; Haque et al., 2012; Kher et al., 2007) and Maillard reactions in the presence of lactose (Haque et al., 2010; Haque et al., 2011, 2015; Le et al., 2011a; Le et al., 2011b; Le et al., 2013) are the most frequently cited causes for the loss of rehydration properties at molecular level.

Development of insolubility of the two high-micelle-casein-content powders, MC and MPC, is believed to share similar mechanisms, nevertheless MC has been less studied and the exact relationship between storage and changes in rehydration properties is not yet precisely known.

From analysis of state of the art, it also appears that the colorimetric change in MC powder with ageing (browning index) has been poorly documented.

Further work is thus needed to establish the influence of storage temperature and time on the ageing of MC powders and to illustrate the link between rehydration time and other ageing indicators (solubility, fragmentation time and browning index). Providing experimental data on this issue and evaluating the feasibility to predict rehydration time from colorimetric measurements, fragmentation time values or solubility changes of the powders are the main aims of this study.

For these purposes, two batches of MC powders (P1 and P2) were stored at temperatures ranging from 4 to 60 °C for different periods up to 12 months. Changes in various indicators (browning index, solubility, fragmentation time and total rehydration time) upon ageing were evaluated. The first part of this article focuses on analysing the changes in these indicators over time at different storage temperatures and assessing the relationships between these

changes and ageing conditions for the two MC powders. The second part of this paper focuses on investigating whether correlations exist between rehydration time and the 3 indicators, i.e., browning index, solubility and fragmentation time and discuss how these could be used to rapidly quantify and predict the increase in rehydration time of MC powders upon ageing.

2. Materials and methods

2.1. Dairy powders manufacture: physicochemical analysis at inlet and outlet of the spray drying tower and process parameters

MC concentrates were obtained through microfiltration (pore size = 0.1 μm) of skimmed milk from Ingredia (Arras, France).

Powders were obtained by spray drying an MC concentrate in a pilot workshop (GEA, Niro Atomizer, St Quentin en Yvelines, France) at Bionov (Rennes, France). The same operating conditions as described by Pierre, Fauquant, Legraet, Piot, & Maubois (1992) and Schuck et al. (1994a) were used for the spray drying. The inlet temperature was 180 °C ± 10 °C and the outlet temperature was 65 ± 5 °C.

Two batches of MC powder were prepared (P1 and P2). Various physico-chemical analyses were performed on the initial microfiltration retentates and the resulting dairy powders. The nitrogen contents (total nitrogen, non-casein nitrogen, non-protein nitrogen) of initial state-fresh powder were determined as described by Schuck et al., (2012). The total nitrogen content, non-casein nitrogen content corresponding to the soluble fraction at pH 4.6, and non-protein nitrogen content corresponding to the insoluble fraction after their precipitation were determined by the Kjeldhal method. Nitrogen contents were converted into protein contents using 6.38 as multiplying factor. These analyses are reported in **Table 1**.

MC powders were packed into individual 380 g tin cans after manufacture. The powders were stored at controlled temperatures of 4 °C (referred to as "reference powder"), 20 °C, 40 °C and 60 °C for various durations to a maximum of 12 months.

Table 1. Physico-chemical properties of MC retentates and powders

	Total dry matter ($g.kg^{-1}$)	Total nitrogen (% N x 6.38)	Non Protein Nitrogen (% N x 6.38)	Non Casein Nitrogen (% N x 6.38)	Ash ($g.kg^{-1}$)	Lactose ($g.kg^{-1}$)	Particle size (µm) Dv50	Water activity
Batch 1								
Retentate 1	137.60	11.9	0.01	1.16	11.00			
Powder 1	944.40	81.55	1.02	7.43	81.49	25.50	53.51	0.25
Batch 2								
Retentate 2	125.00	11.10	0.01	1.03	10.00			
Powder 2	935.00	83.60	0.97	7.05	75.20	11.30	39.01	0.29

2.2. Determination of solubility

To determine solubility, aqueous solutions of 5 % (w/w) MC powder were firstly prepared in distilled water at room temperature for 1 hour under stirring. Then, 50 ml of the MC solution was transferred into 50 ml centrifugation tubes and centrifuged using a Sigma 6K15 refrigerated centrifuge (Sigma, Labozentrifugen GmbH, Osterode am Harz, Germany) at 700 × g at 20 °C for 20 min. The supernatant was placed in a preweighed moisture dish and weighed. After that, the moisture dish (filled with the supernatant) was dried for 24 h in an oven at 105 °C before being cooled down to room temperature in a desiccator containing dry silica gel to avoid condensation and then reweighed. The percentage of soluble material (σ) of the MC powder was calculated using the following equation (Anema et al., 2006):

$$\sigma = \frac{\text{Weight of dry material}}{\text{weight of solution}} \times 100 \qquad \text{Eq. (1)}$$

It represents the solids in the ultracentrifugal supernatant, expressed as a percentage of total soluble solids in the whole solution. The σ values of aged powder samples were compared to that of the reference powder, and this σ(aged)/σ(reference) ratio in percentage was used to study the solubility evolution during ageing.

2.3. Colour measurement of MC powder and browning index determination

The colour measurements were carried out during storage. A CR-300 Minolta colorimeter (Konica Minolta, Osaka, Japan) run in the L-a-b space was used. Prior to measurements, values of L*, a* and b* were calibrated with a standard reference: 96.03, 4.71 and 7.24, respectively. After dispersion of powder on a Petri dish, colour measurement was carried out on 3 different points on the surface of the receptacle. The browning index (BI), used to assess

the intensity of the brown colour of the samples, was calculated from formula combining the L*a*b* (Oliveira et al., 2012):

$$BI = \frac{[100(x - 0{,}31)]}{0{,}17} \qquad \text{Eq. (2)}$$

$$\text{With} \quad x = \frac{(a^* + 1{,}75 \times L^*)}{(5{,}645 \times L^* + a^* - 3.012 \times b^*)} \qquad \text{Eq. (3)}$$

2.4. Rehydration tests

The equipment used for performing rehydration tests has been previously described by Richard et al. (2013; 2012b).

MC powders were reconstituted at 8 % (w/w) dry matter content. This concentration is commonly used in dairy applications at industrial scale. The dairy powder was added manually to the free liquid surface. The powder rehydration protocol was described in a previous publication (Jeantet et al., 2010). The rehydration protocol and sampling site were kept constant throughout all the experiments.

After the addition of powder to the liquid surface (at a stirring rate of 900 rpm), the impeller rotational speed of the agitator N was set at 1,200 rpm for 45 seconds to ensure that the powder was fully incorporated into the liquid. Then, N was set at a constant speed of 900 rpm until the end of the rehydration test. It should be noted that a turbulent flow regime and full suspension of powder particles were achieved at this N value.

2.5. Granulomorphometer measurements and determination of fragmentation time

Digital images of powder particles during the course of the rehydration tests were obtained using the Flow-Cell272 200 S-M (Occhio, Angleur, Belgium) granulomorphometer. This

microscopy technique makes it possible to analyse samples, diluted or not, of the MC suspension pumped into the vessel of the rehydration test. The principle of this granulomorphometer was described in a previous publication (Richard et al., 2012b). Readers can refer to this article for more details.

As previously described by Richard et al. (2012b), the fragmentation time has been defined as the time required to reach the maximum number of particles during the rehydration test.

2.6. Static light scattering and determination of total rehydration time

As described by Richard et al. (2012b), particle size distribution, measured by SLS (Static Light Scattering, Mastersizer 2000, Malvern Instruments Ltd, England) was used to determine the total rehydration time for each test. Sampling was done as follows: 0.5 mL of the powder suspension was withdrawn with a 16-mm-diameter syringe from the rehydration vessel. Samples were diluted in the Malvern cell (volume: 100 mL) with distilled water to reach appropriate obscuration (25 %). The solvent and particle refractive indices were set at 1.33 and 1.3, respectively, for particle size distribution measurements. The absorption index was set at 0.1. The class size limits were 0.02–2,000 μm. Particle size distribution was measured every 5 minutes until the fragmentation time was complete, and then approximately every 30 minutes as long as the size of the particles was larger than 15 μm and finally every 5 minutes until the end of the experiment when reaching the total rehydration time. According to Richard et al. (2012b), the total rehydration time was defined as the time required for Dv50, calculated from the particle size distributions, to reach 0.2 μm after the addition of powder.

The mean standard deviation of total rehydration time results was equal to 11.4%. Similar values of standard deviation for different powders were obtained by Jeantet et al.,(2010).

2.7. Statistical analysis

Student's *t*-tests with a 0.05 level of significance were used to measure the significance of the differences between samples stored and reference powder none stored. As widely known, the differences are statistically nonsignificant when $p > 0.05$ and statistically significant when $p < 0.05$.

3. Results & discussion

Firstly, experimental data collected for MC P1 for the 4 different storage temperatures are presented and discussed.

3.1. Browning index evolution with ageing conditions

The change of colour visually observed for the MC P1 as a function of storage time for different storage temperatures is illustrated in **Figure 1A**. As expected, it clearly appears that browning is strongly influenced by the storage temperature set. Browning occurred faster when the storage temperature increased. At a storage temperature of 60 °C, the increase of the brown coloration of the powder is so significant that it is clearly detectable even by naked eyes.

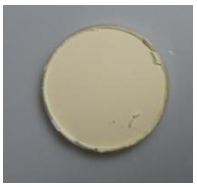
The evolution of the BI for MC P1 stored at different temperatures as a function of time is illustrated in **Figure 1B**. The BI of the MC P1 stored at 4 °C and 20 °C evolves slightly from 8.34 to 8.43 and 8.72, respectively, after 365 days of storage ($p<0.05$). However, the BI indicator of the MC P1 stored at 40 °C showed a significant slight linear evolution ($p<0.05$) over the whole range of storage time studied (360 days). At 60 °C, the evolution of BI with storage time for MC P1 was so drastic that an increase of 50 % was achieved after only 5 days of storage. In fact, the change in BI over time at 60 °C is characterised by a two-step mechanism: During the first 60 days of storage, BI increased linearly with storage time (with

a slope equal to 0.46) ($p<0.05$). Then for the storage period from 60 to 360 days, the increasing rate of BI decreased down to nearly 0 and a plateau was observed ($p>0.05$).

In the literature, it is often suggested that the presence of small quantities of lactose is sufficient to trigger a Maillard reaction. Indeed, interactions between lactose and lysine can occur, leading to food properties evolution like colour, flavour and nutritional value (Martins et al., 2000).

Typically, both the temperature and the storage time have been proven to impact the Maillard reaction. For the MC P1 investigated, it is highly probable that such phenomena were also involved in the browning process.

A.

Reference powder stored at 4 °C	Stored at 20 °C	Stored at 40 °C	Stored at 60 °C	
				**15 days**
				**1 month**
				**12 months**

B.

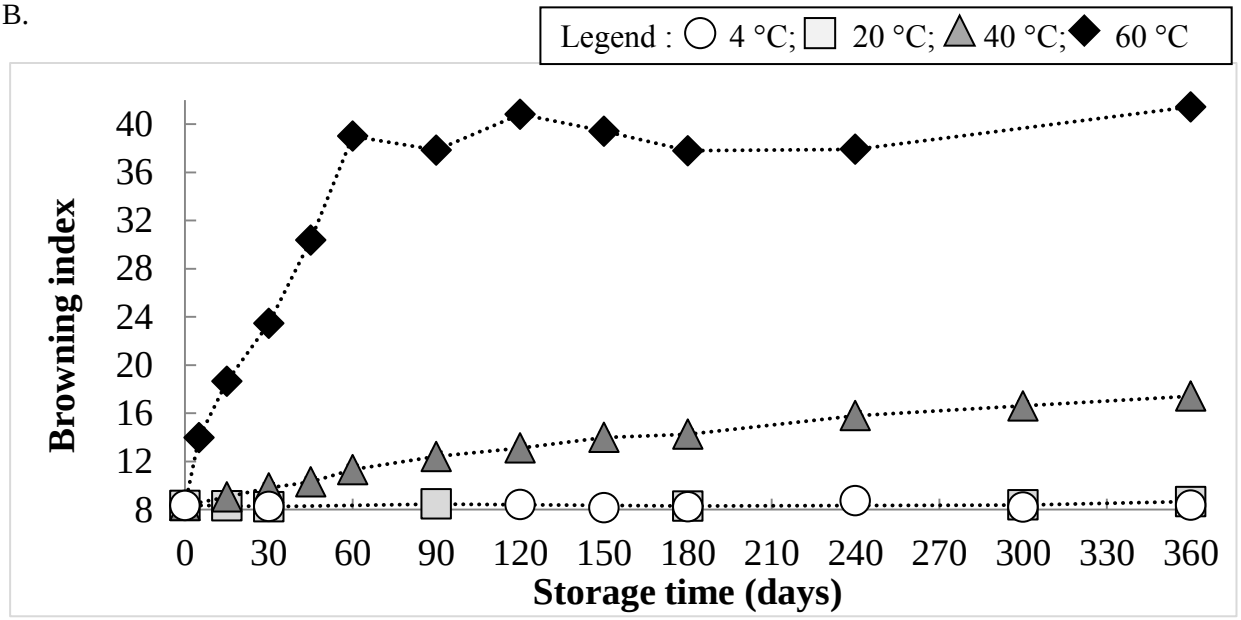


Figure 1. (A) Pictures of MC P1 and visualisation of browning of powder during storage (B) Browning index as a function of storage time for MC P1 stored at different temperatures

3.2. Solubility evolution with ageing conditions

The solubility of MC P1 stored at different temperatures is plotted as a function of time in **Figure 2**. Like what was observed for BI, evolution of solubility with storage time also reveals to be highly dependent on the storage temperature.

The MC P1 stored at 20 °C showed little change in solubility over a storage time of 180 days. It could be observed that the solubility slightly and linearly decreased from 100 % to ~80 % over that period of storage.

A different solubility evolution upon ageing is observed when storage temperature increases. Indeed, the solubility of MC P1 stored at 40 °C drastically decreased in the first days of ageing, then after 60 days of storage at this temperature a plateau was reached and the solubility became constant at around 25 % of the initial value. This drastic decrease of solubility at the first instant of storage at a higher storage temperature was amplified when ageing occurred at 60 °C. It could be noticed that the solubility of the MC P1 stored at 60°C decreased dramatically after 8 h of storage and reached an asymptotic value of ~15 % at 60 days of storage. For these two most severe storage conditions tested, the solubility was first subjected to an exponential decay then reached an asymptotic value.

The loss of solubility observed for MC P1 at higher storage temperatures was in agreement with both the expected trend (increase in quantity of insoluble material with time and storage temperature) and previous studies of high-protein dairy powder (Anema et al., 2006; Gazi and Huppertz, 2015; Le et al., 2011a; Le et al., 2011b)

From further analysis of the experimental data, it appeared that solubility and browning index didn't follow the same evolution with storage time: decay with time is observed for solubility whereas a growth is noticed for BI. For a given storage temperature, the monotonicity and rates of change of these two indicators vary differently with storage time. Indeed, for a storage temperature fixed at 40 °C, the change in solubility followed an exponential decrease contrary

to the change in BI, which followed a linear growth at this temperature (Figure 1). Similarly, the BI values did not change with time during storage at 20 °C, whereas solubility decreased by 23 %. This kinetic difference clearly indicates that loss of solubility and increase in browning during storage are not induced by the same phenomena and consequently the evolution of these two indicators are unlikely to be governed by the apparition of the same chemical species at molecular level.

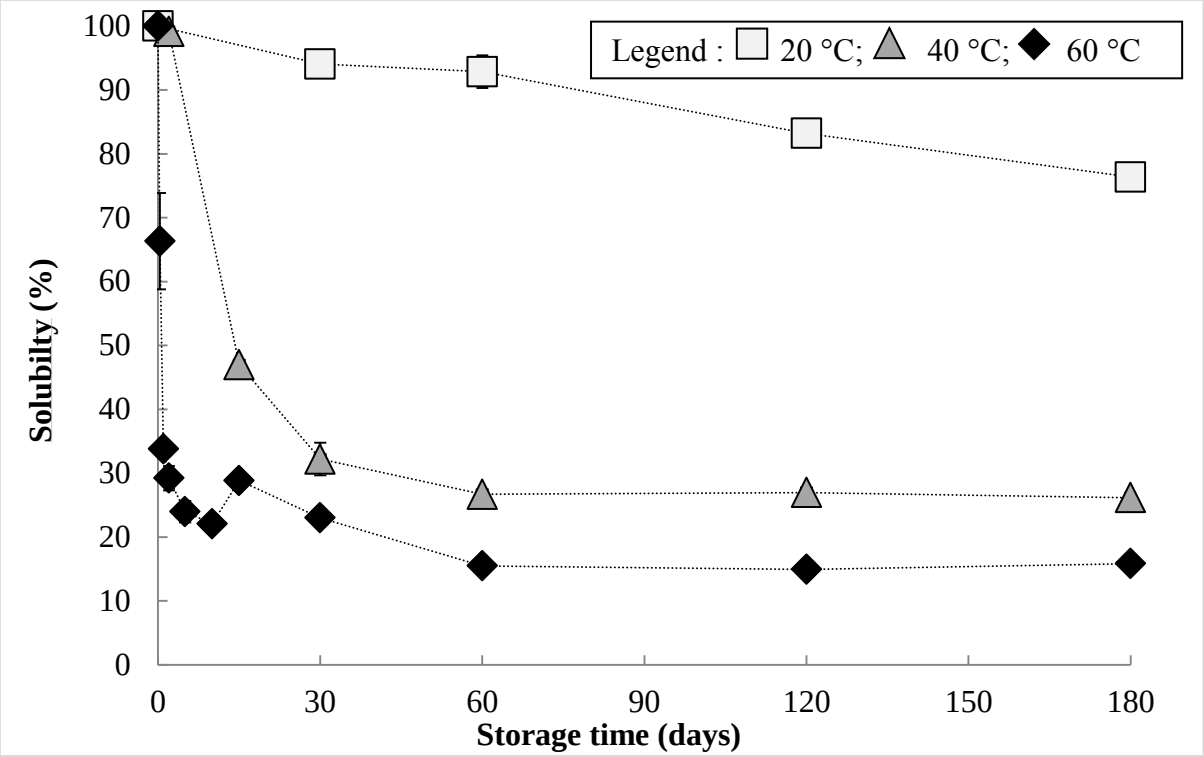


Figure 2. Change in solubility as a function of storage time for MC P1 stored at different temperatures

3.3. Fragmentation time evolution with ageing conditions

Figure 3 shows the change in fragmentation time of MC P1 stored at various temperatures for varying storage times. It can be observed that the storage temperature modify strongly the shape of the fragmentation time / storage time curve. Indeed, the fragmentation time of MC P1 stored at 4 °C doesn't evolve with storage time ($p>0.05$), and barely evolves at 20 °C ($p<0.05$). In contrast, the fragmentation time of MC P1 stored at 40 °C exhibited a 10-fold increase after 15 days of storage and increase significantly until 2 months of storage. The

change in the fragmentation time of MC P1 stored at 60 °C was even more drastic over time, with a 15-fold increase after only 2 days of storage and increase significantly until 5 days of storage.

To our knowledge, there is yet no study reported on fragmentation time evolution upon ageing. We are the first group to report this indicator evolution with storage time for various storage temperatures. Consequently, it is difficult to do further discussion and comparison on the experimental data obtained. Nevertheless, the change in fragmentation time should be regarded as an indicator which quantifies the ease of dissolution of the solid bridges between cross-linked micellar caseins. Consequently, the increase of fragmentation time with the severity of ageing that we observed is actually in agreement with the work of Anema et al., (2006), who have reported a cross-linking increase with storage time and temperature. Indeed, the increase of protein cross-linking during storage makes it more difficult to fragment and dissolve the particles.

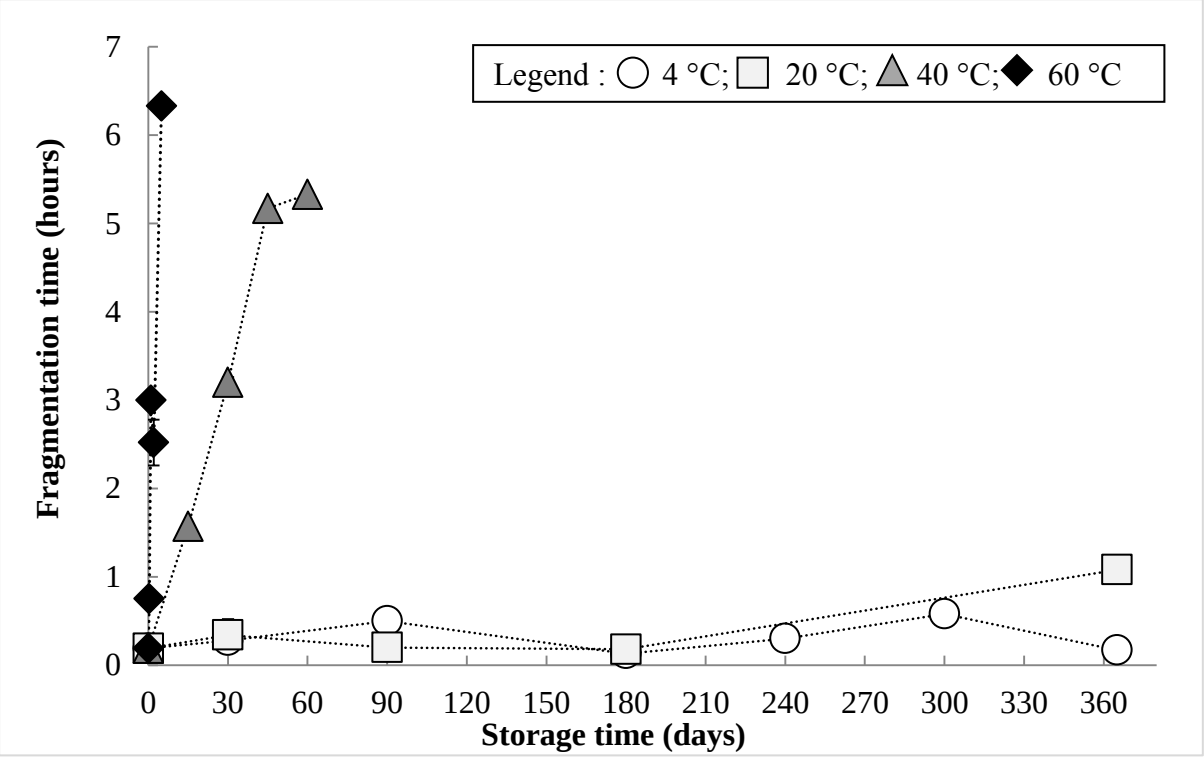


Figure 3. Fragmentation time as a function of storage time for MC P1 stored at different temperatures

3.4. Rehydration time evolution with ageing conditions

The rehydration time of MC P1 is plotted against storage time in **Figure 4**. Again, temperature was observed to have a major impact on the evolution of total rehydration time upon ageing.

The total rehydration time, after 12 months of storage, increased from ~3.5 h to ~5 h for the powder stored at 4 °C ($p<0.05$) and from ~3.5 h to ~7 h ($p<0.05$) for the powder stored at 20 °C. It is worth re-mentioning that a 23 % decrease in solubility was observed after 6 months of storage for the powder stored at 20 °C (Figure 2).

These changes in total rehydration time over storage period are relatively mild when compared to storage at 40 °C and 60 °C. Indeed, the rehydration time doubled for MC P1 stored at 40 °C after 15 days ($p<0.05$) and reached ~29 h after 2 months of storage ($p<0.05$). The change in rehydration time of MC P1 stored at 60 °C was even more drastic. Its value has more than doubled after only 24 h of storage ($p<0.05$) and reached ~35 h after only 5 days of storage ($p<0.05$). These results indicate that basically insolubility developed under all storage conditions, but at different rates depending on the storage temperature.

It seemed that the storage-temperature dependent rehydration time and solubility changes are intimately linked; the lower the solubility, the longer the rehydration time. This is in agreement with several works that have previously referred to this trend for MPC powder (Anema et al., 2006; Gazi and Huppertz, 2015).

As suggested by Fyfe et al., (2011) and Mimouni et al., (2011; 2010a) insolubility can be caused by the formation of a network of cross-linked proteins at the surface of the powder. These surface cross-links act as a barrier to the transport of water which inhibits the hydration of the particles, their subsequent disruption and dissolution, leading to the increase of both fragmentation time and total rehydration time. The increase in the degree of cross-linking

with increasing storage time and temperature reported by Anema et al., (2006) is consistent with this interpretation.

The greater the severity of storage conditions, the more the indicators change. However, it is still difficult at this stage to assess whether indicator parameters (fragmentation time, solubility loss and browning index) could be used to rapidly predict the increase in rehydration times due to ageing. The objective of the next section is to address this issue.

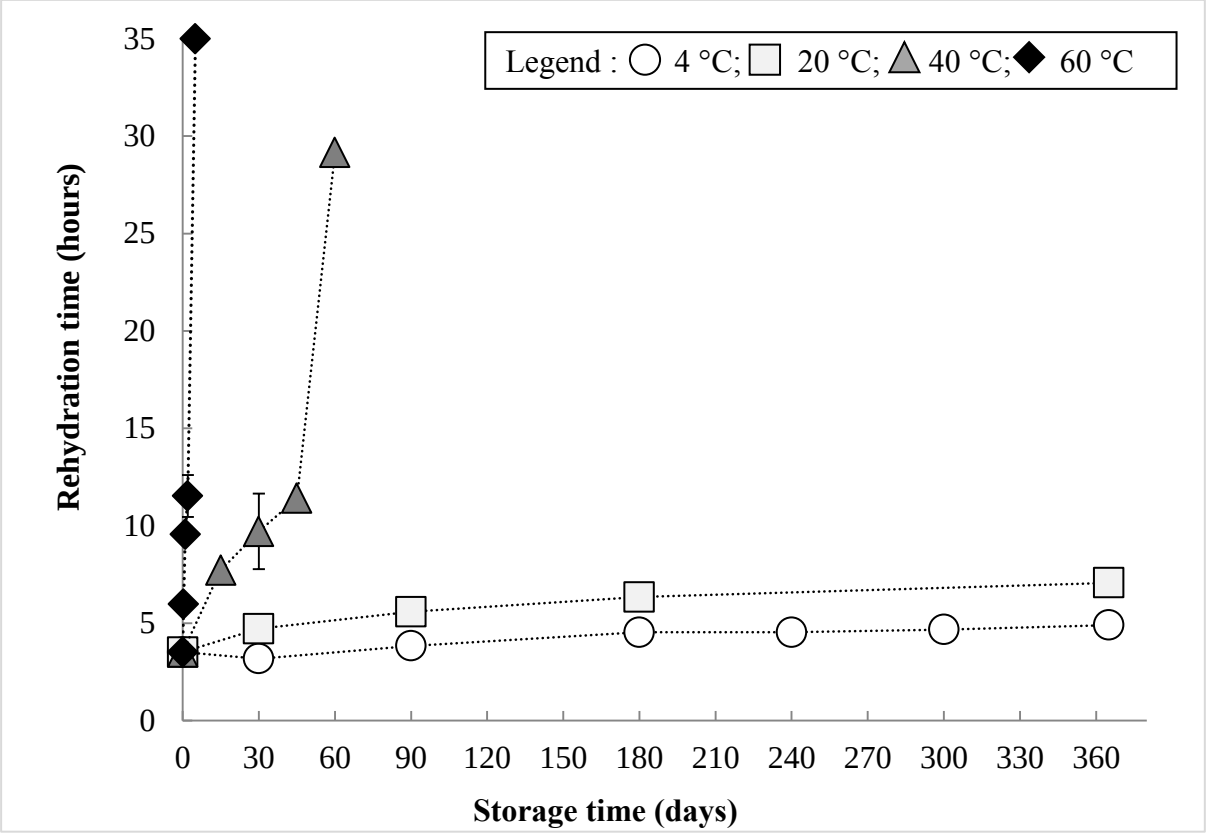


Figure 4. Rehydration time as a function of storage time for MC P1 stored at different temperatures

3.5. Correlation between indicators (fragmentation times, solubility and Browning Index) and rehydration time for MC P1

Firstly, rehydration time versus fragmentation time (**Figure 5A**) and rehydration time versus solubility were plotted (**Figure 5B).** These two indicators (fragmentation times and solubility) were analysed first rather than browning index since the underlying phenomena involved in the evolution of these two indicators are expected to be the same as those governing the

rehydration time evolution during ageing. As evoked previously, it is not so evident that the BI change is closely connected to rehydration properties evolution. Indeed, browning and insoluble formation are not necessarily induced by the same chemical modification pathway. Analysis of **Figures 5A and 5B reveals** that each pair of indicators ([fragmentation time; rehydration time] and [solubility; rehydration time] respectively) follows a reference ageing curve, which could be described using a linear function independent of the storage time and temperature set during ageing. The fitting functions are given by Equation 4 (A&B) for Figure 5A and by Equation 5(A&B) for Figure 5B.

$$\text{Rehydration time} = -0.08 \times \text{solubility} + 12.13 \text{ with } R^2 = 0.96 \text{ if solubility} > 33\ \% \quad \text{Eq. (4A)}$$

$$\text{Rehydration time} = -2.83 \times \text{solubility} + 101.58 \text{ with } R^2 = 0.86 \text{ if solubility} < 33\% \quad \text{Eq. (4B)}$$

$$\text{Rehydration time} = 2.06 \times \text{fragmentation time} + 4.13 \text{ with } R^2 = 0.81 \text{ if fragmentation time} < 4.80$$

Eq. (5A)

$$\text{Rehydration time} = 15.20 \times \text{fragmentation time} - 60.09 \text{ with } R^2 = 0.61 \text{ if fragmentation time} > 4.80$$

Eq. (5B)

In a second time, the rehydration time versus browning index was plotted (**Figure 5C**). Similarly, the analysis of this figure also reveals the existence of an ageing curve independent of the storage time and temperature for this indicator (Equation 6).

$$\text{Rehydration time} = 0.0004 \times \text{Browning Index}^{4.4} \text{ with } R^2 = 0.90 \quad \text{Eq. (6)}$$

The existence of these reference ageing curves and fitting functions (Equations 4-5-6) are of interest. The time-consuming and tedious-experimental-protocol requiring rehydration time determination process could be simplified since the rehydration time could be rapidly

predicted through the determination of fragmentation time or solubility. This is to say that it would be sufficient to carry out fragmentation time or solubility measurements, which are among the easiest tests, to rapidly estimate the rehydration time for the solid-liquid suspension. Based on this method, process parameters (for example rotational impeller speed, N for rehydration in agitated vessel equipped with vertical agitator centrally mounted) can be easily adjusted to achieve the desired product quality and functionality (Jeantet et al., 2010; Richard et al., 2013).

The discovery that reference ageing curves exist and gather all the aged samples of the MC P1 studied is of interest. Indeed, this fact suggests that changes occurred at the highest temperatures over a short storage period could mimic changes occurred under milder temperature conditions over a longer period of time. Therefore, it can be envisaged to establish, under accelerated ageing conditions, the ageing curves for MC P1 which are also valid for less severe conditions. For example, the approximate rehydration time / solubility ageing curve of MC P1 can be plotted using 3 experimental points: initial state and after 2 and 5 days of storage at 60 °C.

Linear regression of the 2 straight lines obtained by connecting the three experimental points leads to the following equations (Equations 7-8-9) :

$$\text{Rehydration time} = -0.11 \times \text{solubility} + 14.84 \text{ with } R^2 = 1 \text{ if solubility} > 29.21\ \% \quad \text{Eq. (7A)}$$

$$\text{Rehydration time} = -4.50 \times \text{solubility} + 142.74 \text{ with } R^2 = 1 \text{ if solubility} < 29.21\% \quad \text{Eq. (7B)}$$

$$\text{Rehydration time} = 3.44 \times \text{fragmentation time} + 2.86 \text{ with } R^2 = 1 \text{ if fragmentation time} < 2.52$$

Eq. (8A)

$$\text{Rehydration time} = 6.16 \times \text{fragmentation time} - 3.99 \text{ with } R^2 = 1 \text{ if fragmentation time} > 2.52$$

Eq. (8B)

$$\text{Rehydration time} = 3.17 \times \text{Browning Index} - 21.90 \text{ with } R^2 = 0.93 \text{ if IB} < 10.8 \text{ Eq. (9A)}$$

$$\text{Rehydration time} = 7.40 \times \text{Browning Index} - 68.43 \text{ with } R^2 = 1 \text{ if IB} > 10.8 \text{ Eq. (9B)}$$

It could be noticed on **Figure 5** that "the approximated ageing curves", established using only 3 accelerated ageing points (dotted line) are not far away from the reference ageing curves (obtained with all the data points tested, in solid line). In this demonstration, only 3 points were used to obtain the approximated ageing curve, because they are sufficient to cover all the experimental data. Obviously, the greater the number of accelerated ageing points used, the more accurate the approximated ageing curve will be.

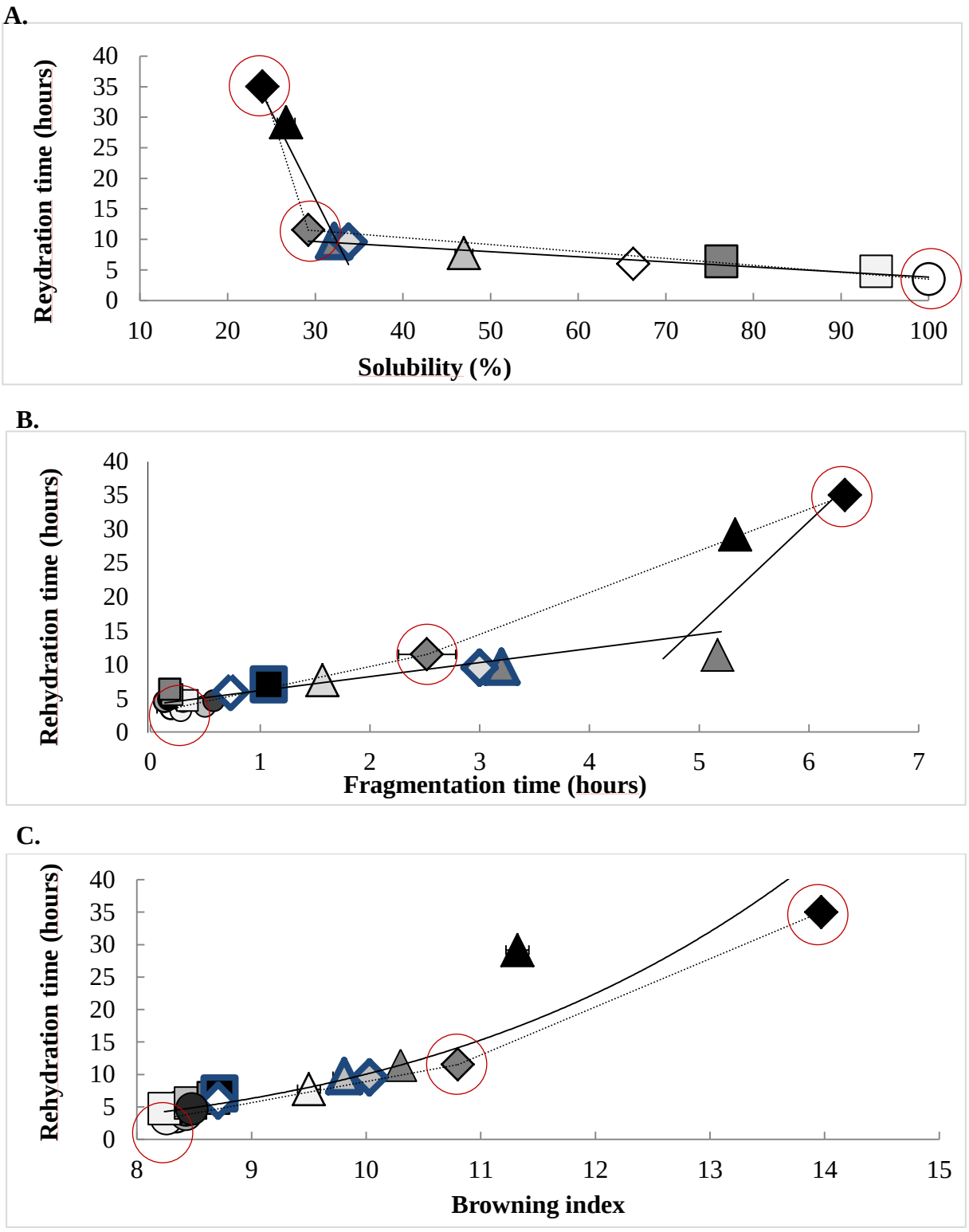


Figure 5. Rehydration time as a function of (A) solubility (B) fragmentation time (C) browning index, of MC P1 stored at 4°C (circle), 20 °C (square), 40 °C (triangle) and 60°C (diamond shape); For the legend, the darker the inner colour of the symbol, the longer the storage time. Reference ageing curves using all ageing points tested are in solid lines, approximate ageing curves established with 3 ageing points (surrounded in red) are in dotted line. Ageing similarities between severe storage and moderate storage are outlined in blue.

3.5. Confirmation of the existence of reference ageing curves and possibility to establish ageing curves using accelerated ageing conditions for MC P2

To confirm the existence of reference ageing curves independent of temperature and storage time for each indicator (solubility, fragmentation time, browning index), as obtained for MC P1, experimental study with another MC powder derived from a different retentate, named MC P2, was performed.

The functional properties (browning index, solubility, fragmentation and total rehydration times) of the two MC powders (reference powder stored at 4 °C and aged powder stored for 5 days at 60 °C) are reported in **Table 2**. It was observed that the final browning index of the aged MC P2 powder is lower than that of the aged MC P1 powder. Moreover, it turned out that rehydration properties of the two aged powders are markedly different after 5 days of storage at 60 °C: a total rehydration is achieved more quickly for powder 2 compared to powder 1.

Firstly, since lactose is responsible for the brown colour of the powders (via the Maillard reaction), the lower lactose content of MC P2 explains its lower browning index.

Secondly, the dry matter compositions of the two retentates are comparable. The lower dry extract of MC P2 implies hence a lower viscosity of retentate 2 (Devilder and Moermans, 1983; Schuck et al., 2005; Snoeren et al., 1982) explaining the smaller particle size of the powder produced by spray drying (Baldwin et al., 1980; Masters, 2002), as shown in Table 1. When the viscosity of the feed liquid increases, more energy is dissipated in the spray tower nozzle to overcome viscous forces. Thus the energy available for breaking up the droplets is reduced, resulting in larger droplets. Therefore, smaller droplets are formed for a less viscous liquid.

Finally, it is widely recognised since the pioneer work of Noyes–Whitney, (1897) that the dissolution rate of chemical particles is faster for smaller ones because the specific surface

area increases when particle size decreases. The review of Dokoumetzidis & Macheras, (2006) and the work of Gaiani et al. (2007) have also confirmed this physical law respectively for biopowders and for milk derivative powders. It is evidenced that the dispersion step (corresponding to the dispersion of single particles throughout the water) is favoured when particle size decreases. As dispersion is known to be the limiting step of MC powder rehydration (Gaiani et al., 2007b), it is logical to consider the smaller particle size of MC P2, resulting from viscosity difference in the drying operation, to be responsible for its faster rehydration.

The relationships between the different indicators and the rehydration times were plotted for MC P2. The reference ageing curves obtained for this second batch MC P2 are presented in **Figure 6**. The corresponding fitting functions are as follows (Equations 10 to 12):

$$\text{Rehydration time} = 8834.2 \times \text{solubility}^{-2.2} \text{ with } R^2 = 0.98 \quad \text{Eq. (10)}$$

$$\text{Rehydration time} = -0.1 \times \text{fragmentation time}^2 + 2.2 \times \text{fragmentation time} + 0.3 \text{ with } R^2 = 0.94 \quad \text{Eq. (11)}$$

$$\text{Rehydration time} = 0.5 \times \text{Browning Index}^2 + 11.1 \times \text{Browning Index} - 55 \text{ with } R^2 = 0.94 \quad \text{Eq. (12)}$$

Unsurprisingly, the shape of reference ageing curves and type of mathematical equations for MC P2 were very different from MC P1. But this new set of results confirmed again anyway the possibility of obtaining reference ageing curves that link the rehydration time with each individual indicator (fragmentation time, browning index and solubility), regardless of the ageing conditions adopted during storage. Experimental data obtained for MC P2 also corroborate the idea that applying accelerated ageing conditions is an interesting way to

predict the changes that would occur under milder conditions (lower temperatures). As for previous MC P1 investigated, three conditions of storage seem particularly interesting to examine the predictive trajectory of ageing curves: initial state and after 2 and 5 days' storage at 60 °C.

The equations for the ageing curves of MC P2 established on these three ageing points using a linear regression per interval are as follows:

$\text{Rehydration time} = -0.09 \times \text{solubility} + 9.67$ with $R^2 = 1$ if solubility > 27 % Eq. (12A)

$\text{Rehydration time} = -0.63 \times \text{solubility} + 24.05$ with $R^2 = 1$ if solubility < 27 % Eq. (12B)

$\text{Rehydration time} = 3.71 \times \text{fragmentaion time} + 0.16$ with $R^2 = 1$ if fragmentation time < 1.87 Eq. (13A)

$\text{Rehydration time} = 0.61 \times \text{fragmentation time} + 5.98$ with $R^2 = 1$ if fragmentation time > 1.87 Eq. (13B)

$\text{Rehydration time} = 5.14 \times \text{browning index} - 34.78$ with $R^2 = 1$ if browning index < 8.15 Eq. (14A)

$\text{Rehydration time} = 1.85 \times \text{browning index} - 7.96$ with $R^2 = 1$ if browning index > 8.15 Eq. (14B)

Again, it could be remarked on **Figure 6** that the ageing curves established using these 3 accelerated ageing conditions (dotted line) give good approximation of reference ageing curves obtained using all experimental data points (solid line).

Further observations of the reference ageing curves (Figures 5 & 6) made it possible to identify and confirm several ageing similarities between severe and moderate ageing conditions. For example, ageing after a period of 8 hours at 60 °C was approximately equivalent to ageing after a period of 12 months at 20 °C, the condition commonly encountered during powder storage and delivery. In the same way, ageing after a 24 hour period at 60 °C is equivalent to that after 1 month period at 40 °C. These equivalences are outlined in blue bold in Figure 5 & 6. These [storage time; storage temperature] concordances are of great help to rapidly predict property changes of the powder during storage.

In previous section, it has been suggested to rapidly identify the shapes of reference ageing curves for the MC powder studied (by using accelerated ageing conditions). It is then necessary to assess the accuracy of predicted rehydration times. The objective of the next section 3.6. is to address this issue. A reverse engineering approach is proposed based on the approximate ageing curves obtained and the measurements of indicators.

Table 2. Functional properties of the two powders studied at reference state (storage at 4°C) and after 5 days' storage at 60 °C

	Powder 1		**Powder 2**	
	Reference powder	Powder stored 5 days at 60 °C	Reference powder	Powder stored 5 days at 60 °C
Browning index	8.34	13.97	6.82	11.86
Solubility (%)	100.00	24.00	100.00	16.10
Fragmentation time (Hours)	0.19	6.33	0.04	13.10
Rehydration time (Hours)	3.51	35.00	0.29	14.00

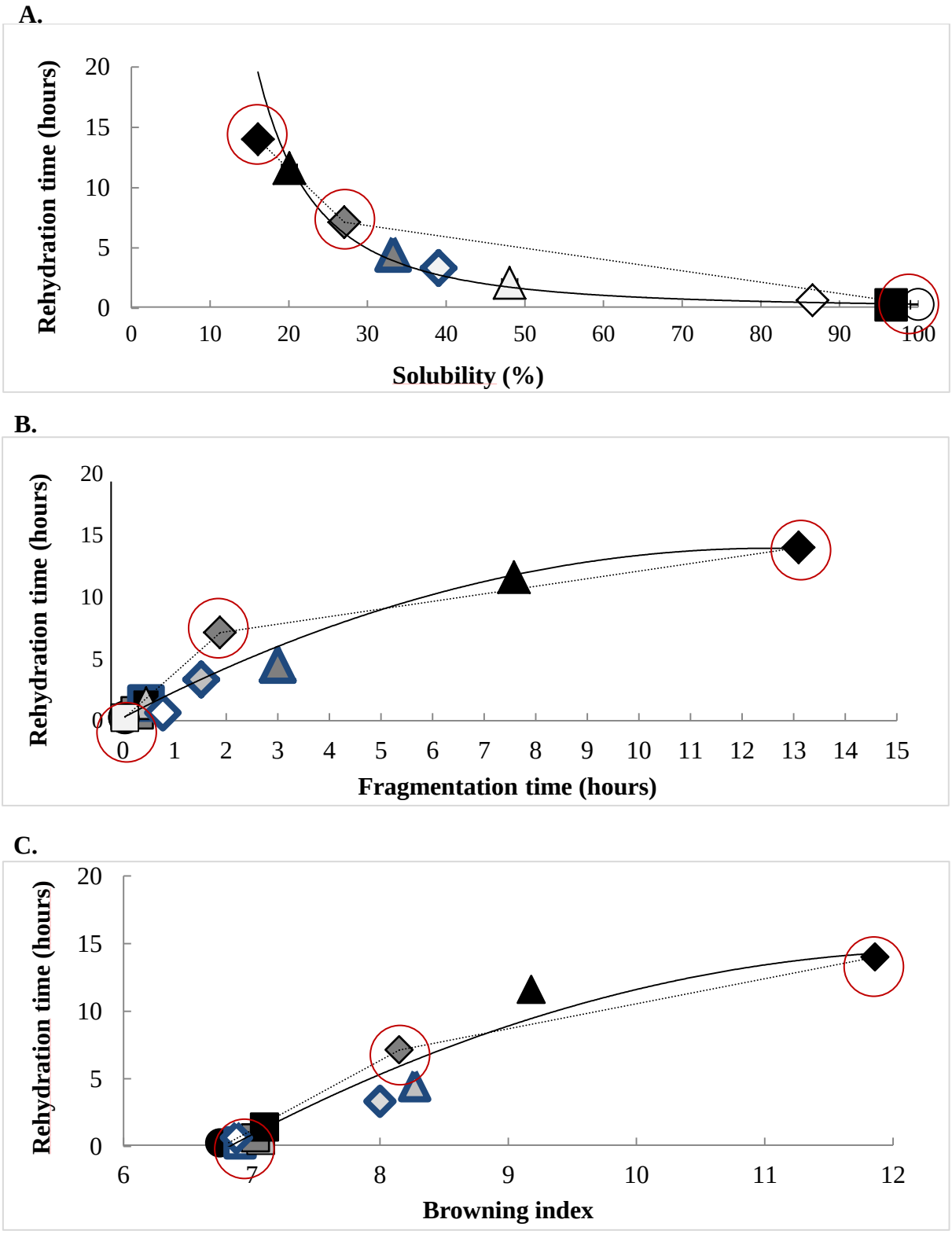


Figure 6. Rehydration time as a function of (A) solubility (B) fragmentation time (C) browning index, of MC P2 stored at 4°C (circle), 20 °C (square), 40 °C (triangle) and 60°C (diamond shape);); For the legend, the darker the inner colour of the symbol, the longer the storage time. Ageing curves using whole ageing points are in solid lines, ageing curves established with 3 ageing points (surrounded in red) are in dotted line. Ageing similarities between severe storage and moderate storage are outlined in blue

3.6 Comparison between experimental and predicted rehydration time

In order to assess the feasibility of predicting the rehydration time for aged samples, experimental and predicted values have been compared for the two MC powders studied. The 4-step procedure used to determine the predicted rehydration time is as follows:

Step 1. Indicator (solubility, fragmentation time or BI) and rehydration time values of the powder studied are measured for three particular ageing conditions (initial state-fresh powder and two accelerated ageing conditions -2 and 5 days of storage at 60 °C)

Step 2. The three experimental points obtained [indicator; rehydration time] are plotted to obtain a first approximated ageing curve

Step 3. The equations of the approximate ageing curve are obtained through linear regression of the 2 straight lines connecting the 3 points.

Step 4. The indicator is measured for an arbitrary aged sample and the corresponding rehydration time value is evaluated using the set of equations obtained at step 3 by inverse engineering approach

Comparisons between experimental and predicted values of rehydration time for MC powders P1 and P2 are presented in Figure 7. Namely, Figures 7A, 7B and 7C give the comparisons based respectively on different indicators (solubility, fragmentation and browning index). Predicted values were obtained using approximated ageing curves previously established (Figures 5A-5B-5C for MC P1 and Figures 6A-6B-6C for MC P2).

Dark circle symbols in Figure 7A, 7B and 7C represent results for MC P1 while grey square symbols represent results for MC P2. Additional experiments under controlled storage conditions were carried out to ascertain the validity and accuracy of the proposed procedure for the prediction of rehydration time. These experimental points are presented in Figure 7A, 7B and 7C as cross symbols and will be commented more in details further in the text.

Whatever the indicator used, it could be observed in **Figures 7A to 7C** that there is a good agreement between predicted and experimental values of rehydration times. Indeed all the data are located in the proximity of the bisector line and the maximum percentage of error is inferior to 25 %. The predicted values are rather good since the standard deviation for experimental determination of rehydration time is relatively high (around 11.4%). Of course, if more numerous accelerated ageing conditions are applied and used to plot the approximate ageing curves, a more exact approximation can be achieved with appreciably reduced deviations between predicted and experimental values. In this case, the equations of the approximate ageing curves will become similar to that describing the reference ageing curves (Equations 7-9 and 10-12, depending on the MC powder studied and the indicators considered). In this context, it is obvious that a compromise between the accuracy of predicted values and experimental time consumed to build ageing curves should be chosen. Nevertheless, from a practical aspect, the feasibility to apply the proposed procedure for fast prediction of rehydration time has been demonstrated for the two powders studied.

To go further, it was decided to check the procedure with an independent series of data. 6 additional samples of aged powder were studied for this purpose. The independent series of data was obtained by subjecting MC P1 powder to storage accidents (short exposure to high temperatures close to 60 °C). Note that this kind of storage accidents are episodes that a powder is proved to undergo during its ship expedition (Leinberger, 2006). Consequently, predicting the effect on powder properties of such short exposure to high temperature is of great interest for both industries and consumers.

6 different accidents of storage were imposed for the 6 aged samples and measurements of indicator parameters and rehydration times were carried out. The ageing conditions of these additional samples and the exact instantaneous time for storage accident are presented in detail Figure 8. Sample 1 has been stored for 5 months at 20 °C with no storage accident.

Samples 2, 5 and 6 have been stored for 5 months at 20 °C including one storage accident (a rise in temperature to 60 °C and then maintained at 60 °C for 8 h after the temperature rise) at different storage times. Sample 5 and 6 have been stored for 5 months at 20 °C including respectively two and three storage accidents. **(Figure S1).**

Predicted rehydration time values of these additional samples subjected to storage accidents have been obtained using the procedure described above. Again, the experimental and predicted values of rehydration time for these additional samples are in good agreement. This further validates our rehydration time prediction procedure based on the determination of indicator parameters and corresponding ageing curves (which could be established by imposing accelerated ageing conditions).

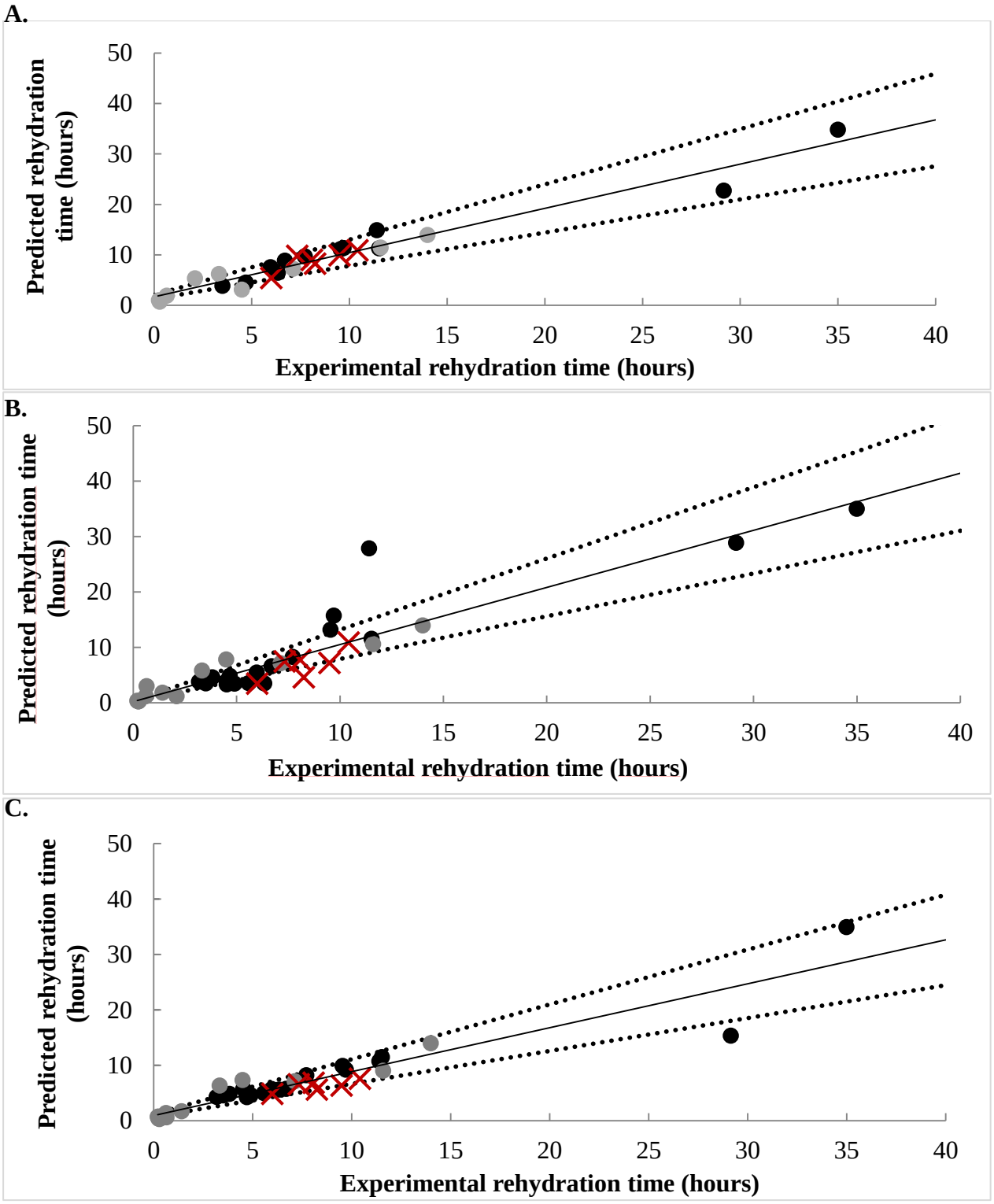


Figure 7. Comparison between rehydration times experimentally measured and predicted using approximate ageing curves based on the measurements of (A) solubility (B) fragmentation time (C) browning index . Measurements for MC P1 are in dark, measurements for MC P2 are in grey, measurements for samples going through storage accidents are represented by red cross. Dotted line represent percentage error.

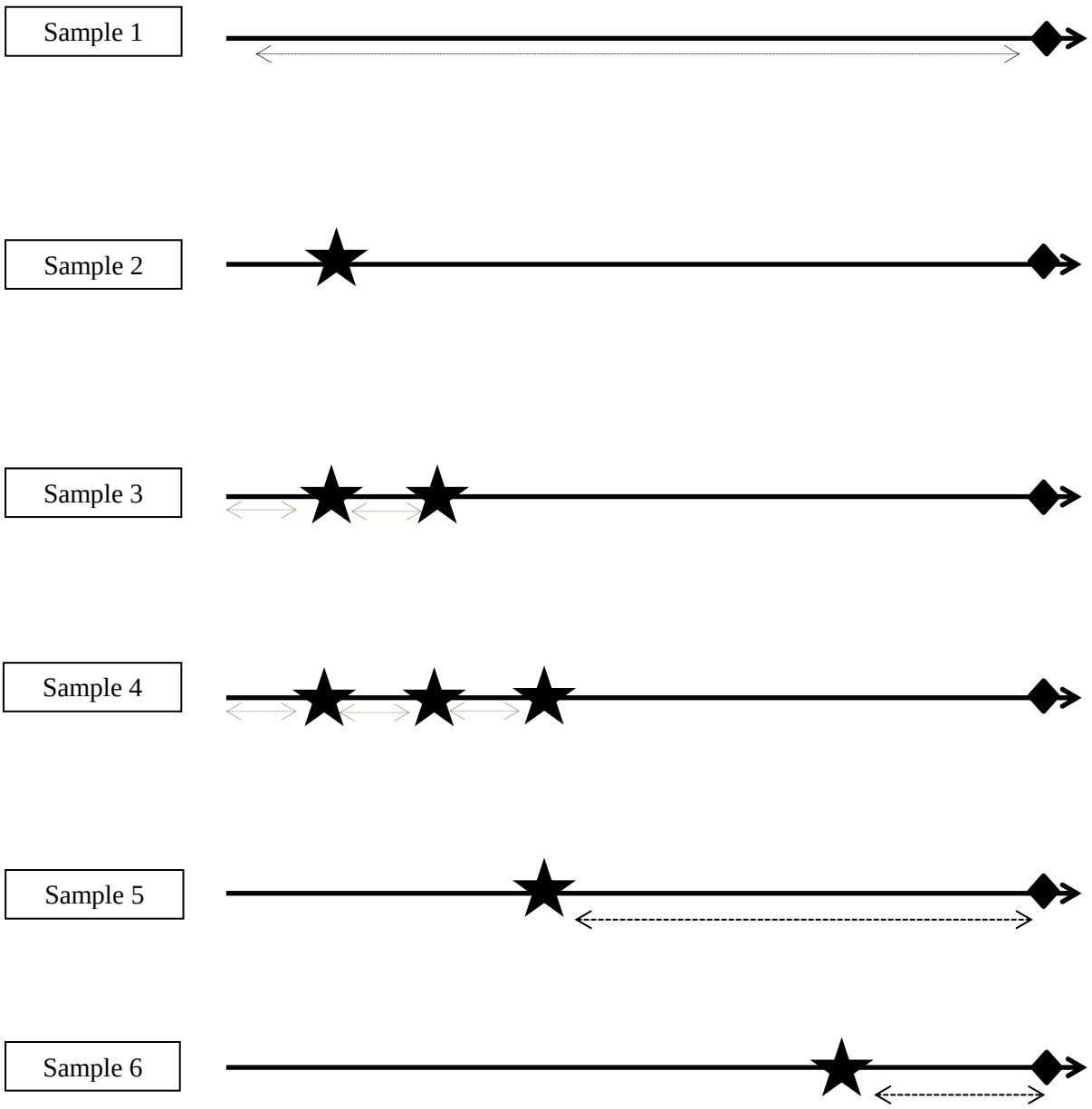


Figure S1. Ageing conditions of supplementary samples stored for 5 months at 20 °C. Stars correspond to storage accidents which is a rise in temperature to 60 °C and maintained at 60°C for 8 h. Full diamond correspond to the end of storage and time instant for analysis

4. Conclusion

To conclude, it has been shown that the characteristic rehydration times (total rehydration time and fragmentation time) as well as the browning index increased with storage time and temperature, whereas solubility decreased for both MC powders studied.

Ageing curves were plotted for each indicator, linking total rehydration time with fragmentation time, browning index and solubility. Several ageing similarities between severe storage and moderate storage have been demonstrated. The shape of each ageing curve depends only on the MC powder, not on ageing conditions.

It has then been shown that applying accelerated ageing conditions made it possible to visualize the reference ageing curves of the MC powders to be characterised. 3 samples subjected to three particular ageing conditions (initial state-fresh powder and two accelerated ageing conditions - 2 and 5 days of storage at 60 °C) were used for this purpose.

With the approximate ageing curves, the rehydration time can be rapidly predicted by measuring one indicator.

Finally, feasibility of obtaining predicted values of rehydration times was also demonstrated for a MC powder having undergone storage accidents (one or more temperature rises to 60 °C in the course of 5 months of storage).

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Bibliography

Anema, S. G., D. N. Pinder, R. J. Hunter, and Y. Hemar, 2006, Effects of storage temperature on the solubility of milk protein concentrate (MPC85): Food Hydrocolloids, v. 20, p. 386-393.

Baldwin, A. J., A. G. Baucke, and W. B. Sanderson, 1980, The effect of concentrate viscosity on the properties of spray-dried skim milk powder: New Zealand Journal of Dairy Science and Technology, v. 15, p. 289-297.

Carr, A., G. Bhaskar, and S. Ram, 2002, Monovalent salt enhances solubility of milk protein concentrate, WO02/096208.

De Castro-Morel, M., and W. J. Harper, 2002, Basic functionality of commercial milk protein concentrates: Milchwissenschaft-Milk Science International, v. 57, p. 367-370.

Devilder, J., and R. Moermans, 1983, The continous measurement of the viscosity of the concentrate during the production of milk powder: Milchwissenschaft-Milk Science International, v. 38, p. 449-452.

Dokoumetzidis, A., and P. Macheras, 2006, A century of dissolution research: From Noyes and Whitney to the Biopharmaceutics Classification System: International Journal of Pharmaceutics, v. 321, p. 1-11.

Fang, Y., C. Selomulya, S. Ainsworth, M. Palmer, and X. D. Chen, 2011, On quantifying the dissolution behaviour of milk protein concentrate: Food Hydrocolloids, v. 25, p. 503-510.

Fyfe, K. N., O. Kravchuk, T. Le, H. C. Deeth, A. V. Nguyen, and B. Bhandari, 2011, Storage induced changes to high protein powders: influence on surface properties and solubility: Journal of the Science of Food and Agriculture, v. 91, p. 2566-2575.

Gaiani, C., J. Scher, J. J. Ehrhardt, M. Linder, P. Schuck, S. Desobry, and S. Banon, 2007a, Relationships between dairy powder surface composition and wetting properties during storage: Importance of residual lipids: Journal of Agricultural and Food Chemistry, v. 55, p. 6561-6567.

Gaiani, C., P. Schuck, J. Scher, S. Desobry, and S. Banon, 2007b, Dairy powder rehydration: Influence of protein state, incorporation mode, and agglomeration: Journal of Dairy Science, v. 90, p. 570-581.

Gazi, I., and T. Huppertz, 2015, Influence of protein content and storage conditions on the solubility of caseins and whey proteins in milk protein concentrates: International Dairy Journal, v. 46, p. 22-30.

Haque, E., B. R. Bhandari, M. J. Gidley, H. C. Deeth, S. M. Moller, and A. K. Whittaker, 2010, Protein Conformational Modifications and Kinetics of Water-Protein Interactions in Milk Protein Concentrate Powder upon Aging: Effect on Solubility: Journal of Agricultural and Food Chemistry, v. 58, p. 7748-7755.

Haque, E., B. R. Bhandari, M. J. Gidley, H. C. Deeth, and A. K. Whittaker, 2011, Ageing-induced solubility loss in milk protein concentrate powder: effect of protein conformational modifications and interactions with water: Journal of the Science of Food and Agriculture, v. 91, p. 2576-2581.

Haque, E., B. R. Bhandari, M. J. Gidley, H. C. Deeth, and A. K. Whittaker, 2015, Change in molecular structure and dynamics of protein in milk protein concentrate powder upon ageing by solid-state carbon NMR: Food Hydrocolloids, v. 44, p. 66-70.

Haque, E., A. K. Whittaker, M. J. Gidley, H. C. Deeth, K. Fibrianto, and B. R. Bhandari, 2012, Kinetics of enthalpy relaxation of milk protein concentrate powder upon ageing and its effect on solubility: Food Chemistry, v. 134, p. 1368-1373.

Havea, P., 2006, Protein interactions in milk protein concentrate powders: International Dairy Journal, v. 16, p. 415-422.

Hunter, R. J., Y. Hemar, D. N. Pinder, and S. G. Anema, 2011, Effect of storage time and temperature of milk protein concentrate (MPC85) on the renneting properties of skim milk fortified with MPC85: Food Chemistry, v. 125, p. 944-952.

Jeantet, R., P. Schuck, T. Six, C. Andre, and G. Delaplace, 2010, The influence of stirring speed, temperature and solid concentration on the rehydration time of micellar casein powder: Dairy Science & Technology, v. 90, p. 225-236.

Jimenezflores, R., and F. V. Kosikowski, 1986, Properties of ultrafiltered skim milk retentate powders: Journal of Dairy Science, v. 69, p. 329-339.

Kher, A., P. Udabage, I. McKinnon, D. McNaughton, and M. A. Augustin, 2007, FTIR investigation of spray-dried milk protein concentrate powders: Vibrational Spectroscopy, v. 44, p. 375-381.

Le, T. T., B. Bhandari, and H. C. Deeth, 2011a, Chemical and Physical Changes in Milk Protein Concentrate (MPC80) Powder during Storage: Journal of Agricultural and Food Chemistry, v. 59, p. 5465-5473.

Le, T. T., B. Bhandari, J. W. Holland, and H. C. Deeth, 2011b, Maillard Reaction and Protein Cross-Linking in Relation to the Solubility of Milk Powders: Journal of Agricultural and Food Chemistry, v. 59, p. 12473-12479.

Le, T. T., J. W. Holland, B. Bhandari, P. F. Alewood, and H. C. Deeth, 2013, Direct evidence for the role of Maillard reaction products in protein cross-linking in milk powder during storage: International Dairy Journal, v. 31, p. 83-91.

Leinberger, D., 2006, Temperature and humidity in ocean containers, USA, Proceedings of Dimensions. International Safe Transit Association: East Lansing.

Marella, C., P. Salunke, A. C. Biswas, A. Kommineni, and L. E. Metzger, 2015, Manufacture of modified milk protein concentrate utilizing injection of carbon dioxide: Journal of Dairy Science, v. 98, p. 3577-3589.

Martins, S., W. M. F. Jongen, and M. van Boekel, 2000, A review of Maillard reaction in food and implications to kinetic modelling: Trends in Food Science & Technology, v. 11, p. 364-373.

Masters, k., 2002, Spray Drying in Practice: Spray Dry Consult International ApS.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari, 2010a, Investigation of the microstructure of milk protein concentrate powders during rehydration: Alterations during storage: Journal of Dairy Science, v. 93, p. 463-472.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari, 2010b, Rehydration of high-protein-containing dairy powder: Slow- and fast-dissolving components and storage effects: Dairy Science & Technology, v. 90, p. 335-344.

Noyes, A., and W. Whitney, 1897, The rate of solution of solid substances in their own solutions: Journal of the American Chemical Society, v. 19, p. 930-934.

Oliveira, F., M. J. Sousa-Gallagher, P. V. Mahajan, and J. A. Teixeira, 2012, Evaluation of MAP engineering design parameters on quality of fresh-sliced mushrooms: Journal of Food Engineering, v. 108, p. 507-514.

Paracha, G. M. U., 2011, Characterisation of the rehydration of milk protein concentrates in the presence of sugar, Massey University, Manawatu campus.

Pierre, A., J. Fauquant, Y. Legraet, M. Piot, and J. L. Maubois, 1992, Native micellar casein separation through cross flow membrane microfiltration: Lait, v. 72, p. 461-474.

Richard, B., J. F. Le Page, P. Schuck, C. Andre, R. Jeantet, and G. Delaplace, 2013, Towards a better control of dairy powder rehydration processes: International Dairy Journal, v. 31, p. 18-28.

Richard, B., M. Toubal, J.-F. Le Page, G. Nassar, E. Radziszewski, B. Nongaillard, P. Debreyne, P. Schuck, R. Jeantet, and G. Delaplace, 2012, Ultrasound tests in a stirred

vessel to evaluate the reconstitution ability of dairy powders: Innovative Food Science & Emerging Technologies, v. 16, p. 233-242.

Schuck, M. Piot, S. Méjean, Y. Le Graet, J. Fauquant, G. Brulé, and J. L. Maubois, 1994, Déshydratation par atomisation de phosphocaséinate natif obtenu par microfiltration sur membrane: Le lait, v. 74, p. 375-388.

Schuck, P., R. Jeantet, and A. Dolivet, 2012, Analytical Methods for Food and Dairy Powders, John Wiley & Sons.

Schuck, P., S. Mejean, A. Dolivet, E. Beaucher, and M. H. Famelart, 2005, Pump amperage: a new method for monitoring viscosity of dairy concentrates before spray drying: Lait, v. 85, p. 361-367.

Schuck, P., S. Mejean, A. Dolivet, C. Gaiani, S. Banon, J. Scher, and R. Jeantet, 2007, Water transfer during rehydration of micellar casein powders: Lait, v. 87, p. 425-432.

Selomulya, C., and Y. Fang, 2013, Food powder rehydration, *in* B. Bhandari, N. Bansal, M. Zhang, and P. Schuck, eds., Handbook of Food Powders: Processes and Properties: Woodhead Publishing Series in Food Science Technology and Nutrition: Cambridge, Woodhead Publ Ltd, p. 379-408.

Sikand, V., P. S. Tong, J. Walker, T. Wang, and L. E. Rodriguez-Saona, 2016, Short communication: Effect of storage temperature on the solubility of milk protein concentrate 80 (MPC80) treated with NaCl or KCl: Journal of Dairy Science, v. 99, p. 1791-1795.

Singh, H., 2002, Milk Proteins : Functional properties: Encyclopedia of Dairy Science, p. 1976-1982.

Snoeren, T. H. M., A. J. Dammam, and H. J. Klok, 1982, The viscosity of whole milk concentrates: Neth. Milk Dairy J, v. 36, p. 305-316.

Udabage, P., A. Puvanenthiran, J. A. Yoo, C. Versteeg, and M. A. Augustin, 2012, Modified water solubility of milk protein concentrate powders through the application of static high pressure treatment: Journal of Dairy Research, v. 79, p. 76-83.

Chapitre 2. Etude de l'évolution de la structure secondaire, par IRTF et DCRS, d'une poudre de PPCN lors d'un stockage

Une altération des propriétés de réhydratation de la poudre de PPCN lors du stockage a clairement été mise en évidence dans le chapitre précédent. Toutefois, les éventuels changements de structure à mettre en lien avec les évolutions de propriétés fonctionnelles répertoriées n'ont pas été discutés. Le présent chapitre vise à combler en partie ces lacunes et représente une contribution pour mieux décrypter les mécanismes sous-jacents à l'échelle moléculaire responsable de l'altération des propriétés fonctionnelles.

Il a été suggéré à plusieurs reprises dans la communauté scientifique que la perte de solubilité d'une poudre de protéines de lait pouvait être expliquée par une modification conformationnelle de la protéine lors du stockage. En effet, le stockage entraînerait une dénaturation des protéines, favorisant les structures en feuillet β et l'exposition des régions hydrophobes

Cependant aucune corrélation directe entre la perte de la solubilité et l'évolution de la structure secondaire de la caséine n'a été clairement exposée.

Une des difficultés majeures pour suivre l'évolution des structures secondaires réside dans le fait que les poudres de PPCN sont composées en moyenne de 90 % de caséines qui vont s'assembler entre elles et que la structure des MC n'est pas complétement homogène et connue. De surcroît, la proportion de structure secondaire dans cet édifice est limitée.

Par conséquent, il existe un réel manque de connaissance sur l'évolution de la structure secondaire des poudres PPCN pendant le stockage et leur influence sur les propriétés de réhydratation.

L'objectif de ce chapitre est d'apprécier une évolution de la structure secondaire pendant le stockage et de quantifier, si possible, cette évolution grâce à la

combinaison de 2 techniques : la spectroscopie infrarouge à transformée de Fourier (IRTF) et le dichroïsme circulaire à rayonnement synchrotron (DCRS).

Pour cela, une poudre de PPCN a été stockée à 4 °C, 40 °C et 60 °C pendant 6 mois. L'évolution des propriétés fonctionnelles lors du stockage a été caractérisée par un suivi de la solubilité et la structure secondaire a été étudiée via les techniques d'IRTF et de DCRS. Des analyses des composantes principales ont été menées sur les spectres d'IRTF.

Un suivi de l'évolution structurale de la poudre à l'état sec a été réalisé par IRTF. Il n'a pas été possible de détecter des évolutions de la structure secondaire à l'état sec. Cependant, en solution, une diminution des structures en hélice α et une légère augmentation en feuillet β et structure aléatoire ont été quantifiées via les spectres de DCRS lors d'un stockage à 40 et 60 °C. Lors du stockage à 60 °C, un décalage du spectre IRTF (bande amide I) vers les basses fréquences a été observé, laissant supposer un réarrangement de la structure secondaire de la protéine lors du stockage. Les deux techniques se révèlent ainsi concordantes et complémentaires.

L'analyse des composantes principales (ACP) des spectres IRTF a permis une séparation des échantillons de PPCN en fonction du temps de stockage avec un pourcentage très élevé de variance expliqué. Comme la perte de solubilité dépend du temps de stockage, l'ACP suggère que l'évolution des pourcentages de structures secondaires et la solubilité sont étroitement liées.

Enfin, en utilisant des données quantitatives fournies par l'analyse de spectres de DCRS, il a été démontré que la perte de structure en hélice α pouvait être corrélée à une perte de solubilité. La diminution des structures en hélice α lors d'un stockage pourrait donc être un marqueur de la perte de solubilité.

Pour conclure, la perte importante de solubilité est associée à un faible changement de conformation, visible uniquement lorsque la poudre est en solution. De plus, les caséines ne comportant que très peu de structures secondaires, il peut être supposé

que le dépliement de la protéine n'est pas l'unique responsable de la perte de solubilité mais n'est qu'une étape initiale. Cette étape pouvant favoriser l'exposition des régions hydrophobes et d'autres modifications à l'origine d'une perte de solubilité.

Investigation of secondary structure evolution of micellar casein powder upon ageing by FTIR and SRCD: Consequences on solubility

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Abstract

Synchrotron Radiation Circular Dichroism (SRCD) and Fourier transform infrared (FTIR) spectroscopy were used to examine the conformation evolution of micellar casein (MC) proteins powder during storage and to determine whether the spectral changes could be related to solubility evolution of these powders.

Loss in intensity of SRCD spectra as a function of storage time has been observed. Quantification of secondary structures for each storage condition revealed losses of α-helix content during storage at 40 and 60 °C. Moreover, a redshift of amid I band in the FTIR spectrum towards low frequencies was demonstrated during the storage of MC powder at 60 °C and was interpreted as a rearrangement of the secondary structure of the protein, which is in line with the CD results.

The qualitative results obtained by FTIR clearly support the quantitative evolution of the secondary structure obtained by analysis of SRCD spectra.

Finally, solubility loss of MC powders during storage has been correlated to changes observed in SRCD and FTIR spectra.

Principal component analysis (PCA) of FTIR spectra permits a good separation of samples according to the storage time, explaining the very high percentage of variance. As solubility loss depends on storage time, PCA suggests that the evolution of secondary structures and solubility loss are closely linked.

Finally with the quantitative data provides by SRCD spectra, it was demonstrated that whatever the storage conditions a unique curve exists between loss of α-helix content and loss in solubility, showing that loss of α-helix content is a marker of solubility loss for MC powders studied.

1. Introduction

High-protein content dairy powder is increasingly used worldwide in many food applications for their high protein content (to standardize milk protein content, make dietary products), texturizing (to be used in melted cheese and fresh dairy products) or emulsifying properties (to be used in cooked sausage).

For these applications the rehydration of powder should be fast and complete. Thus, rehydration ability of the powder is an essential quality attribute to display its underlying functionalities at the end of the reconstitution operation (Crowley et al., 2015; Mimouni et al., 2010c). Unfortunately, drying of dairy protein powders is known to cause a loss in solubility and rehydration ability (Havea, 2006; Mimouni et al., 2010c), especially for powders with high protein content and casein-dominant powders (Crowley et al., 2015), like micellar casein (MC) powder. MC powder is obtained by removing whey proteins from the milk using microfiltration (0.1–0.2 μm), which leads to around 90% of micellar casein containing colloidal calcium phosphate (Schokker et al., 2011). Alterations of the powder occurs gradually during storage and may cause changes in viscosity, gelling ability, foaming and emulsifying properties (DeCastro and Harper, 2001). Study of Nasser et al., (2017b) have shown that the solubility and rehydration properties of MC powders were negatively impacted by severe ageing conditions (temperature, humidity and storage time).

The loss in rehydration properties of a milk powder during its storage is the key issue to be addressed when setting up reliable reconstitution processes on an industrial scale. It is

therefore necessary to investigate in depth the underlying mechanisms governing the decrease of rehydration ability during storage.

Generally, it is believed that the loss of solubility is due in part to conformational modification of protein during processing and storage (Kher et al., 2007). Hydrophobic regions are exposed when proteins are denatured, resulting in a greater proportion of β-sheet structures or less alpha helix, or new interaction between proteins and other component (lactose, forming schiff bases) (Anema, 2014; Singh and Latham, 1993). Such mechanisms could induce protein instability and eventually lead to increased protein–protein interactions and aggregation, which are irreversible (Dickinson and Parkinson, 2004).

Two major techniques are often used to probe conformation of proteins.

The first one, Fourier Transform InfraRed (FTIR) spectroscopy, which provides a wealth of information on structure and environment of protein (Arrondo and Goni, 1999), is a well suited method to study the protein structure including conformational change in dairy products (Haque et al., 2010; van der Ven et al., 2002). 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 conformational amide I region (1600–1700 cm^{-1}) is commonly used to determine the secondary structure of proteins (Barth, 2007). Several authors have tried to understand the relationship between solubility and conformation of high protein content dairy product (Haque et al., 2010; Kher et al., 2007; Schokker et al., 2011). For example Haque et al., (2010) showed that the solubility of milk protein concentrate (MPC) decreased significantly upon aging while only minor changes (indicating some degree of unfolding in protein secondary structure) were observed in FTIR. Moreover, Kher et al., (2007) demonstrated changes in intensity and/or position of the bands at 1630 cm^{-1} in FTIR when the

solubility of a stored sample of MPC powder decreased substantially. Finally, Schokker et al., (2011) compared the protein structure of MC powders at fresh state and those stored for 54 days at 30 °C and also detected slight protein unfolding. They conclude that changes in extended β-sheet structure are related to variability in MC reconstitutability.

The second one, Synchrotron Radiation Circular Dichroism (SRCD) is another spectroscopic technique recognised as an excellent method to evaluate the secondary structure, folding and binding properties of proteins (Greenfield, 2006). It could also provide complementary information about structural changes during storage. Nevertheless, it was more often used to predict a structure of protein (Farrell et al., 2001; Greenfield, 2006; Greenfield, 2015) than to monitor an evolution of secondary structures of milk powder during storage.

However, all these results of secondary structure relate to MPC and not to MC. The MPC powders have a high protein content, ranging from 40 to 90 % of the total solid content (De Castro-Morel and Harper, 2002) and contain milk proteins in the same ratio as in raw milk (80% caseins and 20% whey proteins). Another difficulty for detecting evolution of secondary structure arising of the specificity in composition of the MC powders. Indeed, as explained above, MC powders are obtained through separation of casein from whey proteins and are therefore enriched in caseins. However, caseins have limited secondary structures and the exact content of secondary structures is not totally established, which means that there is a real lack of study on the evolution of the secondary structure of MC powders during storage.

The aim of this work was to get further insight into evolutions of the secondary structures during storage. These evolutions will be quantified to put in light a possible link between those evolutions and solubility by combining the 2 techniques (FTIR and SRCD) and to assess whether such factor could be a marker predicting change in solubility.

2. Material and method

2.1. Dairy powder manufacture: Physicochemical analysis and process parameters

MC (Promilk 872 B1) concentrate was obtained by tangential membrane microfiltration (0.1 µm) of skimmed milk at Ingredia (Arras, France). The retentate was spray dried using the same operating conditions as described previously by Pierre et al., (1992) and Schuck et al., (1994b), in a three-stage spray drying pilot, Bionov (GEA, Niro Atomizer, St Quentin en Yvelines, France). Inlet temperature was 180 ± 10 °C, the fluid bed air temperature was 70 ± 1 °C and outlet temperature was 65 ± 5 °C.

MC powders were packaged in individual cans of 380g under vacuum after manufacture (Water activity of 0.25). MC powder was stored at 40 °C and 60 °C for different periods up to 2 months. Although 40 °C and 60 °C may seem extreme temperatures, it has been shown that it exists a correspondence between storage at ambient temperature for a long period and that at a higher temperature for shorter time periods (Nasser et al., 2017c; Norwood, 2016).

2.2. Synchrotron Radiation Circular Dichroism (SRCD): Secondary structure determination

SRCD extends the spectral range of conventional CD spectroscopy, increase signal-to-noise ratio and accelerate data acquisition.(Wallace, 2000).

10 % w/w MC solutions in H_2O were prepared. CD spectra of liquid samples were recorded between 190 and 260 nm on the SRCD setup (Refregiers et al., 2012) of DISCO beamline (Giuliani et al., 2009) at SOLEIL synchrotron radiation facility, France. Calcium fluoride cuvette with 10 µm path length were used (Wien, 2005). Spectra were recorded 3 times, averaged, and subtracted from the appropriate buffer blank spectrum.

The study is based on a protein mixture and not a protein isolate as usual when using BeStSel. As the sample can be considered as a mixture of several proteins, the results must be interpreted with caution. For the analysed powder samples, the mean standard deviation of structure content was equal to 7.5 %.

Statistical analysis of BestSel results

Student's *t*-tests with a 0.05 level of significance were used to measure the significance of the differences between stored samples and reference powder stored at 4 °C. As is widely known, the differences are statistically nonsignificant when $p > 0.05$ and statistically significant when $p < 0.05$.

2.3. Fourier Transform Infrared Spectroscopy - Attenuated Total Reflectance: Secondary structure determination

2.3.1. Sample and material preparation

2.5 mg of MC powder was dissolved in 22.5 mg of D_20 to obtain a 10% w/w solution. D_2O was used in order to shift the bending band of water out of the amide I spectral region. 9 spectra were recorded for each individual sample of reference and aged powders: after 0.5 months, 2 months and 6 months of storage at 40 °C and after 0.3 day, 1 day, 15 days, 30 days of storage at 60 °C. Fourier Transform Infrared (FTIR) spectra were collected between 650 and 4000 cm^{-1} at room temperature (~ 24 °C) using a Nicolet Magna IR 860 spectrometer, with an attenuated total reflection (ATR) cell (Universal ATR). Purging of the sample chamber with dry air was realized in order to reduce water vapour to a minimum. The spectral resolution was 4 cm^{-1} and 128 scans were collected.

2.3.2. Data pre-processing

The absorption spectrum of the water vapour was routinely subtracted to avoid variations in sample spectra due to the presence of water vapour. A background spectrum of a blank ATR cell has been realized and subtracted from sample spectra. Each spectrum was composed of 4096 double-sidedinterferograms, coadded, phase-corrected, apodized with a Happ–Genzel function, and fast-Fourier-transformed.

2.3.3. Spectral treatment

Figure 1A presents FTIR spectra of MC powders stored at 60 °C from 650 to 3930 cm^{-1}. To avoid the noise of D_2O absorption around 1200 and around 2840 cm^{-1}, attention was focused on the range of 1350 - 1700 cm^{-1} with base line correction and integrated intensity normalization between 1600 and 1700 cm^{-1} (**Figure 1B**).

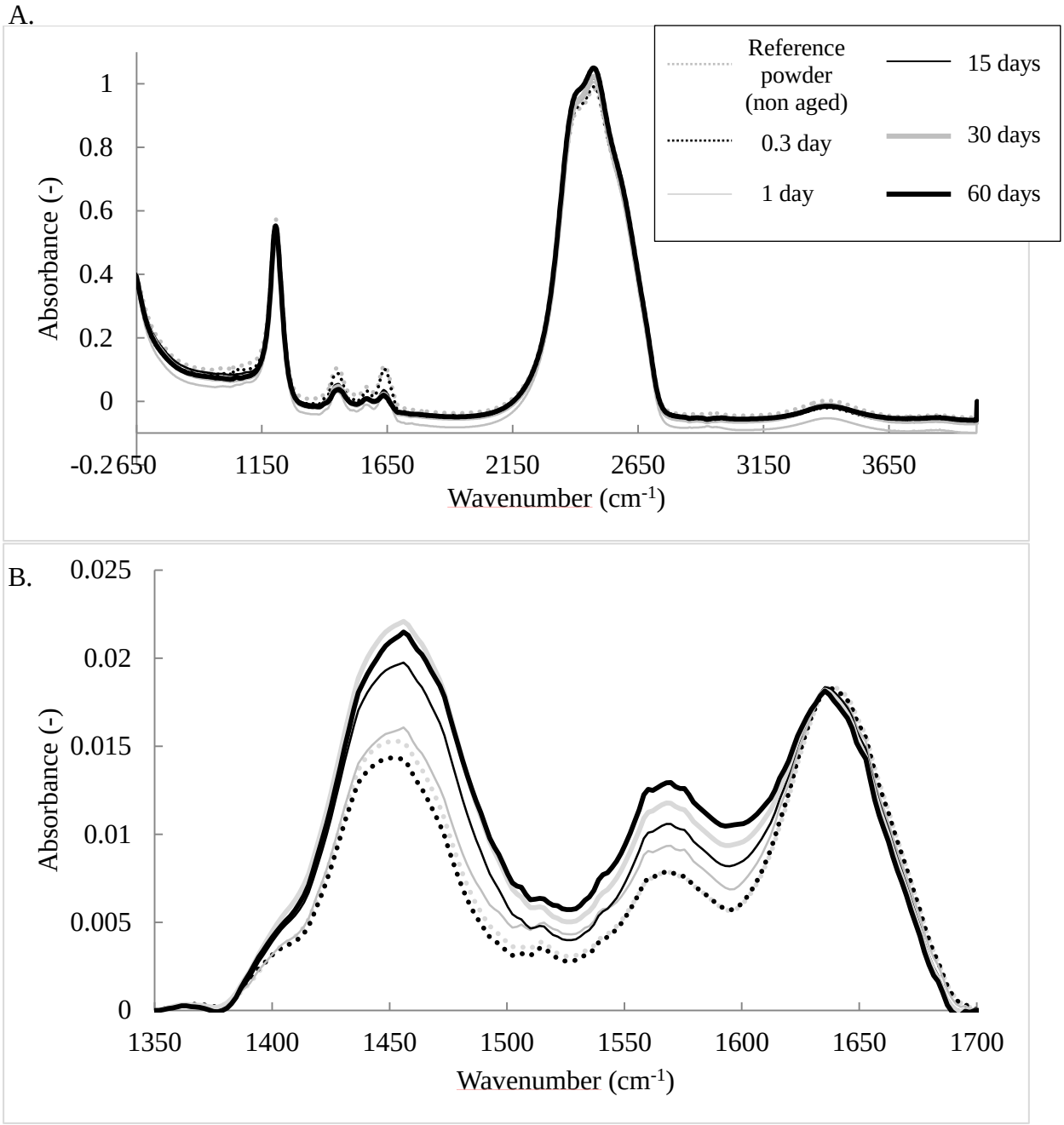

Figure 1. (A) FTIR spectra of MC stored at 60°C (B) Spectra between 1350-1700 cm^{-1} (Corrected baseline and area normalised between 1600 and 1700 cm^{-1})

2.3.4. Data statistical analysis

In order to focus on changes in secondary structure, the spectral domain from 1600 to 1700 cm^{-1} was analysed using principal component analysis (PCA) and Unscrambler® software (version 10.2, CAMO, Norway). PCA is a multivariate technique that describes observations by several inter-correlated quantitative dependent variables when analysing a data table. The goal is to extract the important information from the table, to represent it as a set of new orthogonal variables called principal components, and to display the pattern of similarity of the observations and variables as points in maps (Abdi and Williams, 2010). PCA score plots were used to show similarity maps, making it possible to compare spectra regardless of sample categories. PCA loading derived from first principal component were used to highlight the wavelengths that contribute to the highest variation described by the principal components (PC).

2.4. Determination of solubility

To determine solubility, aqueous solutions of 5 % (w/w) MC powder were firstly prepared under stirring in distilled water at room temperature for 1 hour. Then, 50 ml of the MC solution sample was placed in 50 ml centrifugation tubes and centrifuged using a Sigma 6K15 refrigerated centrifuge (Sigma, Labozentrifugen GmbH, Osterode am Harz, Germany) at 700 × g at 20 °C for 20 min. Secondly, the supernatant was placed in a preweighed moisture dish and weighed. The moisture dish was then dried for 24 h in an oven at 105 °C, cooled to room temperature in a desiccator containing dry silica gel to avoid condensation and reweighed.

The amount of soluble material (σ) in the MC powders was calculated using the following equation (Anema et al., 2006) :

$$\sigma = \frac{\text{Weight of dry material}}{\text{weight of solution}} \times 100 \qquad (1)$$

It represents the solids in the ultracentrifugal supernatant, expressed as a percentage of total soluble solids in the whole solution. The σ values of aged powder samples were compared to that of the reference powder, and this σ(aged)/σ(reference) ratio in percentage was used to study the solubility evolution during ageing.

3. Results

3.1. SRCD spectral analysis of MC powder during storage

3.1.1. Spectral analysis

As shown in **Figure 2**, the spectra of MC solutions reveal minima at 208 nm, and a positive maximum around 190 nm. Broad negative peaks at 208 nm correspond to the absorption of α-helix (Holzwarth, 1985). A typical feature of the SRCD spectrum for a protein with α-helices is the presence of a positive peak at 193 nm and negative peak at 222 nm. Since MC is a protein mixture, the SRCD spectral features of MC proteins differ from typical spectral features of a protein that contains exclusively α-helix.

Despite the difference in storage temperature (40 or 60 ° C), the evolution of the spectra is comparable. The intensities of the peak at 220 nm and 190 nm decreased with storage time at both 40 °C and 60 °C. These changes upon ageing suggested an increase of disorder and a decrease of α-helix content in the protein.

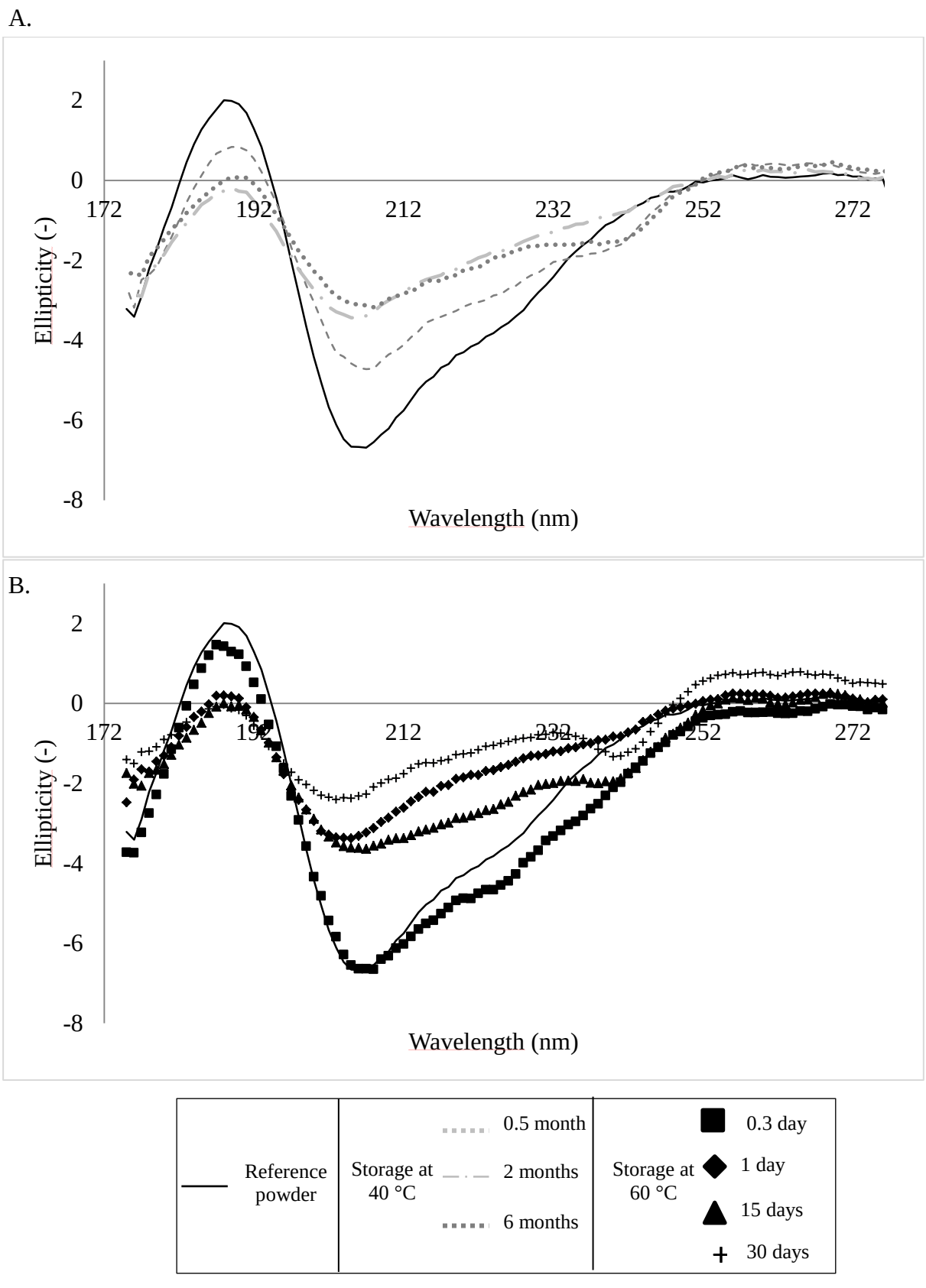

Figure 2. Circular dichroism spectra of MC powder stored at (A) 40 °C and (B) 60°C

3.1.2. BestSel analysis

Evolution of secondary structure content upon ageing obtained by BeStSel is described in **Figure 3**. For the analysed powder samples, the error bars are also presented. A decrease of α-helix from 14.7% to respectively 10.0 % ($p<0.05$) and 7.3 % ($p<0.05$) were observed after 15 days of storage at 40 °C and 60 °C. A increasing trend starting to be significant is noted for the others evolutions of secondary structure at 60 °C ($0.05<p<0.07$), including small increases of β-sheet, from 22.3 % to 26.3 % and of β-turn structure from 16.6 to 18.1 %, in the same period of time. At 40 °C, A rise of β-sheet, random and β-turn structures was observed but could not be considered significant, according to our criteria ($0.05<p<0.06$).

It can be concluded that the higher the temperature, the highest changes in the structure. According to BeStSel quantification, there is a significant loss of α-helix with ageing whatever the temperature of storage. Simultaneously a tendency of β-sheet, β-turn and random structure increase with ageing could be supposed.

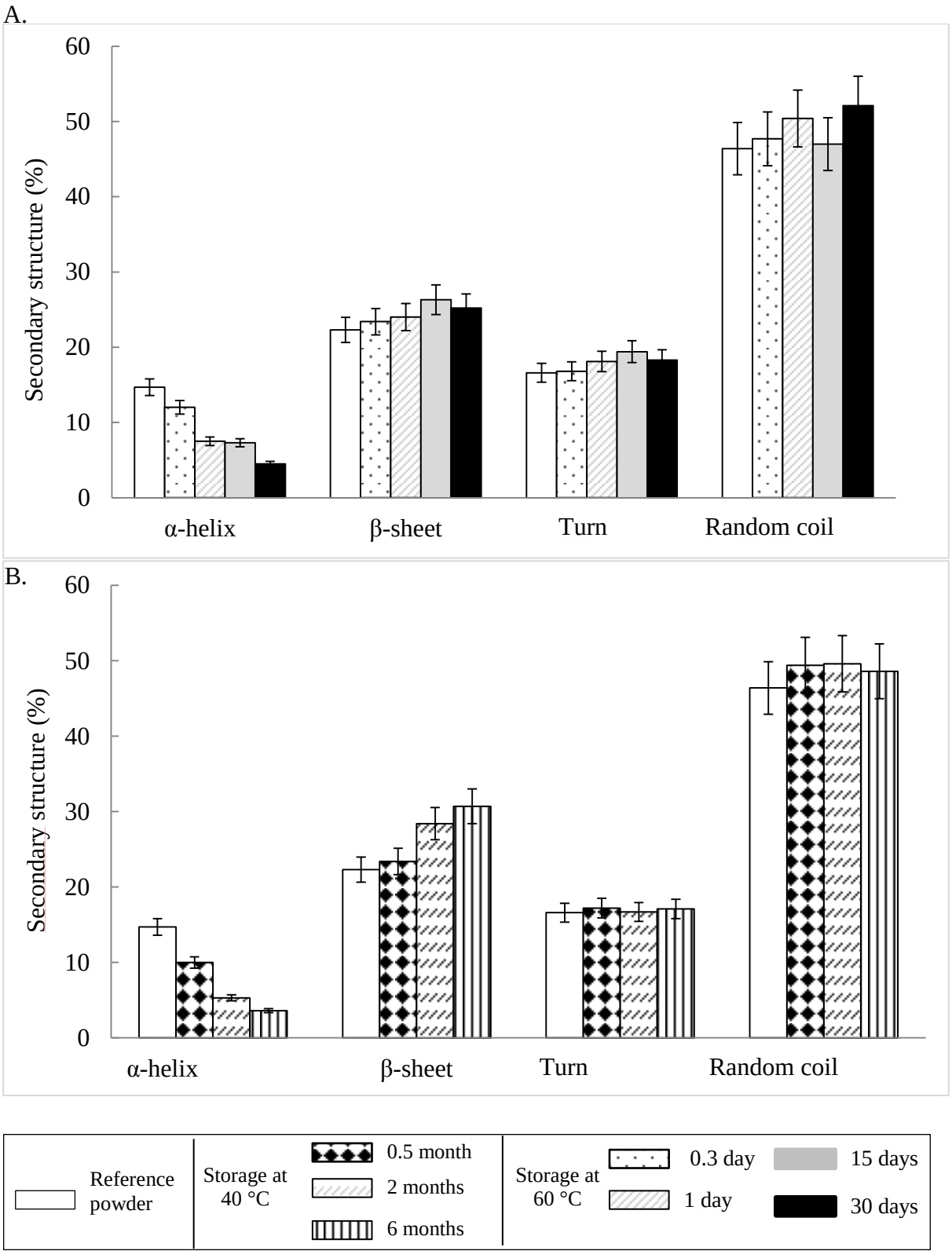

Figure 3. Determination of the secondary structure content of MC powder after storage at (A) 40 °C and (B) 60°C

3.2. FTIR spectral analysis of MC powder during storage

Analyzes were carried out to monitor the structural evolution of the powder in the dry state. They showed the absence of transformation in the dry state (datas not shown).

The following results concern the monitoring of the structural evolution of a powder dissolved in solution.

Figure 4A presents the treated FTIR spectrum between 1600 and 1700 cm^{-1}. A redshift of amide I band was detected during storage. A shift from 1640 cm^{-1} to 1638 cm^{-1} had also been observed by Schokker et al., (2011) during the storage of MC powder, which was attributed to the partial loss of protein secondary structures.

In order to have a better visualization of the influence of aging on the amide I region, the spectrum of the reference powder was subtracted from the spectra collected for samples at different aging time (**Figure 4B)**. Spectra of difference are obtained. As previously underlined, each spectrum observed at a precise storage time corresponds to the mean of 9 repetitions.

Upon aging, the intensity of the band between 1600 and 1630 cm^{-1} increased and that between 1640 and 1680 cm^{-1} decreased.

The spectral region ranging from 1620 to 1640 cm^{-1} has been assigned to β–sheet (Kong and Yu, 2007; Krimm and Bandekar, 1986; Susi and Byler, 1983, 1986). Features centred between approximately 1649 and 1657 cm^{-1} have been assigned to α–helix (Kong and Yu, 2007; Krimm and Bandekar, 1986; Susi and Byler, 1983, 1986). Hence, the increase in intensity between 1600 and 1630 cm^{-1} is tentatively associated with the formation of β-sheet upon aging, whereas, the decrease of intensity between 1640 and 1680 cm^{-1} is proposed to arise from loss of α–helix (**Figure 4B)**. These results complete studies carried out by Haque et al., (2010) who studied Amide I band by FTIR during storage of MPC at 24 and 45 °C. A

decrease of α-helix and increase of β-sheet were reported after 10 weeks at 24 °C. The storage at 45 °C demonstrated a stronger impact on the decrease of α-helix (Haque et al., 2010).

The FTIR results are not quantitative but clearly in agreement with SRCD results. Both techniques demonstrated an evolution of secondary structure of MC during storage.

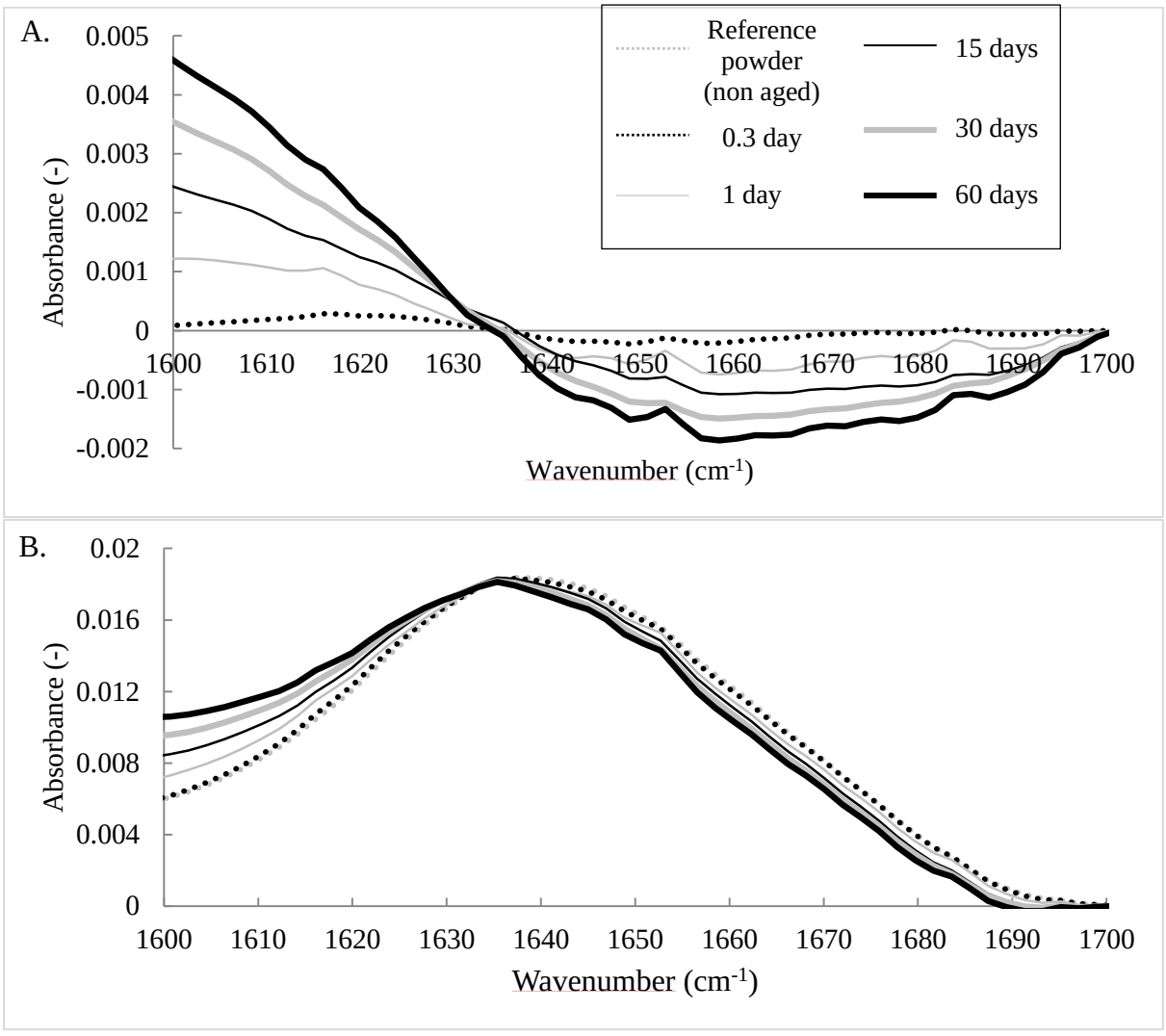

Figure 4. (A) FTIR Spectra between 1600-1700 cm^{-1} (baseline corrected and area normalised) (B) Difference spectra between aged powder and reference powder

3.3 Link between secondary structure evolutions and the solubility

3.3.1. Solubility evolution with aging conditions

The solubility evolution as a function of time of the MC powder stored at 40 and 60 °C is illustrated in **Figure 5**. The solubility was first subjected to an exponential decrease and then reached an asymptotic value. The solubility of the powder stored at 40 °C decreased rapidly in the first 60 days of storage. Then, the loss of solubility became moderate with the solubility leveling off at around 25 %. This sharp decrease of solubility at the first instant of storage was amplified at higher storage temperature (when aging occurred at 60 °C, more precisely). The solubility of the powder stored at 60 °C decreased drastically in the first 10 days of storage. Then, loss of solubility is moderate after this period.

The development of insolubility observed at elevated storage temperature for MC powder is in agreement within our expectation (namely, increase of insoluble matter upon ageing due to the insolubilization of micellar caseins), given the previous studies about MPC powder (Anema et al., 2006; Gazi and Huppertz, 2015; Le et al., 2011c). It is generally suggested that the loss of secondary structure of a protein can lead to the alteration of its functional properties such as solubility (Fyfe et al., 2011; Haque et al., 2010; Kher et al., 2007).

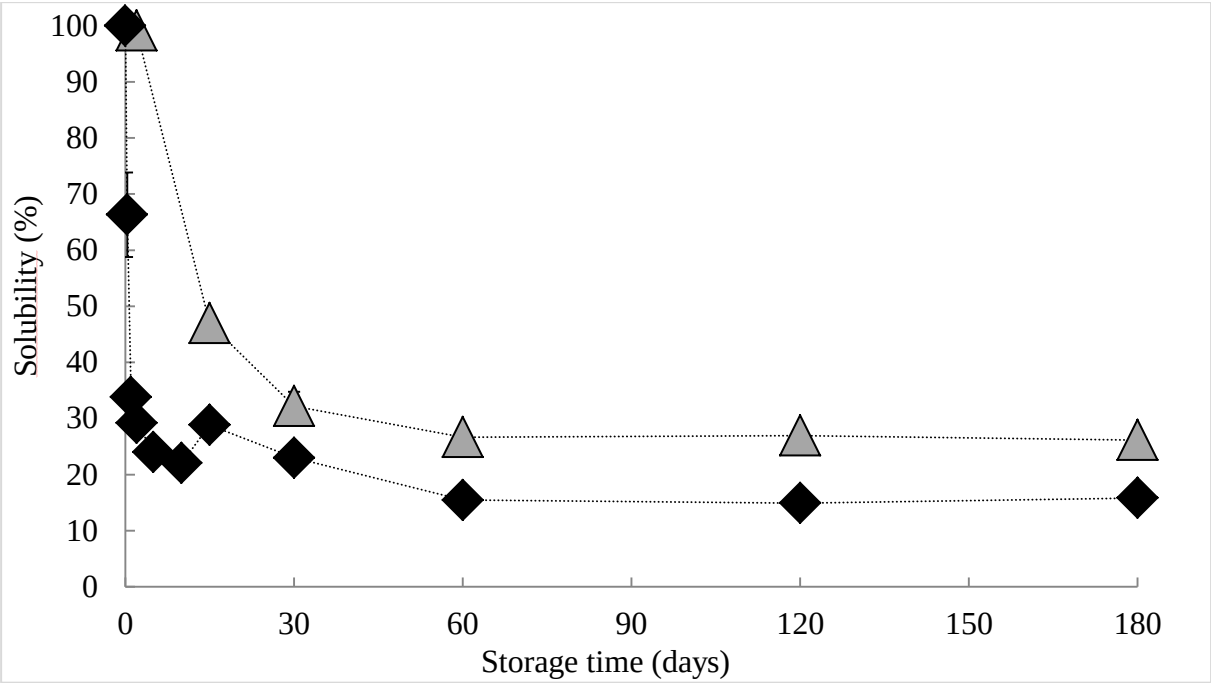

Figure 5. Evolution of solubility as a function of storage time at (△) 40 °C and (◆)60 °C

3.3.2. Link between FTIR spectrum evolution and solubility loss

Figure 6 presents the score plot of principal component analysis (PCA) of treated amide I band (**Figure 4A**) for powder aged at 60 °C. It is shown in the score plot that samples gradually separate from each other along the PC1 axis, which explains 96% of the sample variance. PC2 explains only 2 % of the variance. It is important to specify that the scale chosen for PC2 axis is much smaller than that used for PC1 and that PC2 is definitely not discriminant. PCA permits a significant separation of samples according to storage time..

It could be noted on **Figure 6** that samples with significant solubility changes and very aged powder (to the right of PC1 axis) are well separated from the reference powder (to the left of PC1 axis). However, powders with the shorter storage times, those with the minimum solubility variations showed poorer separation from the reference powder (cf. **Figure 5**). Thus, it can be assumed that there is a relation between loss of solubility and evolution of secondary structure.

PCA analysis of FTIR spectra of MC powder samples is in agreement with previous works of Kher et al., (Kher et al., 2007) on MPC powders , in which the existence of a relation between changes in solubility and FTIR spectra have been also suggested.

PCA loading of FTIR spectra are given in **Figure 7**. It is shown that the first principal component is oriented to 1600 and 1630 cm^{-1} for positive value and 1640 and 1680 cm^{-1} for negative value. These two peaks could be respectively associated to higher random coil, α–helix and β–turns content and lower β-sheet content in the aged samples. This PCA loading confirms previous assessment obtained by analysing the spectra of difference (**Figure 4B**), evidencing an increase of β-sheet structure and a decrease of α-helix during storage.

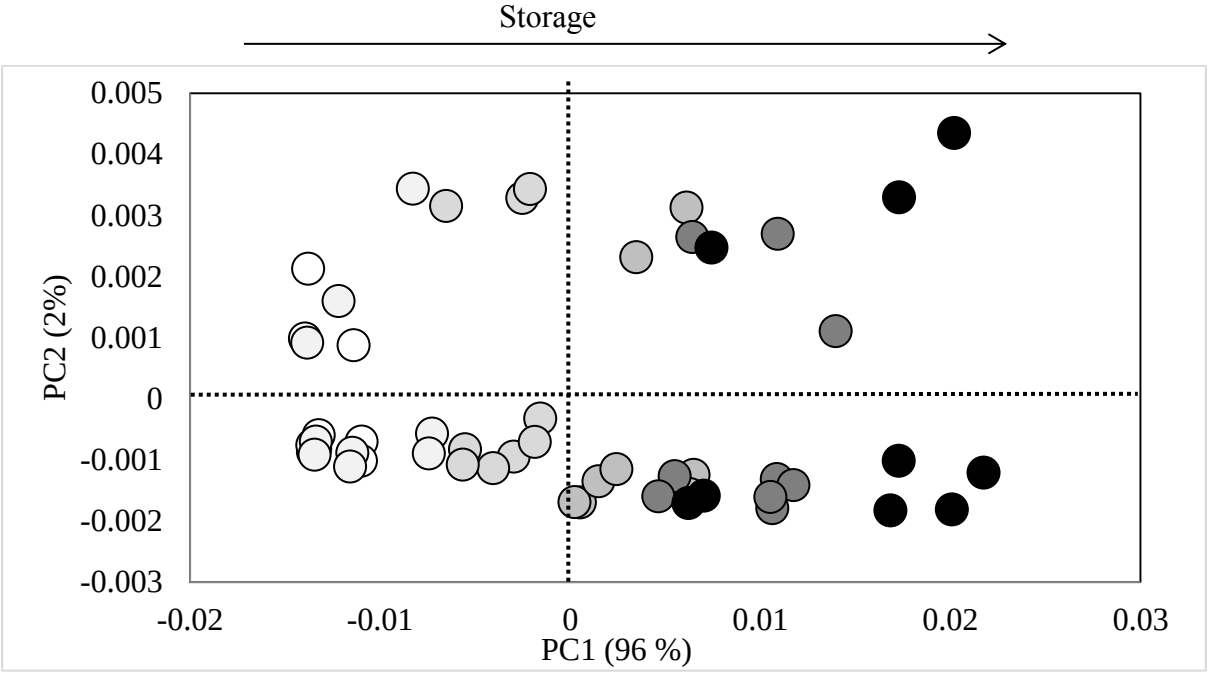

Figure 6. Score plot of the samples characterized by FTIR analysis in the region of amide I bands for the MC powders studied; the darker the inner colour, the longer the storage time at 60°C.

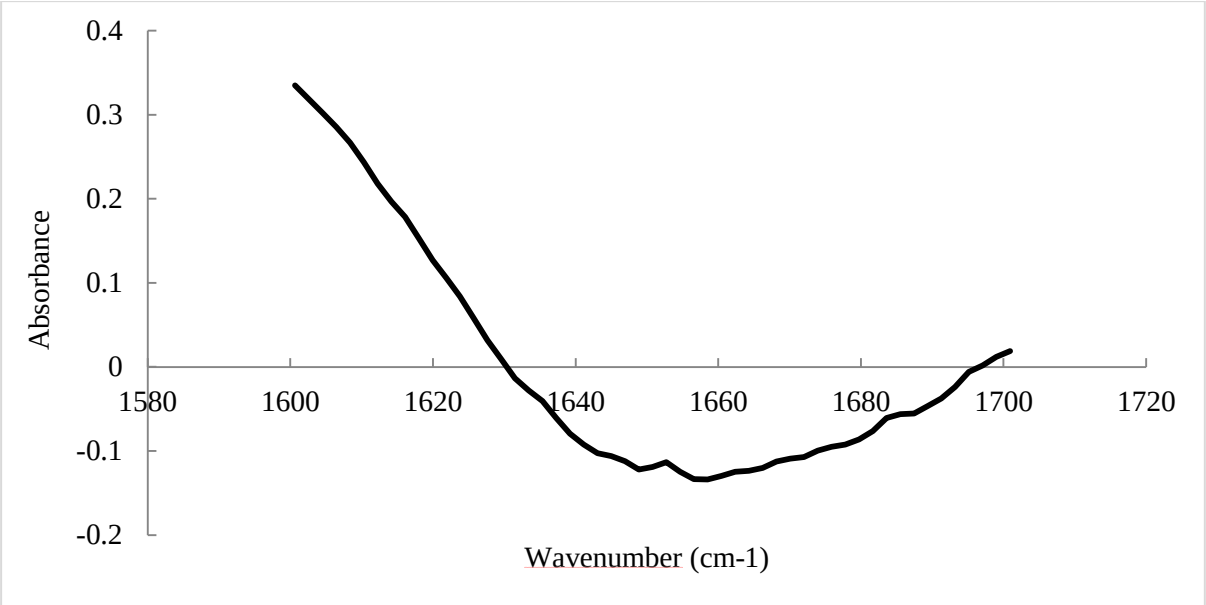

Figure 7. PCA loading of the FTIR spectra of MC powder in solution

3.3.3. Correlation between SRCD analysis and loss in solubility

In **Figure 8**, a plot showing the relation between α-helix content of the samples and their solubility is presented.

A large solubility loss of ~54 % and a less marked α-helix loss of ~5 % compared to the reference powder were observed for the sample stored at 60 °C for 0.3 day. Similarly, for the sample stored for 30 days at 60 °C with a solubility loss of 77 %, only ~10 % loss of α-helix is reported. The same phenomenon is noted for the powder stored at 40 °C. Thus, it could be deduced that a slight evolution of the structure would generate a significant loss of solubility

Obviously, the loss in solubility during storage is strongly correlated with the loss in α-helix ($R^2 > 0.97$). It is striking that all the aged samples lie on a unique curve whatever the storage conditions (temperature and time of storage). The decrease in α-helix content could be a marker indicating a loss in solubility At first glance, it could be easily suggested that the decrease in α-helix content, detected when rehydrating powder, could be the key factor at the molecular level leading to loss in solubility and hence the slow dissolution kinetics of high

protein content milk powder. However, it is important to specify again that micellar casein have limited secondary structure and loss in solubility is very important compared to change in secondary structure. Regards to the results, it seems more appropriate to argue that that loss in solubility must be related to higher order structural changes.

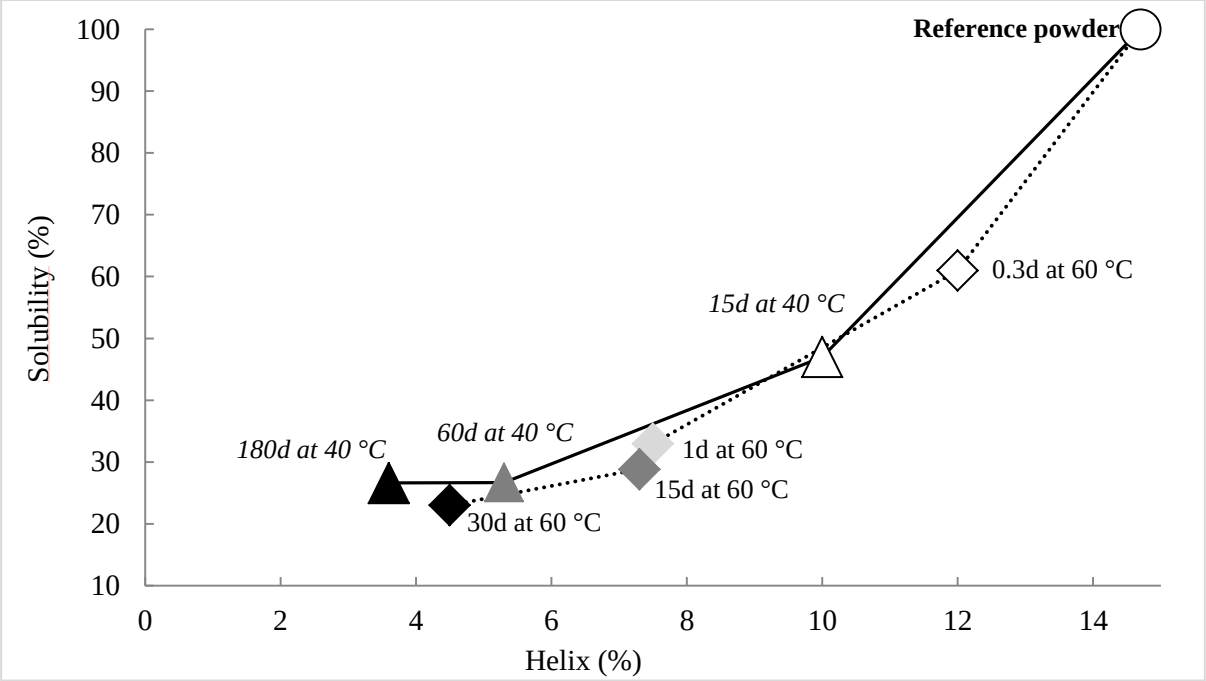

Figure 8. Solubility as a function of helix content evaluated from SRCD after different storage time at 40 and 60 °C.

Conclusion

The main aim of this work was to study the evolution of secondary structures of MC powder as a function of storage time via FTIR and SRCD spectroscopies. Overall, both techniques indicate that MC powder loses its folded structural elements, especially its α-helix content during storage.

No structural transformation was detected in the dry state. Indeed, it is difficult to conceive of an unfolding of a secondary structure in the dry state; the unfolding being generally induced by the penetration of the solvent into the tertiary structure.
The decrease in solubility is therefore associated with a very small conformational change of the protein in solution.-This can give rise to 2 complementary suggestions. First, based on our

previous study (Nasser et al., 2017b), this may suggest that only a small fraction of casein (casein on the powder particle surface) is affected by an evolution of the secondary structure. But this would be sufficient to increase cross-linking between caseins and thicken the thin layer situated exclusively at the powder particle surface (Burgain et al., 2016; Mimouni et al., 2010a; Nasser et al., 2017a). This thin layer of cross-linked caseins would delay the rehydration steps and was hence assumed to be responsible for the slow dissolution of stored milk powders. The second suggestion is in agreement with previous studies (Haque et al., 2010; Qi, 2007): protein unfolding may be not the only factor responsible for the loss of solubility, instead, it may be the initiation step favouring the exposition of hydrophobic regions. This would increase the probability of modification of those less well-structured proteins by introducing more amino acid modification sites and could thus affect protein functions, such as solubility.

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Bibliography

Abdi, H., and L. J. Williams, 2010, Principal component analysis, Wiley Interdisciplinary Rev. Comput. Statist., p. 433-459.

Anema, S. G., 2014, The Whey Proteins in Milk: Thermal Denaturation, Physical Interactions, and Effects on the Functional Properties of Milk, *in* H. Singh, M. Boland, and A. Thompson, eds., Milk Proteins: from Expression to Food, 2nd Edition: Food Science and Technology-International Series: San Diego, Elsevier Academic Press Inc, p. 269-318.

Anema, S. G., D. N. Pinder, R. J. Hunter, and Y. Hemar, 2006, Effects of storage temperature on the solubility of milk protein concentrate (MPC85): Food Hydrocolloids, v. 20, p. 386-393.

Arrondo, J. L. R., and F. M. Goni, 1999, Structure and dynamics of membrane proteins as studied by infrared spectroscopy: Progress in Biophysics & Molecular Biology, v. 72, p. 367-405.

Barth, A., 2007, Infrared spectroscopy of proteins: Biochimica Et Biophysica Acta-Bioenergetics, v. 1767, p. 1073-1101.

Burgain, J., J. Scher, J. Petit, G. Francius, and C. Gaiani, 2016, Links between particle surface hardening and rehydration impairment during micellar casein powder storage: Food hydrocolloids, v. 61, p. 277-285.

Crowley, S. V., B. Desautel, I. Gazi, A. L. Kelly, T. Huppertz, and J. A. O'Mahony, 2015, Rehydration characteristics of milk protein concentrate powders: Journal of Food Engineering, v. 149, p. 105-113.

De Castro-Morel, M., and W. J. Harper, 2002, Basic functionality of commercial milk protein concentrates: Milchwissenschaft-Milk Science International, v. 57, p. 367-370.

DeCastro, M., and W. J. Harper, 2001, Effect of drying on characteristics of 70% milk protein concentrate: Milchwissenschaft-Milk Science International, v. 56, p. 269-272.

Dickinson, E., and E. L. Parkinson, 2004, Heat-induced aggregation of milk protein-stabilized emulsions: sensitivity to processing and composition: International Dairy Journal, v. 14, p. 635-645.

Farrell, H. M., E. D. Wickham, J. J. Unruh, P. X. Qi, and P. D. Hoagland, 2001, Secondary structural studies of bovine caseins: temperature dependence of beta-casein structure as analyzed by circular dichroism and FTIR spectroscopy and correlation with micellization: Food Hydrocolloids, v. 15, p. 341-354.

Fyfe, K. N., O. Kravchuk, T. Le, H. C. Deeth, A. V. Nguyen, and B. Bhandari, 2011, Storage induced changes to high protein powders: influence on surface properties and solubility: Journal of the Science of Food and Agriculture, v. 91, p. 2566-2575.

Gazi, I., and T. Huppertz, 2015, Influence of protein content and storage conditions on the solubility of caseins and whey proteins in milk protein concentrates: International Dairy Journal, v. 46, p. 22-30.

Giuliani, A., F. Jamme, V. Rouam, F. Wien, J. L. Giorgetta, B. Lagarde, O. Chubar, S. Bac, I. Yao, S. Rey, C. Herbeaux, J. L. Marlats, D. Zerbib, F. Polack, and M. Refregiers, 2009, DISCO: a low-energy multipurpose beamline at synchrotron SOLEIL: Journal of Synchrotron Radiation, v. 16, p. 835-841.

Greenfield, N. J., 2006, Using circular dichroism spectra to estimate protein secondary structure: Nature Protocols, v. 1, p. 2876-2890.

Greenfield, N. J., 2015, Circular Dichroism (CD) Analyses of Protein-Protein Interactions, *in* C. L. Meyerkord, and H. Fu, eds., Protein-Protein Interactions: Methods and Applications, 2nd Edition: Methods in Molecular Biology, v. 1278: Totowa, Humana Press Inc, p. 239-265.

Haque, E., B. R. Bhandari, M. J. Gidley, H. C. Deeth, S. M. Moller, and A. K. Whittaker, 2010, Protein Conformational Modifications and Kinetics of Water-Protein Interactions in Milk Protein Concentrate Powder upon Aging: Effect on Solubility: Journal of Agricultural and Food Chemistry, v. 58, p. 7748-7755.

Havea, P., 2006, Protein interactions in milk protein concentrate powders: International Dairy Journal, v. 16, p. 415-422.

Holzwarth, G., 1985, The ultraviolet circular-dichroism of polypeptides: Current Contents/Engineering Technology & Applied Sciences, p. 12-12.

Kher, A., P. Udabage, I. McKinnon, D. McNaughton, and M. A. Augustin, 2007, FTIR investigation of spray-dried milk protein concentrate powders: Vibrational Spectroscopy, v. 44, p. 375-381.

Kong, J., and S. Yu, 2007, Fourier transform infrared spectroscopic analysis of protein secondary structures: Acta Biochimica Et Biophysica Sinica, v. 39, p. 549-559.

Krimm, S., and J. Bandekar, 1986, Vibrational spectroscopy and conformation of peptides, polypeptides and proteins: Advances in Protein Chemistry, v. 38, p. 181-364.

Le, T. T., B. Bhesh, and H. C. Deeth, 2011, Chemical and physical changes in milk protein concentrate (MPC80) powder during storage: Journal of Agricultural and Food Chemistry, v. 59, p. 5465-5473.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari, 2010a, Investigation of the microstructure of milk protein concentrate powders during rehydration: Alterations during storage: Journal of Dairy Science, v. 93, p. 463-472.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari, 2010b, Rehydration of high-protein-containing dairy powder: Slow- and fast-dissolving components and storage effects: Dairy Science & Technology, v. 90, p. 335-344.

Nasser, S., R. Jeantet, P. De-Sa-Peixoto, G. Ronse, N. Nuns, F. Pourpoint, J. Burgain, C. Gaiani, A. Hédoux, and G. Delaplace, 2017a, Microstructure evolution of micellar casein powder upon ageing : Consequences on rehydration dynamics: In press, Journal of Food Engineering.

Nasser, S., A. Moreau, T. Croguennec, F. Tessier J, F. Bray, C. Rolando, A. Hédoux, and G. Delaplace, 2017b, Storage of micellar casein powder with and without lactose : Consequences on color, solubility and chemical modifications, Submitted to Journal of Agricultural and Food chemistry.

Nasser, S., A. Moreau, A. Hédoux, R. Jeantet, and G. Delaplace, 2017c, Influence of storage conditions on the functional properties of Micellar Casein powder: Submitted to LWT-Food Science and Technology.

Norwood, E.-A., 2016, Molecular and functional changes occurring during ageing of whey protein isolate powders, PhD Thesis.

Pierre, A., J. Fauquant, Y. Legraet, M. Piot, and J. L. Maubois, 1992, Native micellar casein separation through cross flow membrane microfiltration: Lait, v. 72, p. 461-474.

Qi, P. X., 2007, Studies of casein micelle structure: the past and the present: Lait, v. 87, p. 363-383.

Refregiers, M., F. Wien, H. P. Ta, L. Premvardhan, S. Bac, F. Jamme, V. Rouam, B. Lagarde, F. Polack, J. L. Giorgetta, J. P. Ricaud, M. Bordessoule, and A. Giuliani, 2012, DISCO synchrotron-radiation circular-dichroism endstation at SOLEIL: Journal of Synchrotron Radiation, v. 19, p. 831-835.

Schokker, E. P., J. S. Church, J. P. Mata, E. P. Gilbert, A. Puvanenthiran, and P. Udabage, 2011, Reconstitution properties of micellar casein powder: Effects of composition and storage: International Dairy Journal, v. 21, p. 877-886.

Schuck, P., M. Piot, S. Mejean, J. Fauquant, G. Brule, and J. L. Maubois, 1994, Dehydration of ultra-clean milk and micellar casein enriched milks: Lait, v. 74, p. 47-63.

Singh, H., and J. M. Latham, 1993, Heat Stability of Milk: Aggregation and Dissociation of Protein at Ultra-high Temperatures: International Dairy Journal, v. 3, p. 225-237.

Susi, H., and D. M. Byler, 1983, Protein-structure by Fourier-transform infrared-spectroscopy - 2nd derivative spectra: Biochemical and Biophysical Research Communications, v. 115, p. 391-397.

Susi, H., and D. M. Byler, 1986, Resolution-enhanced Fourier-transform infrared spectroscopy of enzymes: Methods in Enzymology, v. 130, p. 290-311.

van der Ven, C., S. Muresan, H. Gruppen, D. B. A. de Bont, K. B. Merck, and A. G. J. Voragen, 2002, FTIR spectra of whey and casein hydrolysates in relation to their functional properties: Journal of Agricultural and Food Chemistry, v. 50, p. 6943-6950.

Wallace, B. A., 2000, Synchrotron radiation circular-dichroism spectroscopy as a tool for investigating protein structures: Journal of Synchrotron Radiation, v. 7, p. 289-295.

Chapitre 3. Etude de l'évolution de la microstructure d'une poudre de PPCN lors d'un stockage et les conséquences lors d'un stockage.

Le chapitre 1 de la partie « résultats » a permis de quantifier les évolutions des propriétés de réhydratation (solubilité, temps caractéristiques du processus de réhydratation : temps de fragmentation et temps de réhydratation total) des poudres de PPCN induit par un stockage.

Le chapitre 2 de la partie « résultats » nous a ensuite permis d'aborder l'étude de quelques-uns des mécanismes pouvant être à l'origine de l'augmentation du temps de réhydratation lors du stockage. Notamment, les changements de structures secondaires se produisant au sein des poudres de PPCN.

Dans ce chapitre 3 de la partie « résultats », nous poursuivons nos approches visant à identifier les phénomènes se produisant au cours du vieillissement des poudres de PPCN et limitant leurs aptitudes à la réhydratation.

De précédents travaux ont démontré que la microstructure de surface des grains de poudres à l'état sec et en cours de réhydratation évoluait en fonction des conditions de stockage. Ils ont établi qu'il existait une peau à la surface des grains de poudre, formée de MC agrégées, devenant de plus en plus compacte et rigide lors du stockage. Ces études suggèrent que les évolutions microstructurales de cette peau influenceraient le processus de réhydratation et notamment le déroulement des différentes étapes qui le compose. Ces auteurs avancent que l'évolution des propriétés de cette peau impacterait l'étape de mouillabilité (pénétration de l'eau dans la particule), l'étape de fragmentation des particules, ou encore l'étape finale de dissolution des particules fragmentées (ie. dispersion des MC individuelles au sein du solvant)

Néanmoins, beaucoup de lacunes persistent encore sur le rôle exact de de cette peau à la surface des grains dans le processus de réhydratation. Par exemple, l'influence de la peau dans l'étape de mouillabilité ou de fragmentation lors du processus de réhydratation n'est pas connue puisque ces étapes n'ont jamais fait l'objet d'études exhaustives et la durée exacte de ces étapes n'a jamais été quantifiée pour des poudres de PPCN jeunes ou vieillies.

De plus, on ne sait pas exactement dans quelle proportion respective les différentes étapes du processus de réhydratation (mouillabilité-fragmentation-dissolution des particules fragmentées-dispersion des MC individuelles) sont influencées par l'évolution des propriétés de cette peau au cours du vieillissement.

Enfin, on se demande quels sont les mécanismes sous–jacents à l'échelle moléculaire se produisant dans les grains de poudre à l'état sec lors du vieillissement à l'origine de l'évolution des propriétés de cette peau. Y a-t-il une migration de lipides vers la périphérie du grain de poudre ou/et des modifications chimiques qui peuvent expliquer une aptitude plus faible des poudres de PPCN vieillies à se réhydrater ?

L'objectif de ce chapitre est donc i) d'étudier les évolutions de composition en surface de la poudre lors d'un stockage ii) de quantifier précisément les évolutions de durée des étapes clés de la réhydratation, à savoir le temps de mouillage, de fragmentation et de réhydratation total iii) de visualiser par microscopie électronique à balayage (MEB) les différences structurales de la peau des grains à différents instants du processus de réhydratation et ceux pour les poudres de PPCN jeunes et vieillies iv) de déterminer le lien entre l'évolution de durée des étapes du processus de réhydratation et l'évolution de la microstructure de surface des grains observée v) d'identifier les étapes limitantes du processus de réhydratation pour les poudres de PPCN vieillies.

Pour cela, des conditions de vieillissement contrôlées (4, 20, 40 et 60 °C, jusqu'à 12 mois) sous humidité contrôlée ont été appliquées à une poudre de PPCN. Les

différentes durées caractéristiques des 3 étapes déterminées du processus de réhydratation ont été évaluées par des tests ultrasonores (temps de mouillabilité), granulomorphométrie (temps de fragmentation) et granulométrie à diffraction laser (temps de réhydratation total). Des images de poudres vieillies et non vieillies acquises au cours du processus de réhydratation ont également été obtenues par MEB. Enfin, l'étude de la composition en surface à l'état sec a été suivie en utilisant l'ensemble de techniques suivantes : Iatroscan, résonance magnétique nucléaire (RMN), spectrométrie photoélectronique X (XPS) et spectrométrie de masse des ions secondaires à temps de vol (Tof Sims).

Les résultats montrent une augmentation de la mobilité des lipides et leur migration vers la surface des particules lors d'un stockage à une température supérieure ou égale à 40 °C.

Une augmentation du temps de mouillage, des temps de fragmentation et de réhydratation total des particules a été quantifiée lors du stockage.

Il a été montré que les fonctions représentant l'évolution de la quantité des lipides en surface des grains avec le temps de stockage (à température fixée) et l'évolution du temps de mouillage avec le temps de stockage ont la même monotonie. Ces dynamiques d'évolutions similaires nous ont permis de corréler l'augmentation du temps de mouillage à l'augmentation de la migration des lipides. En revanche, l'augmentation du temps de mouillage n'explique pas à lui seul l'augmentation plus conséquente du temps de réhydratation total des poudres de PPCN observée après vieillissement. D'autres phénomènes que la migration des lipides prennent place et probablement des liaisons entre les MC. Ces réticulations supplémentaires entre caséines expliquent aussi l'augmentation de temps observée pour fragmenter les particules primaires

Ainsi, il a été établi sans ambiguïté que l'augmentation du temps nécessaire à la libération des MC dans le solvant était le principal mécanisme pouvant expliquer l'augmentation du temps de réhydratation total.

Microstructure evolution of Micellar Casein powder upon ageing: consequences on rehydration dynamics

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Abstract

Micellar casein (MC) powder must be completely dispersed and dissolved in water to fully exhibit their functional properties. However, the rehydration properties of these powders decline strongly during storage, leading to loss of solubility and longer rehydration time. In this work, controlled ageing was applied to a MC powder in order to better understand the mechanisms responsible for the deterioration of rehydration properties in the course of storage. The objective was to investigate evolutions of powder surface structure and composition as well as the link between these changes and the decline in rehydration properties, which were evaluated through characteristic times of different steps constituting the full rehydration process. Lipid migration (towards the particle surface) and increase of interactions between surface micellar particles during storage were proven to be responsible for major changes in different rehydration stages. First, the delay in water penetration into particles was quantified; then, the increase of particle fragmentation time was determined and finally, an extended total rehydration time was evidenced. Analysis of the characteristic times of different rehydration stages shows unambiguously that the main step increasing the total rehydration time of aged powder is not lipid migration but the crosslink formation during storage, which can thus be considered as the rate-limiting stage for rehydration of aged powder.

Key-words

Micellar casein powder; storage; rehydration; microstructure; surface

Abbreviations

MPC : Milk protein concentrate ; MC : Micellar casein ; SEM : Scanning Electron Microscopy; NMR : Nuclear magnetic resonance; XPS : X-ray photoelectron spectroscopy; Tof-SIMS : Time-of-Flight Secondary Ion Mass Spectrometry; PSD : Particle size distribution

1. Introduction

During the latest years, high-protein dairy powders such as Milk Protein Concentrates (MPC) have been increasingly used as ingredients by food manufacturers because of their nutritional and functional qualities (Gaiani et al., 2007b; Kelly et al., 2016; Selomulya and Fang, 2013). In MPC, casein / whey protein ratio is the same as in skimmed milk. For that reason, MPC is often used to standardize the protein content in normal milk and in many dairy products including cheese and yoghurt.

For these applications, instant dissolution, or at least good rehydration ability is a desired property, while wettability, sinkability, dispersibility and solubility are the essential prerequisite features for the dairy solution to display its underlying functionalities at the end of the reconstitution operation (Crowley et al., 2015; Mimouni et al., 2010c).

However, it has been reported in several studies that high-protein dairy powders have poor rehydration properties (Fyfe et al., 2011; Gaiani et al., 2007b; Gaiani et al., 2009; Schuck et al., 2007), especially for MPC with increased protein content and casein-dominant powders (Crowley et al., 2015).

Moreover, it has also been observed that rehydration properties alter significantly during storage (Anema et al., 2006; Gazi and Huppertz, 2015; Le et al., 2011c; Thomas et al., 2004), particularly under severe conditions (e.g., high temperature and humidity).

The decline in rehydration properties of a given powder during its storage is the key issue to be addressed when setting up reliable reconstitution processes on an industrial scale. Storage-induced ageing effects should be taken into consideration in the development of upstream processing routes in

order to increase powder rehydration ability in reconstitution operations. Consequently, it is necessary to investigate in depth the underlying mechanisms governing the decrease of rehydration ability during storage and to evaluate their consequences on the three theoretical phases of the rehydration process (Davenel et al., 1997; Gaiani et al., 2007b; Ji et al., 2016):

i) wetting and immersion of particles, including the penetration of the liquid into the pore matrix due to capillary forces

ii) dissolution of the solid bridges between casein micelles constituting the particles, leading to particle fragmentation and release of casein micelles within the liquid volume

iii) dissolution of fragmented particles and consequent reduction in size

At the present state of the art, the understanding of powder reconstitution is insufficient to provide clear guidelines regarding which steps are more drastically impacted by storage conditions. But some researchers tried to approach the problem by illustrating the surface microstructure evolution of hydrated high-protein dairy powder particles under different storage conditions, Mimouni et al., (2010a, c) have shown that an evolution of the particle surface microstructure occurred upon ageing. The monolayer skin (composed of casein micelles) formed on the particle surface during storage exerts a stabilizing effect on particle integrity by making its release of individual micelles difficult and slowing down the complete dissolution of the particle. Burgain et al., (2016) recently confirmed this point as they observed a particle surface hardening phenomenon during storage. It is often mentioned that this skin formation at the surface of primary particles prevents powder to fully reconstitute, even after extended periods of rehydration, due to inhibited transfer of water into powder particles and/or slowed release of cross-linked casein micelles (Mimouni et al., 2010c).

Unfortunately, a lot of questions are still not elucidated concerning this skin formation during storage and its influence on the successive steps constituting the powder rehydration process:

- Could the slowed water penetration due to the presence of the formed skin around the particle (explain alone the rehydration property decline of casein-dominant powders)?

- If not, which is the rate-limiting stage of rehydration, namely the diffusion of water into the particle or the final release of surface cross-linked casein micelles into the surrounding liquid?

- Is the retarded release of casein micelles due to cross-linking the only cause for the extended rehydration time after storage, or other rearrangements at the molecular scale (such as migration of lipid components) could also contribute to an additional delay?

To answer these questions and fill the gap, a high-protein micellar casein (MC) powder was elaborated by membrane filtration of skimmed milk followed by spray-drying to obtain a casein-dominant powder (>90 % casein). Such MC powders, initially used in cheese making (Maubois and Brulé, 1982), are nowadays added to foodstuffs to enhance certain techno-functionalities (heat stability, viscosity, gelation, emulsifying and foaming properties) (Broyard and Gaucheron, 2015). As a second step, different controlled storage conditions (various temperatures at a fixed level of relative humidity) were applied to the MC powder for increasing time period. The evolution of rehydration properties with storage time were assessed through ultrasound attenuation tests, granulomorphometry and static light scattering to determine the three characteristic rehydration times: relaxation, fragmentation and total rehydration time.

Then, Scanning Electron Microscopy (SEM) imaging was carried out for aged and non-aged powder particles during the course of rehydration. The evolution of MC powder bulk/surface/extreme surface composition during storage at dry state was followed using a set of techniques (Iatroscan, Nuclear magnetic resonance (NMR), X-ray photoelectron spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectrometry (Tof-SIMS)). Finally, these experimental data were analysed in order to:

i) find out the link between the rehydration property decline and the storage-condition dependent microstructure evolution of the MC powder particle surface;

ii) identify which step(s) is/are more drastically impacted by the storage condition, constituting thus the rate limiting stage in rehydration.

2. Materials and methods

2.1 Dairy powder manufacture and controlled storage conditions

Micellar Casein (MC - Promilk 872 B1) concentrate was obtained from microfiltration (pore size = 0.1 µm) of skimmed milk at Ingredia (Arras, France). The retentate was spray-dried as previously described in Pierre et al., (1992) and Schuck et al., (1994a) in Bionov (Rennes, France) pilot plant (GEA, Niro Atomizer, St Quentin en Yvelines, France). Inlet air temperature was 180 ± 10 °C, fluid bed air temperature was 70 ± 1 °C and outlet air temperature was 65 ± 5 °C. MC powder was packaged in individual cans of 380 g directly after manufacture (Water activity: 25 %). Cans were stored under a controlled temperature of 4 °C, 20, 40 or 60 °C until 12 months. It will be shown later that for a storage temperature of 4°C, few evolutions of the rehydration properties could be observed even for the longest storage time (12 months). Therefore, this storage condition will be considered in the following as the reference condition and powder stored at this temperature will be named "reference powder". Although 40°C and 60°C may seem extreme temperatures, it has been shown that these temperatures could be achieved in course of powder shipment (Leinberger, 2006), and were thus considered in this study. Another reason for the choice of bigger temperatures is that there exists a correspondence between storage at ambient temperature for longer time period and at these two higher temperatures for a shorter time, as shown by Nasser et al., (2017c) and Norwood., (2016).

2.2 Analysis of structure and composition of casein powder surface

2.2.1 Iatroscan analysis

Iatroscan apparatus was used to quantify polar lipid and triglycerides in the core and on the surface of the powder.

Lipid extraction and analysis were performed as described by Gaiani et al. (2007a). Briefly, lipids were extracted according to the Folch method and collected in chloroform (5 mg mL^{-1}). Lipids of different classes were separated on chromarods-SIII and quantified using a thin layer flame ionization detection Iatroscan apparatus MK V (Iatroscan Laboratory Inc., Tokyo, Japan). To this end, a 1 µL

glass minicap pipet (Hirschmann Laborgerate, Germany) was employed to spot samples onto the chromarods. Lipids were submitted to migration in hexane/diethyl ether/formic acid (80:20:0.2 v/v/v) at 20 °C in order to separate polar lipids and triglycerides. The air and hydrogen flow rates were set at 200 and 160 mL min^{-1}, respectively. The scanning speed was 30 cm $scan^{-1}$. Data acquisition and processing were performed by a compatible personal computer equipped with dedicated software (SCPA GmbH, ChromStar Integrator, version 4.14).

For the non-aged powder, the total lipid content was 1 %, comprising triacylglycerols and polar lipids (73.5 % and 26.5 % respectively).

2.2.2. X-ray photoelectron spectroscopy (XPS) analysis

XPS analysis was carried out to quantify elemental composition of MC powder surface to a depth of approximately 10 nm. Spectra were obtained with a KRATOS Axis Ultra X-ray photoelectron spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromated Al Kα X-ray (hν = 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.

2.2.3 Nuclear magnetic resonance (NMR) analysis

In order to get information about lipid mobility, NMR experiments were performed using a 9.4 T Bruker spectrometer equipped with an AVANCE-II console. Spectra were recorded with a 4 mm double-channel ^{1}HX probe, spinning at 5 kHz. HETCOR (heteronuclear correlation). The RF-field strength for the $\pi/2$ pulse on the ^{1}H channel was set at 59 kHz. For the cross-polarization transfer, the ^{31}P RF-field amplitude was 40 kHz, while the ^{1}H amplitude was linearly ramped from 30 to 60 kHz. The contact time was 1 ms. SPINAL-64 (Small Phase Incremental Alternation with 64 steps) ^{1}H decoupling was applied during acquisition with a RF-field amplitude of 59 kHz (Fung et al., 2000). The data were acquired with a recycle delay of 5 s since it allowed a full relaxation between acquisitions. The number of scans was 704, which led to an overall experimental time of 6 h and 40 min. ^{1}H spectrum was referenced to the tetramethylsilane set at 0 ppm while for the ^{31}P spectrum, it was the H_3PO_4 (85%) set at 0 ppm.

2.2.4 Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) analysis

ToF-SIMS was carried out to provide information on the amount of amino-acids (AA) and lipids at the extreme surface (around 1 nm to 3 nm in depth) of MC powder after storage. Measurements were realised using a TOF.SIMS 5 spectrometer (IONTOF GmbH Germany) equipped with a bismuth liquid metal ion gun (LMIG). The compacted samples were analysed in positive mode with pulsed Bi_3^+ primary ion beam (25 keV, 0.25 pA) rastered over a 500 µm × 500 µm surface area with 256 x 256 pixels during 10 scans. Charging effects, due to the primary ion beam, were compensated by means of a 20 eV pulsed electron flood gun. In these experiments the mass resolution (m/Δm) was about 3000 at m/z=56 for $C_3H_6N^+$.

Presence of protein at extreme surface was monitored by tracking the evolution of mass spectrum area of major amino-acids of the MC powder proteins such as: Glycine (CH_4N^+ at *m/z* = 30), Alanine ($C_2H_6N^+$ at *m/z* = 44), Lysine ($C_3H_7N^+$ at *m/z* = 57), Serine ($C_2H_6NO^+$ at *m/z* = 60), Proline ($C_4H_6N^+$ at *m/z* = 68) , Valine ($C_4H_{10}N^+$ at *m/z* = 72), Threonine ($C_3H_8NO^+$ at *m/z* = 69), Cysteine ($C_2H_{6S}N^+$ at *m/z* = 76), Leucine ($C_5H_{12}N^+$ at *m/z* = 86), Aspartate ($C_3H_6N0_2^+$ at *m/z* = 88), Glutamate ($C_4H_8NO_2^+$ at *m/z* = 88), Histidine ($C_5H_8N_3^+$ at *m/z* = 110) (Henry et al., 2003; Lhoest et al., 2001; Sanni et al., 2002; Schilke and McGuire, 2011; Tyler et al., 2011).

Presence of lipid at extreme surface was monitored by tracking the secondary ion signal of phosphocholine ($C_5H_{15}NOP_4^+$ at *m/z* =184) (Vaezian et al., 2010). As a polar lipid belonging to the phospholipid class and a byproduct in the synthesis of phosphatidylcholine (PC), phosphocholine seems well appropriate for several reasons:

-Firstly, it has been demonstrated that PC is one of the two major milk phospholipids. Indeed, it was reported by Rombaut et al., (2006) that its concentration was in the range of 19.2–37.3 w/w %, slightly below that of phosphatidylethanolamine (19.8–42.0 w/w %).

-Then, it has been proven that PC are phospholipids with very low solubility, which swell in water (Pichot et al., 2013). In this study, swelling phenomena and low solubility properties were reported for MC powder.

-Last, it was established that the choline-containing phospholipids, PC and sphingomyelin, are largely located on the outer surface of the fat globule membrane (Pichot et al., 2013). So, it could be supposed that phosphocholine is the best candidate to detect lipid concentration change at extreme surface.

Each element detection by ToF-SIMS was carried out on 5 different regions of the surface particle. Based on ToF-SIMS analysis, Equation (1) and (2) were used to obtain indicators of relative intensities protein and lipid at extreme surface:

$$\text{Intensity of protein at extreme surface (a.u)} = \frac{\text{The sum of area of mass spectrum of protein fraction}}{(\text{The sum of area of mass spectrum of protein fraction} + \text{mass spectrum area of lipid fraction})} \quad (1)$$

$$\text{Intensity of lipid at extreme surface (a.u)} = \frac{\text{Mass spectrum area of lipid fraction}}{(\text{The sum of area of mass spectrum of protein fraction} + \text{mass spectrum area of lipid fraction})} \quad (2)$$

2.3 Determination of powder rehydration properties

2.3.1. Determination of total rehydration time

The equipment used is identical to that used by Richard et al., (2013; 2012a). The solid/liquid concentration of the powder solution was 8 w/w %, corresponding to the concentration frequently used in industrial applications. The powder incorporation protocol was previously described in Jeantet et al., (2010) and identical throughout all the experiments. The moment of incorporation was defined as t_0 of the rehydration process.

Particle size distribution (PSD) was monitored by Static Light Scattering measurements in the course of rehydration in order to determine the total rehydration time. Particle size distribution was measured every 5 min until the particle size stops increasing by reaching a maximum, then around every 30 min till particle size decreases to 15 µm and finally every 5 min until the end of the experiment. The mean diameter Dv50 appeared as a pertinent parameter to follow particle size reduction, since all samples displayed a monomodal PSD. As previously defined by Richard et al., (2012a), the total rehydration time was the time required for Dv50 to reach 0.2 µm (from t_0) starting from initial time.

The mean standard deviation of total rehydration time results was equal to 11.4%. Similar values of standard deviation for different powders were obtained by Jeantet et al., (2010).

2.3.2. Determination of fragmentation time

Determination of fragmentation time was based on digital imaging of powder particles in the course of rehydration using a Flow-Cell272 200 S-M granulomorphometer (Occhio, Angleur, Belgium) coupled with an image analysis software (Callisto™). The principle of this granulomorphometer for particle detection and counting was described in details in Richard et al., (2012a). The particle detection is based on the difference in grey level between the particle and the background. All the particles detected on acquisition (9 acquisitions per sample) were counted and the total number of particles in the sample was calculated. The analysis processing started immediately after the incorporation of powder in the vessel (t_0). The fragmentation time was defined as the time interval from t_0 to the moment the particle number reached a maximum.

The mean standard deviation of fragmentation time results was equal to 26.7%.

2.3.3. Determination of relaxation time

The relaxation time was obtained using an ultrasound (US) test developed by Richard et al., (2012a), which measures the kinetics of air release from the internal of particles after immersion in water and thus reflects the resistance encountered by water to diffuse towards the core of the particles.

For this test, MC powder was rehydrated at 0.2 % w/w in 1 L of distilled water in a beaker, using a magnetic stirrer (SI Analytics GmBH, Mainz, Germany) at 450 rpm. The water temperature was set at

30 °C and controlled throughout the experiment. The ultrasound probe was immersed in the beaker and the powder was added once the sound wave amplitude baseline was stable. The conditions of rehydration here were different from those applied for total rehydration time determination because higher solid/liquid fractions would lead to the saturation of recorded signals.

Figure 1 shows typical ultrasound amplitude curves versus time for three replicates. The release of air entrapped in particles has been found to be responsible for the extinction of US signal. As water progressively replaced the internal air of the particles, the air release flowrate decreased and the signal moved toward an asymptotic plateau. The relaxation time was defined as the lapse of time from signal extinction upon powder incorporation to its recovery to 90% of the initial value.

The mean standard deviation of relaxation time results was equal to 7.7 %.

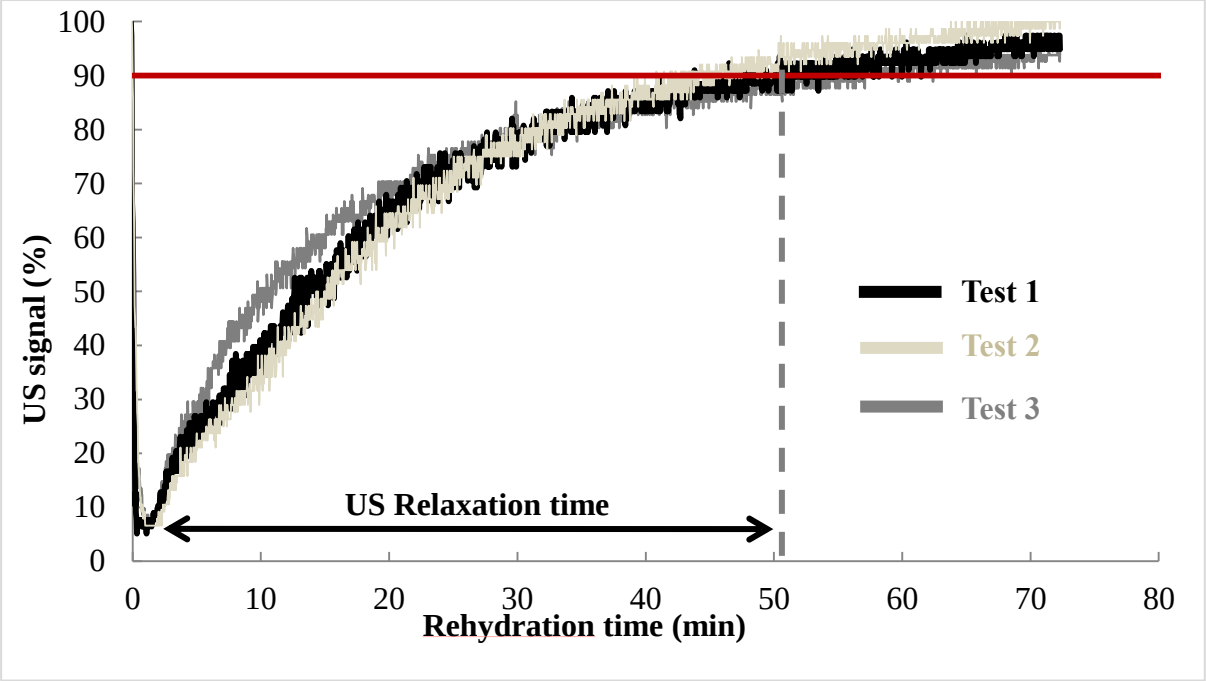


Figure 1: Typical curves of US measurements for the reference MC powder. Test 1 to 3 are three repetitions

2.3.4. Scanning Electron Microscopy (SEM) images of hydrated MC powder

SEM was used to visualize the surface structure of hydrated MC powder (aged and reference) in course of rehydration process. These images allowed us to monitor the presence of the cross-linked casein micelle skin upon ageing and its progressive erosion during rehydration. SEM imaging was realised with a Philips XL30 S-FEG SEM (The Netherlands) operating at an accelerating voltage of 5

kV, at four rehydration times (2 min, 40 min, 2h, 6h) under the same rehydration conditions as those used for relaxation time determination. The sample preparation protocol was the same as that described by Mimouni et al., (2010a).

2.4 Statistical analysis

Student's *t*-tests with a 0.05 level of significance were used to measure the significance of the differences between samples stored and reference powder non stored. As widely known, the differences are statistically nonsignificant when $p > 0.05$ and statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Effect of storage condition on lipid mobility and its migration to the surface

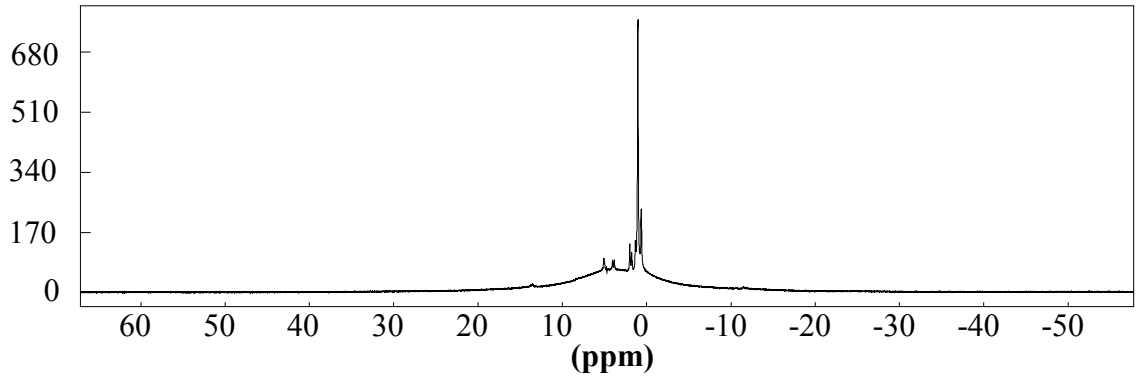
Figure 2a shows ^{1}H NMR spectrum of the reference MC powder (signal intensity vs chemical shift in ppm). In the chemical shift range of 20 to −10 ppm, narrow and sharp peaks were observed. These narrow peaks correspond to the NMR signature of lipids (Belloque and Ramos, 1999; Brescia et al., 2004). Thus, our results show that proteins and lipids were simultaneously present in the sample. In NMR, it is known that the smaller (thinner) peak widths, the higher the dynamic range (Keeler, 2010). Thus, it is demonstrated that lipids display relatively fast dynamics compare to protein in the sample, resulting in smaller peak widths in comparison with protein.

Figure 2b shows ^{1}H NMR spectrum for the reference MC powder and the powder stored for 2 months at 60 °C in the chemical shift range of 5 to -1 ppm where eight narrow peaks corresponding to lipids were observed. The aged MC powder showed significantly sharper peaks compared to the reference powder, indicating that lipids become more and more mobile upon ageing. As previous studies have suggested the possibility of lipid migration towards the particle surface during storage of milk powder (Gaiani et al., 2009; Kim et al., 2002, 2005; Nijdam and Langrish, 2006), we decided to probe this lipid migration to the extreme particle surface by ToF-SIMS **(Figure 3)**.

For the non-aged powder, the intensity of lipid at the surface was 0.090 and the intensity of protein was 0.991. After 3 months of storage at 20 °C, no significant differences between the non-aged powder and aged powder were observed ($p>0.05$). After 3 months of storage at 40 °C, a significant increase in intensity of lipid at the surface (0.100) was noted ($p<0.05$). The lipid migration to the surface seemed faster at 60 °C. Indeed, the surface intensity of lipid increased to 0.122 ($p<0.05$) and 0.160 ($p<0.05$) respectively after 0.5 month and 3 months of storage at 60 °C ($p<0.05$). Therefore, the increase in lipid migration upon ageing could only be assessed at storage temperatures higher than or equal to 40 °C.

The particle surface composition (in terms of atomic concentration and local bonding of atoms) was evaluated by XPS after storage under different conditions, whereas no significant differences between fresh and aged powder were observed **(Figure S1 & S2).** In fact, XPS and ToF-SIMS differ in sampling depth and surface area. XPS measurements are performed at a depth ranging up to 10 nm while ToF-SIMS only probes the extreme particle surface (depth of 1 nm to 3 nm). Another difference between these two techniques lies in the information they provide: XPS measures the elemental composition while ToF-SIMS can detect parts of amino acids and lipids. These significant differences are likely the reasons why lipid migration could only be evidenced by ToF-SIMS.

A.



B.

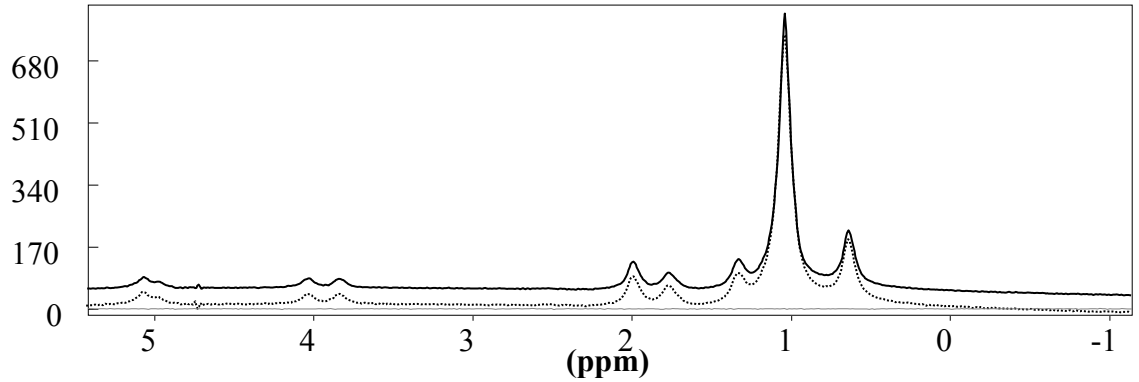


Figure 2: A: NMR spectra of reference powder from 70 to -60 ppm; B: NMR spectra of reference powder from 5.5 to -1 ppm of reference powder (full line) and powder stored for 2 months at 60°C (dotted line)

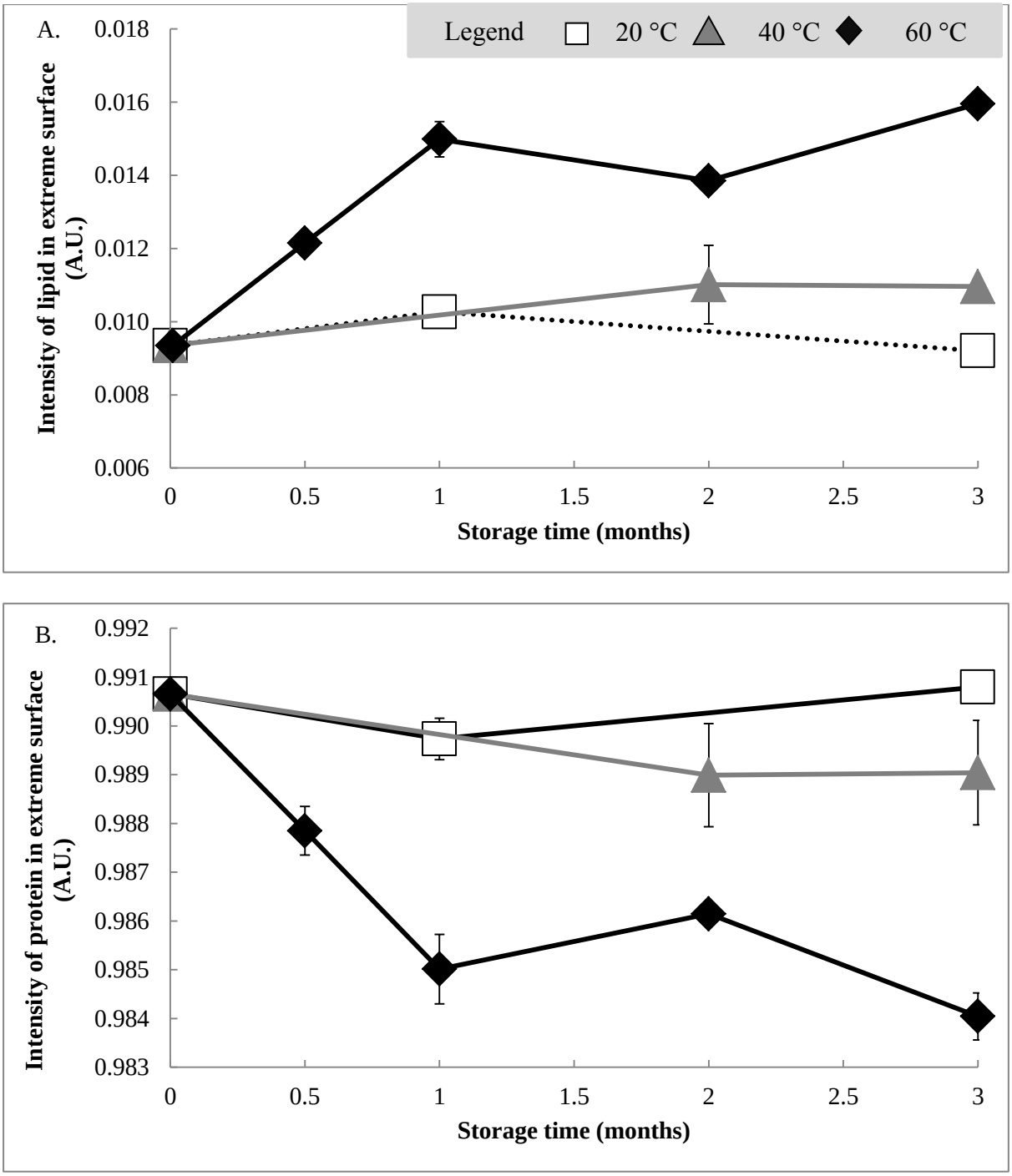


Figure 3: Intensities of protein (A) and Lipid (B) fraction on particle extreme surface as a function of storage time for MC powders stored at different temperatures 20 °C, 40 °C and 60 °C

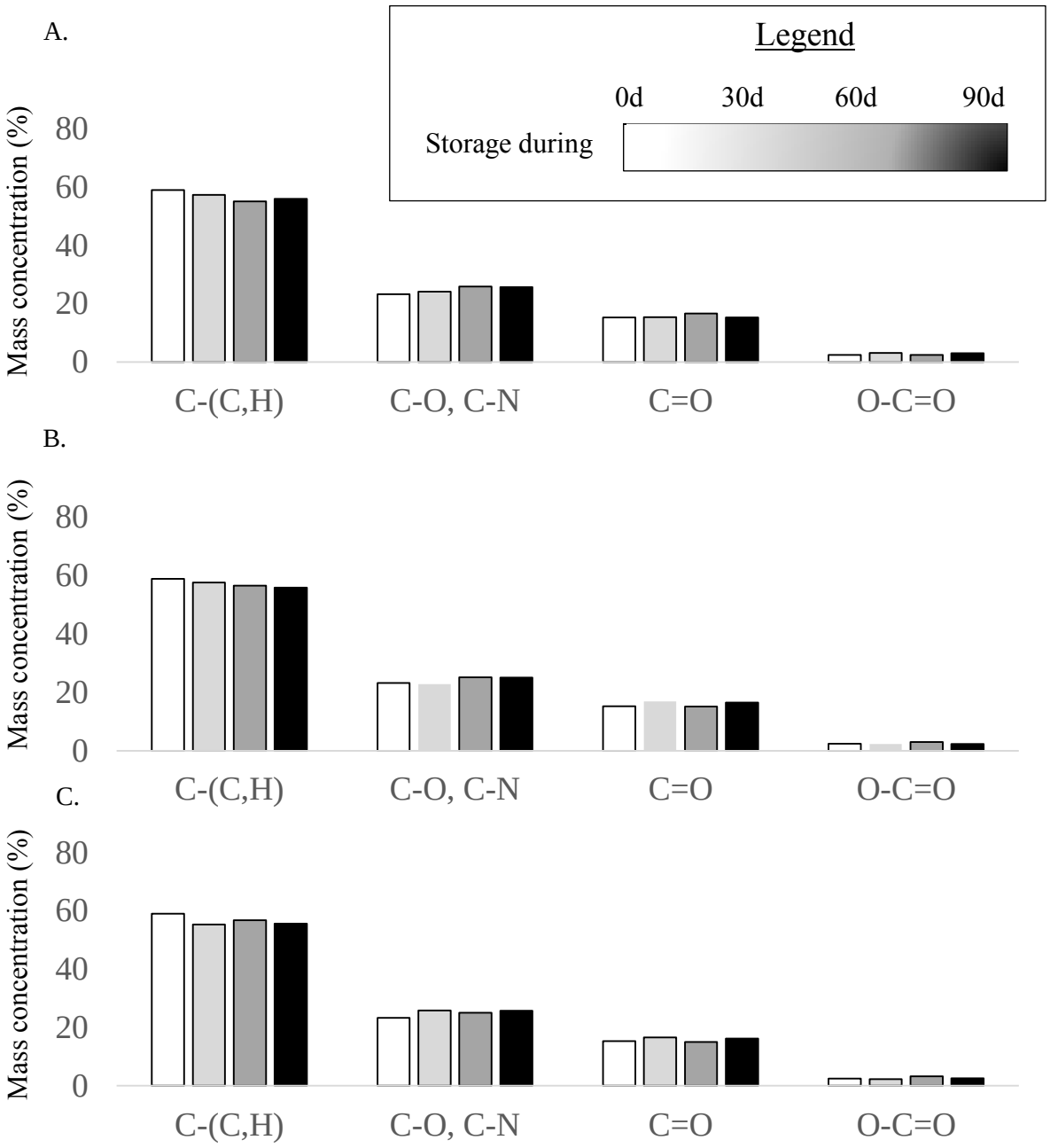


Figure S1. Monitoring of the bonds on the particle surface by XPS, during storage at (A) 20 °C (B) 40 °C (C) 60 °C

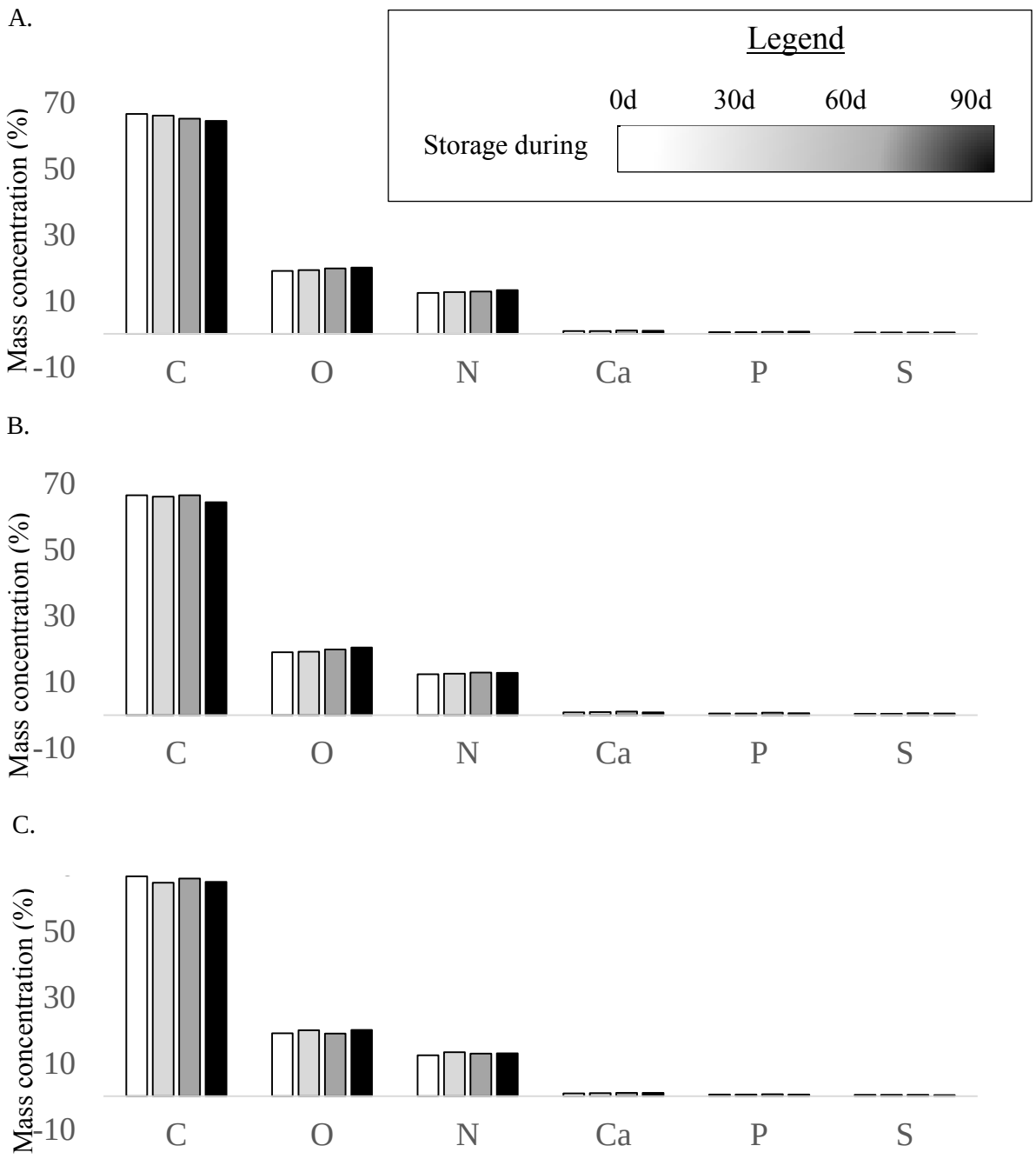


Figure S2. Monitoring of the atome composition on the particle surface by XPS, during storage at (A) 20 °C (B) 40 °C (C) 60 °C

3.2. Effects of storage conditions on the microstructure of hydrated particles

Figure 4 shows SEM images of reference and aged powder in the course of rehydration.

Fresh powder: reference case

As shown in Figure 4A, particles with a diameter of approximately 15 µm (hereafter named large particles) could be observed (arrows Figure 4A) after 2 min of rehydration. The microstructure of the hydrated particles appeared porous. Higher magnifications (not shown here) revealed that particles were formed by a loose assembly of micelles, in the order of 0.2 µm size, separated by pores of various sizes. This structure is similar to that reported by Mimouni et al., (2010a). Since the size of casein micelles is between 0.02 and 0.25 µm, it can be suggested that the spheres observed (examples surrounded Figure 4A) at the surface of the particles were composed of casein micelles linked by intermicellar interactions which were probably formed during drying of MC concentrate. Indeed, Sadek et al., (2016) showed that a thin layer was formed on the particle surface in the early stage of drying, or more precisely, when the sol-gel transition was reached at the droplet surface. The specific mechanical properties of the so-called skin enabled it to deform under drying stress, leading to the deflated and wrinkled aspect of the particle, as was clearly observed on Figure 4. It may be suggested here that this skin refers to the one observed by Mimouni et al., (2010a) upon rehydration of aged MPC, and that even non-aged skin has the ability to resist immediate erosion and retard the release of individual micelles. This would explain why, at early stage of rehydration, no dispersed individual micelles could be observed.

In contrast to the early stage of rehydration, most of the particles were disaggregated and few intact large particles could be detected anymore after 40 min of rehydration (Figure 4B). The skin-like structure seemed to have been partially solubilized at that time, although traces of its previous presence were still apparent (See circle in Figure 4B). Actually, despite the fact that large particles could no longer keep their initial organization in the skin-like structure, they could not be completely dissolved because of the strong interactions between casein micelles. Nevertheless, it is clear that the erosion of the skin-like structure progressively enhanced the release of individual micelles (Arrows in

Figure 4B). Indeed, the silicon wafer became covered by dispersed casein micelles simultaneously with skin erosion.

After 2 h of rehydration, the remains of the outer surface of large particles were still detectable, while the concentration of dispersed casein micelles covering the silicon wafer increased (Figure 4C). This remaining intermicellar network appeared even looser after 6 h of rehydration (Figure 4D), suggesting that complete dissolution of individual micelles may be hard to achieve in rehydration.

Aged powders

Figure 4E shows the surface microstructure of the MC powder particles (of 2-day-storage at 60 °C) after 2 min of rehydration. The difference from fresh particles (Figure 4A) is striking: a smooth surface is clearly visible, suggesting that ageing has induced a considerable rearrangement at the powder particle surface (protein/ lipid concentration change in the outer peripheral layer as well as the above-mentioned reinforced intermicellar interactions). These changes seriously impacted the rehydration process. Indeed, after 40 min of rehydration (Figure 4F), large particles were far less damaged than the reference powder at the same rehydration time (Figure 4B): the skin-like structure was almost intact and only few dispersed casein micelles were visible on the silicon wafer for the aged powder. Judging from these observations, it is clear that this ageing-induced dissolving-resistant crust acted as the barrier that restrained the release of individual micelles into the surrounding liquid. After 2h of rehydration (Figure 4G), solubilisation of the ageing-induced crust considerably progressed but remained visible. Two phases seemed to spread on the silicon wafer, one dense and the other porous. It is supposed that the dense phase contained the remaining crust over disintegration, while the porous phase corresponded to dispersed individual micelles. After 6 h of rehydration (Figure 4H), no more trace of large particles could be detected, however dense intermicellar network was still visible, highlighting the poor dissolving ability of the crust.

Moreover, the presence of this crust modified the swelling behaviour of the particles. Indeed, swelling of the particles could be observed only for aged powder (4F), not for the fresh powder, suggesting that

the dissolving resistant feature of the ageing-induced crust, coupled with appropriate mechanical properties, has made it possible to maintain the particle structure long enough for swelling to happen.

The rehydration of the powder stored for 10 days at 60 °C is presented in Figure 4 (I-L).

After 2 min of rehydration (Figure 4I), the 10-day-aged particles appeared smoother than the 2-day-aged powder particles (Figure 4E), but still kept their characteristic deflated and wrinkled aspect. After 40 min (Figure 4J) and 2 h of rehydration (Figure 4K), large particles with intact crust were still present and no individual micelles could be observed on the silicon wafer. This also suggests that the skin formed during drying was reinforced by strong micelle interactions after storage at 60 °C for increasing time period. Moreover, particles have become swollen, like the powder stored at 60 °C for 2 days. Indeed, particle size increased from the initial 15 µm to around 25 µm at these stages. The rehydration process should be heterogenous since both smooth (Dotted line arrow) and porous aspects (Full line arrow) were observed on the same particle. After 6 h, particles were still visible but their mean size has decreased around 9 µm (Figure 4L), which means that dissolution was still at its beginning stage.

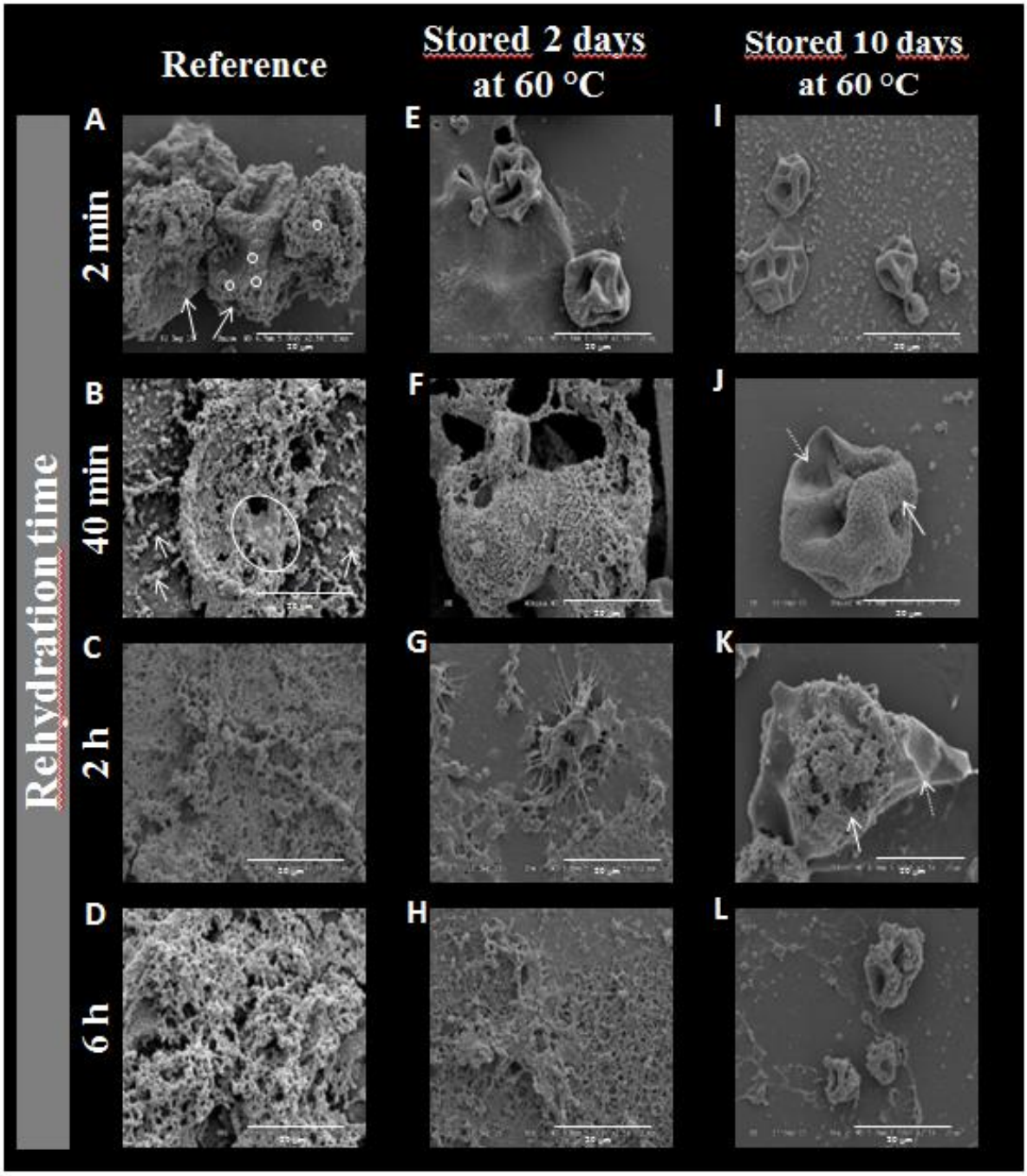


Figure 4: Emission scanning electron pictures of reference MC powder particles, after 2-day-storage at 60 °C and after 10-day-storage at 60 °C. Pictures were obtained at magnification ×2,500 at different rehydration time (2 min; 40 min; 2h; 6 h)

3.3. Effect of storage conditions on rehydration properties of MC powder

3.3.1. US relaxation time

Figure 5 shows the US relaxation time evolution as a function of storage time for the different ageing temperatures (4 °C, 20 °C, 40 °C and 60 °C). Relaxation times obtained by US tests can give indirect evidence for the kinetics of water penetration in the particles.

When the powder was stored at 4 °C, the relaxation time decreased from 50 min to around 40 min ($p<0.05$), regardless of ageing time up to 12 months of storage. For powder stored at 20 °C, the relaxation time decreased over the first 4 months of storage to approximately 45 min ($p=0.11$), and then slightly increased to 58 min ($p=0.07$). These trends were confirmed to be almost significant.

A monotonous increase of relaxation time was observed for powder stored at 40 °C. No initial decrease was detected (the first sample was at 15 days of storage). The relaxation time finally reached about 100 min, after 12 months of storage.

An even stronger upward trend of the relaxation time was observed at 60 °C, reaching 574 min after only 5 months of storage. However, a close analysis on short ageing times revealed that a decrease of relaxation time down to 35 min occurred during the first day of storage ($p<0.05$) (data not shown).

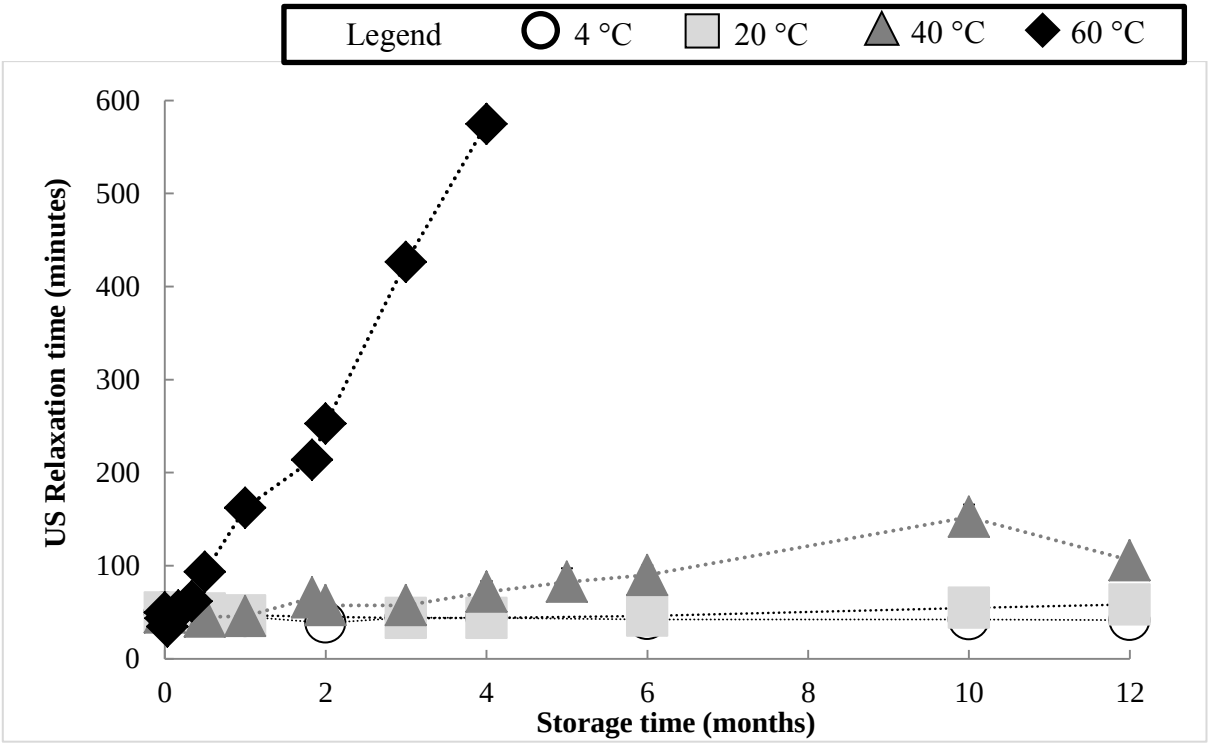


Figure 5: US relaxation time as a function of storage time at different temperatures: 4 °C, 20 °C, 40 °C and 60 °C

3.3.2. Fragmentation times

Figure 6 shows the evolution of fragmentation time as a function of storage time for different ageing temperatures (4°C, 20 °C, 40 °C and 60 °C). It represents the time needed for particle fragmentation, reflecting the ease of dissolving the solid bridges between the micelles casein. The latter depends on water penetration and cohesion of the crust. It turned out that storage temperature had a strong impact on the evolution of this indicator. When the powder was stored at 4 °C, the fragmentation time remained almost constant around 12 min over the 12 months of storage. The evolution is not significate ($p>0.05$). Only a slight upward trend was observed for powder stored at 20 °C ($p<0.05$), with the fragmentation time reaching 1 h after 12 months of storage.

For powder stored at 40 °C, the fragmentation time increased monotonously upon ageing, reaching around 5 h after 12 months. It is noteworthy that the fragmentation time was already as long as 2 h (a 10-fold increase compared to the reference powder) at 15 days of storage. The fragmentation time evolution of MC stored at 60 °C was even more drastic over time, reaching a 15-fold increase after only 2 days of storage and up to 6.5 h after 5 days of storage.

Study on the evolution of fragmentation time during ageing has not yet been conducted. Nevertheless, the evolutions reported here are in agreement with the work of Anema et al., (2006) who reported the increase of cross-linking with storage time and temperature. Indeed, the cross-linking of proteins in the course of storage could explain why it becomes more and more difficult to fragment the large particles and dissolve them.

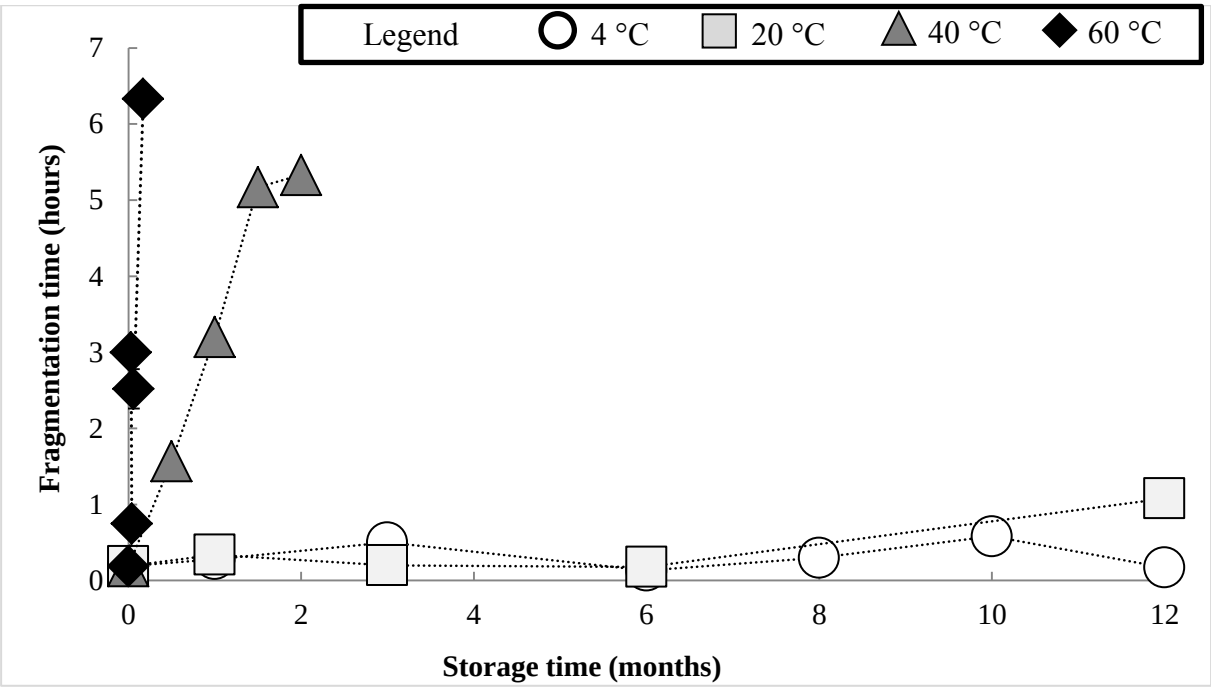


Figure 6: Fragmentation time as a function of storage time for MC stored at different temperatures

3.3.3. Total rehydration time

Figure 7 shows evolutions of total rehydration time as a function of storage time for different ageing temperatures (4°C, 20 °C, 40 °C and 60 °C). It was found that total rehydration time of MC stored at 4 °C was only slightly affected by ageing. Although that the total rehydration time could be observed as nearly constant in the range of the storage time investigated (up to 12 months), the increase is analysed as statistically significate ($p<0.05$)

At 20 °C, the evolution of total rehydration time with storage time was clearer. The total rehydration time doubled after 6 months, and then remained around 6.4 h up to 12 months ($p<0.05$).

The evolution of total rehydration time was much more significant for MC powder stored at 40 °C and 60 °C ($p<0.05$). As a matter of fact, the total rehydration time has already doubled after only 15 days of storage at 40 °C and more than doubled after only 24 h of storage at 60 °C.

Note that experimental conditions did not allow us to run tests with a rehydration time longer than 35 h. As total rehydration time for MC powder of 5-day-storage at 60 °C and of 2-month-storage at 40 °C was longer than 35 h, it was thus not possible to quantify them.

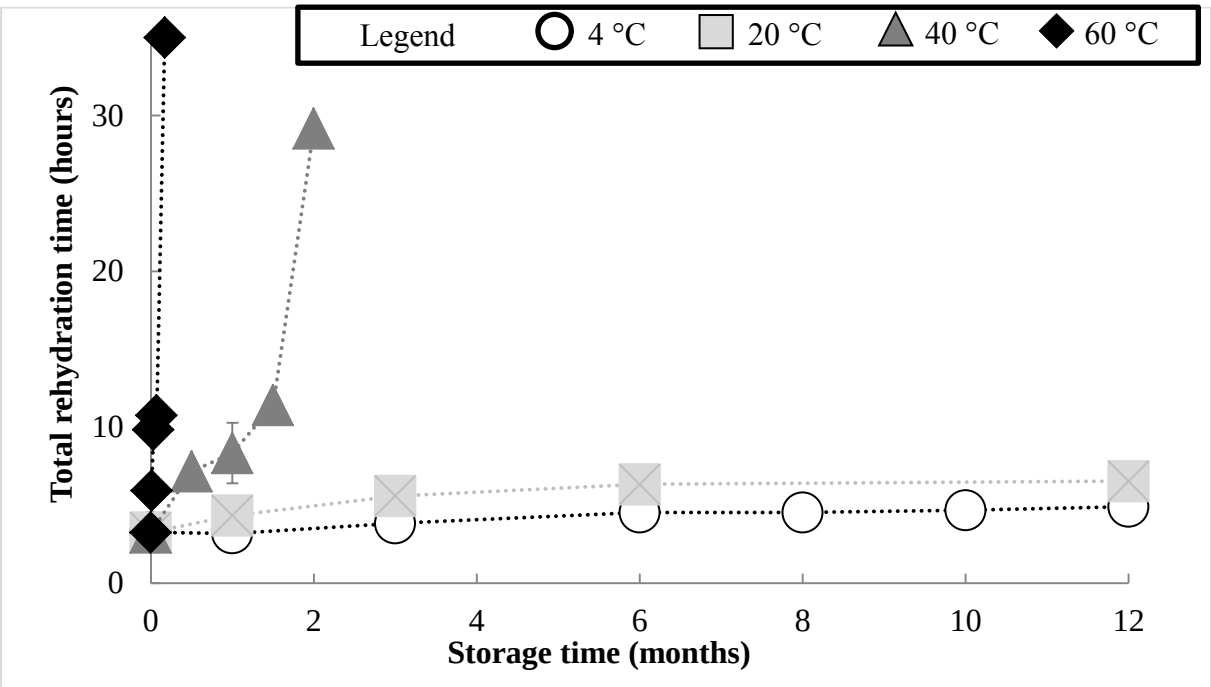


Figure 7: Total rehydration time as a function of storage time and for MC stored at different temperatures: 4°C, 20 °C, 40 °C and 60 °C.

4. Discussion

In this study, surface migration of lipids was observed using Tof-Sims for storage temperatures greater than or equal to 40 °C. Lipid migration kinetics were affected by both temperature and storage time**.** On the contrary, **s**torage at 20 °C and below induced no or only slight evolution in lipid migration with ageing, even for a long storage time period. These results seem coherent with most of the trends reported in the literature using other techniques. Indeed, changes in surface composition (in terms of proteins and lipids) assessed by XPS have also been reported for different kinds of milk powders at

high temperatures (Kim et al., 2002, 2005; Nijdam and Langrish, 2006) whereas no change was detected for several milk powders after 6 months of storage at 20 °C (Kim et al., 2005). Gaiani et al., (2009) also found by XPS measurements that lipid content at the particle surface of MC powder increased from 6 to 17 % after 60 days of storage, regardless of the temperature, with a concomitant decrease of the protein percentage at the surface from 94 % to 83 %. Our study is in overall agreement with their work except that lipid and protein migration kinetics were revealed to be dependent on temperature in our work. This difference could be explained by the lower lipid content of the MC powder used in our study, namely phospholipids. Since the migration ability depend firstly on the concentration gradient within the particle, more drastic storage conditions are necessary to induce noticeable lipid clustering and surface migration for powder with lower lipid content.

Likewise, Faldt et al., (1993) observed by SEM the formation of an irregular and wrinkled surface during storage of caseinate powder at 20, 30, 35, 40 and 50 °C, for periods up to 60 days. This was associated with the presence of fat at the surface, once again supporting the hypothesis of lipid release at the particle surface.

Evolution of particle surface was observed in course of rehydration using SEM. The dissolution mechanism of MC powder has been described in the following steps: swelling of particles, dissolution of the solid bridges between micelles casein, gradual release of cross-linked micelles from the surface into the solvent, dissolution of fragmented particles. It completes the study of Ji et al. (2016) which describes steps of contact of powder particles with water, namely de-agglomeration, continuous release of materials from the surface of particles and finally erosion of the external layer of particles to obtain full dissolution. Extended swelling phenomena, observed as the first step of rehydration, could be due to water penetration into the external layer of micellar caseins (Ji et al., 2016). The increase of swelling phenomena with ageing suggests that the ability of the crust to resist to dissolution became higher, delaying the fragmentation of the particles after water penetration and the subsequent release of individual micelles. This hypothesis was recently confirmed by Burgain et al., (2016), who highlighted the particle surface hardening phenomenon during storage.

In our work, smoother surface of the particles was reported for powder aged at higher temperatures, resulting in a compact microstructure that slows down the rehydration process. This effect of storage

conditions on the microstructure of the hydrated particles is clearly in accordance with the work of Mimouni et al., (2010a, c) and could be explained by the increase of micelle interactions and the consequent compaction of the particle surface. Our study also shed light on the point that the rehydration of particles is a highly heterogeneous phenomenon. This fact was also underlined in the work of Burgain et al., (2016) and Mimouni et al., (2010a).

It was proven herein that ageing did not only affect the total rehydration time but also the characteristic times of the different stages composing the whole rehydration process, namely water penetration and particle fragmentation. Indeed, it was established that the US relaxation time (related to the wetting step) encountered a non-monotonous evolution. The storage-temperature dependent decrease of US relaxation time at the beginning of storage could be explained by the formation of pores that allowed an easier water penetration, as advanced by Mimouni et al., (2010a). For longer and more severe storage conditions, the increase of relaxation time can be explained by the compaction of micelles at the particle surface, preventing penetration of water into the particle core. This study sheds light on underlying phenomenon like formation of pores, compaction of micelles occurred during storage, and modified interaction between particle surface and water.

A parallel may be drawn between the migration of lipids to the particle surface and US relaxation time during storage. Indeed, a good exponential relationship ($R^2 > 0.90$) was found between the US relaxation time and the relative intensity of lipid in extreme surface **(Figure 8)**. This study consolidates previous results, suggesting that hydrophobic components such as lipids impede wetting (Faldt and Bergenstahl, 1996). However, our experiments indicate that the limiting step for total rehydration was not the wetting step, hence neither was the migration of lipid to the external layer. Indeed, the ageing-induced increase in water penetration time remained weak compared to the strong increase in total rehydration time. It explains fully the low coefficient correlation ($R^2 = 0.55$, results not shown) between US relaxation time (related to water penetration) and total rehydration time. Moreover, total rehydration time could still double even when lipid migration and evolution of US relaxation time were not noticeable (eg; storage at 20°C). This is in agreement with the assumption of Mimouni et al., (2010c): other additional mechanisms than surface composition modification and wetting limitation occur, exerting a key impact on rehydration ability. As such, cross-link formation

appeared as the main phenomenon responsible for total rehydration time increase. It was clearly shown by SEM that interactions between caseins were reinforced upon ageing, inducing significant increase of both fragmentation and total rehydration time. Consequently, the delayed casein micelle release probably due to cross-link formation during storage should be considered as the rate-limiting stage of rehydration for aged powder.

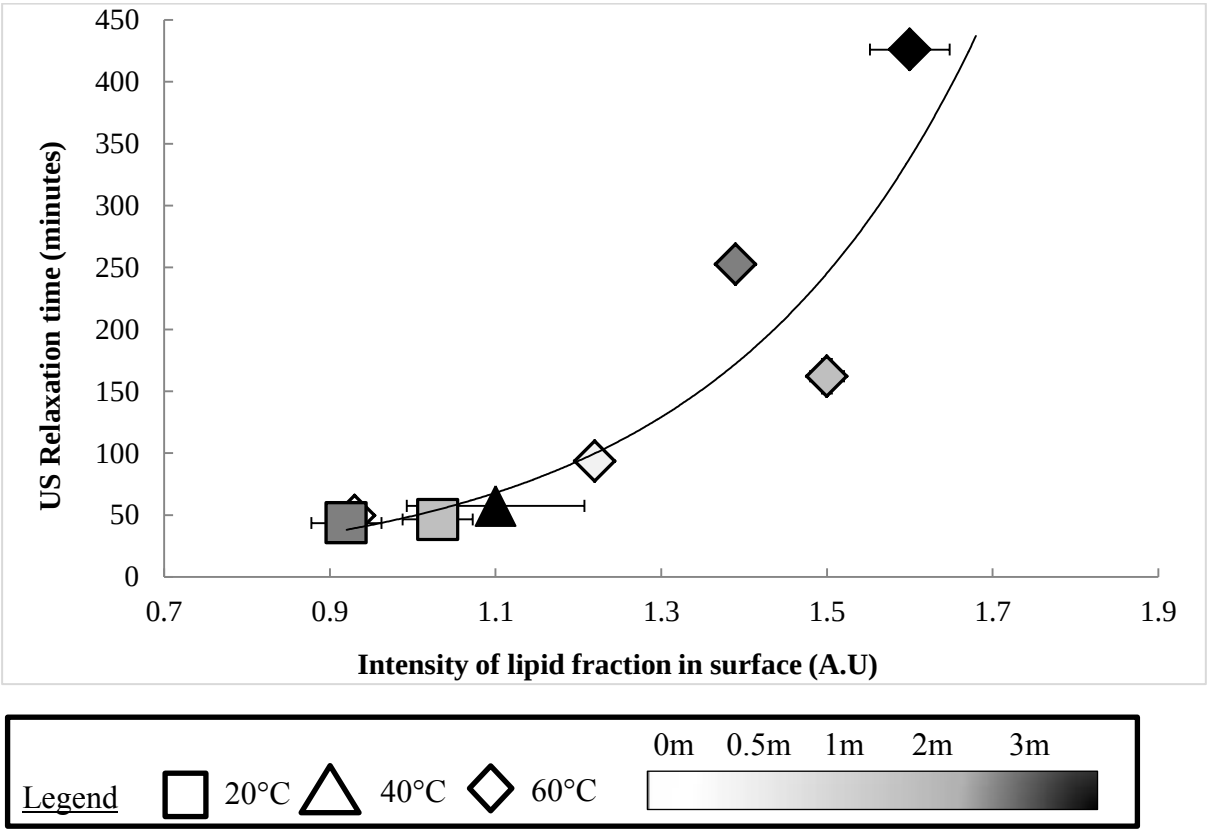


Figure 8: US Relaxation time as function of intensity of lipid fraction in surface at different time/temperatures pairs of storage

5. Conclusion

The effect of ageing on the microstructure of MC surface particles in course of rehydration and the evolution of surface particle composition (lipids and proteins) during storage were studied. In the same time, evolution of wetting, fragmentation and total rehydration time upon ageing were monitored.

For powder stored at a temperature greater than or equal to 40 °C, increases in the migration of surface lipids, surface crust resistance and rehydration characteristic times were demonstrated. As demonstrated by Nasser et al., (2017c), a catching up behaviour from the changes obtained at lower temperatures to those at highest temperatures reflecting a clear and relevant ageing evolution curve

(ageing path) of the MC powder. It suggests that changes observed at 40 and 60 °C will also be visible at 4 and 20 °C but at more advanced storage times.

A strong correlation was demonstrated between migration of lipids to the surface and evolution of wetting time. Nevertheless, it was clearly established that wetting step was not the main reason for extended total rehydration time upon ageing. On the contrary, it was shown that delayed casein micelles release induced drastic increase of fragmentation and total rehydration time probably due to cross link formation, and should therefore be considered as the key mechanism extending total rehydration time.

Acknowledgements

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Bibliography

Anema, S. G., D. N. Pinder, R. J. Hunter, and Y. Hemar, 2006, Effects of storage temperature on the solubility of milk protein concentrate (MPC85): Food Hydrocolloids, v. 20, p. 386-393.

Belloque, J., and M. Ramos, 1999, Application of NMR spectroscopy to milk and dairy products: Trends in Food Science & Technology, v. 10, p. 313-320.

Brescia, M. A., V. Mazzilli, A. Sgaramella, S. Ghelli, F. P. Fanizzi, and A. Sacco, 2004, H-1 NMR characterization of milk lipids: A comparison between cow and buffalo milk: Journal of the American Oil Chemists Society, v. 81, p. 431-436.

Broyard, C., and F. Gaucheron, 2015, Modifications of structures and functions of caseins: a scientific and technological challenge: Dairy Science & Technology, v. 95, p. 831-862.

Burgain, J., J. Scher, J. Petit, G. Francius, and C. Gaiani, 2016, Links between particle surface hardening and rehydration impairment during micellar casein powder storage: Food hydrocolloids, v. 61, p. 277-285.

Crowley, S. V., B. Desautel, I. Gazi, A. L. Kelly, T. Huppertz, and J. A. O'Mahony, 2015, Rehydration characteristics of milk protein concentrate powders: Journal of Food Engineering, v. 149, p. 105-113.

Davenel, A., P. Schuck, and P. Marchal, 1997, A NMR relaxometry method for determining the reconstitutability and the water-holding capacity of protein-rich milk powders: Milchwissenschaft-Milk Science International, v. 52, p. 35-39.

Faldt, P., and B. Bergenstahl, 1996, Spray-dried whey protein/lactose/soybean oil emulsions .2. Redispersability, wettability and particle structure: Food Hydrocolloids, v. 10, p. 431-439.

Faldt, P., B. Bergenstahl, and G. Carlsson, 1993, The surface coverage of fat on food powders analyzed by ESCA (Electron-spectroscopy for chemical- analysis): Food Structure, v. 12, p. 225-234.

Fung, B. M., A. K. Khitrin, and K. Ermolaev, 2000, An improved broadband decoupling sequence for liquid crystals and solids: Journal of Magnetic Resonance, v. 142, p. 97-101.

Fyfe, K. N., O. Kravchuk, T. Le, H. C. Deeth, A. V. Nguyen, and B. Bhandari, 2011, Storage induced changes to high protein powders: influence on surface properties and solubility: Journal of the Science of Food and Agriculture, v. 91, p. 2566-2575.

Gaiani, C., J. Scher, J. J. Ehrhardt, M. Linder, P. Schuck, S. Desobry, and S. Banon, 2007a, Relationships between dairy powder surface composition and wetting properties during storage: Importance of residual lipids: Journal of Agricultural and Food Chemistry, v. 55, p. 6561-6567.

Gaiani, C., P. Schuck, J. Scher, S. Desobry, and S. Banon, 2007b, Dairy powder rehydration: Influence of protein state, incorporation mode, and agglomeration: Journal of Dairy Science, v. 90, p. 570-581.

Gaiani, C., P. Schuck, J. Scher, J. J. Ehrhardt, E. Arab-Tehrany, M. Jacquot, and S. Banon, 2009, Native phosphocaseinate powder during storage: Lipids released onto the surface: Journal of Food Engineering, v. 94, p. 130-134.

Gazi, I., and T. Huppertz, 2015, Influence of protein content and storage conditions on the solubility of caseins and whey proteins in milk protein concentrates: International Dairy Journal, v. 46, p. 22-30.

Henry, M., C. Dupont-Gillain, and P. Bertrand, 2003, Conformation change of albumin adsorbed on polycarbonate membranes as revealed by ToF-SIMS: Langmuir, v. 19, p. 6271-6276.

Jeantet, R., P. Schuck, T. Six, C. Andre, and G. Delaplace, 2010, The influence of stirring speed, temperature and solid concentration on the rehydration time of micellar casein powder: Dairy Science & Technology, v. 90, p. 225-236.

Ji, J. F., J. Fitzpatrick, K. Cronin, P. Maguire, H. Z. Zhang, and S. Miao, 2016, Rehydration behaviours of high protein dairy powders: The influence of agglomeration on wettability, dispersibility and solubility: Food Hydrocolloids, v. 58, p. 194-203.

Keeler, J., 2010, Understanding NMR Spectroscopy ,2nd edition, Wiley, 526 p.

Kelly, G. M., J. A. O'Mahony, A. L. Kelly, T. Huppertz, D. Kennedy, and D. J. O'Callaghan, 2016, Influence of protein concentration on surface composition and physico-chemical properties of spray-dried milk protein concentrate powders (vol 51, pg 34, 2015): International Dairy Journal, v. 52, p. 125-125.

Kim, E. H. J., X. D. Chen, and D. Pearce, 2002, Surface characterization of four industrial spray-dried dairy powders in relation to chemical composition, structure and wetting property: Colloids and Surfaces B-Biointerfaces, v. 26, p. 197-212.

Kim, E. H. J., X. D. Chen, and D. Pearce, 2005, Melting characteristics of fat present on the surface of industrial spray-dried dairy powders: Colloids and Surfaces B-Biointerfaces, v. 42, p. 1-8.

Le, T. T., B. Bhesh, and H. C. Deeth, 2011, Chemical and physical changes in milk protein concentrate (MPC80) powder during storage: Journal of Agricultural and Food Chemistry, v. 59, p. 5465-5473.

Leinberger, D., 2006, Temperature and humidity in ocean containers, USA, Proceedings of Dimensions. International Safe Transit Association: East Lansing.

Lhoest, J. B., M. S. Wagner, C. D. Tidwell, and D. G. Castner, 2001, Characterization of adsorbed protein films by time of flight secondary ion mass spectrometry: Journal of Biomedical Materials Research, v. 57, p. 432-440.

Maubois, J. L., and G. Brulé, 1982, Utilisation des techniques à membrane pur la séparation, la purification et la fragmentation des protéines laitières, Le Lait, p. 484-510.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari, 2010a, Investigation of the microstructure of milk protein concentrate powders during rehydration: Alterations during storage: Journal of Dairy Science, v. 93, p. 463-472.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari, 2010b, Rehydration of high-protein-containing dairy powder: Slow- and fast-dissolving components and storage effects: Dairy Science & Technology, v. 90, p. 335-344.

Nasser, S., A. Moreau, A. Hédoux, R. Jeantet, and G. Delaplace, 2017, Influence of storage conditions on the functional properties of Micellar Casein powder: Submitted to LWT-Food Science and Technology.

Nijdam, J. J., and T. A. G. Langrish, 2006, The effect of surface composition on the functional properties of milk powders: Journal of Food Engineering, v. 77, p. 919-925.

Norwood, E.-A., 2016, Molecular and functional changes occurring during ageing of whey protein isolate powders, PhD Thesis.

Pichot, R., R. L. Watson, and I. T. Norton, 2013, Phospholipids at the Interface: Current Trends and Challenges: International Journal of Molecular Sciences, v. 14, p. 11767-11794.

Pierre, A., J. Fauquant, Y. Legraet, M. Piot, and J. L. Maubois, 1992, Native micellar casein separation through cross flow membrane microfiltration: Lait, v. 72, p. 461-474.

Richard, B., J. F. Le Page, P. Schuck, C. Andre, R. Jeantet, and G. Delaplace, 2013, Towards a better control of dairy powder rehydration processes: International Dairy Journal, v. 31, p. 18-28.

Richard, B., M. Toubal, J.-F. Le Page, G. Nassar, E. Radziszewski, B. Nongaillard, P. Debreyne, P. Schuck, R. Jeantet, and G. Delaplace, 2012, Ultrasound tests in a stirred vessel to evaluate the reconstitution ability of dairy powders: Innovative Food Science & Emerging Technologies, v. 16, p. 233-242.

Rombaut, R., and K. Dewettinck, 2006, Properties, analysis and purification of milk polar lipids: International Dairy Journal, v. 16, p. 1362-1373.

Sadek, C., P. Schuck, Y. Fallourd, N. Pradeau, R. Jeantet, and C. Le Floch-Fouere, 2016, Buckling and collapse during drying of a single aqueous dispersion of casein micelle droplet: Food Hydrocolloids, v. 52, p. 161-166.

Sanni, O. D., M. S. Wagner, D. Briggs, D. G. Castner, and J. C. Vickerman, 2002, Classification of adsorbed protein static ToF-SIMS spectra by principal component analysis and neural networks: Surface and Interface Analysis, v. 33, p. 715-728.

Schilke, K. F., and J. McGuire, 2011, Detection of nisin and fibrinogen adsorption on poly(ethylene oxide) coated polyurethane surfaces by time-of-flight secondary ion mass spectrometry (TOF-SIMS): Journal of Colloid and Interface Science, v. 358, p. 14-24.

Schuck, M. Piot, S. Méjean, Y. Le Graet, J. Fauquant, G. Brulé, and J. L. Maubois, 1994, Déshydratation par atomisation de phosphocaséinate natif obtenu par microfiltration sur membrane: Le lait, v. 74, p. 375-388.

Schuck, P., S. Mejean, A. Dolivet, C. Gaiani, S. Banon, J. Scher, and R. Jeantet, 2007, Water transfer during rehydration of micellar casein powders: Lait, v. 87, p. 425-432.

Selomulya, C., and Y. Fang, 2013, Food powder rehydration, *in* B. Bhandari, N. Bansal, M. Zhang, and P. Schuck, eds., Handbook of Food Powders: Processes and Properties:

Woodhead Publishing Series in Food Science Technology and Nutrition: Cambridge, Woodhead Publ Ltd, p. 379-408.

Thomas, M. E. C., J. Scher, S. Desobry-Banon, and S. Desobry, 2004, Milk powders ageing: Effect on physical and functional properties: Critical Reviews in Food Science and Nutrition, v. 44, p. 297-322.

Tyler, B. J., C. Bruening, S. Rangaranjan, and H. F. Arlinghaus, 2011, TOF-SIMS imaging of adsorbed proteins on topographically complex surfaces with Bi-3(+) primary ions: Biointerphases, v. 6, p. 135-141.

Vaezian, B., C. R. Anderton, and M. L. Kraft, 2010, Discriminating and Imaging Different Phosphatidylcholine Species within Phase-Separated Model Membranes by Principal Component Analysis of TOF-Secondary Ion Mass Spectrometry Images: Analytical Chemistry, v. 82, p. 10006-10014.

Chapitre 4. Etude du stockage des poudres de PPCN avec et sans lactose : Conséquences sur la couleur, la solubilité et les modifications chimiques

Les chapitres précédents ont permis de quantifier l'évolution des propriétés fonctionnelles d'une poudre de PPCN lors d'un stockage (chapitre 1) et de démontrer l'évolution d'une couche mince à la surface des grains de poudres, de plus en plus compacte et uniforme au cours du stockage (chapitre 3). Ces chapitres ont suggéré que cette couche mince ralentissait la fragmentation des particules primaires de poudres immergées au sein du solvant, retardait la libération des micelles individuelles, induisant une augmentation du temps de réhydratation.

Ce chapitre 4 a pour volonté d'étendre les connaissances sur les mécanismes à l'échelle moléculaires à l'origine de la déstabilisation des protéines et d'alimenter la réflexion sur les mécanismes d'insolubilisation d'une poudre de PPCN lors d'un stockage. Il est un complément au chapitre 2 de la partie « résultats » (évolution des structures secondaires) car il creuse notamment le rôle du lactose dans les mécanismes de déstabilisation.

De précédents travaux ont démontré la présence de complexes à hauts poids moléculaires, formés par des liaisons non covalentes et des ponts disulfures, lors du stockage de poudres. L'augmentation de complexes à hauts poids moléculaires au cours du stockage a été positivement corrélée à la perte de solubilité.

Il a également été suggéré dans ces travaux que la lactosylation de la protéine pouvait être une modification chimique majeure responsable de l'agrégation des protéines via la formation de produits de réaction de Maillard, conduisant à un brunissement de la poudre. Cependant, l'implication du lactose dans la formation d'insolubles et son rôle dans les mécanismes sous-jacents est loin d'être pleinement compris. De plus, ces

études pionnières ne proposent pas un aperçu de l'ensemble des réactions chimiques éventuellement impliquées dans la déstabilisation des protéines.

L'objectif de ce chapitre est de préciser le rôle du lactose dans les modifications chimiques des poudres de PPCN pendant le vieillissement et d'établir son implication dans la formation de liaisons entre protéines et la perte de solubilité observée lors du stockage.

Pour cela, deux poudres de PPCN ayant une teneur en lactose différente ont été stockées à 4 et 60 °C.

L'évolution des propriétés fonctionnelles lors du stockage des poudres avec ou sans lactose, a été caractérisée par l'indice de brunissement (IB) et la solubilité. L'influence du lactose dans la formation de complexes à haut poids moléculaires et les types de liaisons chimiques (covalentes ou non covalentes) reliant les protéines ont été étudiées par électrophorèse sous conditions réductrices et non réductrices.

Enfin, pour évaluer la contribution du lactose dans la déstabilisation des protéines, les modifications chimiques comme la lactosylation, déphosphorylation, la désamidation et l'acétylation ont été évaluées pour les deux types de poudres par spectrométrie de masse couplée à une méthode de quantification.

Les évolutions des propriétés fonctionnelle (solubilité) et sensorielle (couleur-IB) mesurées au cours du stockage ont démontré sans ambiguïté que :

i) le lactose était responsable du brunissement de la poudre lors du stockage.

ii) l'absence de lactose n'empêchait pas de développement d'insolubles.

A l'échelle moléculaire, il a été démontré que l'évolution des modifications chimiques était différente en fonction de la présence de lactose. En effet, en présence

de lactose, une augmentation de la lactosylation, de la désamidation et de l'acétylation des protéines a été quantifiée.

En revanche, seule une déphosphorylation et une augmentation importante de la désamidation des protéines ont été quantifiées lors du stockage de poudre délactosée. La diminution du pH en présence de lactose, due à la RM, pourrait être à l'origine des différentes modifications chimiques observées.

L'analyse de ces résultats nous amène à suggérer que la lactosylation, initiant la RM, peut déstabiliser les protéines par l'augmentation de l'acétylation mais n'est pas l'unique voie impliquée. En effet, d'autres modifications chimiques se mettent en place en absence de lactose et peuvent favoriser la réticulation entre les caséines pendant le stockage d'une poudre de PPCN.

Les techniques électrophorèses SDS Page n'ont pas permis de détecter la formation de complexes à hauts poids moléculaires via des liaisons covalentes au cours du stockage. Ainsi aucun lien entre l'analyse des liaisons covalentes des caséines et la perte de solubilité n'a pu être clairement établi, et ce quelle que soit la teneur en lactose. Le fait que ces liaisons puissent être difficilement détectables par électrophorèse nous amène à penser que les liaisons créées entre les protéines, responsables de l'insolubilité ne concernent qu'une faible fraction des caséines. Compte tenu des résultats exposés au chapitre 3 de la partie « résultats », on est en droit de supposer que cette faible fraction implique exclusivement les caséines réparties en couche mince à la surface des grains.

Storage of micellar casein powders with and without lactose: Consequences on color, solubility and chemical modifications

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Abstract

During storage, a series of changes occur for dairy powders such as protein lactosylation, formation of Maillard reaction products (MRP) leading to powder browning and increase of insoluble matter. The kinetics of protein lactosylation and MRP formation are influenced by lactose content of the dairy powder. However, influence of lactose in the formation of insoluble and its role in the underlying mechanisms is far from being fully understood.

This paper aims to investigate the role of lactose in the formation of insoluble matter in a more comprehensive way than the existing literature. For that, two casein powders with different lactose content, standard micellar casein (MC) powder (MC1) and delactosed MC powder (MC2), were prepared and stored under controlled conditions for different periods of time.

Powder browning index measurements and solubility tests on reconstituted powders were performed to study the evolution of the functional properties of MC powder during aging, with or without lactose. Proteomic approaches (one-dimensional electrophoresis and Liquid Chromatography Mass Spectrometry (LCMS) and innovative Label-Free Quantification methods were used to track and quantify the chemical modifications occurring during the storage of the powders and to better understand lactose-content-dependent protein destabilization.

The evolution of the functional properties indicated that reducing the amount of lactose limited the browning of MC powders but had no effect on the loss of solubility of proteins after storage. It is proved that lactose is not at the origin of the formation of insoluble matter. Electrophoresis analysis did not reveal any link between the formations of covalent bonds between caseins and loss in solubility, regardless of the lactose content. However, LCMS analyses have shown that different levels of chemical modifications occur during the MC powder storage, depending on the presence of lactose. An increase of protein lactosylation and acetylation was observed for the powder with higher lactose content while an increase of protein deamidation and dephosphorylation was observed for that containing lower lactose. The decrease of pH in the presence of lactose, due to Maillard reaction (MR), may explain the difference in the chemical modifications of the two powders. In view of the present results, it can be suggested that lactosylation in early MR is not responsible for the development of insolubility and cross-links between caseins during storage.

Key-words : Micellar casein; storage; lactose, functionality, chemical modifications

1. Introduction

During storage, milk powder behaves as a complex reaction system. A large number of chemical, physical and biochemical reactions can occur, especially under harsh conditions (e.g. long storage time, high temperature) and with increasing powder water activity (Fyfe et al., 2011; Guyomarc'h et al., 2000; Le et al., 2011c; Mimouni et al., 2010a; Thomas et al., 2004). These reactions have negative impacts on the functional properties (Nasser et al., 2017c) and nutritional quality (Haque et al., 2015) of the powder. Some changes, such as those in color, can be detected with naked eyes and browning index can be used as markers of

the powder quality (Le et al., 2011a; Nasser et al., 2017c). Other functional changes are more difficult to identify without reconstituting the powder in solution. This is for instance the case for the determination of dispersion and solubilisation properties of the milk protein powder (Anema et al., 2006). A fast and complete dispersion and solubilisation of the milk proteins is a prerequisite for the effective expression of other functionalities (gelling, emulsifying, etc.). Sadek et al., (2016) showed that a thin layer was formed on the particle surface in the early stage of drying. Mimouni et al., (2010a) also observed that this thin layer played a role in resisting the immediate erosion of the particle surface and retarding the release of individual micelles. This thin surface layer became more and more compact and uniform during storage. The reinforced intermicellar interactions and the delayed steps of rehydration (Mimouni et al., 2010a; Nasser et al., 2017a) were assumed to be responsible for the slow dissolution of stored milk powders. These changes are particularly marked during the storage of micellar casein (MC) powder, a high-protein dairy powder with a content of micellar caseins up to 90 % (Nelson and Barbano, 2005). Several studies were conducted to identify the underlying mechanisms at the molecular level for the loss of protein solubility of dairy powders, among which are micellar casein powders. Attempts for i) characterizing the protein cross-links formed during storage (Anema et al., 2006; Le et al., 2012) ii) identifying the chemical modifications that destabilize protein structure (Gazi and Huppertz, 2015; Holland et al., 2011; Le et al., 2011a; Le et al., 2011b; Le et al., 2012; Le et al., 2013) were carried out. Anema et al., (2006) and Le at al., (2012) demonstrated the formation of high-molecular-weight (HMW) complexes during the storage of high-protein dairy powders and UHT milk using two-dimensional gel electrophoresis *(*2-DE). Proteins aggregate through non-covalent and covalent interactions, the latter involve either disulfide bonds (sensitive to reducing agents) or covalent non-disulfide cross-links (resistant to reducing agents). Anema et al., (2006) and Le at al., (2012) established that HMW complexes were mainly stabilized by non-

covalent cross-links and covalent non-disulfide cross-links. Their presence is positively correlated to the loss of protein solubility.

It was suggested that milk protein lactosylation and dephosphorylation could be the major chemical modifications responsible for protein aggregation (Le et al., 2011b; Le et al., 2013). The first major modification proposed is protein lactosylation. After the reaction between lactose and a lysine residue of the protein (protein lactosylation) a complex series of reactions (van Boekel, 1998) leading to Maillard Reaction Products (MRP) were initiated (Nahid and Niaz, 2015). Some led to the browning of the powder and others are suspected to be responsible for the formation of HMW complexes and the loss of solubility of the proteins (Le et al., 2011a).

The second major chemical modification is protein dephosphorylation. It consists in the β-elimination of cysteine or phosphoserine, forming dehydroalanine (DHA) that can react with nucleophilic amino acids (e.g., lysine, cysteine, histidine) in the same or adjacent proteins, producing lysinoalanine (LAL), lanthionine or histidinoalanine cross-links (Pellegrino et al., 2011).

Unfortunately, these pioneer studies did not give an overview on the set of chemical reactions possibly involved in protein destabilization. For instance, protein acetylation was rarely studied during the storage of dairy products. Protein acetylation is responsible for the neutralization of the positive charges and the modification of the size of lysine side chain, resulting in changes of conformation and interactions between proteins (Drazic et al., 2016). Although protein deamidation during storage has been reported (Holland et al., 2011; Le et al., 2012), further study on this aspect is still needed. The deamidation consists in the cyclisation of an asparagine residue into a succinimide group in a flexible zone of the protein structure. This reaction is favoured at pH > 6 and requires preferentially a glycine residue immediately after the asparagine residue in the polypeptide chain.

The state of the art analysis raised the question whether lactosylation is the main route leading to inter-protein cross-links and subsequent formation of insoluble matter in stored protein powders. An increase of the protein lactosylation during the storage of milk products has been definitively demonstrated by Anema et al., (2006) and Le et al., (2011a; 2011b; 2012; 2013). Although these authors suggested that MR was at the source of protein cross-linking and an alteration of the solubility of high-protein dairy powders stored at elevated temperature (up to 50 °C) and/or at elevated relative humidity (44-86 %), it has not been formerly demonstrated that the loss of protein solubility during storage could not occur in the absence of lactose. This ambiguity was recently underlined by Gazi and Huppertz (2015) who mentioned that since lactose content in high-protein dairy product is only between 1 and 3 %, it could be speculative that it was responsible for the initiation of protein cross linking. In addition, it was demonstrated that kappa-casein (κ-csn) was very weakly lactosylated during aging (Anema et al., 2006), whereas it is generally admitted that κ-casein is the most involved in protein cross linking reaction. It appears clearly that the relationship between lactosylation and the loss of protein solubility has to be clarified.

To sum up, the role of lactose in the chemical changes of milk protein powders during aging and its link with the loss of protein solubility need to be further studied. To fill this gap, two casein powders with different lactose contents: standard micellar Casein (MC) powder (MC1) and delactosed MC powder (MC2) were prepared and stored under controlled conditions at 60 °C for different periods.

The functional properties of MC powders with or without lactose during aging were characterized by browning index and protein solubility test (after powder reconstitution in water). The influence of lactose in the formation of HMW complexes and the types of inter-protein chemical bonds (covalent or non-covalent) were studied by a combination of reducing

(R) / non- reducing (NR) one dimension-electrophoresis. Finally, to evaluate the contribution of lactose in the chemical modifications occurring during storage, protein lactosylation, dephosphorylation, deamidation and acetylation of the two powders have been compared with the aid of proteomic techniques (one-dimensional electrophoresis and Liquid Chromatography Mass Spectrometry (LCMS)) coupled to Label-Free Quantification method.

2. Materials & Methods

2.1 Dairy powder manufacture and physicochemical analysis

MC concentrate (Promilk 872 B1) from Ingredia was obtained from microfiltration of a skimmed milk followed by a concentration step by ultrafiltration (UF).

Ultrafiltration and diafiltration

The UF process allowed to remove small components such as minerals, lactose and non-protein nitrogen compounds from skimmed milk while retaining larger components such as the micellar caseins (Singh, 2007). The process was described by Goudedranche et al., (1980). A pilot UF installation (Carbosep TECH-SEP, Rhône-Poulenc, France) equipped with dual ceramic membranes (Tami Industries, Nyons, France) with a total membrane surface area of 12.6 m^2 and a molecular weight cut-off of 8 kDa was used. The flow rate of the retentate was set at 200 $L.h^{-1}$. MC was concentrated to 75% of its initial volume in the UF step.

The UF retentate was separated into two batches of equivalent volume. One batch was diafiltered with osmosis water in order to reduce the amount of residual lactose. The osmosis water was added in continue and the rate of diafiltration was equal to 3. Then, both retentates were spray dried.

Spray drying

The retentates were spray-dried according to the protocol described by Pierre et al., (1992) and Schuck et al., (1994a). A pilot plant spray dryer (GEA Niro A/S, Søeborg, Denmark) type Mobile Minor (UMR STLO, Rennes, France) was used. Inlet and outlet temperatures were set at 250 °C +/- 2 °C and 80 °C +/- 2 °C, respectively. The retentates were injected in the spray drier at 30 $mL.min^{-1}$ using a peristatic pump (Watson Marlow 520 S). The UF retentate batch spray dried right after ultrafiltration gave the standard MC powder (MC1). The UF retentate batch delactosed by diafiltration before spray drying gave a delactosed MC powder (MC2).

Physicochemical analysis of retentates and dairy powders

The powders were analysed for their composition, water activity, and powder particle size. Total nitrogen, non-protein nitrogen and non-casein nitrogen (expressed as protein equivalents) were quantified by Kjeldhal method. The ash content were determined according to standard 27/1964 IDF. The total dry matter was determined as described by (Schuck et al., 2012) and 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 content 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, with 5 mM H_2SO_4 buffer as elution flow at a flow rate of 0.4 $mL.min^{-1}$. Free lactose content was detected using a differential refractometer (model RI 2031 plus, Jasco). The cations and the anions were quantified by 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). The characteristics of the powders are reported in **Table 1.**

Table 1. (A) Physicochemical properties and (B) Mineral composition of MC powders

A.	Total nitrogen	Non Protein Nitrogen	Non Casein Nitrogen	Total dry matter	Ash	Particle size (Dv50)	Water activity	pH
	%	%	%	%	%	µm	a_w	
MC1	80.1	0.5	7.3	94.1	7.8	30.3	0.25	6.90
MC2	83.6	0.8	9.5	95.5	8.3	26.8	0.27	6.97

B.	Lactose	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	PO_4^{3-}	Citrate
	%	%	%	%	%	%	%	%
MC1	2.620	0.083	0.294	2.316	0.097	0.864	2.941	0.382
MC2	<10ppm	0.005	0.049	2.654	0.100	0.111	2.672	0.161

2.2. Storage of MC powders

After manufacture, MC powders were packaged in individual cans of 50g and stored either at a controlled temperature of 4 °C (further named as “reference powder”) or at 60 °C for different periods up to 3 months. This latter temperature condition was selected because it could actually be encountered during the shipment of milk derivative powders toward tropical countries.

2.3. Characterization of changes occurring during powder storage

One-dimensional electrophoresis and gel scanning

The formation of covalent interactions (disulfide bonds and non-disulfide bonds) between proteins during the storage of MC powders were monitored by Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (Tricine-SDS–PAGE) under non-reducing (NR) and reducing (R) conditions, as performed previously by Anema, (2000). Under NR conditions, the disulfide-linked protein complex remained intact whereas they were dissociated under R conditions.

Tricine-SDS-PAGE was performed at room temperature in a SE 600 Series vertical Slab Gel Unit (Hoefer Scientific instrument, San Francisco, US). Sample and gel preparation were based on the procedure of Pardo and Natalucci, (2002). SDS PAGE was performed at 30 V until the samples had completely left the stacking gel and then the voltage was increased to 90 V until the tracking dye reached 4/5 of the gel. Immediately after the protein migration, the gel was fixed, stained and destained, also according to Pardo and Natalucci, (2002).

The image of the gel was captured with a PC scanner and saved in JPEG format. The image was processed using gel analysis module of ImageJ software to obtain densitograms. Integrated intensities of the protein bands were determined using ImageJ. The relative quantities of the proteins were estimated by measuring the intensity of each protein band and expressed as a percentage of the total band intensity. All Tricine-SDS-PAGE analysis was carried out in triplicate.

Mass spectrometry

Nano HPLC coupled to Q Exactive plus Mass Spectrometry was used to identify and to get relative quantification of some selected chemical modifications occurring during the storage of MC powders.

Separation/identification/quantification of peptides

The quantity of proteins was measured by BCAssay method. 100 µg of proteins of the MC powders were processed in the 10 kDa filtration units (Millipore) using eFASP protocol (enhanced Filter Aided Samples Preparation) (Erde et al., 2014). Trypsin was used for the digestion of proteins with a ratio of 1/50 (trypsin/protein). Peptides were extracted and the solvent was evaporated. Samples were diluted in buffer A of nanoHPLC and analysed in triplicate.

The peptides mixtures were analysed using a nanoflow HPLC instrument (U3000 RSLC Thermo Fisher Scientific) coupled on-line to a quadrupole-Orbitrap mass spectrometer (Q Exactive^{TM} Plus, Thermo Scientific) with a nanoelectrospray ion source. 1 µL of peptide mixture (corresponding to 500 ng of proteins) was loaded onto the pre-concentration trap (Thermo Scientific, Acclaim PepMap100 C18, 5 µm, 300 µm i.d × 5 mm) using partial loop injection, for 5 min at a flow rate of 10 µL/min with buffer A (5% acetonitrile and 0.1% formic acid). Peptides were separated on analytical column (Acclaim PepMap100 C18, 3 µm, 75 mm i.d. × 500 mm) with a linear gradient of 5–50% buffer B (75% acetonitrile and 0.1% formic acid) at a 250 nL/min flow rate and a 45 °C controlled temperature. The total time for a LC MS/MS run was about 240 min long. MS data were acquired using a top-20 data-dependent method dynamically choosing the most abundant precursor ions from the survey scan for HCD fragmentation (Sun et al., 2013). Dynamic exclusion duration was 60 s. Isolation of precursors was performed with a 1.6 m/z window and MS/MS scans were acquired with a starting m/z of 80. Survey scans were acquired in the Orbitrap analyzer with m/z range of 350–1600 with 70,000 resolutions at m/z 200. Resolution for HCD spectra was set to 35,500 at m/z 200 and 28 eV normalized collision energy.

LFQ intensities treatment: Identification and quantification of chemical modifications

LFQ is based on the comparison of the different MS signal intensity to determine the relative abundance of peptides present in each sample.

The acquired raw files were analyzed with MaxQuant software (version 1.5.3.30) using the Andromeda search engine (Cox and Mann, 2008). Proteins were identified by searching MS and MS/MS data of peptides in the decoy UniProt-Bovine database (Version June 2016, 59,345 entries) supplemented with 262 frequently observed contaminants and forward/reverse sequences. The precursor mass and fragment mass were identified with an initial mass tolerance of 10 ppm and 20 ppm, respectively. The search included variable modifications, i.e. the oxidation of methionine and proline, deamidation of asparagine and glutamine, lactosylation of arginine and lysine, level of phosphorylation of serine residue. Minimal peptide length was set to six amino acids and a maximum of three mis-cleavages was allowed. The false discovery rate (FDR) was set to 0.05 for peptide and protein identifications. MS runs from casein powder were analyzed with the "match between runs" option and a 20 min retention time window. In the case of identified peptides that are all shared between two proteins, these were combined and reported as one protein group. Proteins matching to the reverse database were filtered out. For the proteins that were identified with single peptide, detailed information about the MS/MS spectrum, peptide sequence, precursor m/z is provided (**Table 2**). LFQ intensities for respective protein groups were uploaded in Perseus and analyzed (Tyanova et al., 2016). Briefly, contaminant proteins, resolved by post traductional modification-sites only and identified by reverse decoy were removed from the dataset. Raw LFQ intensities were logarithmized by Log2. At least three LFQ values per protein group needed to be present for the analysis. Non-quantified values were replaced by 0.

Statistical analysis

Significant evolutions were determined using ANalyse Of VAriance (ANOVA) test with Permutation-based FDR < 0.05 method to perform testing corrections.

Table 2. Information about chemical modifications

Modifications	Delta mass (Da)	Composition	Site of modifications
Deamidation	0.984016	H(-1) N(-1) O	N, Q
Acetylation	42.010565	H(2) C(2) O	K, N-term
Phosphorylation	79.966331	H O(3) P	S, T, Y
Lactosylation	324.105647	H(20) C(12) O(10)	K

Color measurement of MC powders and browning index determination

Browning of powders was monitored with browning index (BI), calculated from a formula combining the L*a*b* values (Oliveira et al., 2012) :

$$BI = \frac{[100(x - 0{,}31)]}{0{,}17} \quad (1)$$

$$\text{With } x = \frac{(a^* + 1{,}75 \times L^*)}{(5{,}645 \times L^* + a^* - 3.012 \times b^*)} \quad (2)$$

More details for browning index determination of stored powder can be found in Nasser et al,.(Nasser et al., 2017c).

Determination of solubility

The amount of soluble material (σ) in the MC powders was calculated using the following equation (Anema et al., 2006):

$$\sigma = \frac{\text{Weight of dry material}}{\text{weight of solution}} \times 100 \qquad (3)$$

It represents the solids in the ultracentrifugal supernatant, expressed as a percentage of total soluble solids in the whole solution. The σ values of aged powder samples were compared to that of the reference powder, and this σ(aged)/σ(reference) ratio in percentage was used to study the solubility evolution during ageing.

More details for solubility determination of stored powder can be found in Nasser et al., (2017c).

Statistical analysis

Student's *t*-tests with a 0.05 level of significance were used to measure the significance of the differences between aged samples and reference powder. As is widely known, the differences are statistically nonsignificant when $p > 0.05$ and statistically significant when $p < 0.05$

3. Results & discussion

3.1. Influence of lactose on the changes of powder functional properties

Browning index

BI evolution of the MC powders stored at 60 °C as a function of time is illustrated in **Figure 1**. The colour of MC2 did not evolve, even after 3 months of storage. In contrast, the browning index of MC1 increased rapidly during the first 20 days of storage, and then levelled off after 60 days of storage. Thus, the presence of lactose is essential for the browning of the stored powders. This result is coherent with the study of Al-Saadi et al., (2013), who compared the BI of 2 types of liquid milk, with and without lactose, subjected to a heat treatment (8 h 95 °C). This is also coherent with study of Norwood et al., (2017), who observed an increase in the BI of whey protein isolate powder with storage time, and concluded that the higher the lactose content, the greater the evolution of the BI. In spite of the differences in raw material and processing parameters, the presence of lactose is essential for browning to occur in both heat-treated liquid milk and stored powders. Our result also demonstrated that browning of the MC powders did occurred even in the presence of a very low lactose content (about 2.6 % w/w). This result is in agreement with the works of Anema et al.,(Anema et al., 2006) who suggested that the presence of small quantities of lactose was sufficient for browning to be observed during storage.

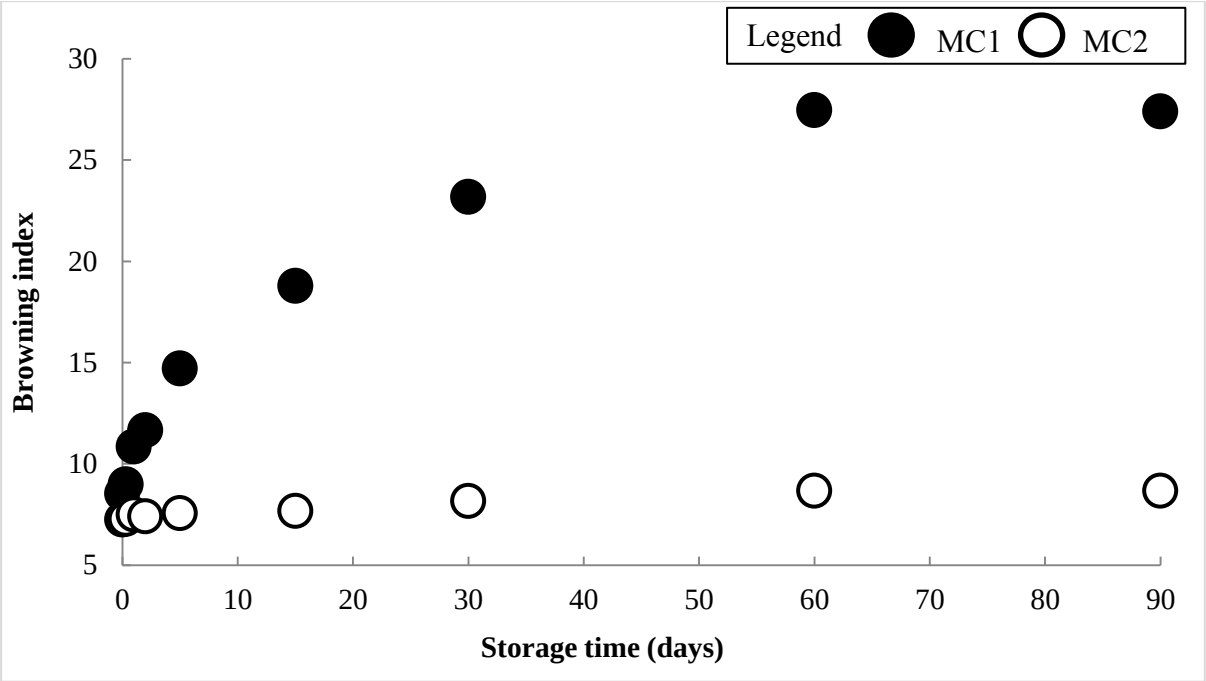

Figure 1. Browning index of MC powders as a function of storage time at 60 °C

Solubility

The protein solubility of MC powders stored at 60 °C is plotted as a function of storage time in **Figure 2**. A sharp decrease of protein solubility was observed in the first 8 hours of storage. The decrease then slowed down until reaching a plateau at 25% after 10 days of storage. The decrease of protein solubility with storage time was nearly identical for the two powders in spite of their different lactose content. This demonstrates that protein solubility loss of stored MC powders was not correlated to the amount of lactose, and hence neither to formation of MRP. After 60 days of storage at 60 °C, MC powders without lactose exhibited no browning but a protein solubility loss of 75 %, in the same range as the MC powder containing lactose. This observation is contrary to the hypothesis put forward by Le et al., (2011b) based on their study on protein solubility change of stored whole milk powder, skim milk powder (high lactose/protein ratio) and whey protein concentrate powder (low lactose/protein ratio). These authors suggested that the increase of MR indicators (furosine

and HMF contents, b* value) of stored powders could be indicative of MR product-mediated casein cross-linking being the source of protein solubility decrease.

As protein solubility loss during storage was not induced by the presence of lactose, the objective of the next chapter will be to evaluate influence of lactose on the protein cross-linking during storage.

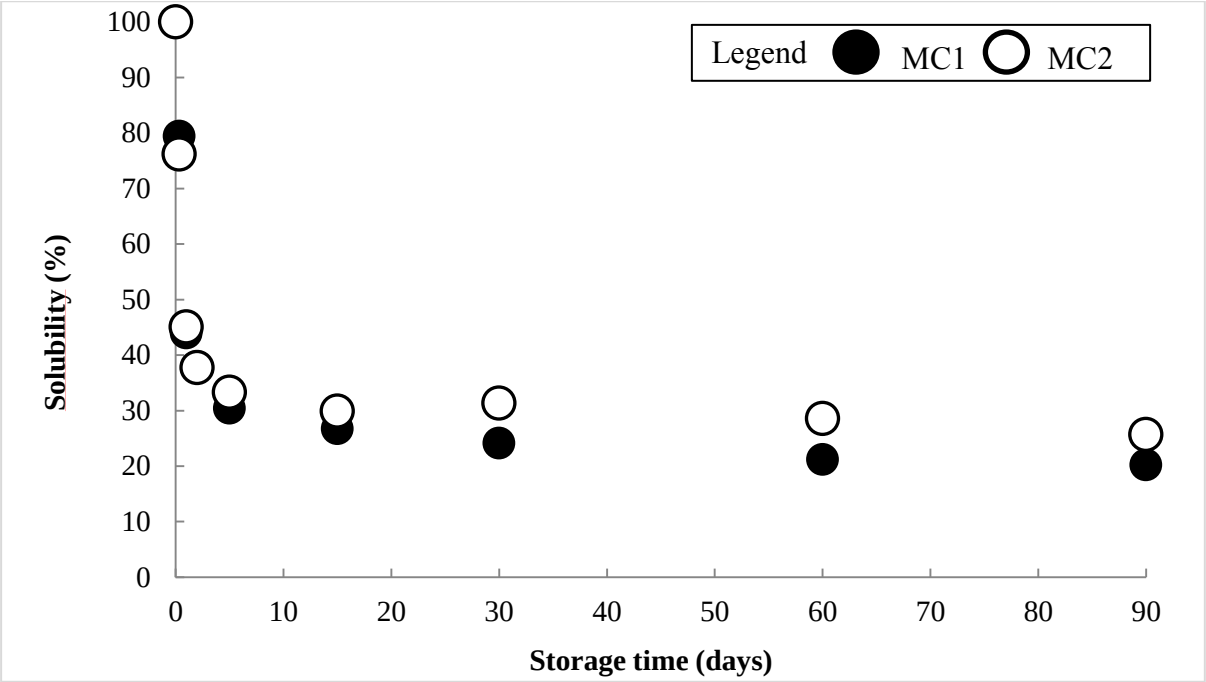

Figure 2. Change in protein solubility of MC powders stored at 60 °C as a function of storage time

3.2. Influence of lactose on the protein cross-linking during storage

Figure S1 shows the electrophoresis separation under NR **(Figure S1A)** and R conditions **(Figure S1B)** of the major proteins (α_{S1}-casein, α_{S1}-csn; α_{S2}-casein, α_{S2}-csn; β-casein, β-csn; and κ-casein, κ-csn) in MC1 and MC2 powder stored at 60 °C.

To progress on the investigation of mechanisms involved in protein solubility loss, the caseins participating in the formation of covalent HMW complexes during powder storage were quantified by SDS-Page analyses. Results are presented according to the lactose content in the MC powders and powder storage time (**Figure 3**). Interestingly, the content of the HMW

complex is already high prior to storage, which can be attributed to the spray drying step. Moreover, neither the decrease of the amount of individual caseins and nor the increase of the amount of HMW complexes was marked enough to be correlated to protein solubility loss during storage ($p>0.05$). Except for β-csn, the individual casein contents were higher under R conditions than under NR conditions (results not shown).

These results were unable to demonstrate any formation of cross-link between caseins even after 5 days of storage at 60 °C, which is known to happen under severe conditions of aging ($p>0.05$). Moreover, it indicates that the intermolecular disulfide bridges are either naturally present as a structure element of the micellar casein or formed during the skimmed milk processing into MC powders. Anyway, they are not formed during powder storage. It concerns all the caseins except β-csn.

Previous works have demonstrated the formation of a skin layer at the surface of casein powder particles during storage (Mimouni et al., 2010a; Nasser et al., 2017a). Burgain et al., (2016) recently confirmed this point as they observed a particle surface hardening phenomenon during storage. This skin was suggested to result from extensive interactions between proteins at the surface of the powder particles and the consequent formation of insoluble HMW complexes. The present result indicates that the intermolecular interactions established during the storage of the MC powders leading to insoluble HMW complexes are mainly non-covalent, regardless of the presence of lactose. It is in agreement with the studies of Anema et al., (Anema et al., 2006) who argued that HWM complexes formed during the aging of high-protein dairy powders are mainly constituted of non-covalent bonds. Another possibility could be that the intermolecular cross-linking concerned only a few percentages of the caseins, situated at the surface of the powder particle and the techniques used in this study is not sensitive enough to detect these evolutions.

The objective of the next chapter is to evaluate the contribution of lactose by tracking and quantifying the protein chemical modifications occurring during the storage of the MC powders.

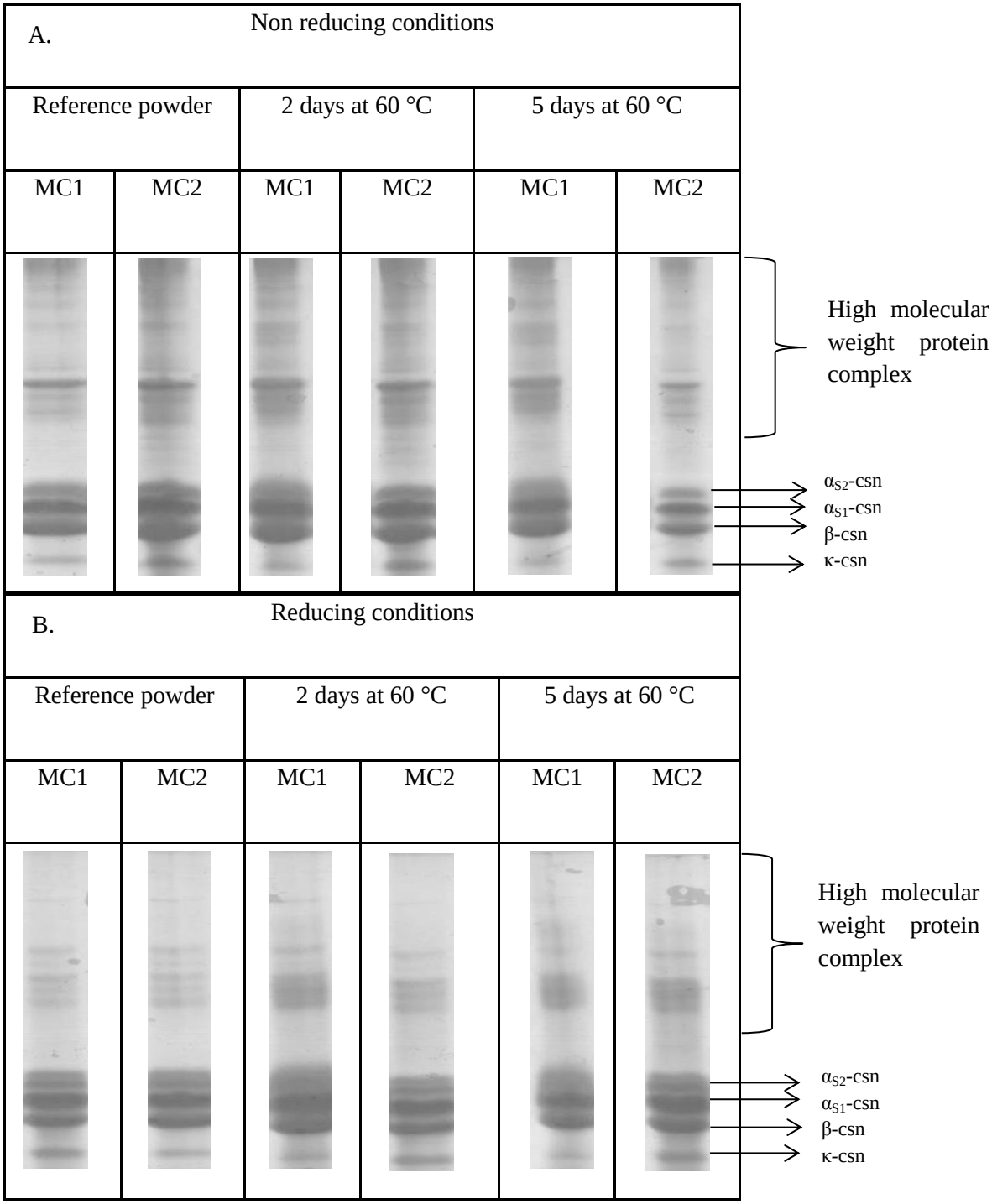

Figure S1. SDS-Page gel for MC powders at different time of storage under (A) non reducing conditions (B) reducing conditions and comparison with reference powders.

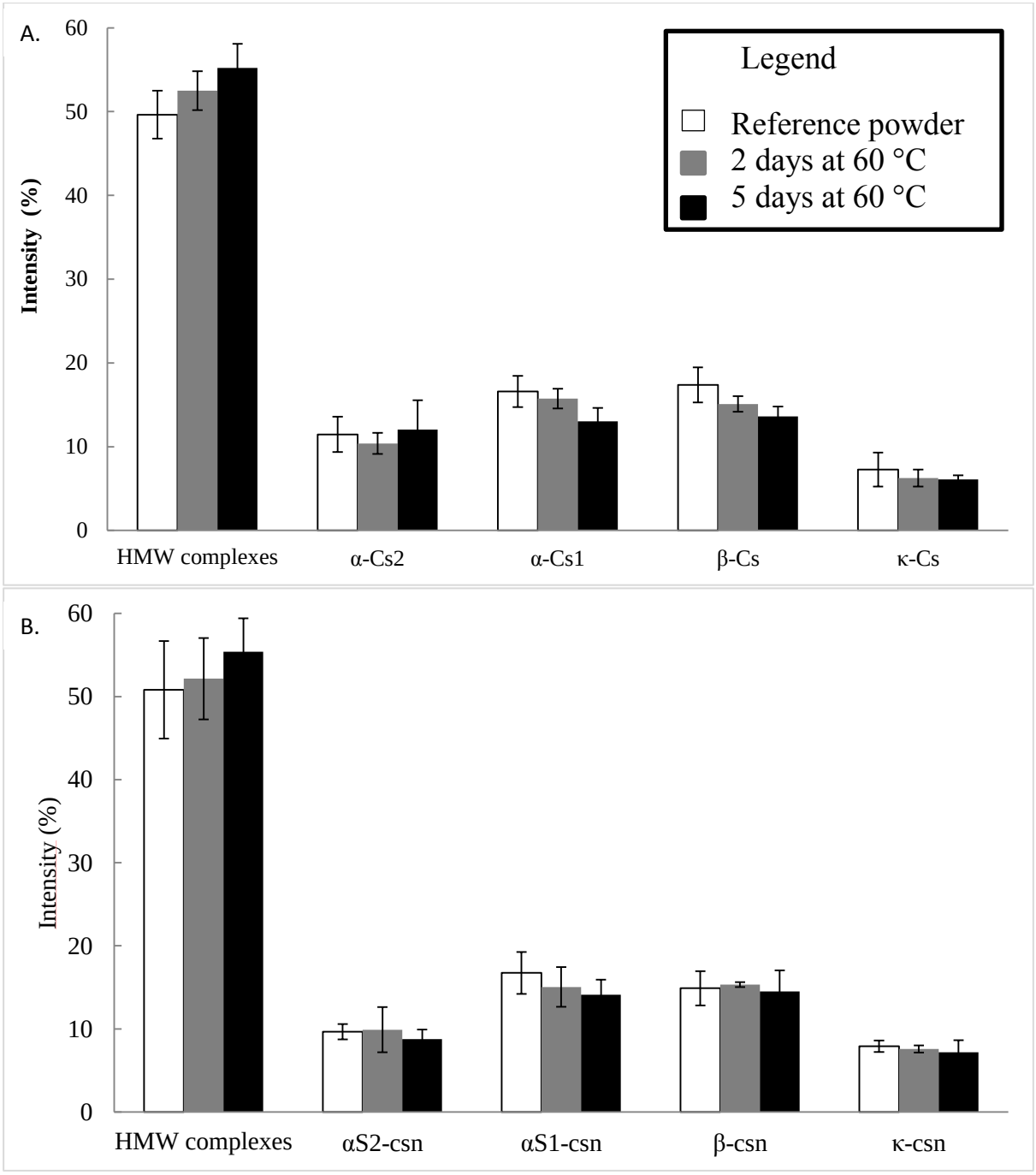

Figure 3. Evolution of intensities of HMW complexes and individual caseins for (A) MC1 and (B) MC2 during storage at 60 °C under non reducing conditions

3.3. Influence of lactose in protein chemical modifications

LFQ was used to compare the relative intensity of modified peptides for MC1 and MC2 powders. LFQ analyses were performed with the reference powder and aged powders (stored for 15 days and 60 days at 60 °C).

Study of lactosylation

Relative intensities of lactosylated peptides during the storage of MC1 and MC2 powders are represented in **Figure 4**. As expected, the intensity of lactosylated peptides is lower for MC2 than for MC1 powders. This difference is already marked for the fresh powders (prior to storage). As the two powders were prepared from the same UF retentate, the difference of lactosylation rate between the fresh powders was assigned to the spray-drying before storage, in accordance with Fox et al,.(2015).

The evolutions in lacosylation protein rate during storage at 60 °C were at the limit of being significant. Nevertheless, the trends observed were discussed. The intensity related to protein lactosylation decreased during the storage of MC2 powder (Figure 4). As MC2 powder was free of lactose (<10 ppm), proteins could not be further lactosylated. The decrease of the level of protein lactosylation of MC2 powder can be explained by a series of degradation reactions involving the bound lactose molecules (Tamine, 2009). Such degradations usually lead to various MRP, among which were small acids (formic acid, acetic acid), and other colored MRP (McSweeney and O'Mahony, 2016). The amount of products formed during the storage of MC2 was probably too small to bring about noticeable changes in BI or in pH of the reconstituted solution. (The latter maintained at 6.97 after 60 days of storage).

For MC1 powder, the relative intensity of lactosylation slightly increased after 15 days and 60 days of storage (Figure 4). In the first days of storage, the increase of protein lactosylation can be explained by the early stages of MR. Mainly ε-amino groups of lysine and the N-term amino acids of the protein react with lactose molecules to form a Schiff base and then a more

stable Amadori product, named lactulosyllysine when lysine and lactose are involved (van Boekel, 1998). Then, the Amadori product decomposed through various reactional routes leading to the MRP, as mentioned earlier for the storage of MC2 powder. For MC1, formation of MRP was expected to occur as well during the storage. Indeed, the browning of the powder intensified and the pH of the reconstituted water solution of MC1 decreased to 6.53 after 60 days' stortage at 60 °C. However, no significant decrease in the intensity of lactosylation was observed during the storage of MC1. It is assumed that the decrease is compensated by the lactosylation of the proteins by consuming the free lactose in the powder (2.6%).

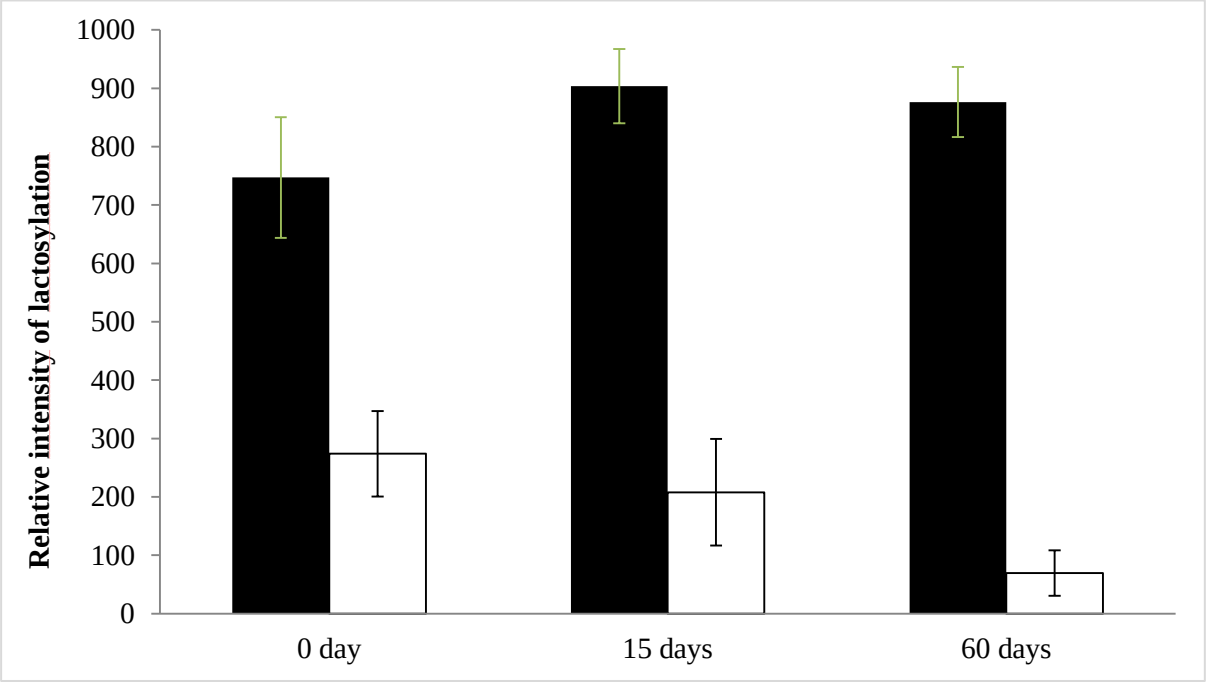

Figure 4. Evolution of the protein lactosylation relative intensity for ■ MC1 and □ MC2 powders during storage at 60°C

Study of phosphorylation

The relative intensity of the phosphorylated serine was tracked during the storage of MC1 and MC2 powder (**Figure 5).** The protein phosphorylation level in MC1 powder was not affected by the storage conditions whereas a significant decrease was observed for MC2 powder after

60 days of storage ($p<0.05$). The chemical release of the phosphate moiety from serine residues is pH dependent. The reaction is favoured at alkaline pH. The pH decrease during the storage of MC1 could retard the degradation of the phosphorylated serine into DHA. The DHA are extremely reactive and was reported to be at the source of LAL cross-links. Therefore, the formation of LAL would be probably more prevalent during the storage of MC2 powder than of MC1 powder.

Our results complete the works of Holland et al.,(Holland et al., 2011) Le et al,.(Le et al., 2012; Le et al., 2013) who looked for cross-linked phosphorylated peptides formed during milk product storage by 2-DE/proteomics approaches. Unfortunately, detection of phosphorylated peptide complexes required efficient and specific materials and methods and do not allow unambiguous interpretations (Holland et al., 2011; Steen et al., 2006). To avoid study on protein complexes, Le et al., (2013) worked on isolated α_{S1}-csn. Phosphorylated and non-phosphorylated samples of α_{S1}-csn mixed with lactose at different concentrations were compared during heat treatment. They demonstrated that the amount of cross-linked proteins was independent of the dephosphorylation level of α_{S1}-csn. Dephosphorylation observed on MC2 can destabilize the protein without inducing insolubilty.

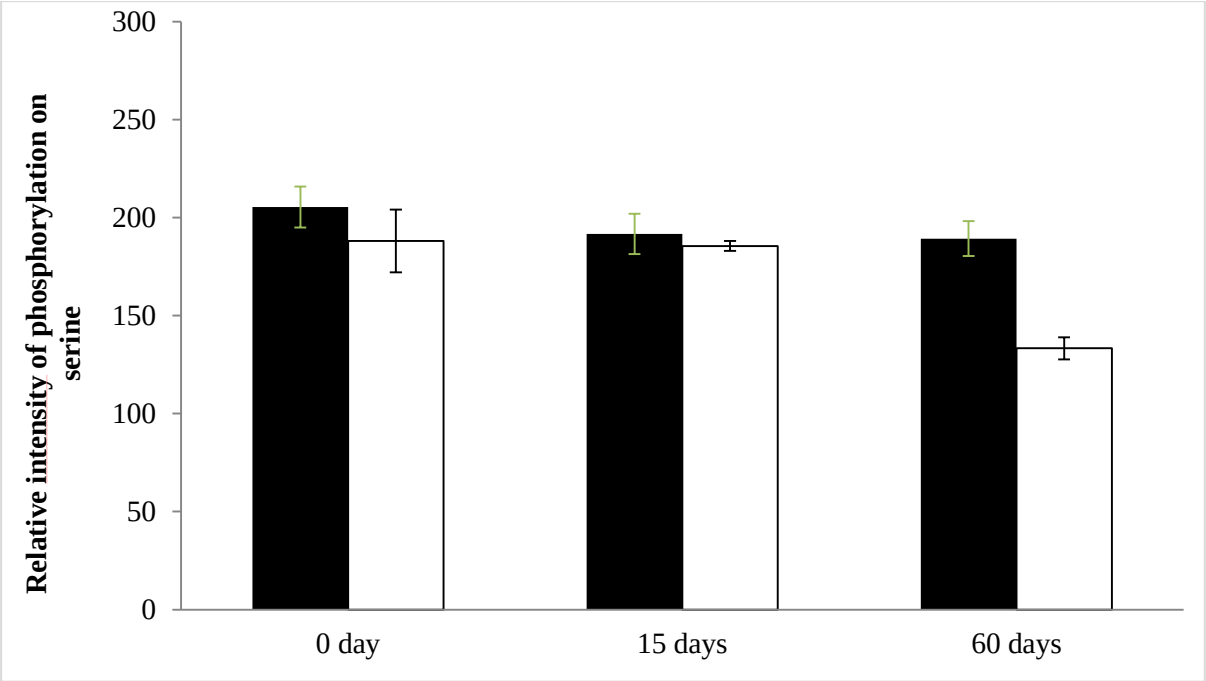

Figure 5. Evolution of the protein phosphorylation relative intensity for ■ MC1 and □ MC2 powders during storage at 60°C

Study of acetylation

The relative intensities of lysine acetylation for MC1 and of MC2 during storage are presented in **Figure 6.** An increase of the amount of acetylated lysine was observed for MC1 after 60 days of storage ($p<0.05$), while no evolution of acetylation level is noted for MC2 under the the same storage condition ($p>0.05$).

The increase of the amount of acetylated lysine during the storage of protein powders was barely investigated. The acetylation of lysine is the addition of CH_3-C=O group to lysine (Drazic et al., 2016). More presumably in the present case, it consists in the reaction between an acetic acid molecule with the ε-amine of lysine. The abundance of acetic acid is expected to be higher in MC1 since it is one of the reaction products of MR. Lakkis and Villota.,(1992) demonstrated that, acylation (which means adding R-C=O group) enhanced the denaturation and improved the surface hydrophobicity of proteins. According to Drazic et al.,(2016), this

chemical modification has a more drastic effect on multicomponent proteins, possibly by perturbing their protein-protein interaction mode. N-terminal acetylation of proteins can affect protein stability but its mechanism and resulting consequences is still not very clear.

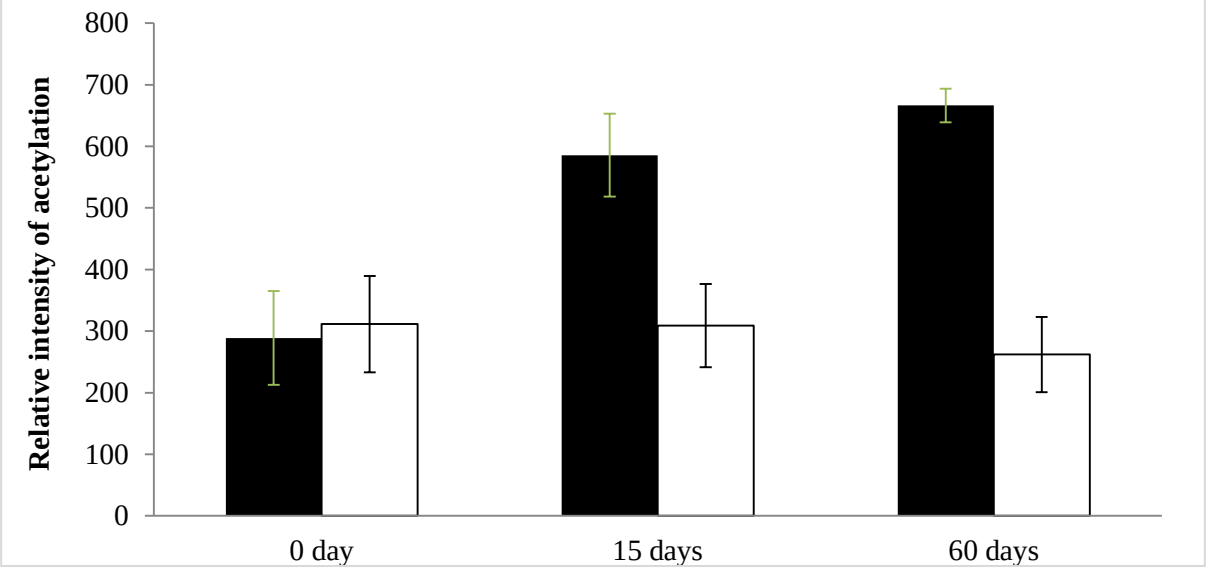

Figure 6. Evolution of the protein acetylation relative intensity for ■ MC1 and □ MC2 powders during storage at 60°C

Study of deamidation

The relative intensities of protein deamidation during the storage of MC1 and MC2 are presented in **Figure 7**. An increase in protein deamidation was observed for MC1 and MC2 powder after 60 days of storage ($p<0.05$), but this modification was less important in the presence of lactose. Protein deamidation is accelerated at alkaline pH conditions through a proposed cyclic imide intermediate (Baertschi et al., 2011). The decrease of pH during the storage of MC1 may explain the limited protein deamidation during drying and storage.

Holland et al.,(Holland et al., 2011) and Le et al,.(Le et al., 2012) have also demonstrated the occurrence of protein deamidation during the storage of milk product without really correlating it to the formation of cross-links or the loss of protein solubility. Since this

reaction is the common degradation pathway of protein (Baertschi et al., 2011), the presence of lactose can hence limit the reaction of deamidation and prevent the destabilization of the proteins during powder storage.

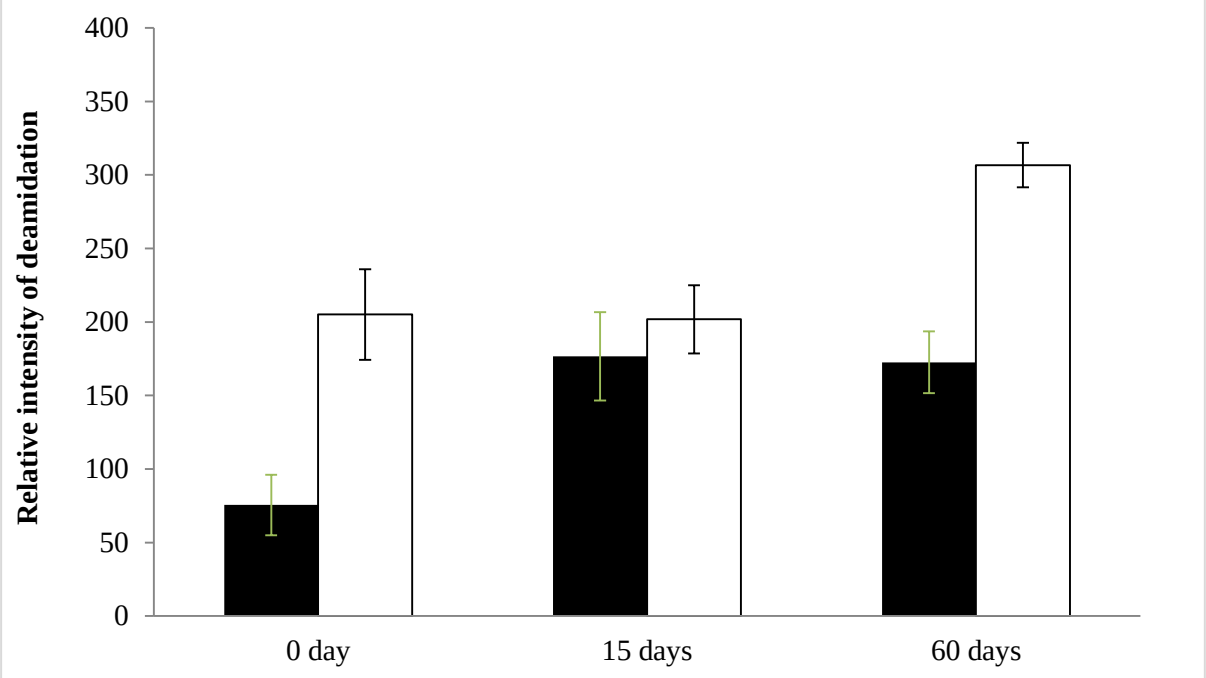

Figure 7. Evolution of the protein deamidation relative intensity for ■ MC1 and □ MC2 powders during storage at 60°C

This study has demonstrated that the absence of lactose could prevent powder browning but has no effect on the loss of protein solubility. The presence of small quantities of lactose is sufficient to trigger MR, involving interaction between lactose and lysine and the degradation of lactosylated residues into a variety of MRP, some of which responsible for powder colour change and pH decrease during storage. Lactose and MR are not main contributors to the loss of protein solubility during powder storage.

The present results suggest that protein cross-linking are mainly non-covalent and/or concern only a small fraction of the caseins, since they were not detected by electrophoresis. If these

cross-linked caseins formed a thin layer situated exclusively at the surface of the powder particles, solubility property changes observed during powder storage could be easily explained: The thin layer of cross-linked caseins would delay the penetration of water into the powder particle and the release of the micellar caseins located in the core of the powder particles into the solvent.

To the best of our knowledge, no precedential study has ever been carried out on the influence of lactose on the aging of milk powders and protein destabilization. In view of the present results it can be suggested that lactosylation in early MR is not responsible for the development of insolubility and cross-links between caseins during storage.

The extent of protein chemical modifications occurring during MC powder storage depended on powder lactose content, more specifically, on the lactose induced MRP and the decrease of pH during the storage. In presence of lactose, an increase of lactosylation and acetylation were noted. While in delactosed powder, protein deamidation and protein dephosphorylation were more dominant. To conclude, lactose is not directly responsible for the formation of insoluble matter. Whereas some MRP can destabilize protein via the increase of acetylation, the absence of lactose does not prevent protein destabilization and the formation of insoluble matter after powder storage. Reducing the amount of lactose in MC powders, for instance by introducing a diafiltration step, will limit powder browning but not its solubility loss.

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Bibliography

Al-Saadi, J. M. S., A. M. Easa, and H. C. Deeth, 2013, Effect of lactose on cross-linking of milk proteins during heat treatments: International Journal of Dairy Technology, v. 66, p. 1-6.

Anema, S. G., 2000, Effect of milk concentration on the irreversible thermal denaturation and disulfide aggregation of beta-lactoglobulin: Journal of Agricultural and Food Chemistry, v. 48, p. 4168-4175.

Anema, S. G., D. N. Pinder, R. J. Hunter, and Y. Hemar, 2006, Effects of storage temperature on the solubility of milk protein concentrate (MPC85): Food Hydrocolloids, v. 20, p. 386-393.

Baertschi, S., K. Alsante, and A. Reed, 2011, Pharmaceutical Stress Testing: Predicting Drug Degradation, 2nd Edition: Pharmaceutical Stress Testing: Predicting Drug Degradation, 2nd Edition, v. 210, p. 1-612.

Burgain, J., J. Scher, J. Petit, G. Francius, and C. Gaiani, 2016, Links between particle surface hardening and rehydration impairment during micellar casein powder storage: Food hydrocolloids, v. 61, p. 277-285.

Cox, J., and M. Mann, 2008, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification: Nature Biotechnology, v. 26, p. 1367-1372.

Drazic, A., L. M. Myklebust, R. Ree, and T. Arnesen, 2016, The world of protein acetylation: Biochimica Et Biophysica Acta-Proteins and Proteomics, v. 1864, p. 1372-1401.

Erde, J., R. R. O. Loo, and J. A. Loo, 2014, Enhanced FASP (eFASP) to Increase Proteome Coverage and Sample Recovery for Quantitative Proteomic Experiments: Journal of Proteome Research, v. 13, p. 1885-1895.

Fox, P., T. Uniacke-Lowe, P. L. H. McSweeney, and O. M. J.A., 2015, Heat-Induced Changes in Milk, Dairy chemistry and biochemistry, p. 345-375.

Fyfe, K. N., O. Kravchuk, T. Le, H. C. Deeth, A. V. Nguyen, and B. Bhandari, 2011, Storage induced changes to high protein powders: influence on surface properties and solubility: Journal of the Science of Food and Agriculture, v. 91, p. 2566-2575.

Gazi, I., and T. Huppertz, 2015, Influence of protein content and storage conditions on the solubility of caseins and whey proteins in milk protein concentrates: International Dairy Journal, v. 46, p. 22-30.

Goudedranche, H., J. L. Maubois, P. Ducruet, and M. Mahaut, 1980, Utilization of the new mineral UF membranes for making semi-hard cheeses: Desalination, v. 35, p. 243-258.

Guyomarc'h, F., F. Warin, D. D. Muir, and J. Leaver, 2000, Lactosylation of milk proteins during the manufacture and storage of skim milk powders: International Dairy Journal, v. 10, p. 863-872.

Haque, E., B. R. Bhandari, M. J. Gidley, H. C. Deeth, and A. K. Whittaker, 2015, Change in molecular structure and dynamics of protein in milk protein concentrate powder upon ageing by solid-state carbon NMR: Food Hydrocolloids, v. 44, p. 66-70.

Holland, J. W., R. Gupta, H. C. Deeth, and P. F. Alewood, 2011, Proteomic Analysis of Temperature-Dependent Changes in Stored UHT Milk: Journal of Agricultural and Food Chemistry, v. 59, p. 1837-1846.

Lakkis, J., and R. Villota, 1992, Effect of acylation on substructural properties of proteins - A study using fluorescence and circular-dichroism: Journal of Agricultural and Food Chemistry, v. 40, p. 553-560.

Le, T. T., B. Bhandari, and H. C. Deeth, 2011a, Chemical and Physical Changes in Milk Protein Concentrate (MPC80) Powder during Storage: Journal of Agricultural and Food Chemistry, v. 59, p. 5465-5473.

Le, T. T., B. Bhandari, J. W. Holland, and H. C. Deeth, 2011b, Maillard Reaction and Protein Cross-Linking in Relation to the Solubility of Milk Powders: Journal of Agricultural and Food Chemistry, v. 59, p. 12473-12479.

Le, T. T., B. Bhesh, and H. C. Deeth, 2011c, Chemical and physical changes in milk protein concentrate (MPC80) powder during storage: Journal of Agricultural and Food Chemistry, v. 59, p. 5465-5473.

Le, T. T., H. C. Deeth, B. Bhandari, P. F. Alewood, and J. W. Holland, 2012, A proteomic approach to detect lactosylation and other chemical changes in stored milk protein concentrate: Food Chemistry, v. 132, p. 655-662.

Le, T. T., J. W. Holland, B. Bhandari, P. F. Alewood, and H. C. Deeth, 2013, Direct evidence for the role of Maillard reaction products in protein cross-linking in milk powder during storage: International Dairy Journal, v. 31, p. 83-91.

McSweeney, P. L. H., and J. A. O'Mahony, 2016, Volume 1B: Proteins: Applied Aspects, Advanced Dairy Chemistry, 179-196 p.

Mimouni, A., H. C. Deeth, A. K. Whittaker, M. J. Gidley, and B. R. Bhandari, 2010, Investigation of the microstructure of milk protein concentrate powders during rehydration: Alterations during storage: Journal of Dairy Science, v. 93, p. 463-472.

Nahid, T., and M. Niaz, 2015, Food Processing and Maillard Reaction Products: Effect on Human Health and Nutrition: International Journal of Food Science, v. 2015, p. 6.

Nasser, S., R. Jeantet, P. De-Sa-Peixoto, G. Ronse, N. Nuns, F. Pourpoint, J. Burgain, C. Gaiani, A. Hédoux, and G. Delaplace, 2017a, Microstructure evolution of micellar casein powder upon ageing : Consequences on rehydration dynamics: In press, Journal of Food Engineering.

Nasser, S., A. Moreau, A. Hédoux, R. Jeantet, and G. Delaplace, 2017b, Influence of storage conditions on the functional properties of Micellar Casein powder: Submitted to LWT-Food Science and Technology.

Nelson, B. K., and D. M. Barbano, 2005, A microfiltration process to maximize removal of serum proteins from skim milk before cheese making: Journal of Dairy Science, v. 88, p. 1891-1900.

Norwood, E. A., S. Pezennec, J. Burgain, V. Briard-Bion, P. Schuck, T. Croguennec, R. Jeantet, and C. Le Floch-Fouere, 2017, Crucial role of remaining lactose in whey protein isolate powders during storage: Journal of Food Engineering, v. 195, p. 206-216.

Oliveira, F., M. J. Sousa-Gallagher, P. V. Mahajan, and J. A. Teixeira, 2012, Evaluation of MAP engineering design parameters on quality of fresh-sliced mushrooms: Journal of Food Engineering, v. 108, p. 507-514.

Pardo, M., and C. Natalucci, 2002, Electrophoretic analysis (Tricine-SDS-Page) of bovine caseins, Acta Farm. Bonaerense, p. 57-60.

Pellegrino, L., S. Cattaneo, and I. De Noni, 2011, Effects of processing on protein quality of milk and milk products, San Diego, USA, Encyclopedia of dairy sciences.

Pierre, A., J. Fauquant, Y. Legraet, M. Piot, and J. L. Maubois, 1992, Native micellar casein separation through cross flow membrane microfiltration: Lait, v. 72, p. 461-474.

Sadek, C., P. Schuck, Y. Fallourd, N. Pradeau, R. Jeantet, and C. Le Floch-Fouere, 2016, Buckling and collapse during drying of a single aqueous dispersion of casein micelle droplet: Food Hydrocolloids, v. 52, p. 161-166.

Schuck, M. Piot, S. Méjean, Y. Le Graet, J. Fauquant, G. Brulé, and J. L. Maubois, 1994, Déshydratation par atomisation de phosphocaséinate natif obtenu par microfiltration sur membrane: Le lait, v. 74, p. 375-388.

Schuck, P., R. Jeantet, and A. Dolivet, 2012, Analytical Methods for Food and Dairy Powders, John Wiley & Sons.

Singh, H., 2007, Interactions of milk proteins during the manufacture of milk powders: Lait, v. 87, p. 413-423.

Steen, H., J. A. Jebanathirajah, J. Rush, N. Morrice, and M. W. Kirschner, 2006, Phosphorylation analysis by mass spectrometry - Myths, facts, and the consequences for qualitative and quantitative measurements: Molecular & Cellular Proteomics, v. 5, p. 172-181.

Sun, L. L., G. J. Zhu, and N. J. Dovichi, 2013, Comparison of the LTQ-Orbitrap Velos and the Q-Exactive for proteomic analysis of 1-1000 ng RAW 264.7 cell lysate digests: Rapid Communications in Mass Spectrometry, v. 27, p. 157-162.

Tamine, A. Y., 2009, Milk Processing and Quality Management, *in* M. J. Lewis, and H. C. Deeth, eds., Heat Treatment of Milk, Dairy Science & Technology Consultant.

Thomas, M. E. C., J. Scher, S. Desobry-Banon, and S. Desobry, 2004, Milk powders ageing: Effect on physical and functional properties: Critical Reviews in Food Science and Nutrition, v. 44, p. 297-322.

Tyanova, S., R. Albrechtsen, P. Kronqvist, J. Cox, M. Mann, and T. Geiger, 2016, Proteomic maps of breast cancer subtypes: Nature Communications, v. 7.

van Boekel, M., 1998, Effect of heating on Maillard reactions in milk: Food Chemistry, v. 62, p. 403-414.

Partie 3. Conclusion et perspectives

Conclusion et perspectives

Cette thèse est une contribution pour mieux comprendre les évolutions de fonctions ou propriétés sensorielles des poudres de lait lors de leur stockage et les évolutions de structures associées.

Plus précisément, nous avons étudié le vieillissement au stockage de 3 poudres de lait concentrées en caséines.

La fonctionnalité étudiée a été l'aptitude à la réhydratation. La propriété sensorielle examinée a été la couleur. Les évolutions de structures explorées ont été : les structures secondaires, la composition en surface des grains, les modifications chimiques.

Notre démarche a consisté :

i) à imposer aux poudres de PPCN des variations contrôlées de température, à humidité relative constante, pour différentes durées de stockage.

ii) à caractériser pour ces poudres de PPCN plus ou moins vieillies les évolutions d'aptitude à la réhydratation. Pour cela, les poudres ont été réhydratées en solution aqueuse puis les grandeurs physiques (solubilité, temps de mouillabilité, de fragmentation, de dispersion de toutes les MC) ont été mesurées en utilisant différents appareils et prototypes disponibles aux laboratoires

iii) à identifier et quantifier les changements structuraux. L'étude des changements structuraux a été obtenue en utilisant différentes techniques analytiques (IRTF, DCRS, XPS, ToF-SIMS, MEB, Tricine SDS-Page, chromatographie liquide couplée à un spectromètre de masse (CLSM), RMN) à différentes échelles (mésocopique à moléculaire)

En ce qui concerne l'évolution des propriétés fonctionnelle et sensorielle au stockage, des augmentations du temps de mouillage, du temps de fragmentation, du

temps de réhydratation total, de l'indice de brunissement ont été observées. A contrario une diminution de la solubilité a été observée lors du stockage. Il a été démontré que l'altération des propriétés fonctionnelles et sensorielles augmentait avec la sévérité du stockage (température et durée).

La température de stockage apparaît donc comme le principal paramètre altérant les poudres de PPCN. Il paraît donc essentiel de stocker les poudres à une température inférieure à 20 °C, afin de minimiser l'altération des propriétés fonctionnelle des poudres.

Il a également été démontré qu'il était possible d'obtenir des courbes de vieillissement (courbes représentant l'évolution du temps de réhydratation total avec l'indice de brunissement, ou l'évolution du temps de réhydratation total avec le temps de fragmentation ou l'évolution du temps de réhydratation total avec la solubilité) qui rassemblaient l'ensemble des conditions de stockage testées (durée et température) et ce quelle que soit la composition initiale de la poudre.

Il a également été établi que les équations mathématiques décrivant les courbes de vieillissement étaient dépendantes de la composition du retentât atomisé.

Basée sur ces observations et notamment l'existence de trajectoire de vieillissement unique par type de poudre de PPCN, une méthode d'ingénierie reverse a été proposée pour prédire le temps de réhydratation totale. Cette méthode consiste :

i) à réaliser un vieillissement accéléré (par application de température de stockage de 60°C) pour quelques échantillons de poudres (trois échantillons suffisent pour les poudres testées)

ii) mesurer l'évolution du temps de réhydratation et de l'indicateur utilisé en abscisse de la courbe de vieillissement (solubilité, temps de mouillabilité, de fragmentation) pour identifier l'allure de la courbe de vieillissement

iii) à réaliser une mesure de l'indicateur pour l'échantillon vieilli et à se baser sur la courbe de vieillissement préalablement approximée pour obtenir la valeur de temps de réhydratation de la poudre

L'analyse comparative du comportement au vieillissement de 3 types de poudre de PPCN a permis de démontrer que la composition du retentât (matière sèche) pouvait avoir un impact sur la taille des particules de poudres formées en atomisation. De ce fait, les poudres dont la taille de grains est plus petite se réhydratent plus rapidement. Néanmoins la conservation lors du stockage ne se trouve pas optimisée.

De plus, il a été démontré que « diminuer le lactose d'une poudre de PPCN jusqu'à l'état de trace » n'était pas une voie pour endiguer la perte de solubilité au cours du stockage (constatée lors de la réhydratation des poudres). En revanche, l'élimination du lactose permet d'éviter le brunissement.

En ce qui concerne l'évolution des propriétés structurales, des marqueurs de structures ont été identifiés.

Il a ainsi été démontré par 2 techniques différentes (IRTF et DCRS) que la perte de structure secondaire en hélice α était corrélée avec une perte de solubilité lors du stockage.

Toutefois, la structure secondaire des caséines est limitée et aucune transformation structurale n'a été détectée à l'état sec. Cela semble logique car il est difficile de concevoir un dépliement d'une structure secondaire à l'état sec ; le dépliement étant généralement induit par la pénétration du solvant au sein de la structure tertiaire.

La diminution de solubilité est donc associée à un très faible changement conformationnel de la protéine en solution.

Dès lors, il apparait que le dépliement de la protéine ne peut être l'unique facteur responsable de la perte de solubilité mais constitue une étape initiale favorisant l'exposition des régions hydrophobes et d'autres modifications à l'origine d'une perte de solubilité.

Les évolutions de modifications chimiques avec le temps de stockage ont été étudiées. Des augmentations de la lactosylation, de l'acétylation et de la désamidation ont été démontrées lors du stockage d'une poudre de PPCN. Cependant, on observe que la grande majorité des modifications chimiques sont présentes dès le début du stockage. Encore une fois, il est étonnant que si peu d'évolution engendre de si grandes pertes de solubilité.

Au vu des faibles évolutions au vieillissement constatées sur les structures secondaires et les modifications chimiques, il est fort probable d'imaginer que la baisse d'aptitude à la réhydratation des poudres est liée à des changements se produisant en surface du grain (en périphérie du grain et non volumique) et non le volume entier.

Il a également été démontré que la migration des lipides vers la surface du grain de poudre et l'évolution du temps de mouillage étaient corrélées. Ce phénomène ne semble toutefois pas en mesure d'expliquer à lui seul l'augmentation importante du temps de réhydratation total au cours du vieillissement. En revanche, l'augmentation du temps nécessaire à la libération des MC dans le solvant, dû à l'agrégation des MC formant une couche mince en surface des particules, peut l'expliquer. L'augmentation du temps de fragmentation des particules primaires semble donc être un indicateur pertinent pour indiquer que les micelles se sont agrégées et prédire l'augmentation du temps de réhydratation total.

Enfin, il a été démontré que l'évolution des modifications chimiques se mettant en place lors du vieillissement était différente suivant la présence de lactose ou non. La diminution du pH en présence de lactose, due à la RM, pourrait être à l'origine des différents schémas réactionnels initiés.

Ainsi, les évolutions des marqueurs de structures des poudres de PPCN stockées à températures élevées semblent peu marquées face à l'altération drastique des propriétés de réhydratation. Nous suggérons alors que seule une faible fraction des

caséines exclusivement située en surface serait concernée par ces modifications de structures, comme illustré **Figure 15**. Ces caséines modifiées au cours du stockage s'agrégeraient et rendraient la couche mince de MC, en surface des particules, de plus en plus compacte et difficile à hydrater. L'augmentation du temps de libération des micelles étant l'étape responsable de l'augmentation du temps de réhydratation total, cela pourrait pleinement expliquer comment une faible évolution des marqueurs structuraux est à l'origine de l'altération importante des propriétés de réhydratation des poudres de PPCN.

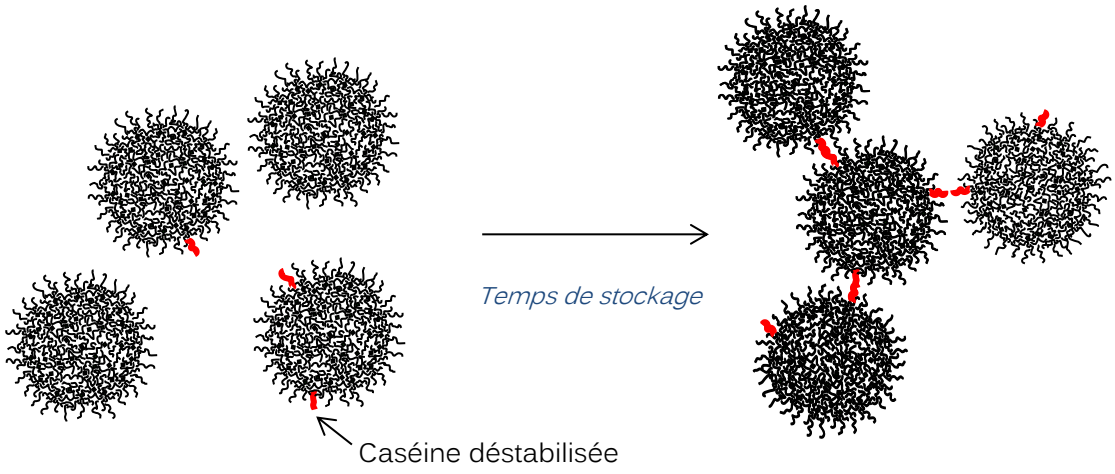

Figure 15. Agrégation des micelles de caséines au cours de stockage via déstabilisation de quelques caséines situées en surface.

Suite aux résultats acquis lors du projet de thèse, plusieurs perspectives peuvent être envisagées.

Il pourrait être intéressant d'étudier l'influence de l'humidité relative (Hr) sur les propriétés fonctionnelles et structurelles lors d'un stockage. On peut s'attendre à ce que l'augmentation de l'Hr de stockage accélère l'altération des poudres. L'objectif serait de déterminer une Hr de stockage limite évitant une dégradation trop rapide des poudres de protéines de lait. Grâce à ces informations supplémentaires, il pourrait être

envisagé d'ajuster l'Hr lors du stockage en mettant en place des déshumidificateurs et ainsi éviter une altération trop rapide des poudres.

Une deuxième perspective concerne directement l'application des matériels et méthodes développés lors de ce projet à de nouvelles poudres de lait.

En effet, afin de caractériser les poudres de PPCN, de nombreux matériels et méthodes (test de réhydratation, de colorimétrie, test ultrasonore pour la détermination du temps de mouillabilité, d'indice de solubilité, suivi de l'évolution de la structure secondaire, de la surface (composition et structure), suivi de la quantification des modifications chimiques et des tests statistiques) ont été sélectionnés, mis en place ou adaptés lors de ce projet. Ils pourront être directement réutilisés pour caractériser de nouvelles poudres à la composition différente et dont le comportement reste encore inconnu.

On peut penser par exemple à appliquer ces matériels et méthodes aux poudres de protéines de lait dont le lactose a été hydrolysé. En effet, la conservation de ces nouvelles poudres de protéines de lait adaptées aux intolérants au lactose et aux demandes/besoins des industriels/consommateurs a été, jusqu'à aujourd'hui, très peu étudiée.

Une autre perspective émane des analyses et interprétations de ce projet et propose d'approfondir les connaissances concernant les procédés d'obtention d'une poudre de lait afin d'optimiser sa fonctionnalité et sa conservation.

En effet, il semblerait que les protéines des poudres de PPCN soient déjà déstabilisées (présence de nombreuses modifications chimiques) et que des réticulations entre caséines aient déjà eu lieu (peau déjà formée en surface des particules) avant toute période de stockage. Les opérations unitaires nécessaires à l'obtention d'une poudre de PPCN seraient donc à l'origine d'une première

déstabilisation des protéines. On peut se demander quel serait l'impact d'un traitement plus doux sur la conservation des poudres. De plus, il a été observé que la composition du retentât (teneur en caséines, en lactose…) impactait les caractéristiques de la poudre obtenue après séchage (ex. taille de grains). Afin d'être capable d'adapter l'étape de séchage (températures d'entrée et de sortie) à la composition du retentât à sécher, des études complémentaires reliant composition/comportement au séchage/fonctionnalités des poudres/conservation sont donc nécessaires. Par la suite, ces connaissances supplémentaires pourraient permettre d'effectuer des actions correctives (redéfinition des corrections de séchage..) afin de minimiser les modifications lors du séchage et de la conservation.

Enfin, une dernière perspective résultant également des analyses et interprétations de ce projet, propose de prolonger les études sur la structure exacte des poudres de PPCN. La connaissance du modèle de structure de départ, de l'évolution de la structure lors des procédés d'obtention de la poudre et lors de la conservation peuvent permettre de solutionner l'agrégation à toutes les étapes.

En effet, la connaissance de la structure moléculaire des caséines est indispensable dans la compréhension des mécanismes d'insolubilisation des poudres. Afin d'enrayer l'agrégation, il faudrait dans un premier temps : déterminer l'agencement exacte entre les composés des PPCN. Puis déterminer les composés concernés par des modifications de structure, par RMN par exemple, afin de minimiser leur présence (exemple κ caséine) tout en conservant les propriétés désirées des caséines. Cependant, pour contrôler la composition des caséines, une maitrise des étapes de filtration avant séchage sera primordiale. De plus, une représentation exacte du rôle de chaque composant de la caséine sera également nécessaire.

Annexe 1. Revue Bibliographique

Storage of high protein dairy powders: changes in protein structures and functions. A review

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Abstract

Background: Milk, being a highly perishable product, is converted into a wide range of dairy powders in order to increase its shelf life and to be stored for long periods of time (0.5 – 2 years). These powders must keep their quality and functional properties during storage. Within the milk protein ingredients, high protein dairy powders present a class of ingredients that are enriched in protein and depleted in minerals and lactose. High protein dairy powders are used in the formulation of a wide range of high added value food products thanks to their specific and controlled functionalities.

Scope and approach: However, high protein dairy powders undergo adverse changes during storage, with both functional and structural changes. As these powders represent a significant share in the global market of milk, it is of prime interest to understand the mechanisms involved in their ageing in order to control their end use properties.

Key findings and conclusions: Powders are relatively stable product compared to non-dehydrated products but they are still subjected to molecular mobility enhanced at temperature above the powder's glass transition temperature. The molecular mobility is responsible for mesoscopic structural changes, local molecular changes and also for interaction reactions with non-protein components in the powder, in particular through the Maillard reaction. These modifications are believed to be at the origin of the changes in the dairy powders' functions occurring during storage.

Keywords High protein dairy powders, storage, structure, function

Introduction

In 2014 world milk production was about 802 million tonnes, which represented 3% increase compared to 2013 following a growing global demand and a world trade (without intra-EU trade) of 42 billion USD (Rouyer, 2015). Indeed, milk production and milk consumption zones do not perfectly match. In some countries such as NZ, Ireland, France, etc. milk production exceeds milk consumption whereas the opposite is the standard for other emerging countries such as China, Russia, etc. Thus dairy companies used to increasingly convert milk and milk derivatives into powders to facilitate storage and transportation across the world. Drying keeps dairy products over long periods (0.5 to 2 years) and facilitate trade through exportation by lowering volumes of ×5 to ×15.

The emergence of filtration technologies made it possible for the dairy industry to design increasingly sophisticated preparation processes in order to improve milk component valorisation and meet user needs in term of functional and nutritional properties. This gave rise to the development of a wide variety of high added value milk protein powders or whey proteins concentrates (WPC) and isolate powders (WPI), etc. These latter can be named as high protein powders.

High protein 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 powders and infant formulae powders have become the driving force of investments in the dairy sector worldwide. They contributed significantly to the overall 8.4 billion USD of investments realised for dairy powders between 2012 and 2015 (Rouyer, 2015).

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 conditions their value and uses. This is particularly the case for product 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, recent studies indicate that dairy powders stored under unfavourable temperature and humidity conditions undergo physical (wettability, flowability, browning,...) (Kim, Chen, & Pearce, 2009; Morgan, Appolonia Nouzille, Baechler, Vuataz, & Raemy, 2005; Stapelfeldt, Nielsen, & Skibsted, 1997) and

chemical (lactose crystallization, Maillard reaction, changes in protein structure, fat oxidation ...) (Le, Holland, Bhandari, Alewood, & Deeth, 2013; Morgan et al., 2005; Thomas, Scher, & Desobry, 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 milk protein 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 milk protein powder.

1. Dairy powders

Dairy ingredients are increasingly used throughout the food industry in a dry form. There is a wide range of dairy powders of varying compositions and multiple functions, with regard to the milk and process involved.

1.1. 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, Brulé, & Delaplace, 2011). The challenge is to keep the physical and functional properties of the material unchanged in the dry form, 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 (Pierre 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 affect solubility of the solid particles.

1.2. Main types of dairy powders

The use of membrane filtration makes possible to vary the composition of the dairy fraction prior to spray drying, resulting in a wide range of powders (figure 1) with different physical and functional properties (Pierre Schuck, 2002).

Milk protein concentrate powder, skim milk powder (SMP) or whole milk powder (WMP) contain the same milk proteins in the same ratio than in milk (80% caseins and 20% whey proteins) but with decreasing amount of protein and various amount of lactose, mineral and fat (Pierre Schuck, 2002). 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 powders are obtained through the spray drying of whey protein fraction recovered either from milk microfiltrate, cheese whey or acid whey after casein removal.

2. Powders change during storage

Powders are considered stable as soon as the functional properties of the powder are unchanged. 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 as stable. Molecular mobility and powder stability is dependent on the glass transition temperature (*Tg*) which is specifically the property of an amorphous material (Bhandari & Howes, 1999). When the temperature is above *Tg*, 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 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, Simatos, Champion, Contreras-Lopez, & Le Meste, 2004). Indeed, storage is not as well mastered as any other manufacturing steps and powders can be subjected to high temperatures (above *Tg*) for weeks during delivery (Leinberger, 2006). This could affect the molecular mobility and thus induces functional and structural variabilities (figure 2).

3. Change of functional properties upon storage

Ideally, dehydrated products must have similar properties to those of the original product after rehydration (Walstra, Wouters, & Geurts, 2005). 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 et al., 2004).

3.1. 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" (IB):

$$IB = \frac{[100(x - 0{,}31)]}{0{,}17} \qquad \text{with:} \quad x = \frac{(a + 1{,}75L)}{(5{,}645L + a - 3{,}012b)}$$

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 ($\theta \geq 50°C$) and high humidity ($\geq$ 50% RH). They were able to establish that the powder increasingly browned as a function of heating time (figure 3).

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 0.75 a_w), a dairy powder with a ratio of whey protein 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, Walisinghe, & Chen, 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 found in the particle surface composition. The surface of a whole milk powder particle is mainly covered with a fine layer of fat, leading to a greater cohesion in the powder (Fitzpatrick, Barry, et al., 2007; Fitzpatrick, Iqbal, Delaney, Twomey, & Keogh, 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, Hodnett, et al., 2007; Fitzpatrick et al., 2004). Therefore, SMP form more cohesive cakes than whole milk powders 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).

3.2. 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, Schuck, Six, Andre, & Delaplace, 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, Pinder, Hunter, & Hemar, 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 milk protein concentrate (MPC) powder with a 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 & 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 (P Schuck et al., 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, Jeantet, & Brulé, 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, Knoth, Scherze, & Muschiolik, 2007; Hiller & Lorenzen, 2010). However, all authors do not share these observations as for Baeza, Carrera Sanchez, Pilosof, & Rodríguez Patino (2005) who claimed that the Maillard reaction increases the surface tension. These differences could be due to the type of sugar used, not all have the same properties and to the protein structural change concomitant to sugar 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 & Lorenzen, 2010; Thomas 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, Busti, Verdini, & Delorenzi, 2013). On the other hand, it has been shown that the previous mixtures improved foam stability (Hiller & Lorenzen, 2010). These changes would be due to Maillard reaction products because they result in a steric protection against coalescence (Dunlap & Côté, 2005), or because they are found in the interfacial film which would be thicker and more viscoelastic (Ganzevles, Cohen Stuart, Vliet, & de Jongh, 2006; Wooster & 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 & Lorenzen (2010) observed a low emulsifying activity of sodium caseinate / lactose and glucose, WP / glucose mixtures due to the formation of Maillard reaction products with high molecular weights 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, Melton, & Stanley (2006) with sodium caseinate / glucose or lactose mixtures and French & 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 drops thereby promoting coalescence.

4. Change of 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 govern 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.

4.1. 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 & Roos, 2016). Many milk powders are in an amorphous glassy state (Bhandari & 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 *Tg* (Struik, 1977). However, this condition is not always sufficient to ensure the physical and chemical stability of protein powders during long storage (Zylberman & 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 & 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 (Liu, Bhandari, & Zhou, 2006). The level of physical aging is increasing above *Tg* 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 (Jin Kim, Hagiwara, Kawai, Suzuki, & Takai, 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 ± 1°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, Lauridsen, Skibsted, & Risbo, 2005).

Rearrangements caused by the molecular mobility can therefore affect protein structures and thereby molecular interactions (Abdul-Fattah, Kalonia, & Pikal, 2007).

4.2. Structure modifications

4.2.1. 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, Deeth, Whittaker, Gidley, & Bhandari (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 of powder particles (figure 4, A et B) while the particle surface of fresh milk protein concentrate (MPC) powder (protein content >80%) is smooth (figure 4, C et 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 powder, 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 rearrangement during long-term storage, such as protein crosslinking.

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 (Kim, Chen, & Pearce, 2002; Nijdam & Langrish, 2006). In order to monitor these modifications, X-Ray Photoelectron Spectroscopy (XPS) seems to be the ideal method to perform (Fyfe et al., 2011; Gaiani et al., 2006; Kim et al., 2002). In fact, XPS gives information on an elemental level (from the C, O, and N percentages) within 10 nm of the particle surface. The method described by Fäldt, Bergenstahl, & Carlsson (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 Kim, Chen, & Pearce (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 latters 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 latters. 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, Clerc, Ulrich, & Benet, 2005; Gaiani et al., 2009).

4.2.2. 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 & Goñi, 1999).

Several authors have used Fourier Transform Infrared spectroscopy (FTIR) to monitor changes in protein structure. Schokker et al. (2011) compared the protein structures of fresh and stored MPC (protein content >85%) powder 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 α-helix decrease and β-sheet increase.

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 structure of the casein (Kher, Udabage, McKinnon, McNaughton, & Augustin, 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 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, & 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 structure 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 & Huppertz (2015) believe that the insoluble fraction formed during storage of a MPC at 50°C is a combination of denatured whey protein 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 & Ha, 1993). It is important to note that temperature and storage time have a positive effect on aggregation and this from 25°C (Morr & 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 amongst themselves 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, Schuck, & Croguennec (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 5).

Maillard Reaction. Maillard reaction, or non-enzymatic browning, usually develops during processing and storage of milk protein powders. This reaction modifies proteins 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, Jongen, & van Boekel, 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, Stringano, Carulli, & Palmisano, 2010; Morgan, Léonil, Mollé, & Bouhallab, 1997, 1998). Morgan, Bouhallab, et al. (1998) have identified every lysine which are involved in the lactosylation reaction of β-LG (table 1). 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 after 22h of reaction at 50°C and 65% relative humidity.

Concerning SMP, Guyomarc'h, Warin, Donald Muir, & Leaver (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 proteins crosslinking in SMP from 25% to 50.3% and also in WPI powders, forming compounds of more than 100 kDa (Guyomarc'h et al., 2014; Le et al., 2013). AGE are probably involved in these interactions following lactosylation (Le et al., 2013; Singh, 1991). The greater the amount of lactose, the higher the level of crosslinking increases. Even if lactose is present in small quantities, it is modified through the formation of Maillard reaction products which then contributed to the formation of large aggregates. The effect is more important on caseins, which become insoluble, than on whey proteins which remain soluble (Le et al., 2013). It is noteworthy that lactosylation started during spray drying possibly due to the process temperature 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 with lysines of caseins micelles which lead to further crosslinking affecting the powder solubility (Anema et al., 2006).

According to Hiller & 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) report a study on denaturation and aggregation 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 & Lorenzen (2010) reported that several studies suggested losses of organised tertiary protein structures upon covalent binding of sugar molecules, thus sterically unfolding proteins by diminished intra/inter molecular interactions (Nacka, Chobert, Burova, Léonil, & Haertlé, 1998; Wooster & Augustin, 2007), and upon temperature (Broersen, Voragen, Hamer, & de Jongh, 2004) and humidity (Morgan et al., 1999) effects on proteins. For Báez et al., (2013), glycation did not result in changes in secondary structure of protein, but also leads 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 non-enzymatic browning 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, Naranjo, Leiva, & Malec, 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 aw is between 0.50 and 0.80 (Martinez-Alvarenga et al., 2014; Thomas et al., 2004). Above, the dilution of reactants and high water content lead to a reaction limitation because of the mass action law.

Conclusion

The shelf-life of a dairy powder clearly depends on its composition and storage conditions. Powders are relatively stable product compared to non-dehydrated products but they are still subjected to molecular mobility which is enhanced at temperature above the powder's *Tg*. The molecular mobility is responsible for mesoscopic structural changes (*e.g.* component migration), 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.

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Figure Captions

Figure 1: "Cracking" of milk (from Schuck, 2002)

Figure 2: Properties evolutions of dairy powders upon storage

Figure 3: 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 (°C)/ relative humidity (%)/ time (h)/ and molar ratio, respectively (adapted from Martinez-Alvarenga et al. 2014)

Figure 4: 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)

Figure 5: 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 (from Gulzar, Bouhallab, Jardin, Briard-Bion, & Croguennec, 2013)

Table 1: 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 (adapted from Morgan, Bouhallab, et al., 1998)

Figure 1

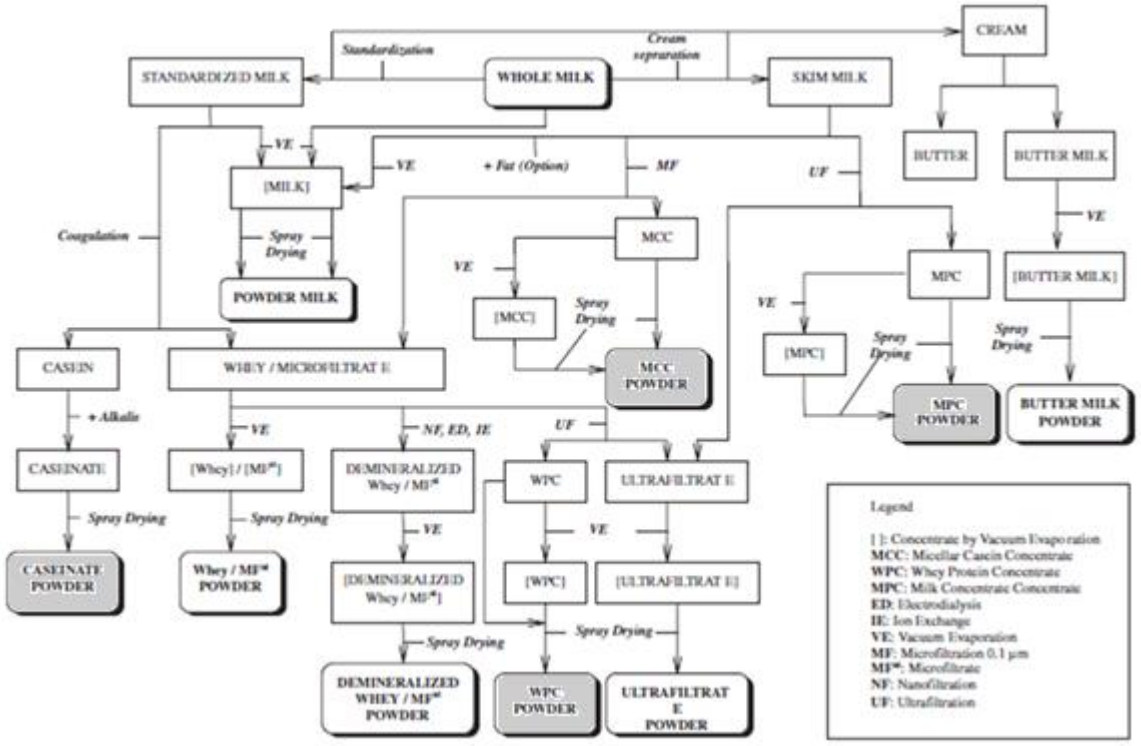

Figure 2

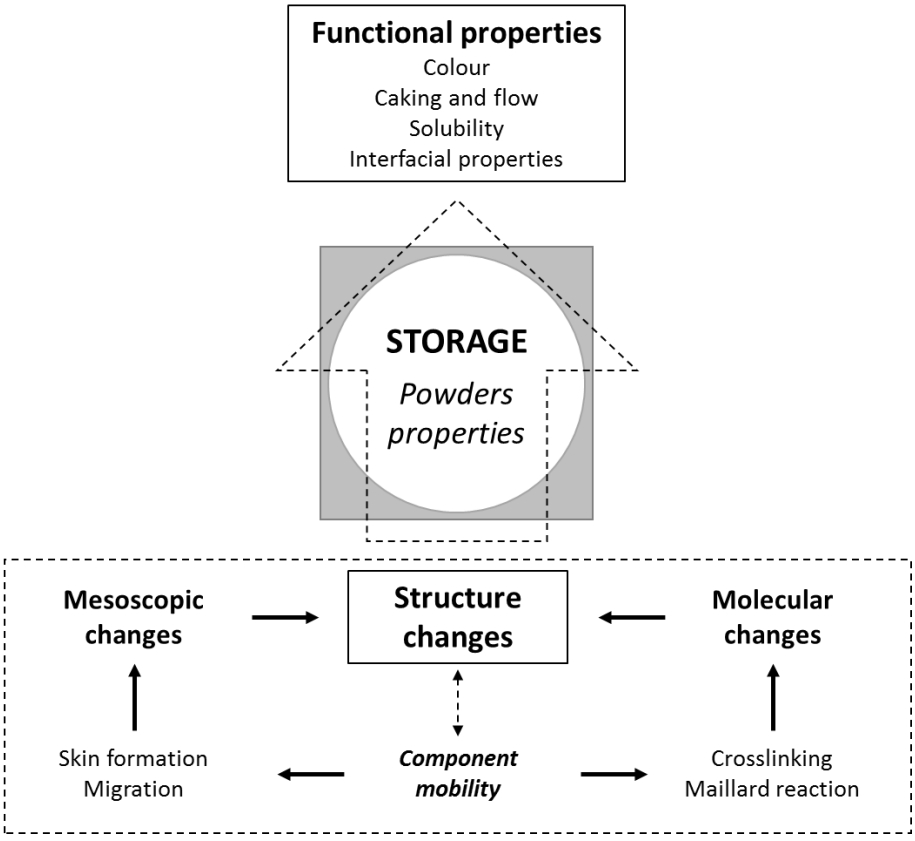

Figure 3

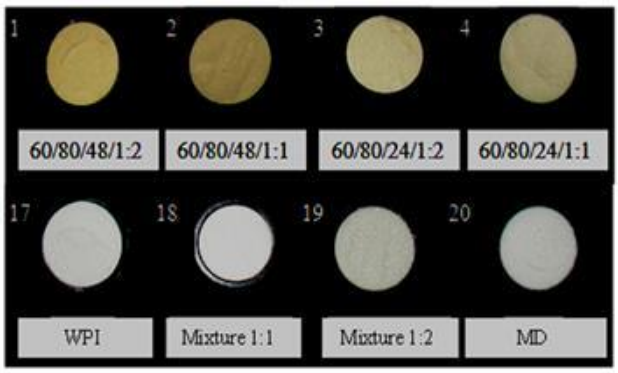

Figure 4

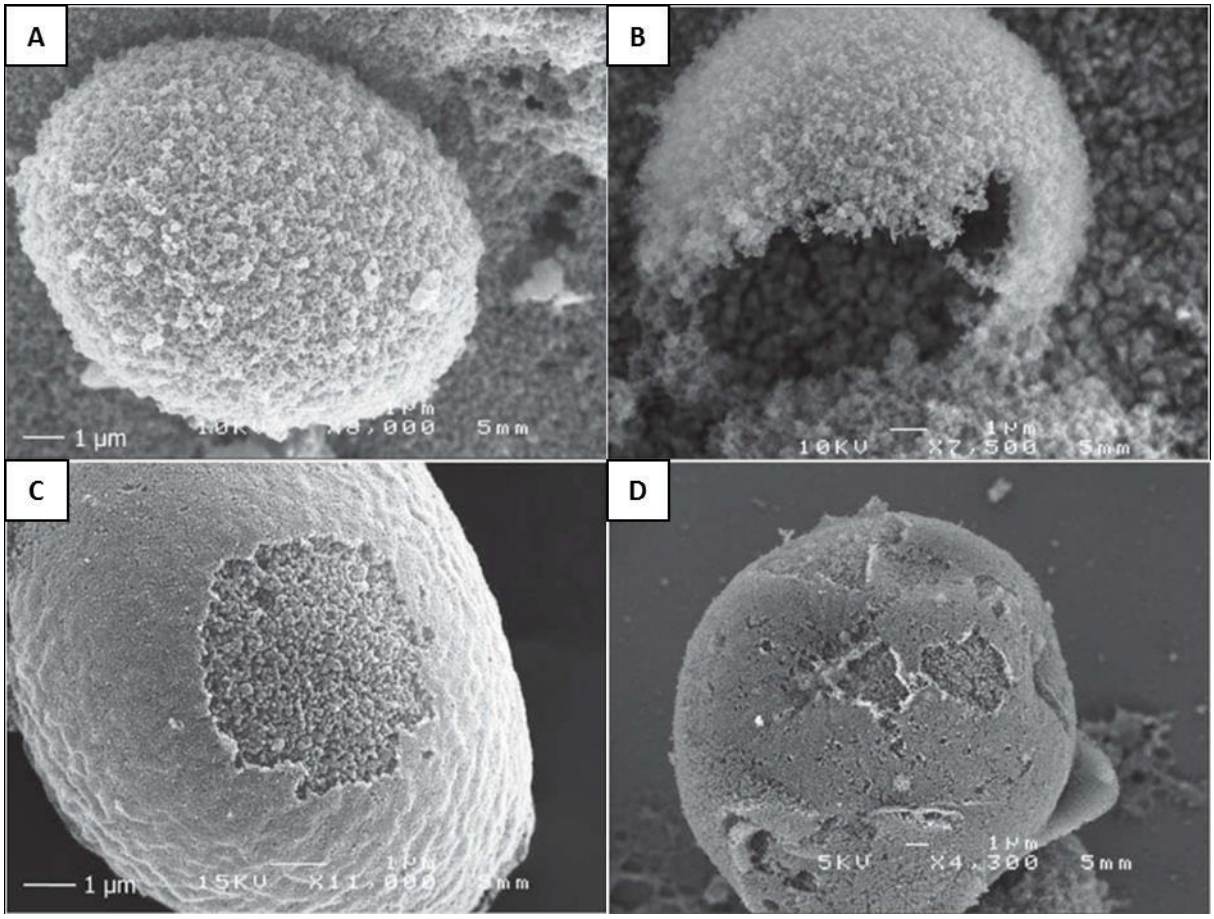

Figure 5

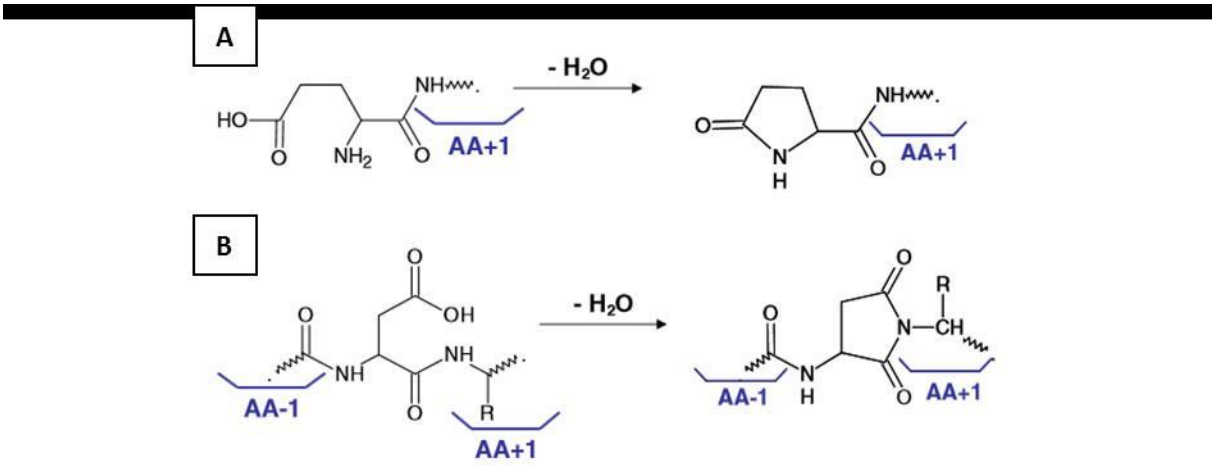

Table 1

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	$Lys_{8, 141}$

Bibliography

Abdul-Fattah, A. M., Kalonia, D. S., & Pikal, M. J. (2007). The challenge of drying method selection for protein pharmaceuticals: Product quality implications. *Journal of Pharmaceutical Sciences*, *96*(8), 1886–1916. http://doi.org/10.1002/jps.20842

Anema, S. G., Pinder, D. N., Hunter, R. J., & Hemar, Y. (2006). Effects of storage temperature on the solubility of milk protein concentrate (MPC85). *Food Hydrocolloids*, *20*(2–3), 386–393. http://doi.org/10.1016/j.foodhyd.2005.03.015

Arrondo, J. L., & Goñi, F. M. (1999). Structure and dynamics of membrane proteins as studied by infrared spectroscopy. *Progress in Biophysics and Molecular Biology*, *72*(4), 367–405.

Baechler, R., Clerc, M.-F., Ulrich, S., & Benet, S. (2005). Physical changes in heat-treated whole milk powder. In *Le Lait* (Vol. 85, pp. 305–314). EDP Sciences.

Báez, G. D., Busti, P. A., Verdini, R., & Delorenzi, N. J. (2013). Glycation of heat-treated β-lactoglobulin: Effects on foaming properties. *Food Research International*, *54*(1), 902–909. http://doi.org/10.1016/j.foodres.2013.08.013

Baeza, R., Carrera Sanchez, C., Pilosof, A. M. R., & Rodríguez Patino, J. M. (2005). Interactions of polysaccharides with β-lactoglobulin adsorbed films at the air–water interface. *Food Hydrocolloids*, *19*(2), 239–248. http://doi.org/10.1016/j.foodhyd.2004.06.002

Bhandari, B. R., & Hartel, R. W. (2005). *Phase transitions during food powder production and powder stability*. Taylor & Francis.

Bhandari, B. R., & Howes, T. (1999). Implication of glass transition for the drying and stability of dried foods. *Journal of Food Engineering*, *40*(1–2), 71–79. http://doi.org/10.1016/S0260-8774(99)00039-4

Broersen, K., Voragen, A. G. J., Hamer, R. J., & de Jongh, H. H. J. (2004). Glycoforms of β-lactoglobulin with improved thermostability and preserved structural packing. *Biotechnology and Bioengineering*, *86*(1), 78–87. http://doi.org/10.1002/bit.20030

Cheriot, S. (2007, December 11). *Rôle des produits de la réaction de Maillard dans l'inhibition de l'oxydation enzymatique des phénols et des lipides*. AgroParisTech.

Croguennec, T., Jeantet, R., & Brulé, G. (2008). *Fondements physicochimiques de la technologie laitière*. Lavoisier TEC & DOC.

Dunlap, C. A., & Côté, G. L. (2005). β-Lactoglobulin−Dextran Conjugates: Effect of Polysaccharide Size on Emulsion Stability. *Journal of Agricultural and Food Chemistry*, *53*(2), 419–423. http://doi.org/10.1021/jf049180c

Fäldt, P. (1995). Surface composition of spray-dried emulsions. Lund University Thesis, Department of Food Engineering, Scandinavia.

Fäldt, P., Bergenstahl, B., & Carlsson, G. (1993). The Surface Coverage of Fat on Food Powders Analyzed by Esca (Electron Spectroscopy for Chemical Analysis). *Food Structure*, *12*(2).

Fan, F., & Roos, Y. H. (2016). Structural relaxations of amorphous lactose and lactose-whey protein mixtures. *Journal of Food Engineering*, *173*, 106–115. http://doi.org/10.1016/j.jfoodeng.2015.10.047

Fechner, A., Knoth, A., Scherze, I., & Muschiolik, G. (2007). Stability and release properties of double-emulsions stabilised by caseinate–dextran conjugates. *Food Hydrocolloids*, *21*(5–6), 943–952. http://doi.org/10.1016/j.foodhyd.2006.10.021

Fitzpatrick, J. J., Barry, K., Cerqueira, P. S. M., Iqbal, T., O'Neill, J., & Roos, Y. H. (2007). Effect of composition and storage conditions on the flowability of dairy powders. *International Dairy Journal*, *17*(4), 383–392. http://doi.org/10.1016/j.idairyj.2006.04.010

Fitzpatrick, J. J., Hodnett, M., Twomey, M., Cerqueira, P. S. M., O'Flynn, J., & Roos, Y. H. (2007). Glass transition and the flowability and caking of powders containing amorphous lactose. *Powder Technology*, *178*(2), 119–128. http://doi.org/10.1016/j.powtec.2007.04.017

Fitzpatrick, J. J., Iqbal, T., Delaney, C., Twomey, T., & Keogh, M. K. (2004). Effect of powder properties and storage conditions on the flowability of milk powders with different fat contents. *Journal of Food Engineering*, *64*(4), 435–444. http://doi.org/10.1016/j.jfoodeng.2003.11.011

Fogliano, V., Monti, S. M., Visconti, A., Randazzo, G., Facchiano, A. M., Colonna, G., & Ritieni, A. (1998). Identification of a β-lactoglobulin lactosylation site. *Biochimica et Biophysica Acta*

(BBA) - Protein Structure and Molecular Enzymology, *1388*(2), 295–304. http://doi.org/10.1016/S0167-4838(98)00177-0

French, S. J., & Harper, W. J. (2003). Maillard reaction induced lactose attachment to bovine β-lactoglobulin: Foam and emulsion functionality. *Milchwissenschaft*, *58*(5–6), 252–256.

Fyfe, K. N., Kravchuk, O., Le, T., Deeth, H. C., Nguyen, A. V., & Bhandari, B. (2011). Storage induced changes to high protein powders: influence on surface properties and solubility. *Journal of the Science of Food and Agriculture*, *91*(14), 2566–2575. http://doi.org/10.1002/jsfa.4461

Gaiani, C., Ehrhardt, J. J., Scher, J., Hardy, J., Desobry, S., & Banon, S. (2006). Surface composition of dairy powders observed by X-ray photoelectron spectroscopy and effects on their rehydration properties. *Colloids and Surfaces B: Biointerfaces*, *49*(1), 71–78. http://doi.org/10.1016/j.colsurfb.2006.02.015

Gaiani, C., Schuck, P., Scher, J., Ehrhardt, J. J., Arab-Tehrany, E., Jacquot, M., & Banon, S. (2009). Native phosphocaseinate powder during storage: Lipids released onto the surface. *Journal of Food Engineering*, *94*(2), 130–134. http://doi.org/10.1016/j.jfoodeng.2009.01.038

Ganzevles, R. A., Cohen Stuart, M. A., Vliet, T. van, & de Jongh, H. H. J. (2006). Use of polysaccharides to control protein adsorption to the air–water interface. *Food Hydrocolloids*, *20*(6), 872–878. http://doi.org/10.1016/j.foodhyd.2005.08.009

Gazi, I., & Huppertz, T. (2015). Influence of protein content and storage conditions on the solubility of caseins and whey proteins in milk protein concentrates. *International Dairy Journal*, *46*, 22–30. http://doi.org/10.1016/j.idairyj.2014.09.009

Gulzar, M., Bouhallab, S., & Croguennec, T. (2011). Structural consequences of dry heating on Beta-Lactoglobulin under controlled pH. *Procedia Food Science*, *1*, 391–398. http://doi.org/10.1016/j.profoo.2011.09.060

Gulzar, M., Bouhallab, S., Jeantet, R., Schuck, P., & Croguennec, T. (2011). Influence of pH on the dry heat-induced denaturation/aggregation of whey proteins. *Food Chemistry*, *129*(1), 110–116. http://doi.org/10.1016/j.foodchem.2011.04.037

Guyomarc'h, F., Famelart, M.-H., Henry, G., Gulzar, M., Leonil, J., Hamon, P., … Croguennec, T. (2014). Current ways to modify the structure of whey proteins for specific functionalities—a review. *Dairy Science & Technology*, 1–20. http://doi.org/10.1007/s13594-014-0190-5

Guyomarc'h, F., Warin, F., Donald Muir, D., & Leaver, J. (2000). Lactosylation of milk proteins during the manufacture and storage of skim milk powders. *International Dairy Journal*, *10*(12), 863–872.

Haque, E., Bhandari, B. R., Gidley, M. J., Deeth, H. C., Møller, S. M., & Whittaker, A. K. (2010). Protein Conformational Modifications and Kinetics of Water−Protein Interactions in Milk Protein Concentrate Powder upon Aging: Effect on Solubility. *Journal of Agricultural and Food Chemistry*, *58*(13), 7748–7755. http://doi.org/10.1021/jf1007055

Haque, E., Whittaker, A. K., Gidley, M. J., Deeth, H. C., Fibrianto, K., & Bhandari, B. R. (2012). Kinetics of enthalpy relaxation of milk protein concentrate powder upon ageing and its effect on solubility. *Food Chemistry*, *134*(3), 1368–1373. http://doi.org/10.1016/j.foodchem.2012.03.034

Havea, P. (2006). Protein interactions in milk protein concentrate powders. *International Dairy Journal*, *16*(5), 415–422. http://doi.org/10.1016/j.idairyj.2005.06.005

Hiller, B., & Lorenzen, P. C. (2010). Functional properties of milk proteins as affected by Maillard reaction induced oligomerisation. *Food Research International*, *43*(4), 1155–1166. http://doi.org/10.1016/j.foodres.2010.02.006

Jeantet, R., Brulé, G., & Delaplace, G. (2011). *Génie des procédés appliqué à l'industrie laitière*. Lavoisier.

Jeantet, R., Schuck, P., Six, T., Andre, C., & Delaplace, G. (2010). The influence of stirring speed, temperature and solid concentration on the rehydration time of micellar casein powder. *Dairy Science & Technology*, *90*(2–3), 225–236. http://doi.org/10.1051/dst/2009043

Jin Kim, Y., Hagiwara, T., Kawai, K., Suzuki, T., & Takai, R. (2003). Kinetic process of enthalpy relaxation of glassy starch and effect of physical aging upon its water vapor permeability property. *Carbohydrate Polymers*, *53*(3), 289–296. http://doi.org/10.1016/S0144-8617(03)00075-4

Kher, A., Udabage, P., McKinnon, I., McNaughton, D., & Augustin, M. A. (2007). FTIR investigation of spray-dried milk protein concentrate powders. *Vibrational Spectroscopy*, *44*(2), 375–381. http://doi.org/10.1016/j.vibspec.2007.03.006

Kim, E. H.-J., Chen, X. D., & Pearce, D. (2002). Surface characterization of four industrial spray-dried dairy powders in relation to chemical composition, structure and wetting property. *Colloids and Surfaces B: Biointerfaces*, *26*(3), 197–212.

Kim, E. H.-J., Chen, X. D., & Pearce, D. (2005). Effect of surface composition on the flowability of industrial spray-dried dairy powders. *Colloids and Surfaces B: Biointerfaces*, *46*(3), 182–187. http://doi.org/10.1016/j.colsurfb.2005.11.005

Kim, E. H.-J., Chen, X. D., & Pearce, D. (2009). Surface composition of industrial spray-dried milk powders. 3. Changes in the surface composition during long-term storage. *Journal of Food Engineering*, *94*(2), 182–191. http://doi.org/10.1016/j.jfoodeng.2008.12.001

Le, T. T., Holland, J. W., Bhandari, B., Alewood, P. F., & Deeth, H. C. (2013). Direct evidence for the role of Maillard reaction products in protein cross-linking in milk powder during storage. *International Dairy Journal*, *31*(2), 83–91. http://doi.org/10.1016/j.idairyj.2013.02.013

Leinberger, D. (2006). Temperature and humidity in ocean containers, Proceedings of Dimensions. International Safe Transit Association: East Lansing, MI, USA.

Li-Chan, E. (1983). Heat-Induced Changes in the Proteins of Whey Protein Concentrate. *Journal of Food Science*, *48*(1), 47–56. http://doi.org/10.1111/j.1365-2621.1983.tb14786.x

Liu, Y., Bhandari, B., & Zhou, W. (2006). Glass Transition and Enthalpy Relaxation of Amorphous Food Saccharides: A Review. *Journal of Agricultural and Food Chemistry*, *54*(16), 5701–5717. http://doi.org/10.1021/jf060188r

Losito, I., Stringano, E., Carulli, S., & Palmisano, F. (2010). Correlation between lactosylation and denaturation of major whey proteins: an investigation by liquid chromatography-electrospray ionization mass spectrometry. *Analytical and Bioanalytical Chemistry*, *396*(6), 2293–2306. http://doi.org/10.1007/s00216-010-3465-6

Martinez-Alvarenga, M. S., Martinez-Rodriguez, E. Y., Garcia-Amezquita, L. E., Olivas, G. I., Zamudio-Flores, P. B., Acosta-Muniz, C. H., & Sepulveda, D. R. (2014). Effect of Maillard reaction conditions on the degree of glycation and functional properties of whey protein isolate – Maltodextrin conjugates. *Food Hydrocolloids*, *38*, 110–118. http://doi.org/10.1016/j.foodhyd.2013.11.006

Martins, S. I. F. S., Jongen, W. M. F., & van Boekel, M. A. J. S. (2000). A review of Maillard reaction in food and implications to kinetic modelling. *Trends in Food Science & Technology*, *11*(9–10), 364–373. http://doi.org/10.1016/S0924-2244(01)00022-X

Maskan, M. (2001). Kinetics of colour change of kiwifruits during hot air and microwave drying. *Journal of Food Engineering*, *48*(2), 169–175. http://doi.org/10.1016/S0260-8774(00)00154-0

Mimouni, A., Deeth, H. C., Whittaker, A. K., Gidley, M. J., & Bhandari, B. R. (2010). Investigation of the microstructure of milk protein concentrate powders during rehydration: Alterations during storage. *Journal of Dairy Science*, *93*(2), 463–472. http://doi.org/10.3168/jds.2009-2369

Morgan, F., Appolonia Nouzille, C., Baechler, R., Vuataz, G., & Raemy, A. (2005). Lactose crystallisation and early Maillard reaction in skim milk powder and whey protein concentrates. *Le Lait*, *85*(4–5), 315–323. http://doi.org/10.1051/lait:2005017

Morgan, F., Bouhallab, S., Mollé, D., Henry, G., Maubois, J.-L., & Léonil, J. (1998). Lactolation of β-lactoglobulin monitored by electrospray ionisation mass spectrometry. *International Dairy Journal*, *8*(2), 95–98.

Morgan, F., Léonil, J., Mollé, D., & Bouhallab, S. (1997). Nonenzymatic Lactosylation of Bovine β-Lactoglobulin under Mild Heat Treatment Leads to Structural Heterogeneity of the Glycoforms. *Biochemical and Biophysical Research Communications*, *236*(2), 413–417. http://doi.org/10.1006/bbrc.1997.6955

Morgan, F., Léonil, J., Mollé, D., & Bouhallab, S. (1998). Modification of Bovine β-Lactoglobulin by Glycation in a Powdered State or in an Aqueous Solution: Effect on Association Behavior and Protein Conformation. *Journal of Agricultural and Food Chemistry*, *47*(1), 83–91. http://doi.org/10.1021/jf9804387

Morgan, F., Mollé, D., Henry, G., Vénien, A., Léonil, J., Peltre, G., … Bouhallab, S. (1999). Glycation of bovine β-Lactoglobulin: effect on the protein structure. *International Journal of*

Food Science & Technology, *34*(5–6), 429–435. http://doi.org/10.1046/j.1365-2621.1999.00318.x

Morr, C. V., & Ha, E. Y. W. (1993). Whey protein concentrates and isolates: Processing and functional properties. *Critical Reviews in Food Science and Nutrition*, *33*(6), 431–476. http://doi.org/10.1080/10408399309527643

Nacka, F., Chobert, J. M., Burova, T., Léonil, J., & Haertlé, T. (1998). Induction of new physicochemical and functional properties by the glycosylation of whey proteins. *Journal of Protein Chemistry*, *17*(5), 495–503.

Nijdam, J. J., & Langrish, T. A. G. (2006). The effect of surface composition on the functional properties of milk powders. *Journal of Food Engineering*, *77*(4), 919–925. http://doi.org/10.1016/j.jfoodeng.2005.08.020

Oliver, C. M., Melton, L. D., & Stanley, R. A. (2006). Functional properties of caseinate glycoconjugates prepared by controlled heating in the "dry" state. *Journal of the Science of Food and Agriculture*, *86*(5), 732–740. http://doi.org/10.1002/jsfa.2406

Özkan, N., Walisinghe, N., & Chen, X. D. (2002). Characterization of stickiness and cake formation in whole and skim milk powders. *Journal of Food Engineering*, *55*(4), 293–303. http://doi.org/10.1016/S0260-8774(02)00104-8

Pereyra Gonzales, A. S., Naranjo, G. B., Leiva, G. E., & Malec, L. S. (2010). Maillard reaction kinetics in milk powder: Effect of water activity at mild temperatures. *International Dairy Journal*, *20*(1), 40–45. http://doi.org/10.1016/j.idairyj.2009.07.007

Roos, Y. H. (2002). Importance of glass transition and water activity to spray drying and stability of dairy powders. *Le Lait*, *82*(4), 475–484.

Roudaut, G., Simatos, D., Champion, D., Contreras-Lopez, E., & Le Meste, M. (2004). Molecular mobility around the glass transition temperature: a mini review. *Innovative Food Science & Emerging Technologies*, *5*(2), 127–134. http://doi.org/10.1016/j.ifset.2003.12.003

Rouyer, B. (2015). Dynamic of investment in dry dairy ingredients worldwide. *CNIEL International press*. p 4.

Schokker, E. P., Church, J. S., Mata, J. P., Gilbert, E. P., Puvanenthiran, A., & Udabage, P. (2011). Reconstitution properties of micellar casein powder: Effects of composition and storage. *International Dairy Journal*, *21*(11), 877–886. http://doi.org/10.1016/j.idairyj.2011.05.004

Schuck, P. (2002). Spray drying of dairy products: state of the art. In *Le Lait* (Vol. 82, pp. 375–382). EDP Sciences.

Schuck, P., Davenel, A., Mariette, F., Briard, V., Méjean, S., & Piot, M. (2002). Rehydration of casein powders: effects of added mineral salts and salt addition methods on water transfer. *International Dairy Journal*, *12*(1), 51–57. http://doi.org/10.1016/S0958-6946(01)00090-5

Singh, H. (1991). Modification of food proteins by covalent crosslinking. *Trends in Food Science & Technology*, *2*, 196–200. http://doi.org/10.1016/0924-2244(91)90683-A

Stapelfeldt, H., Nielsen, B. R., & Skibsted, L. H. (1997). Effect of heat treatment, water activity and storage temperature on the oxidative stability of whole milk powder. *International Dairy Journal*, *7*(5), 331–339.

Struik, L. C. E. (1977). Physical aging in plastics and other glassy materials. *Polymer Engineering & Science*, *17*(3), 165–173. http://doi.org/10.1002/pen.760170305

Struik, L. C. E. (1978). Orientation effects and cooling stresses in amorphous polymers. *Polymer Engineering & Science*, *18*(10), 799–811. http://doi.org/10.1002/pen.760181011

Tamime, A. Y., Robinson, R. K., and Michel, M. (2007). Microstructure of concentrated and dried milk products. In Blackwell Publishing Ltd., Oxford, UK (Ed.), *Structure of Dairy Products* (pp. 104–133).

Thomas, M. E. C., Scher, J., & Desobry, S. (2004). Lactose/β-Lactoglobulin Interaction During Storage of Model Whey Powders. *Journal of Dairy Science*, *87*(5), 1158–1166. http://doi.org/10.3168/jds.S0022-0302(04)73264-6

Thomsen, M. K., Lauridsen, L., Skibsted, L. H., & Risbo, J. (2005). Temperature Effect on Lactose Crystallization, Maillard Reactions, and Lipid Oxidation in Whole Milk Powder. *Journal of Agricultural and Food Chemistry*, *53*(18), 7082–7090. http://doi.org/10.1021/jf050862p

Van Boekel, M. A. J. S. (1998). Effect of heating on Maillard reactions in milk. *Food Chemistry*, *62*(4), 403–414. http://doi.org/10.1016/S0308-8146(98)00075-2

Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2005). *Dairy Science and Technology, Second Edition*. CRC Press.

Wooster, T. J., & Augustin, M. A. (2007). Rheology of whey protein–dextran conjugate films at the air/water interface. *Food Hydrocolloids*, *21*(7), 1072–1080. http://doi.org/10.1016/j.foodhyd.2006.07.015

Zylberman, V., & Pilosof, A. M. R. (2002). Relationship between the glass transition, molecular structure and functional stability of hydrolyzed soy proteins. In H. Levine (Ed.), *Relationship between the glass transition, molecular structure and functional stability of hydrolyzed soy proteins* (pp. 158–168).

Résumé

Au cours du stockage, les propriétés fonctionnelles (aptitude à la réhydratation) et sensorielles (couleur) des poudres de protéines de lait peuvent être altérées, et ce d'autant plus que la teneur en caséines de la poudre est élevée. Comprendre les mécanismes de modifications structurelles à l'échelle moléculaire à l'origine des évolutions des propriétés des poudres concentrées en caséines représente un enjeu important pour l'industrie laitière et l'objectif de cette thèse.

Pour cela, des poudres de phosphocaséinate natif (PPCN), avec ou sans lactose ont été stockées jusqu'à 12 mois à 4, 20, 40 et 60 °C sous humidité contrôlée. Les évolutions de structures secondaires, les migrations de composés, les modifications chimiques ainsi que l'évolution des propriétés fonctionnelles et sensorielles ont été déterminées expérimentalement.

L'analyse des résultats a permis de i) démontrer que l'application de conditions de vieillissement accélérées permettait d'identifier la trajectoire au vieillissement des poudres de PPCN stockées à température ambiante ii) proposer des tests pour prédire les temps de réhydratation total.

Les évolutions de structure secondaires à l'origine de la déstabilisation des protéines ont pu être identifiées ainsi que différentes modifications chimiques (acétylation, désamidation, lactosylation, déphosphorylation) selon la composition. Il a été établi que l'agrégation des caséines localisée en surface des grains de poudre ralentissait leur dispersion au sein du milieu aqueux et conduisait à une augmentation du temps de réhydratation.

Enfin, il a été établi que l'absence de lactose n'évitait pas la déstabilisation des protéines et la formation d'insolubles mais empêchait le brunissement.

Abstract

During storage, the functional (rehydration) and sensory (color) properties of the milk protein powders can alter, especially when the casein content of the powder is high. Understanding the mechanisms of structural changes at the molecular level that are at the origin of those property changes of casein-concentrated powders represents an important challenge for the dairy industry and hence the objective of this thesis.

For this purpose, powders of Micellar casein (MC), with or without lactose were stored up to 12 months at 4, 20, 40 and 60 ° C under constant relative humidity. Evolutions of secondary structures, compound migrations, chemical modifications as well as the evolution of functional and sensory properties were determined experimentally.

The analysis of the results demonstrated the possibility to predict i) the ageing trajectory of the PPCN powders stored at ambient temperature by applying accelerated aging conditions; ii) the total rehydration time using the experimental tests that we proposed.

The secondary structural changes at the origin of protein destabilization as well as different chemical modifications (acetylation, deamidation, lactosylation, dephosphorylation) were identified according to the composition. It was established that the aggregation of the caseins on the particle surface slowed down their dispersion into the aqueous medium, resulting in an increase of rehydration time.

Finally, it has been established that the absence of lactose does not prevent protein destabilization or formation of insoluble matter, but it does prevent browning.