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INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE
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Laboratoire de Science et Génie Alimentaires

THESE

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Spécialité : Procédés Biotechnologiques et Alimentaires

**Effet de l'hydratation d'une formulation infantile modèle
(lactose, β -lactoglobuline, amidon) sur les changements
d'état et les interactions entre constituants**

**Effect of hydration of model infant food (lactose, β -
lactoglobulin, starch) on state changes and interactions
between components**

par

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Preface:

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Contents :

Abbreviations.....	6
Glossaire	8
Résumé (en français).....	9
General introduction.....	13
Chapter I: Literature Review.....	15
I.1 Introduction.....	16
I.2 Human and cow milk composition	17
I.3 Infant foods formulations.....	20
I.3.1 Milk-based formulas	20
I.3.1.1 Casein-predominant formulas	21
I.3.1.2 Whey-predominant formulas	22
I.3.2 Soy protein-based formulas	22
I.3.3 Protein hydrolysate-based formulas.....	23
I.3.4 Amino acid-based formula.....	23
I.4 Ingredients.....	24
I.4.1 Carbohydrate.....	24
I.4.1.1 Lactose	24
I.4.1.2 Starch	28
I.4.1.2.1 Starch granule structure	30
I.4.1.3 Maltose and Maltodextrins	30
I.4.2 Proteins	31
I.4.2.1 Whey proteins	31
I.4.2.2 Human milk proteins and cow milk proteins.....	33
I.4.2.3 Protein hydrolysates.....	33
I.4.3 Fats.....	34
I.5 Stability of infant formulas	35
I.5.1 Proteins/polysaccharides interactions	36
I.5.1.1 Effect of interactions on water adsorption behavior	37
I.5.1.2 Effect of interactions on bioavailability of micronutrients	38
I.5.2 Maillard browning reaction.....	39
I.5.2.1 Chemistry of Maillard reaction	39
I.5.2.2 Maillard reaction in infant food	41
I.5.2.3 Factors affecting Maillard reaction	42
I.5.2.4 Effect of Maillard reaction on nutritional quality	43
I.5.2.5 Beneficial effect of Maillard reaction	44
I.5.2.6 Effect of Maillard reaction on functional properties.....	44
I.5.3 Oxidation.....	45
I.5.3.1 Factors affecting rate of oxidation	45
I.5.3.2 Effect of oxidation on nutritional quality.....	46
I.5.4 Mechanical and physico-chemical properties of infant foods	46
I.5.4.1 Collapse.....	47
I.5.4.2 Stickiness and Caking	47
I.5.4.3 State diagram	48
I.5.4.4 Glass transition temperature	49
I.6 Lactose crystallization during storage	54
I.6.1 Crystallization Models	54
I.6.1.1 Avrami model	54

Contents

I.6.1.2 Hoffman equation	55
I.6.1.3 Williams-Landel-Ferry (WLF) model	56
I.6.2 Crystalline forms of lactose	59
I.6.2.1 Crystallization process	59
I.7 Justification of the study	60
Chapter II: Materials and Methods	62
II.1 Characterization of the powder and formulations preparation	63
II.1.1 Physicochemical properties determination	63
II.1.1.2 BLG purity	64
II.2 Model infant formulation preparation	64
II.3 Characterization of amorphous lactose	66
II.3.1 Water sorption	66
II.3.2 Moisture sorption prediction of formulations	67
II.3.2.1 Procedure	68
II.4 Evaluation of crystallization	68
II.4.1 Gravimetric methods	68
II.4.2 Scanning Electron Microscopy (SEM)	68
II.4.2.1 Principle	68
II.4.2.2 Procedure	69
II.4.3 Melting behavior: Differential Scanning Calorimetry (DSC)	69
II.4.3.1 Principle	69
II.4.3.2 Procedure	72
II.5 Kinetic of lactose crystallization by Dynamic Vapor Sorption (DVS)	73
II.5.1 Principle	73
II.5.2 Procedure	74
II.6 Infrared spectroscopy	74
II.6.1 Principle	74
II.6.2 Fourier transform infrared (FTIR) spectroscopy	76
II.6.3 Procedure	76
II.7 Experimental design	77
II.7.1 What is a mixture design?	77
II.7.2 Model	78
II.7.3 The experimental matrix	79
II.7.4 Entering the mixture components	80
II.7.4.1 Mixture model terms	80
II.7.4.2 Scheffe model form	80
II.7.4.3 Crossed model	80
Chapter III: Results and Discussion	82
III.1 Moisture sorption properties of model infant formulations	83
III.1.1 Moisture sorption isotherm of model infant formulations	83
III.1.2 Lactose Crystallization	86
III.1.3 Comparison between calculated and experimental results	88
III.1.3.1 Binary mixtures	89
III.1.3.2 Ternary mixture	90
III.1.4 Onset and Kinetic of Crystallization	93
III.1.5 Effect of gelatinized starch and BLG on moisture sorption properties of formulations	97
III.1.6 Conclusion	102
III.2 Kinetic of crystallization studied by Dynamic Vapor Sorption (DVS)	103
III.2.1 Conclusion	112

Contents

III.3 Lactose state in model infant formulation.....	113
III.3.1 Differential Scanning Calorimetry (DSC).....	113
III.3.2 Scanning Electronic Microscopy (SEM) of formulations.....	118
III.3.3 Infrared spectroscopy of lactose crystallization.....	125
III.3.3.1 IR spectroscopy of pure lactose	125
III.3.3.2 Influence of BLG and starch on lactose spectra.....	127
III.4 Non-covalent interactions by infrared spectroscopy	129
III.4.1 General spectra.....	129
III.4.2 Spectral region of 3600 to 2800 cm ⁻¹	130
III.4.3 Region spectral of 1700 to 1600 cm ⁻¹	132
III.4.4 Infrared Spectra of formulations	134
III.4.4.1 Influence of lactose and starch on BLG spectrum	136
III.4.5 Conclusion.....	141
III.5 Non-enzymatic browning in model infant formulations.....	142
III.5.1 Conclusion.....	152
Conclusion.....	154
References	156

Abbreviations

AOAC	Association of Official Analysis Chemists
a_w	water activity
Å	Angstrom ($=10^{-10}$ m)
BET	Brunauer-Emmet-Teller model
BLG	β -lactoglobulin
BSA	Bovine Serum Albumin
°C	Celsius degree
ΔC_p	heat capacity increment at glass transition,
Da	Dalton (molecular weight)
db	dry basis
DE	Dextrose Equivalent
DHA	Docosahexaenoic acid
dm/dt	rate of mass changes
DSC	Differential Scanning Calorimetry
DTA	Differential Thermal Analysis
DVS	Dynamic Vapor Sorption
eHF	extensively hydrolyzed formulae
EMC	equilibrium moisture content
FAs	fatty acids
FTIR	Fourier Transform Infrared
g	gram
$g.l^{-1}$	gram/liter
GAB	Guggenheim-Anderson-De Boer model
GC	gas chromatography
HMF	hydroxymethyl furfural
HPLC	high performance liquid chromatography
HTST	high temperature short-time
J/kg·K	Joule/kilogram·Kelvin
$J g^{-1} °C^{-1}$	Joules/gram·degree Celsius
$kJ mol^{-1}$	kilo Joules/mol
k	rate constant (Avrami model)
kCal	kilo Calorie
α -la	α -lactalbumin
min	minute
n	Avrami exponent (Avrami model)
Pa.s	Pascal. second
pHF	partially hydrolyzed formulae
PUFAs	polyunsaturated fatty acids
rh-GH	growth hormone
RH	relative humidity
s	second
SEM	Scanning Electron Microscopy
SH	sulfhydryl group
t	time (Avrami model)
T_g	glassy transition temperature
T_s	suitable reference temperature (WLF model)
T_m	melting temperature

Abbreviations

UHT	ultrahigh temperature
WLF	Williams-Landel-Ferry model
WLF	Williams-Landel-Ferry model
ν	stretching vibrations (in infrared)
$^{\circ}$	degree (specific rotation)
ω	mass fraction
θ	crystallinity (Avrami model)

Glossaire

Caking : mottage

Collapse : effondrement de la structure des particules

Differential Scanning Calorimetry : analyse thermique différentielle

Dispersibility : dispersibilité de particules dans l'eau sous une faible agitation

Dynamic Vapor Sorption : sorption dynamique de la vapeur

Flowability : aptitude à l'écoulement

Glass transition temperature : La transition vitreuse est liée aux modifications de diffusion moléculaire et de viscosité. Elle sépare les états amorphes vitreux et caoutchoutique

Infant formula : formulation de lait infantile

Maillard reactions : réactions de Maillard qui se produisent entre des protéines et des sucres réducteurs

Melting point : point de fusion

Preferential exclusion : exclusion préférentielle

Relative humidity : humidité relative

Scanning Electron Microscopy : microscopie électronique à balayage

Steric hindrance : encombrement stérique

Stickiness: adhérence

Water activity: activité d'eau

Wettability : mouillabilité, correspond à la pénétration du liquide à l'intérieur des pores des particules

Whey proteins : protéines du lactosérum

Résumé (en français)

En 2002, le marché global d'aliments infantiles était d'environ 15 milliards d'euros et seulement 11% des bébés consomment des aliments infantiles (Heinz Company, 2003). Le marché français d'aliments infantiles en poudre a été évalué à 903,9 millions d'euros en 2002, ce qui correspond à une hausse d'environ 5,4 % pour la période 2001-2002. Par ailleurs, les formulations infantiles constitue la plus grosse part du marché d'aliments infantiles, avec 407 M€, viennent ensuite les produits salé avec 251 M€, les produits sucrés avec 127 M€, les produits céréaliers avec 106 M€ et les autres produits avec 35 M€ (Bachelier, 2003). Selon l'association française des aliments diététiques et infantiles, avec une consommation annuelle d'environ 95 kilogramme, les français sont les plus grands consommateurs au monde d'aliments infantiles. Ils sont suivis des allemands et des américains.

Les formulations infantiles contiennent une grande quantité de lactose à l'état amorphe et cristallin. Le lactose amorphe est instable et peut être plastifié par la température et l'eau. Pendant la plastification, lorsqu'on dépasse la température de la transition vitreuse, il y a une diminution de la viscosité, une augmentation de la mobilité moléculaire, et un changement rapide de l'état physique du lactose. Lorsque qu'il y a transition du lactose d'un état amorphe vitreuse à un état amorphe caoutchoutique (la transition vitreuse), la viscosité diminue et donc la mobilité des molécules du lactose augmente et la cristallisation se produit. La transition d'un état amorphe à un état cristallin pendant le stockage cause des changements indésirables tels que la libération d'eau, le motage, l'effondrement et l'accélération du brunissement non-enzymatique se produisent. Ces phénomènes sont d'autant plus significatifs que la quantité de lactose dans les formulations est importante. Dans l'idéal, il est préférable de stocker le lactose dans un état complètement cristallisé. Or, la méthode de séchage par atomisation est généralement utilisée lors de la fabrication d'aliments infantiles. Cette technique ne permet pas l'obtention du lactose dans un état totalement cristallisé.

Dans cette étude, des aliments infantiles modèles ont été préparés par la co-lyophilisation de trois composants : le lactose, la β -lactoglobuline (BLG) et l'amidon. Les objectifs de cette étude étaient :

1- de déterminer les propriétés de sorption d'eau des formulations lyophilisées et de proposer des modèles mathématiques capables de prédire ces propriétés de sorption d'eau,

Résumé

2- d'étudier l'effet de l'humidité relative ou la teneur en eau des formulations sur la cinétique de la cristallisation, l'état physique du lactose, les changements de structure des composants des formulations et le brunissement non enzymatique,

3- d'utiliser des modèles mathématiques afin de prédire les propriétés de sorption d'eau, la cinétique de cristallisation et les changements de couleur et le brunissement non enzymatique qui se produisant pendant le stockage,

Un plan de mélange a été utilisé afin de prédire les effets des composants sur ces propriétés et de mettre en évidence des éventuelles interactions entre ces composants. De plus, la quantité d'eau adsorbée par les formulations peut être calculée à partir des valeurs de sorption d'eau de chaque composant du mélange. Ce calcul n'est toutefois pas applicable lorsque le lactose est cristallisé. En effet, en présence de protéines et de polysaccharides, la taille des cristaux du lactose est plus petite par rapport à celle de cristaux de lactose pure. Par conséquent la surface des cristaux augmente et d'avantage d'emplacements sont disponibles pour adsorber l'eau.

L'humidité relative de la cristallisation du lactose change en présence des protéines ou des saccharides. La stabilisation du lactose contre la cristallisation est attribuée à l'exclusion préférentielle, à l'obstacle stérique de protéine et de polysaccharide et/ou à l'augmentation de la viscosité du système, limitant ainsi le mouvement structural.

Les résultats ont montré que, pendant le stockage des formulations à l'HR élevé, le lactose a migré sur la surface des particules. On a observé ce phénomène en comparant les photos de microscopie électronique à balayage des échantillons juste après cristallisation et après 3 mois de stockage dans les mêmes conditions de température et d'humidité relative. La taille de cristaux de lactose, stockés pendant 3 mois, a augmenté par rapport à ceux stockés pendant quelques heures dans les mêmes conditions d'humidité relative. Il est probable que pendant le stockage, une migration importante du lactose ainsi qu'un changement de composition externe des particules se produisent.

La spectroscopie infrarouge à transformé de Fourier a aussi permis de mettre en évidence des changements pour des formulations contenant du lactose, de la BLG et de l'amidon gélatinisé avant et après stockage à des humidités relatives différentes. En effet, il a été observé pour toutes les formulations, un décalage de la bande d'amide I de BLG vers les plus petites longueurs d'ondes, ce qui indique la présence de liaisons d'hydrogène entre les composants

Résumé

des formulations. En augmentant l'humidité relative de ~ 0 ou 11 % à 75 ou 85 %, la bande d'amide I de BLG se décale vers les plus petites longueurs d'ondes. Il est possible que dans ces conditions, les liaisons d'hydrogène entre la BLG et le lactose et l'amidon gélatinisé aient été rompues par l'eau. Lorsque l'humidité relative est élevée, le lactose est à l'état cristallin et hydrophobe. Ainsi, le nombre de liaisons d'hydrogène actuels entre la BLG et le lactose a diminué. Une autre hypothèse qui pourrait être suggérée pour ce phénomène est que lorsque l'humidité relative est élevée, le brunissement non enzymatique se produit entre le lactose et la BLG, influençant ainsi le déplacement des longueurs d'ondes de la bande d'amide I de la BLG.

Les résultats des modifications du paramètre b (couleur jaune dans le system CIE L*, a*, b*) des aliments infantiles modèles stockés dans des conditions d'humidités relatives différentes, ont montré que l'humidité relative a un effet significatif sur le changement du paramètre b pendant le stockage. La cristallisation du lactose joue aussi un rôle important sur le changement du paramètre b. Une grande différence a été observée entre les formulations dans lesquelles a eu lieu la cristallisation du lactose et celles dans lesquelles le lactose était amorphe. Une augmentation importante du paramètre b s'est produite pendant la première semaine de stockage. Par la suite, les changements de couleur b se produisent linéairement dans des conditions d'humidités relatives élevées. Le plan de mélange a aussi permis d'expliquer l'effet des composants sur le changement de couleur pendant le stockage.

Résumé

Ce travail a donné lieu aux publications et communications suivantes:

Publications:

Nasirpour A., Scher J., Desobry S. 2006. Baby foods; formulations and interactions (a review). *Critical Reviews in Food Science and Nutrition*. 46: 665 - 681.

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Al Mahdi R., Nasirpour A., Banon S., Scher J., Desobry S. 2006. Morphological and mechanical properties of dried skim milk and wheat flour mixtures during storage. *Powder Technology*. 163: 145-151.

Communications:

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Nasirpour A., Scher J., Desobry, S. 2006. Lactose crystallization delay in lactose/ β -lg/starch model infant foods. *IFT 2006, USA*. (Oral presentation)

Nasirpour A., Scher J., Linder M., Desobry S. 2006. Solid state interactions between major components of model infant formulas during storage. *ISFRS 2006, Switzerland*. (Oral presentation)

Nasirpour A., Scher J., Desobry S. 2004. Interactions between lactose/starch/ β -lg during storage of dry infant food. *Séminaire annuel de l'Ecole Doctorale RP2E* (Nancy) with article in congress book. (ISBN 2-9518564-3-1)

General introduction

In 2002, the global baby food market was valued at around 15 billion euros, with developed markets of Western Europe and North America accounting for 58 % of the value, but only 11 % of babies use baby foods (Heinz Company, 2003). The proportion of powdered infant formula sales has increased by 5.4 % in 2002-2003. The French market for baby foods was valued at 903.9 M€ in 2002. This represents growth value of 6.1 % over 2001, and around 34.5 % for the five-year review period 1998-2003). In the infant foods category, infant formulas, with 407 M€, is largest segment followed by salty products with 251 M€, sweet products with 127 M€, milk and cereal products with 106 M€ and others products with 35 M€ (Bachelier, 2003). Frenchs are the world's largest consumers of baby foods, buying about 95 kilos per year. They are followed by consumers in Germany and the United States, according to the French Association of Dietetic and Children Foods.

Infant formula contains a large quantity of lactose that is in amorphous and crystallized state. Amorphous lactose is meta-stable and may be softened or plasticized by temperature and water. In plasticization, resulting in glass transition, viscosity decreases, molecular mobility increases, and physical state of the lactose changes rapidly. Transition from amorphous to crystallized state causes most drastic changes during storage of powder containing lactose. The most important changes that occur are water liberation, stickiness, caking, collapse, acceleration of non-enzymatic browning etc. In industries, it would be better to have 100 % crystallized lactose to prevent these undesirable changes. Spray drying method is used commonly in infant food production, This problem is more important when lactose quantity increases in formulations. This is the case for infant formula.

In this study, model infant foods were prepared by co-lyophilisation of three major components, i.e. lactose, β -lactoglobulin (BLG) and starch. The objectives of this study were to:

- 1- determine moisture sorption properties of the freeze-dried formulations
- 2- investigate the effect of relative humidity or moisture content on crystallization, kinetic of crystallization, physical state of lactose, non-enzymatic browning and structure changes of each component and formulation
- 3- use mathematical model to explain moisture sorption properties, kinetic of crystallization and predict color changes or non-enzymatic browning taking place during storage.

This thesis is organized in three chapters. The first chapter is a literature review explaining infant food formulations, reactions and interactions between components and their effect on nutritional and physical properties of infant foods and the crystallization models. The materials and methods used in this study are presented in the second chapter. Results and discussion follow in chapter three that is divided into five parts. First part talks about moisture sorption properties of model infant formulations. In this part, effects of each component and the interactions between these components are presented as mathematical models.

Lactose crystallization, measured by Dynamic Vapor Sorption (DVS), lactose state in model infant formulations studied by Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM) and Fourier Transform Infrared (FT-IR), non-covalent interactions studied by infrared and nonenzymatic browning are presented in the second, third, fourth and fifth section respectively.

Chapter I: Literature Review

I.1 Introduction

In the European market, baby foods have an important situation. There are a large range of products entitled *Infant formulas* and *baby foods* for different ages. The main ingredients of these products are presented in Table 1. Generally, it contains a source of protein such as milk proteins, milk proteins hydrolysate and soybean protein (in the American market and recently in Europe).

Table 1 Major ingredients of baby foods

Protein sources	Nonfat dry milk, condensed skim milk, partially demineralized whey, Na and Ca caseinates, soy protein isolate and soy milk
Oils	Soy, corn, safflower, sunflower, colza, palm, copra,
Carbohydrates	Lactose, starch, sucrose, corn syrup and corn syrup solids
Minerals	Major Minor
	Calcium carbonate, mono-, di- and tribasic calcium phosphates, dibasic magnesium phosphate, potassium citrate, magnesium chloride
	Potassium iodide, ferrous sulfate, manganous sulfate, cupric sulfate, zinc sulfate
Vitamins	A, E, K, C, B ₁ , B ₂ , B ₆ , B ₁₂ , niacin, folic acid, pantothenic acid, biotin, choline, inositol
Functional ingredients	Soy lecithin, mono- and diglycerides, starch, carrageenan

Adapted from Miles 1982

In addition, in European Union Regulations, soy protein may be used in formulations but with some conditions: these formulas must contain an available quantity of methionin at least equal to that contained in the reference protein and a minimum quantity of L-carnitine (1.8 μ moles/100kJ) (European Communities, 2000).

Lactose, starch, maltodextrins and sucrose, are used as energy sources. Generally, fatty acid sources are vegetable oils such as sunflower, colza, palm, copra, corn and recently animal fat. In Europe, other ingredients may be added to infant formula and follow-on formulas if their suitability for particular use has been established by generally accepted scientific data

(Koletzko *et al.*, 2002). Production, handling and storage of infant foods are considered more carefully than other food formulations, because these foods are sensitive to alteration reactions and interactions modifying physical properties (lactose crystallization, Maillard reaction, oxidation, caking, interactions and reactions between proteins polysaccharides, etc) and nutritional availability of nutrients during storage. Therefore, these stages, especially storage conditions must be controlled to maintain a good quality of product. Storage temperature and relative humidity are two important criteria to preserve physico-chemical properties and microbial quality of infant foods.

In this dissertation, the term baby food comprises categories of food meant for babies such as infant formulas, mixture of milk powder and cereal. In this literature review section, the composition of baby foods, some formulations available in the European market and the regulations enforce in the European Union, the physico-chemical properties of each component, the reactions and interactions between components in infant foods will be described and discussed.

I.2 Human and cow milk composition

Despite technological advances in formula preparation, human milk substitutes lack of many compounds found in human milk, including, anti-infective agents, enzymes and trophic factors (these proteins in human milk are known to stimulate cell growth and division) (Guo *et al.*, 1996). The composition difference between human and cow milk especially in proteins and carbohydrates is now well known (Table 2). Protein content in human milk is three fold less than in cow milk.

In addition, β -lactoglobulin (BLG) is not present in human milk and α -lactalbumin (α -la) and lactoferrin are the major whey proteins. Moreover, caseins predominate in cow milk, whereas soluble whey predominates in human milk. The ratio of caseins to whey proteins is generally stated to be 40:60 in human milk and 82:18 in cow milk (Fomon, 1993). In human milk, nearly all of the casein is β -casein. It is also a major protein in cow milk, but α s1-casein and α s2-casein account for more than half of the caseins in cow milk. In general, bovine caseins are more phosphorylated than human milk casein (Gurr, 1981).

Table 2 Protein composition of human and cow milk

Protein	Cow		Human	
	g/100ml	Total, %	g/100ml	Total, %
Total	3.30	100	0.88	100
Caseins	2.60	79	0.31	35
Total whey:	0.70	21	0.57	65
α -lactalbumin	0.12	3.5	0.15	17
β -lactoglobulin	0.30	9.0	0.00	-
lactoferrin	tr	-	0.15	17
serum albumin	0.03	1.0	0.05	6
lysozyme	tr	-	0.05	6
immunoglobulins	0.10	3.0	0.10	11
others	0.15	4.5	0.07	8

Data from Gurr 1981

tr: trace

Lactose is a major saccharide of human and cow milk as shown in Table 3: Human milk contains 7 % lactose, while cow milk contains only 4.8 %.

Table 3 Major compositions of human and cow and milk

Constituent	Human milk	Cow's milk
Protein (%)	1.0	3.4
Lactose (%)	7.0	4.8
Fat (%)	3.8	4.0
Ash (minerals) (%)	0.2	0.7
Total solids (%)	12.4	13.0

Adapted from Spreer 1998

This difference is very important for baby food manufacturers because of lactose crystallization. Another important difference between human and cow milk is the oligosaccharides content in human milk (approximately 130 compounds). It has been shown that human milk oligosaccharides induce an increase in bifidobacteria number of colonic flora for breast-fed infants, accompanied by a significant reduction in the number of pathogenic bacteria, due to their bifidogenic activity (Grittenden and Playe, 1996).

The 90 major milk oligosaccharides were isolated and their structures determined (Newburg, 1997). In the food industry, composition and structure of human milk

oligosaccharides cannot be reproduced and other oligosaccharides groups of animal and vegetal origin (like fructooligosaccharides and galactooligosaccharides) are used in infant feeding in order to obtain the beneficial effects of human milk oligosaccharides.

Cow-milk allergy can affect several organs and an infant with cow-milk allergy may show more than one symptom. This allergy is mostly prevalent at the age of 1 year and has been reported in 2.8 % of the general child population (Schrandner *et al.*, 1993). Cow milk allergy may be considered as an important initial link in the allergic disease family. This involvement may be due to the immaturity of the immune system and to immature gut defense mechanisms, where the major load of new antigens is encountered at an early age.

Milk and milk products are of particular nutritional importance in infancy. Therefore, in cow milk allergy, it is necessary to use substitute formulas during cow-milk elimination diet. For this purpose, the most common approach is predigestion of bovine casein or whey to provide nitrogen as mixtures of peptides and amino acids (Isolauri *et al.*, 1995). The use of protein hydrolysates by the food industry is increasing to provide essential amino acids in baby nutrition (Peñas *et al.*, 2004).

Due to their superior nutritional quality, hydrolysates of cow milk proteins are the most widely used protein in infant formulas. As early as 1942, a hypoallergenic casein hydrolysate was employed in the manufacture of formula for infants with cow milk allergy (Cordano and Cook, 1985). Whey protein hydrolysates have also been used as the primary nitrogen source in European-manufactured infant formulas, which target reduced-allergenic properties (Pahud and Schwarz, 1984). Extensively hydrolysed proteins have substantially reduced immunological reactivities and are primarily used in hypoallergenic infant formulas. Because of the need to completely eliminate cow proteins allergenicity to avoid sensitization, these proteins hydrolysates are usually comprised of free amino acids and very short peptides (di- and tripeptides) as shown in Table 4 (Mahmoud, 1994).

However, hydrolysis does not give non-allergenic formulas and their use may result in unidentified continued allergen challenge. The completeness of the antigen elimination may be ensured by synthetic amino acid-derived formulas, which have been shown to be safe for children with multiple food allergies (Sampson *et al.*, 1992).

Table 4 Molecular weight profile of different classes of protein hydrolysates

Molecular Weight Fraction (Dalton)	<i>Degree of hydrolysis</i>		
	Slight	Moderate % in each fraction	Extensive
<500	3.8	9.8	90.2
500-1000	1.1	13.5	5.5
1000-2000	1.8	13.7	2.9
2000-5000	3.7	16.6	1.4
>5000	89.7	46.4	0

Data from Mahmoud 1994

Functional properties of protein hydrolysates depend on the degree of hydrolysis. Limited or partial hydrolysis substantially increases hydrolysates solubility, particularly at the isoelectric point of the parent protein (Chobert *et al.*, 1988, Turgeon *et al.*, 1992). Haque and Mozaffar (1992) demonstrated the same results for casein hydrolysates. As infant formulas are protein-stabilized emulsions, the emulsifying properties of the protein hydrolysates are important. Several researchers investigated the emulsifying properties of various enzymatically hydrolysed proteins under different hydrolysis conditions (Chobert *et al.*, 1988, Gauthier *et al.*, 1993, Mahmoud, 1994). They showed that the protein emulsifying properties are improved by carefully controlling the extent of hydrolysis, but extensive hydrolysis, especially performed for the manufacture of hypoallergenic protein hydrolysates, results in drastic loss of protein's emulsifying properties.

I.3 Infant foods formulations

I.3.1 Milk-based formulas

Milk based formulas are consumed by majority of infants and are marketed in powdered, liquid concentrate, and liquid ready-to-eat forms (Table 5).

They are made from cow's milk that has been modified by butterfat removal and vegetable oil addition. To have better taste and nutritional quality, more carbohydrates are added (lactose) and some proteins are removed. Various cow milk-derived products (e.g., non-fat milk,

Chapter I: Literature Review

casein, combinations of casein and whey proteins, or partially hydrolyzed whey protein concentrate) provide proteins. Lactose, corn syrup solids, sucrose, or corn maltodextrin provide carbohydrates and vegetable oil blends provide fat. Milk base formulas fall into two categories; casein-predominant and whey-predominant formulas.

Table 5 Major components (as indicated on packaging) of infant formulas present on the France market

Component	Protein :			Carbohydrates			
	Casein	Whey proteins		Lactose	Starch	Glucose syrup	Maltodextrin
Bledina® 1 st age	10.5	6.3	4.2	45.3	-	-	14
2 nd age	11.7	7	4.7	46.2	-	-	14.6
Gallia® 1 st age	11	6.6	4.4	45.7	-	-	14
2 nd age	12.3	7.4	4.9	46.2	-	-	14.6
Guigoz® 1 st age	11.5	6.3	5.2	35.4	15.3	-	7.1
2 nd age	16.1	12.4	3.7	42.8	14	-	-
Modilac® 1 st age	10.4	5.2	5.2	44	-	18	-
2 nd age	12.1			51	-	12.8	-
Nidal® 1 st age	12.8						
2 nd age	14.3	9	3.8	44	14.1	-	-
		11	3.3	45.6	13.5	-	-
Nutricia® 1 st age	12.9						
2 nd age	12.8			44	-	-	8.1
		10.2	2.6	43	-	-	12

I.3.1.1 Casein-predominant formulas

They consist in dilute bovine skim milk to which fat and other nutrients are added but the casein/whey ratio remains unchanged.

New regulations and standards for infant foods indicate that it is not necessary to have these constant ratios: only minimum and maximum protein content in infant formula must be respected depending on the protein source. The minimum of proteins in infant formula and

Chapter I: Literature Review

follow-on formula for milk based formulations are 10.84 and 13.55 (g/100 g product) respectively and the maximum is 18.07 and 27.1 respectively (Table 6).

Table 6 Essential composition of infant formula manufactured from cow milk proteins

Components	Infant formula		Follow-on formula	
	Min	Max	Min	Max
Energy	600 kcal	800 kcal	600 kcal	800 kcal
Proteins	10.84 g	18.07 g	13.55 g	27.1 g
Carbohydrates	42 g	84 g	42 g	84 g
Lactose	21 g		10.8 g	
Starch ^a		20 g		
Sucrose		20% ^b		20% ^b
Lipid	26.4 g	39 g	19.8 g	39 g

Adapted from European Communities regulations 2000

All of the values are based on 100 grams of finished product

^a Precooked or gelatinized starch

^b of total carbohydrates

I.3.1.2 Whey-predominant formulas

Gurr (1981) reviewed the composition of human and artificial milks for infant. As Table 2 shows, BLG does not exist in human milk and α -la and lactoferrin are the major whey proteins. There are different considerations on the two casein-predominant and whey-predominant formulas and their effects on infant growth. The two categories have quite similar effect. Studies conducted in both full-term and preterm infants showed no effect of altering the whey-to-casein ratio of formula on growth or nitrogen balance. However, these studies consistently showed that plasma threonine concentrations were increased in infants fed formulas with increased whey contents (Darling *et al.*, 1999, Fomon, 1993).

I.3.2 Soy protein-based formulas

These formulas are marketed in powdered, liquid concentrate and liquid ready-to-eat forms are consumed by about 25 % of term infants in the United States (American Academy of Pediatrics Committee on Nutrition, 1999). This category is suitable for infants that have

allergies to cow milk proteins and lactose intolerance. The protein level of soy formula is usually higher than that of milk based formula, because of differences in protein digestibility and differences in amino acid composition (Lonnerdal, 1994). However, an increasing number of infants are being fed with these products because of perceived nonspecific gastrointestinal problems (Committee on Nutrition. American Academy of Pediatrics, 1998). Soy contains phytate and numerous authors showed phytate could form chelates with several divalent mineral ions and make them unavailable for absorption (Davies and Nightingale, 1975) such as zinc (Lonnerdal *et al.*, 1984, Sandström *et al.*, 1983).

I.3.3 Protein hydrolysate-based formulas

Infants with a milk allergy or intolerance to intact protein often consume these formulations. These formulas are marketed in powdered, liquid concentrate, and liquid ready-to-eat forms. Enzymatically hydrolyzed casein, fortified with selected amino acids, provides nitrogen; combinations of corn syrup solids, maltodextrin, or sucrose provide carbohydrate; and vegetable oil blends provide fat. One protein hydrolysate-based product also includes medium chain triglyceride oils as a fat source. Tormo *et al.* (1998) studied the effects of feeding of protein hydrolysate formula on infant growth. They showed the infants receiving the cow milk protein hydrolysate based formula progress as well as those fed with unmodified cow milk based formula, in spite of not receiving any benefit from the functions of unmodified proteins.

I.3.4 Amino acid-based formula

Infants who do not tolerate formulas based on cow milk proteins, soy protein isolate, or casein hydrolysates may consume these formulations. One amino acid-based formula is currently marketed in the United States and it is marketed only in powdered form. This formula contains corn syrup solids, free amino acids, and a vegetable oil blend as sources of macronutrients.

I.4 Ingredients

An infant formula should contain the following nutrients at a level between the minimum level specified and the maximum level specified for each 100 kCal of the infant formula in the form prepared for consumption as directed on the container (Table 6). In this part, the major components of an infant formula such as carbohydrates, proteins and fats and their properties are discussed.

I4.1 Carbohydrate

Only the following carbohydrates may be used in infant formula (Table 6 shows quantity of them); lactose, maltose, sucrose, maltodextrins, glucose syrup or dried glucose syrup, pre-cooked starch naturally free of gluten and gelatinized starch naturally free of gluten (European Communities, 2000).

I.4.1.1 Lactose

Lactose is the largest segment with around 35-50 % and is the most important energy source in infant formula. Moreover, lactose is important in two aspects: nutrition and in infant formulas industries. Lactose promotes absorption of calcium, magnesium and manganese in infant (Ziegler and Fomon, 1983). Generally more than 40 % lactose exist in infant formula and during drying, lactose state plays an important role in physical properties of final product. As lactose content increases in the formulation, product handling becomes more difficult.

Lactose structure

Lactose is a disaccharide consisting of galactose and glucose, linked by a β 1-4 glycosidic bond (Figure 1).

Its systematic name is β -D-galactopyranosyl-(1-4)- α -D-glucopyranose (α -lactose) or β -D-galactopyranosyl-(1-4)- β -D-glucopyranose (β -lactose). The hemiacetal group of the glucose moiety is potentially free (i.e. lactose is a reducing sugar) and may exist as α - or β -anomer. In the structural formula of the α -form, the hydroxyl group on the C, of glucose is cis to the

hydroxyl group at C, (oriented downward). When either isomer is dissolved in water, there is a gradual change from one form to the other until equilibrium is established, i.e. mutarotation. Equilibrium mixture at 20°C is composed of 62.7 % β - and 37.3 % α -lactose. The equilibrium constant, β/α is 1.68 at 20°C.

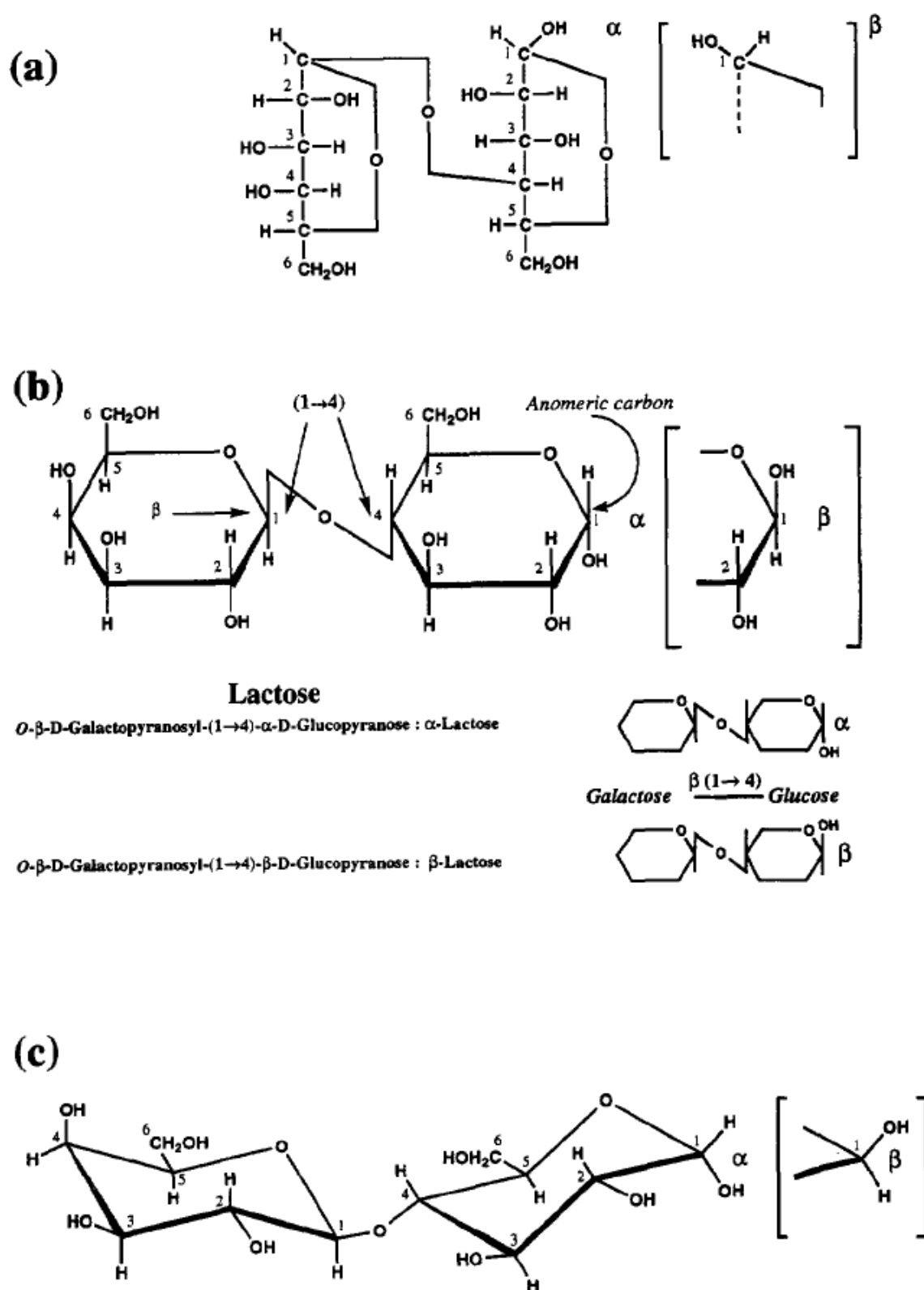


Figure 1 Structural formulae of α- and β-lactose. (a) Fischer projection, (b) Haworth projection and (c) conformational formula

Chapter I: Literature Review

The proportion of lactose in the α -form increases as the temperature is increased and the equilibrium constant consequently decreases. The equilibrium constant is not influenced by pH, but the rate of mutarotation is dependent on both temperature and pH. The change from α - to β -lactose is 51.1, 17.5 and 3.4 % complete at 25, 15 and 0 °C, respectively, in 1 h and is almost instantaneous at about 75 °C (Fox and McSweeney, 1998).

In powder state, lactose can be in crystalline and amorphous state depending on the drying condition. There are different types of crystallized lactose such as; α -hydrate (α -lactose monohydrate) containing 5 % water of crystallization, α -anhydrous may be prepared by dehydration of α -hydrate, β -anhydride and lactose glass is prepared by rapid drying of lactose (spray drying or freeze-drying). The relationship between the different crystalline forms of lactose is shown in Table 7 and Figure 2.

Table 7 some physical properties of two common forms of lactose (Fox and McSweeney, 1998)

Property	α -hydrate	β -anhydride
Melting point (°C)	202	252
Specific rotation	+89.4°	+35°
Solubility in water (g 100 ml ⁻¹) at 20 °C	7	50
Specific gravity (20 °C)	1.54	1.59
Specific heat (J/kg-K)	0.299	0.285
Heat of combustion (kJ mol ⁻¹)	5687	5946

Handling of this carbohydrate is very important in dairy industries. After spray drying of infant formula, lactose is in amorphous state or in a mixture of amorphous and crystalline state. Storage of this product is very difficult because amorphous state is thermodynamically unstable thus during storage, lactose is crystallized and caking phenomenon are observed (Aguilera *et al.*, 1995, Chuy and Labuza, 1994). During this phenomena, amorphous lactose are transformed into α and β crystalline lactose forms, which can be anhydrous, or monohydrated (α -lactose only), according to both powder production and storage conditions. The chemical and physical properties of lactose were explained by numerous researchers (Fox, 1996, Walstra *et al.*, 1999).

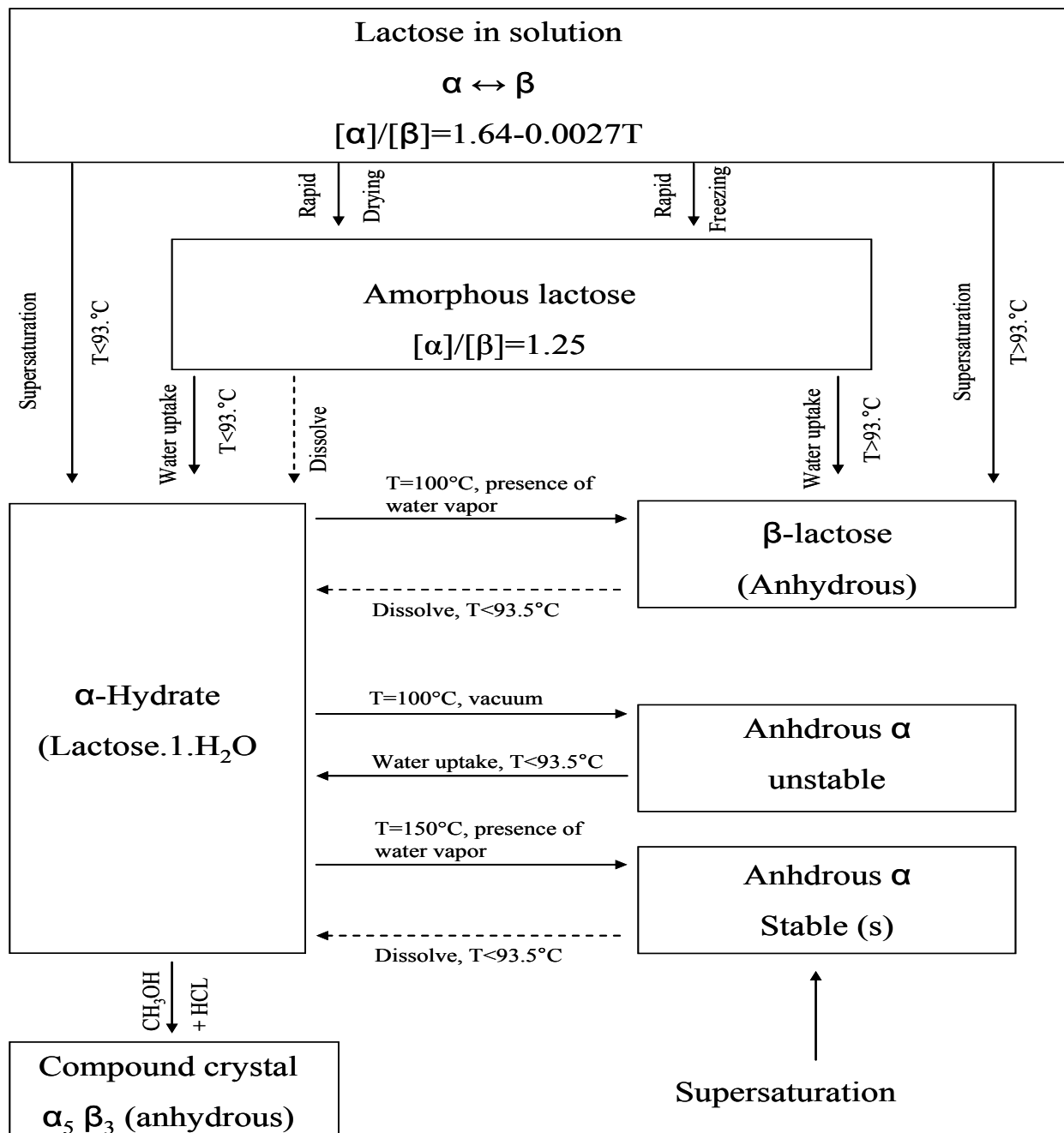


Figure 2 Modifications of lactose from Walstra *et al.* (1999)

I.4.1.2 Starch

Starch is the major form of stored carbohydrate in plants. Starch is composed of a mixture of two substances: amylose, an essentially linear polysaccharide, and amylopectin, a highly branched polysaccharide as shown in Figure 3. Both forms of starch are polymers of α -D-Glucose. Natural starches contain 10-20 % amylose and 80-90 % amylopectin. Amylose

Chapter I: Literature Review

forms a colloidal dispersion in hot water (which helps to thicken gravies) whereas amylopectin is completely insoluble.

Amylose molecules consist typically of 200 to 20,000 glucose units, which form a helix as a result of the bond angles between the glucose units.

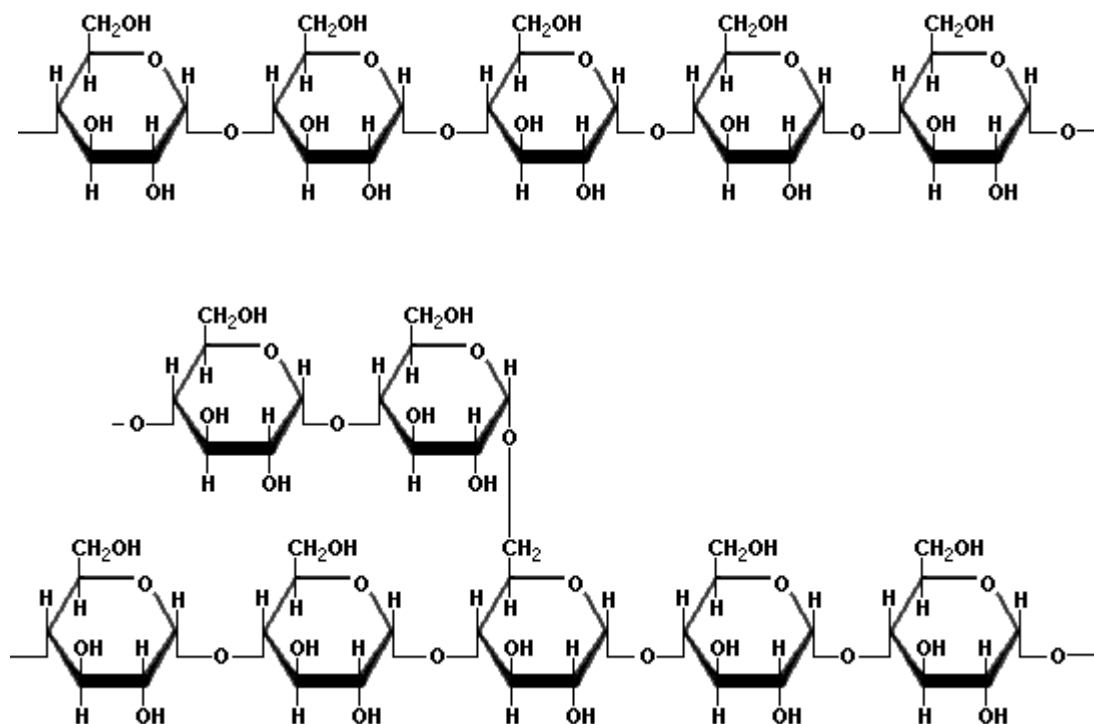


Figure 3 Structure of amylose (above) and amylopectin (below)

Amylopectin differs from amylose in being highly branched. Short side chains of about 30 glucose units are attached with $1\alpha\rightarrow6$ linkages approximately every twenty to thirty glucose units along the chain. Amylopectin molecules may contain up to two million glucose units.

Starch is a complex substance, composed of two separate glucose polymers: amylose and amylopectin. These polysaccharides are arranged in a three-dimensional, semi-crystalline structure known as a starch granule. In most common type of cereal endosperm starches, the relative weight percentage of amylopectin is between 72-82 % and amylose 18-33 %. The linear region of the amylose chain forms a dark blue complex with polyiodide ions in solution at room temperature (Cornell and Hoveline, 1998).

I.4.1.2.1 Starch granule structure

Starch occurs naturally as water-insoluble granules within plant tissues. The shape and size of granules are characteristic of its botanical origin (Parker and Ring, 2001). Granules exist with diameters ranging from 1 μm to more than 100 μm , and shapes of regular (ovoid, spherical, angular) or irregular composition (Imberty *et al.*, 1991). Several plant sources exhibit two separate distributions of granule size and shapes. Wheat, for example, contains granules with diameters from 5-30 μm in both round and lenticular shapes. The starch granule is also partially crystalline with crystallinities in the region of 30 % being reported (Parker and Ring, 2001). Examination of the product has shown that the chain length participating in the crystalline domains is comparable to the short chain fraction of amylopectin, hence the suggestion that it is the short chains of amylopectin which form the double helices (French, 1984, Guilbot and Mercier, 1985, Parker and Ring, 2001).

I.4.1.3 Maltose and Maltodextrins

Maltodextrins are obtained by acid and/or enzymatic hydrolysis of starch, but to a lower extent than that required to produce starch syrups. Among the wide choice of commercially available maltodextrins, choosing the appropriate one for these applications is a matter of compromise between its properties in solution (before the process) and its properties at the glassy state (after the process) (Marchal *et al.*, 1999). Maltodextrins perform multifaceted functions in food systems, including bulking, providing resistance to caking, adding texture and body, binding flavor and fat, serving as oxygen barriers, aiding dispersability and solubility, freezing control and preventing crystallization and as product extenders (Chronakis, 1998). They are also useful to reduce Maillard reactions and are used in micro encapsulation of food components such as fat and oils, vitamins, minerals and colorants (Reinceccius, 1991, Sheu and Rosenberg, 1995). Maltodextrins are usually supplied with the dextrose equivalent value (DE) as the only information from which all properties seem to be empirically guessed. This measure is realized under well-defined conditions and is a rough estimation of the similarity of the compound of interest to glucose and is directly linked to the number average molecular weight. The DE of maltodextrins has, however, been shown to be inadequate to predict product performances in various applications (Chronakis, 1998).

Recently, it has been clearly shown that the molecular weight distribution is, as could be expected, a much more accurate tool to predict maltodextrin fundamental properties but it has only been applied to particular, specific issues so far (Striegel *et al.*, 1998, Wang and Wang, 2000, White *et al.*, 2003).

I.4.2 Proteins

The role of proteins as an ingredient is very important in two aspects: nutrition and functional properties. Milk proteins are widely used in baby food. The properties of many dairy products depend on the properties of milk proteins. The milk proteins are fractionated into two well-defined groups; casein and whey or serum protein. The major compositions of whey proteins are BLG, α -la and serum albumin. The ratio of casein : whey proteins shows large difference between bovine milk proteins and human milk (see Table 4).

I.4.2.1 Whey proteins

The main proteins in whey are BLG, α -la, proteose-peptone, immunoglobulins and bovine serum albumin (BSA) (Aguilera, 1995, Walstra *et al.*, 1999). BLG, α -la and BSA occur naturally as globular proteins folded into compact three-dimensional structures in which the reactive hydrophobic amino acid side chains are buried in the interior of the protein, leaving most of the polar side chains to the exterior (Kinsella and Whitehead, 1989). BLG is a major whey protein and its concentration in cow milk is about 3.2 g.l⁻¹ (Cayot and Lorient, 1998). This protein has been used in many studies, the main reason being stability of this protein in solutions at large range of pH (2-9) and temperatures (Aguilera, 1995).

BLG

BLG was among the first proteins to be crystallized, and since crystallizability was long considered a good criterion of homogeneity, BLG, which is a typical globular protein, has been studied extensively and is very well characterized (Kauffmann *et al.*, 2001).

Table 8 Amino acid composition of the BLG in the milk (Fox and McSweeney, 1998)

Amino acids	ASP	Asn	Thr	Ser	Glu	Gln	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Trp	Lys	His	Arg	Total residues
Number	11	5	8	7	16	9	8	3	14	5	10	4	10	22	4	4	2	15	2	3	162

Four genetic variant of bovine BLG designated A, B, C and D, have been identified in bovine milk. BLG is a small globular protein with 162 amino acids and soluble in water. Total volume of this protein in solution is around 21700 Å. The amino acid composition of BLG is presented in Table 8. It is rich in sulphur amino acids that give it a high biological value. It contains 2 moles of cystine and 1 mole of cysteine per monomer of 18 kDa. The cysteine is especially important since it reacts, following heat denaturation, with the disulphide of κ -casein and significantly affects rennet coagulation and the heat stability properties of milk. BLG exist in 4 structures. First, primary structure consisting of 162 residues per monomer. Secondary structure of BLG consists in 10-15 % α -helix, 43 % β -sheet and 47 % unordered structure, including β -turns. The tertiary structure of BLG has a very compact globular structure in which the β -sheets occur in a β -barrel-type structure or calyx (Figure 4). Each monomer exists almost as a sphere with diameter of about 3.6 nm. The last structure of BLG has a quaternary structure as explained by Fox and McSweeney (1998).

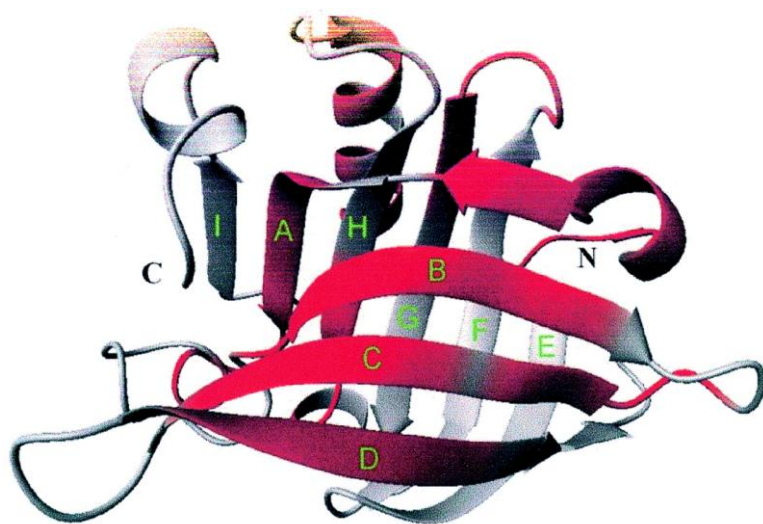


Figure 4 Backbone representation of BLG-A. Regions with high helical preference are red

BLG-A structure in aqueous solution consists of nine antiparallel β -strands (51 %) and one α -helix (7 %) in its native state (Figure 4) (Brownlow *et al.*, 1997). However, this high β -sheet content sharply contrasts with secondary structure predictions for the amino acid sequence that indicate a distribution of 48 % helix and 13 % sheet (Shiraki *et al.*, 1995).

I.4.2.2 Human milk proteins and cow milk proteins

It has long been recognized that the protein concentration of cow milk is much greater than that of human milk. The difference is more than threefold (Table 4). Moreover, caseins predominate in cow milk, whereas whey (the soluble proteins) predominates in human milk. The ratio of caseins to whey proteins is generally stated to be 40:60 in human milk and 82:18 in cow milk (Fomon, 1993). In human milk, nearly all of the casein is β -casein. It is also a major protein in cow milk, but α_{s1} -casein and α_{s2} -casein account for more than half of the caseins in cow milk. In general, bovine caseins are more highly phosphorylated than human milk casein (Gurr, 1981). As Table 4 shows, the major whey proteins of human milk are α -la, immunoglobulins, and lactoferrin, whereas the major whey protein of cow milk is BLG.

I.4.2.3 Protein hydrolysates

The first foreign proteins encountered by infants are usually cow milk-derived proteins. When exclusive breastfeeding is not possible, mother's milk must be supplemented or replaced with bottle feedings, which are often based on unhydrolyzed cow milk formulae. This results in extremely high exposure to cow milk proteins. Mother's milk is the natural nutrition for all newborn infants and usually contains low levels of food allergens from the mother's diet, including BLG, the main allergen in cow milk (Silvy, 1989). Unmodified cow milk itself contains more than 32 proteins of high allergic potential that may induce cow milk protein allergy (Hersey, 1976, Klausner *et al.*, 2000). It has long been known that the allergic properties of many proteins are decreased by enzymatic hydrolysis with digestive enzymes (Klausner *et al.*, 2000). The resulting free amino acids are not allergenic. However, due to their often-bitter taste (Adler-Nissen, 1986), low lipid emulsifiability, high osmolarity, and high cost (Mahmoud, 1994), they are not suitable for use in normal infant formula.

Extensively hydrolyzed formulae (eHF) are almost allergen free, and most of their residual oligo-peptides have molecular weights below 3,000 Daltons. In animal models, they induce neither sensitization nor tolerance to food proteins (Fessas and Schiraldi, 2000).

There are no major differences in the anthropometric and biochemical parameters of infants fed with partially hydrolyzed formulae (pHF) compared with infants fed with an adapted cow milk or human milk (Decsi *et al.*, 1996, Giovannini *et al.*, 1994, Malet, 1992, Odelram *et al.*, 1996, Rigo *et al.*, 1994). There is also no evidence of major differences

between infants fed with eHF and an adapted cow's milk formula on growth parameters and biochemical parameters, except on protein and nitrogen utilization. Blood urea nitrogen concentrations in some investigations (Giovannini *et al.*, 1994, Rigo *et al.*, 1994) have been found to increase at different time points. Total amino acid concentrations were found to be significantly higher in infants fed with pHF (Rigo *et al.*, 1994) and eHF too (Giovannini *et al.*, 1994). On the whole, available data suggest that there is no evidence that feeding the pHF impairs the growth and biochemical parameters of infants. There is also no evidence of differences between infants fed with eHF and an adapted cow's milk formula and/or human milk on growth parameters and biochemical parameters, except on indices of protein metabolism (Hernell and Lonnerdal, 2003).

Peñas *et al.* (2004) studied the effect of combined high pressure and enzymatic treatment on hydrolysis and immunoreactivity of dairy whey proteins. They showed an important decrease of immunochemical reactivity for the combined treatment of high pressure, pepsin and trypsin, associated with a considerable hydrolysis of α -la and BLG and production of small peptides. Their results indicated that high pressure enhances whey protein hydrolysis, and, depending upon the choice of enzymes, reduces the residual antigenicity of the hydrolysates.

I.4.3 Fats

Fats are an essential part of any milk formula. It provides 50 % of the daily energy requirements (Berger *et al.*, 2000). Fats also carry essential fatty acids and fat soluble vitamins along with providing a full and satisfying feeling in the infant's stomach. Formulas usually contain a combination of vegetable and animal fats to give a ratio of unsaturated to saturated fats similar to breast milk especially in two essential fatty acids, Linoleic acid (C18:2n-6) and α -linolenic acid (C18:3n-3). Breast-milk fatty acids usually contain 8–30 % 18:2n-6, 0.5–2.0% 18:3n-3, 0.5–0.8% 20:4n-6, 0.1–0.4% 22:6n-6, and small amounts of other n-6 and n-3 fatty acids (Innis, 1991). It is now known that the variability in breast-milk n-6 and n-3 fatty acid concentrations is largely explained by variations in the fat composition of the mother's diet (Harris *et al.*, 1984, Henderson *et al.*, 1992, Innis, 1991, Makrides *et al.*, 1996). Greater saturated fat content (which may be animal or vegetable sourced) may reduce calcium absorption (Williams *et al.*, 1970). Breast milk contains 44 % saturated fats, 17.1 % polyunsaturated fat, and 38.9 % monounsaturated fat. It is better to respect these ratios of fats to prevent digestive problems. Long chain polyunsaturated fatty acids (PUFAs) are recent

additions to many formulas. Different types of PUFAs are Omega 6, Omega 3. These are essential for good vision and brain development. PUFAs are particularly beneficial in the first three months of life, after this, the digestive system can manufacture its own. Several sources of 20:4n-6 and 22:6n-3 are currently available for supplementation of infant or other diets. These include fish oils, egg total lipid or phospholipids, and oils derived from microalgal and fungal sources (single-cell triacylglycerols).

Recently, infant formulas contain more vegetable oil than milk fat. The main vegetable oils used are coconut oil, soybean oil, corn oil, palm olein, palm kernel oil, palm oil, high oleic safflower oil, peanut oil and in Europe, low-arucic acid rapeseed oil. Milk fat contain higher level of trans fatty acids (FAs; a negative health factor), higher level of conjugated linoleic acid (a positive health factor), higher levels of short-chain FAs (neutral effect), and higher levels of phospholipids than vegetable oil (Berger *et al.*, 2000). When milk fat is replaced with vegetable oils, formulas contain less short-chain FAs and C18:0 and higher C12:0 and C18:1. Human milk contains a low quantity of short-chain FAs (Jensen, 1996) thus, it is not necessary that the infant formulas contain the short-chain FAs. Yang *et al.* (2003) produced human milk fat substitutes by lipase-catalysed acidolysis of lard with soybean fatty acids in a solvent-free stirred reactor on a gram scale. The human milk fat substitute, based on lard and soybean fatty acids, were suitable for infant formula with respect to fatty acid composition, fatty acid distribution of triacylglycerols, and melting properties.

I.5 Stability of infant formulas

Interactions and reactions are very important in infant food systems. They influence quality, structure and food product processing. Numerous researchers have studied interactions in emulsions and other liquid systems but there are not sufficient studies on interactions and their effects in solid state. There are different types of interactions in the food systems including hydrogen bonds, hydrophobic interactions, Van der Waals and electrostatic interactions as showed in Figure 5. Numerous authors (Atkins, 1994, McClements, 1999) explain the basis of these forces. As shown in figure 5, The hydrophobic interactions appear between nonpolar groupings like the aromatic nuclei of some amino acids, which can form a stacking or the groupings methyls (figure 5, B and C), electrostatic interactions occur between

molecular species that possess a permanent electrical charge, such as ions and polar molecules (Rogers, 1989).

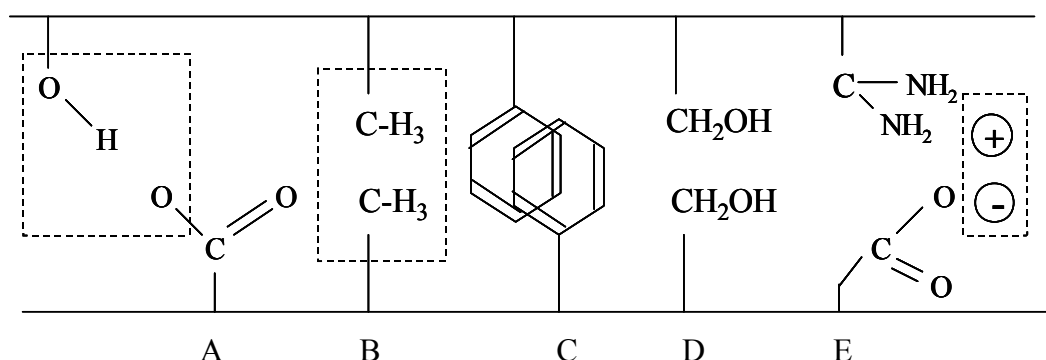


Figure 5 Molecular forces which caused interactions with proteins; A; Hydrogen bonds, B and C; Hydrophobic interactions, D; Van der Waals and E; Electrostatics interactions

Van der Waals forces act between all types of molecular species, whether they are ionic, polar or nonpolar. Hydrogen bonds occur between oxygen or nitrogen and hydrogen. The interactions between two particles depend on the surface to interfacial energy change that occurs when the solids come into contact. The surface energy of a solid is rarely homogeneous. Therefore, the energy change at the true areas of contact will not be a single function of the true area of contact but will vary with the nature of the exact portions of the two solid surfaces in contact (Hiestand, 1966).

I.5.1 Proteins/polysaccharides interactions

Interactions between proteins and polysaccharides have been studied by numerous authors in different systems especially in emulsions and colloids. Solid state interactions are very important to protect the activity and second structure of proteins in pharmaceutical products such as enzymes and hormones. Drying processes like spray drying, drum drying and freeze-drying affect protein structure and nutritional quality of foods. Saccharides protect proteins against some process such as freezing and freeze-drying. Protecting effect of saccharides is clear and has been studied (Carpenter *et al.*, 1998, Dong *et al.*, 1995, Imamura *et al.*, 2003, Souillac *et al.*, 2002). Some saccharides, for example glucose, protect quaternary structure of some enzymes (Anchordoquy *et al.*, 2001). Several conditions are necessary to protect the protein structure by saccharides. First, creation of amorphous sugar network is essential for example mannitol crystallizes during freeze drying and it can not protect the protein structure

such as growth hormone rh-GH (Costantino *et al.*, 1998, Izutsu and Kojima, 2002). Moreover, direct interactions between proteins and saccharides, particularly by hydrogen bonds, are necessary to stabilize the proteins against drying or freezing. It seems that saccharides as lactose interact with polar groups of proteins by hydrogen bonds, and replace water molecules eliminated during drying or freeze-drying (Allison *et al.*, 1999, Arakawa *et al.*, 2001).

Infant formula is very complex. To preserve proteins structure and nutrient bioavailability, it is necessary to optimize the quantity of each component and the process parameters. There is no sufficient information about the effects of interactions on bioavailability of nutrients in infant formulas in the literature. Could it be possible to increase the nutrient bioavailability by interactions control?

Erdogdu *et al.* (1995) studied the interaction between the defatted milk fractions and wheat flour by differential scanning calorimetry (DSC) in solid state and showed that lactose interact with starch and gluten. It increased the endotherm temperatures of gelatinization and enthalpy (ΔH) of starch. Gluten reduces the melting temperatures and ΔH of lactose. Casein did not interact with starches and whey proteins interacted with wheat flour components. The gelatinization temperature of the tailing starch was also (as with prime starch) positively correlated with the lactose concentration. Caseins showed no evidence of interaction with tailings starch.

The chemical modification of proteins by processes such as glycosylation could change their conformational stability because it modifies the balance between the hydrogen bonds, electrostatic and Van der Waals forces. Glycosylation can be an efficient method to increase the hydrophilicity of proteins and modify their solubility, conformational stability and emulsifying properties (Costantino *et al.*, 1998).

I.5.1.1 Effect of interactions on water adsorption behavior

Hydration or rehydration is the first and perhaps most critical step in imparting desired functional properties in food systems. Water interacts with the components and exhibits different properties from those of “free” water (Chou and Morr, 1979). Interactions between water and proteins are important especially when second or tertiary structure of proteins must be kept. Several Researches was carried out to find out the mechanisms of proteins structures

preservation of during drying and after rehydration (Arakawa *et al.*, 2001, Corrigan *et al.*, 2002, Millqvist-Fureby *et al.*, 1999, Thomas *et al.*, 2004b). Various mechanisms have been invoked to describe how sugars improve the stability of lyophilized proteins. For example, Franks (1994) contends that sugars provide a ‘glassy’ matrix in which protein mobility and reactivity are reduced. Another view is that this matrix dilutes the protein in the solid state, reducing intermolecular contacts and thus inhibiting deleterious intermolecular pathways (i.e. aggregation) (Liu *et al.*, 1991).

Costantino *et al.* (1998) studied water sorption behavior of lyophilized protein-sugar systems. They showed that the amount of water adsorbed at the glass transition increases with increasing amount of protein, and proposed that either the sugar was experiencing reduced hydration (due to preferential binding of water by the protein) or protein-sugar interactions did not permit crystallization. In addition, they showed that solid-state interactions between protein and sugar molecules retard sucrose crystallization.

I.5.1.2 Effect of interactions on bioavailability of micronutrients

The interactions between different components of an infant formula reduce absorption and bioavailability of micronutrients. These type interactions affect micronutrients absorption in human by competitive inhibition. Sandström *et al.* (1985) showed that in an aqueous solution, the absorption of zinc is reduced by increasing of iron. However, when iron is added to solid foods or infant formula, no effect on zinc absorption in adults is observed (Davidsson *et al.*, 1995, Sandström *et al.*, 1985).

It was showed by numerous authors that calcium reduces iron absorption in human or animal (Barton *et al.*, 1983, Cook *et al.*, 1991, Dawson-Hughes *et al.*, 1986). Calcium has an important role in nutrition, especially in infant nutrition, and is added to infant formulas. By this finding and important role of calcium in infant nutrition, it is necessary to optimize the quantity of calcium and iron in infant formulas. Wauben and Atkinson (1999) showed that a high calcium diet did not inhibit iron absorption and it can be speculated that there may be an adaptive response to the inhibition of iron absorption by calcium to meet the increased iron needs in the presence of a high calcium diet.

Generally, iron is added to infant formulas and as it was explained, the quantity of iron affect absorption of other micronutrients such as zinc or copper or it catalyses oxidation. Haschke *et al.* (1986) studied the effect of iron quantity (10.2 and 2.5 mg/L) on absorption of nutrients in infant formula. They showed that iron concentration in formula had no effect on absorption and/or retention of nitrogen, fat, calcium, magnesium and zinc. However, other researches showed that iron has a competitive effect on zinc and copper absorption (Sandström *et al.*, 1985). It is necessary to develop some research with real condition to know the interactions between micronutrients and their effects on bioavailability and absorption of micronutrients in infants.

I.5.2 Maillard browning reaction

Past century, food scientists have played a critical role in unraveling the chemistry of the Maillard reaction (Gerrard, 2006), setting the stage for those now focused on the reaction in biological systems. More recently, researchers have isolated and structurally characterized 25 Maillard reaction products from tissues in the body, with more being reported at an increasing rate in the scientific literature (Baynes and Thorpe, 2004). In the medical arena, attention has shifted from an original interest in aging and diabetes, to a broader scope including human health, disease and nutrition. Thus, the boundaries between food scientists and medical researchers are disappearing.

I.5.2.1 Chemistry of Maillard reaction

An outline of the Maillard reaction is given in Figure 6. Maillard reactions have three basic phases. The initial reaction is the carbonyl group condensation of a reducing sugar (aldose) with a free amino group of a protein or an amino acid, which loses a molecule of water to form N-substituted glycosylamine (Step A).

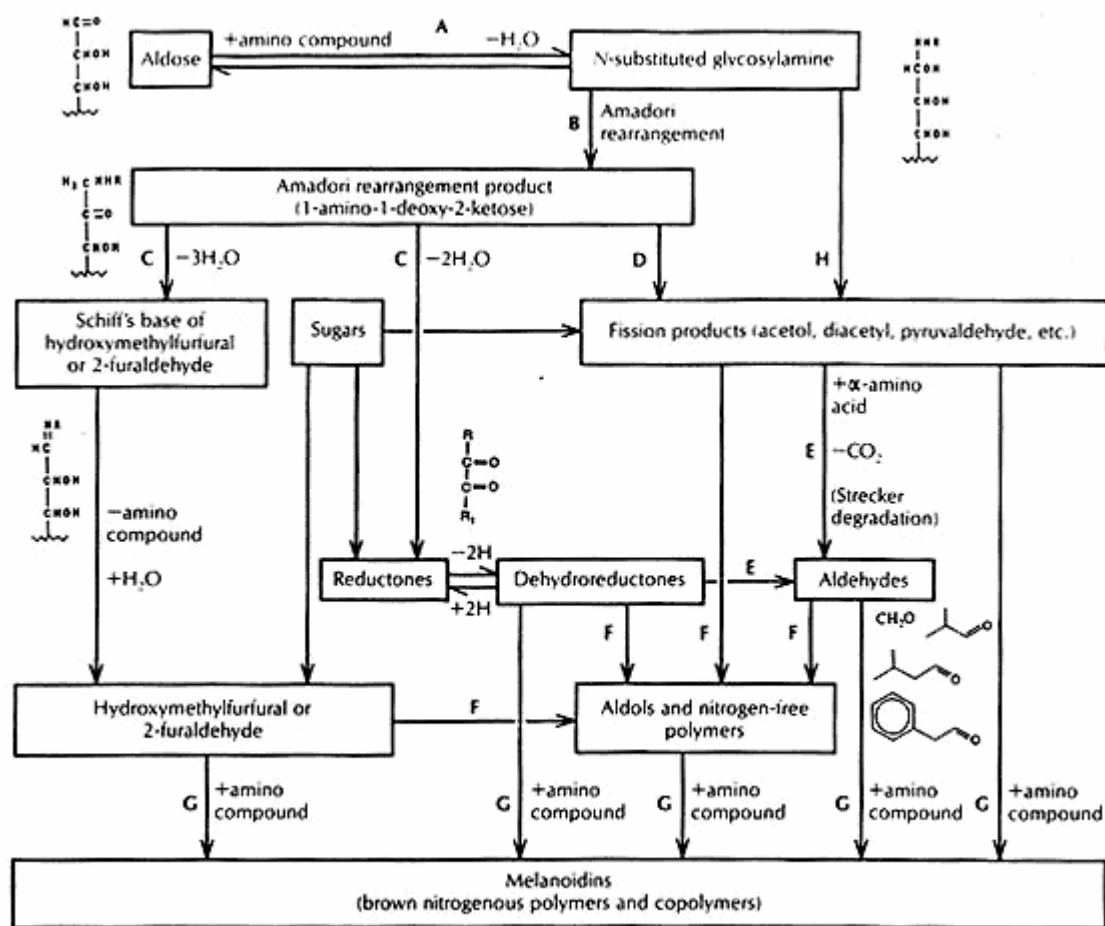


Figure 6 An outline of Maillard reaction adapted from Hodge (1953)

This is unstable and undergoes the "Amadori rearrangement" to form "1-amino-1-deoxy-2-ketoses" (known as "ketosamines") (step B). The ketosamine products of the Amadori rearrangement can then react in three ways in the second phase. One is simply by further dehydration (loss of two water molecules) into reductones & dehydro reductones (step C). These are essentially "caramel" products and in their reduced state are powerful antioxidants. A second is the production of short chain hydrolytic fission products such as diacetyl, acetol, pyruvaldehyde, etc (step D). These then undergo "Strecker degradation" with amino acids to aldehydes (step E) and by condensation to aldols, or they may react in the absence of amino compounds, to give aldols and high molecular weight, nitrogen-free polymers (step F). A third path is the Schiff's base/furfural path. This involves the loss of 3 water molecules (step C), then a reaction with amino acids and water. All these products react further with amino acids in the third phase to form the brown nitrogenous polymers and copolymers called melanoidins (step G). These can be off flavors (bitter), off aromas (burnt, onion, solvent,

rancid, sweaty, cabbage) or positive aromas (malty, bread crust-like, caramel, coffee, roasted). Step H in Figure 6 illustrates a direct route to fission products from N-substituted glycosylamines, without the formation of an ARP (Amadori rearrangement product).

I.5.2.2 Maillard reaction in infant food

Mixing of ingredients, concentration and drying (spray-drying or drum-drying) are the principal processes of preparing infant foods. The two methods of drying (spray and drum) are used in industries but generally spray drying is used to produce infant formulas and drum drying is used for products that are not sensitive to the processing temperature (Desobry *et al.*, 1997). The methods of preparation will be not discussed in this review but only effect of heat treatment on nutritional quality of infant formula will be discussed.

Retort sterilization for liquid products and high temperature short-time (HTST) treatment are used before spray-drying for powder formulas. Ultrahigh temperature (UHT) treated formula has become more commonly used in recent years. This process is very short in duration (3–10 s) at high temperatures (130–150°C). Compared with conventional retort sterilization (118.8°C for 10–15 min), this mild heat treatment has less harmful effects on the digestibility of the protein (Lonnerdal and Hernell, 1998). Several researchers (Huang *et al.*, 1993, Miao and Roos, 2005, Rudloff and Lönnerdal, 1992) showed that the protein digestibility of UHT treated formula is higher than that of powdered and retort-sterilized liquid formulas. Heat treatment has different effect on infant food quality. This process decrease the bioavailability of vitamins and could also reduce the antigenicity of whey proteins considerably (Heppell *et al.*, 1984), but it has virtually no effect on the antigenicity of casein. Therefore heat denaturation alone cannot produce a formula with low allergenicity (Lee, 1992). Despite the little advantage of heat treatment on antigenicity of whey proteins this process has some other disadvantages such as Maillard reaction (reaction of lysine with the dehydroalanine resulting from cystine degradation to form lysinoalanine) (Langhendries *et al.*, 1992), reduction of protein digestibility (Rudloff and Lönnerdal, 1992) and protein solubility, and cooked flavor (Walstra *et al.*, 1999).

Due to the high content of proteins and reducing sugars and its neutral pH, infant food is a very good medium for the occurrence of the Maillard reaction. The early stage of the Maillard reaction has been reported to occur at temperature as low as +5°C and at 45 % RH

(De Block *et al.*, 1998). Jones *et al.* (1998) showed that lactosylation (lactose condensation on amino acid residues) of BLG mainly occurred during the spray-drying step, and during powder storage at 25°C temperature. Important factors that influence lactosylation are the moisture content of input concentrated milk, the inlet and outlet temperatures in the drier and the interactions (Guyomarc'h *et al.*, 2000). By modifying the condition of spray drying, the quality of spray dried milk can improved. Optimum conditions should include a low outlet temperature on the spray dryer with a high inlet temperature to ensure a reasonable drying rate. Preservation of the powder's quality over a long period requires storage at low temperature and low moisture content.

The degree of Maillard reaction is measured as the content of hydroxymethyl furfural (HMF) or other Maillard products such as Furosine (Van Renterghem and De Block, 1996), color and pigments formation (Burin *et al.*, 2000, Morales and Van Boekel, 1998, Schebor *et al.*, 1999) and free monosaccharides (Troyano *et al.*, 1994, Villamiel and Corzo, 2000). HMF is produced during a late stage of Maillard degradation (Friedman, 1996). For milk powder stored at the lower water activities, the color change was less than other conditions (Ahfat *et al.*, 1997).

I.5.2.3 Factors affecting Maillard reaction

Maillard reaction rate is affected by water content and storage temperature, system composition and pH (Buera *et al.*, 1987, Chávez-Servín *et al.*, 2006, Labuza *et al.*, 1970, Roos and Himberg, 1994). Labuza *et al.* (1970) showed that dehydrated foods have a slow browning rate. Maximum browning rate occurred at the medium a_w (around 0.7) or intermediate moisture foods. Moreover, Dattatreya and Rankin (2006) demonstrated that low pH caused increased browning in low a_w sweet whey powder ($a_w=0.182$). At low water content due to low diffusion of moisture, the reaction is controlled and at high water content, the reaction rate decreases due to dilution of the water-soluble reactant. The rate of non-enzymatic browning is extremely low, below T_g because of limited diffusion (Roos and Himberg, 1994) and at temperature more than T_g this rate is higher (Karmas *et al.*, 1992). The diffusion coefficient is inversely proportional to viscosity of system. If the viscosity of system decreases above T_g , the rate of non-enzymatic browning depends on $(T-T_g)$. Karmas and Karel, (1994) showed that browning is retarded by retarding crystallization in the mix systems. However, some other authors showed that retardation of crystallization in a system

containing sugar that is co-lyophilized with polymers and proteins such as growth hormone is not dependent on the increase of the system's T_g (Mazzobre *et al.*, 2001, Stubberud and Forbes, 1998). Thus, there are some others parameters that influence the non-enzymatic browning but their effect was not clearly shown.

I.5.2.4 Effect of Maillard reaction on nutritional quality

Chemical modification of proteins by carbohydrate results in the formation of glycated proteins. These reactions may affect the nutritional and safety of proteins. Maillard reaction decrease nutritional quality of infant foods and the capacity of Maillard reaction products to bind magnesium, inhibit absorption of copper, calcium, and zinc (O'Brien and Morrissey, 1997). Essential amino acid residues of milk proteins especially lysine are less available when linked to lactose and consequently digestibility and quality of milk proteins decrease (El and Kavas, 1997, Friedman, 1996, Guyomarc'h *et al.*, 2000, Langhendries *et al.*, 1992). The availability of lysine decreases during drying process and storage (6 months at 20-30°C), around 14 and 15 % respectively (El and Kavas, 1997). Anderson (1980) compared biological availability of lysine in two different drying methods. The skim milk was dried by roller dryer had less lysine availability than spray dried skim milk. Evangelisti *et al.* (1994) found that for infant formulas, the quantity of lysine blocked as lactulose-lysine increased by the lactose to protein ratio. Moreover, lactose and lactulose influence absorption of calcium by infants. Lactose enhances diffusion rather than the active component of calcium transport, but lactulose enhances calcium absorption more intensively (Brommage *et al.*, 1983). This effect is more important in the liquid infant formulas than powder infant formulas. As numerous authors showed (Langhendries *et al.*, 1992, López-Fandino and Olano, 1999) UHT and especially in-bottle-sterilized infant formulas contain higher levels of lactulose than the corresponding powder infant formulas. Sarriá *et al.* (2001) showed that rats consuming liquid infant formula had a lower calcium intake than those consuming powder infant formulas with the same quantity of calcium. However, O'Brien and Morrissey (1997) showed that Maillard reaction products do not decrease calcium absorption.

Numerous authors studied the effect of Maillard reaction on nutritional quality of different products (Gumbmann *et al.*, 1993) (on casein, glucose and starch). Moreover, Chuyen *et al.* (1991) studied nutritional and physiological effects of casein modified by glucose at 50 °C and 95 °C in aqueous solution. Keyes and Hegarty (1979) studied the effects

of autoclaving of the samples containing lactose, casein and α -la. They showed that casein was less susceptible to damage than α -la. Finot (1990) showed some physiological and pharmacological effects of this reaction on protein, mineral and vitamin. These effects include growth inhibition, protein and carbohydrate digestion, amino acid absorption.

Maillard reaction could increase the allergenicity of some proteins. Toxicological studies have also demonstrated the carcinogenic character of some Maillard degradation compounds (Friedman, 1996). Friedman (1996) reviewed the effect of this reaction on allergenicity and carcinogenicity of product. Due to sensibility of infants, it is better to prevent this reaction in infant formulas by controlling the process and storage conditions.

I.5.2.5 Beneficial effect of Maillard reaction

Besides the disadvantages of Maillard reaction there are some advantages such as the antimutagens compounds (Kada *et al.*, 1985), antioxidants (Hodge *et al.*, 1976) and antibiotics (Einarsson, 1987) formation of antioxidants during Maillard reaction between tryptophan and fructose or glucose (Chiu *et al.*, 1991).

Lactulose is a non-absorbable sugar that does not bind calcium in the small intestine but stimulates the growth of bifidobacteria in the colon of rats and infants, producing short-chain fatty acids by fermentation, which may favor calcium absorption (Trinidad *et al.*, 1996). This effect seems to be specific for calcium because it has not been observed for other elements (Pérez-Granados *et al.*, 2000, Sandström *et al.*, 1983).

I.5.2.6 Effect of Maillard reaction on functional properties

Protein glycation is condensation of a reducing sugar on the amino groups of proteins. It has been demonstrated that glycation of whey proteins could improve their original functional properties (Chevalier *et al.*, 2002, Chevalier *et al.*, 2001). Chevalier *et al.* (2001) showed Maillard reaction improved the functional properties of BLG and the nature of sugars is an essential factor for improving the functional properties of glycated proteins. For example, glycation of BLG with arabinose or ribose (the most reactive sugars) improve its emulsifying properties and foaming properties are better when BLG is glycated with glucose or galactose

(moderately reactive sugars). Numerous authors (Friedman, 1996; Thomas *et al.*, 2004b) reviewed effect of Maillard reaction on functional properties of milk powder.

I.5.3 Oxidation

Milk fat or the other fats are used in infant formulas deteriorate by oxidation of their unsaturated fatty acids. The rate of oxidation is affected by composition of fatty acids, degree of unsaturated fatty acids, presence of antioxidants and process and storage conditions such as temperature, humidity and light exposure. Lipid oxidation is an autocatalysed free radical chain reaction, which is normally divided into three phases: initiation, propagation and termination. The first step is abstracting a hydrogen atom from fatty acid, forming a fatty acid free radical. The second step is the peroxy radical may obtain hydrogen, forming a hydroperoxide and another free radical, which continues the reaction (Fox and McSweeney, 1998). The hydroperoxides are unstable and may break down to various products, including the off-flavors. Baldwin and Ackland (1991) studied the effect of process parameters on storage stability of milk powder. They showed higher preheat treatment increased anti oxidant effect during storage of whole milk powder. Heating of milk leads to denaturation of whey proteins, especially BLG, thus exposure of sulfhydryl (SH) groups which can react with free radicals of the unsaturated fatty acids resulting in a decrease of oxidation rate (Van Mil and Jans, 1991). Reducing the oxygen content in the package prevents milk fat oxidation. As it was explained, in human milk, around 55 % of fatty acids are unsaturated and among this quantity more than 17 % of fatty acids are polyunsaturated and this ratios are respected in infant formulas. Thus, infant formulas are sensitive to oxidation and must be protected against oxidation.

I.5.3.1 Factors affecting rate of oxidation

Oxygen, light exposure, storage temperature, water content, percentage of unsaturated fatty acids and process parameters are the most important factors that affect oxidation. It was shown by numerous authors that if oxygen in the milk powder or infant formulas packages is replaced by nitrogen and CO₂ the oxidation is not detectable and the peroxide value does not increase (Van Mil and Jans, 1991). Oxidation increases during storage for example whole milk powder have a maximum shelf life of 6 months (Anon, 1989) at room temperature.

However, it was found that whole milk powder could have a shelf life in excess of 12 months if packed in cans under vacuum or an inert gas such as nitrogen to inhibit the development of off-flavors (Kieseker and Aitken, 1993).

I.5.3.2 Effect of oxidation on nutritional quality

To improve the nutritional quality, the long-chain polyunsaturated fatty acids (PUFA) are added to infant formulas. PUFA may be more susceptible to oxidation and consequently degradation of vitamin E under certain conditions (Decsi and Koletzko, 1995, Koletzko *et al.*, 1995). For example, several studies showed that fish oils containing highly unsaturated ω -3 PUFA reduce vitamin E plasma concentrations (Muggli, 1989, Nair *et al.*, 1993). Docosahexaenoic acid (DHA) is the essential structural ingredient of breast milk lacking in infant formulas.

Iron is added to infant formulas for anemia problems in infants and it catalyses oxidation of fatty acids. The risks and benefits of adding iron to infant formulas are not clear. Raghuveer *et al.* (2002) studied the different quantity of iron in infant formula and they showed oxidation and oxidation products increase by increasing the quantity of iron. Other research showed that the quantity of iron has no demonstrable impact on anemia of prematurity occurring in the first months of life (American Academy of Pediatrics Committee on Nutrition, 1999) thus it was proposed to decrease the quantity of iron from 12 to 8 mg/L (Hertrampf *et al.*, 1998).

I.5.4 Mechanical and physico-chemical properties of infant foods

A large amount of infant foods present on markets are in powder state thus flowability and floodability in these products is very important. An infant food that has not a good flowability is considered as a bad quality product. The factors that affect flowability of any given powder are numerous. They include surface properties, shape and size distribution, moisture content, bulk density, compressibility and porosity, etc (Peleg, 1978). High relative humidity, compaction pressure and small particle size are some of the causes for flow difficulties (Peleg *et al.*, 1973). Agglomeration gathers to collapse, stickiness and caking. In the literature, the authors are sometimes in disagreement on the definition of the terms of

agglomeration because the three phenomena: collapse, stickiness and caking, are very dependent and act often simultaneously on powders.

I.5.4.1 Collapse

Collapse is the term used to describe the loss of structure in a dried matrix and is viewed as flow under gravity, while caking and stickiness are terms used to describe similar phenomena in powders during storage (Tsourouflis *et al.*, 1976). Amorphous materials collapse under gravity when its viscosity gets sufficiently low. Gravimetric methods are good methods for the study of collapse phenomena. Collapse is linked to a decrease in porosity and an increase in density (Lloyd *et al.*, 1996). Collapse is related to glass transition phenomenon and to viscosity changes. Above the glass transition temperature, molecular mobility is greatly increased and many amorphous compounds crystallize when viscosity decreases to a critical value of 10^6 - 10^8 Pa.s where material fluidisation is possible (Bhandari and Howes, 1999, Champion *et al.*, 2000, Downton *et al.*, 1982). At 5 gH₂O/100 g dry matter, many food powders have a T_g close to or below room temperature (Roos and Karel, 1991b). In amorphous dry foods, this leads to rapid collapse of structure (Thomas *et al.*, 2004b).

I.5.4.2 Stickiness and Caking

Infant foods and milk powder are hygroscopic and adsorb water during their preservation cause stickiness and caking. Lactose releases water during crystallization and cause stickiness and caking in products containing large quantity of lactose. Water on particle surface decreases local viscosity and favor particle cohesion (Lloyd *et al.*, 1996, Prentice, 1992, Rennie *et al.*, 1999). Water forms interparticle liquid bridges, which is fifty times more important than Van der Waals interactions force (Aguilera *et al.*, 1995, Netto *et al.*, 1998). The molecular diffusion and the crystallization of lactose are more important especially on the level of the liquid bridges. Crystallized lactose between particles could also form solid bridge, which increase caking in powders (Özkan *et al.*, 2002, Özkan *et al.*, 2003).

I.5.4.3 State diagram

The state diagram gives some important information about state, stability and other physical properties of powders. The simple state diagram shows the physical state of food materials as a function of concentration and temperature (Roos and Karel, 1991a). These state diagrams describe the effect of food composition on stability, show the effect of temperature and moisture content on material characteristics and they show the formation of the amorphous state in food processing (Roos, 1995a). State diagrams are not available for infant foods because of different formulations that exist on market, but as it was explained, the main components of infant food formulations is milk powder, starch, maltodextrins, sucrose and some other micro elements. The state diagram of milk powder is therefore useful. Figure 7 shows the state diagram of milk powder.

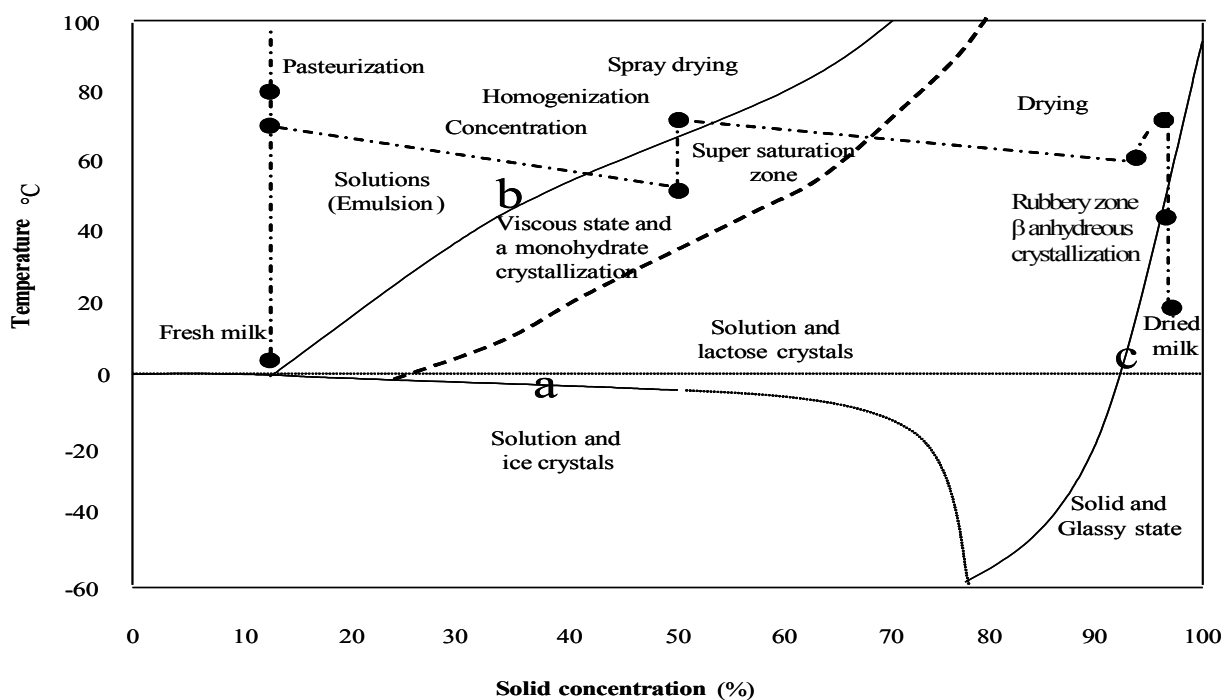


Figure 7 Simplified state diagram of model milk powder (Lactose and water state changes). a is the freezing curve of water, b is lactose solubility curve adapted for milk, c is lactose glass transition temperature (T_g) curve.

Adapted from Vuataz, (1999)

In this figure, there are three curves illustrating freezing (a), lactose solubility that was adapted for whole milk by the interactions of water with all solids without fats and crystallized materials (b), and glass transition temperature curves (c). The second zone is the most important zone of this figure since lactose has a risk of crystallization and it is very important in drying process of milk. Lactose crystallization needs time because there is a lactose nucleation and growth kinetic (Hartel and Shastry, 1991). Moreover, lactose crystals exist in two forms of α and β with different solubility, which change the solubility, curves.

In this diagram, dash lines show the spray drying process. The first stage is pasteurization, afterwards milk is concentrated until 50 %, and product temperature is decreased to the viscous state and crystallization zone (less than 50°C) for lactose crystallization. In spite of this stage, lactose is never crystallized at 100% by spray drying method (Jayaprakasha *et al.*, 1995). Lactose crystallization during storage causes serious problems such as collapse and caking. The last part is drying that is situated at the viscoelastic and β lactose crystals formation zone. When milk powder temperature decreases, it comes in the solid glassy state. This zone is stable and lactose state does not change.

State diagram could be used for predicting the storage conditions. This diagram shows critical values for a_w and water content that could be used in selecting packaging materials and storage conditions (Roos, 1995a).

I.5.4.4 Glass transition temperature

A glass is a supercooled liquid whose viscosity is greater than 101 Pa.s (Sperling, 1986). Due to its high viscosity, it exists in a metastable solid state, in which it is capable of supporting its own weight against flow due to the force of gravity. Ferry, (1980) stated that glass forms when a typical liquid, with a disordered molecular structure, is cooled to a temperature of about 100 °C below the equilibrium crystalline melting temperature or freezing point, at a rate sufficiently high to avoid crystallization. The transition from the liquid state to the glassy state is characterized by discontinuities in certain physical properties, such as coefficient of expansion, free volume, dielectric constant, viscosity, while other properties change in a more or less smooth manner (White and Cakebread, 1966). Figure 8 summarizes these changes.

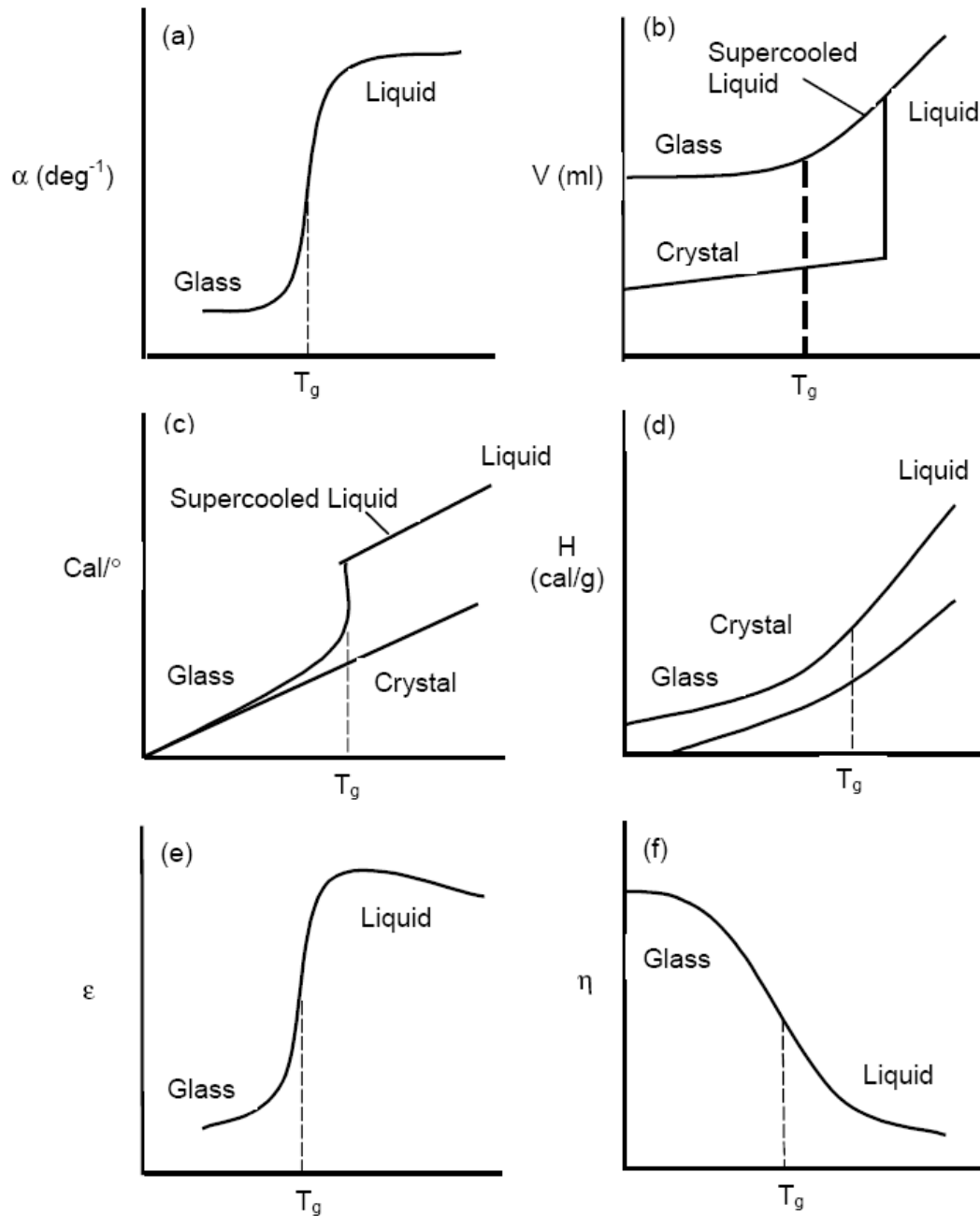


Figure 8 Characteristics of transition from liquid to glass (a) Coefficient of expansion, (b) volume, (c) specific heat, (d) heat content, (e) dielectric constant, and (f) viscosity. As presented by White and Cakebread, 1966

In complex foods, T_g is usually not a discrete temperature and may have an onset to end point range of 20°C. The glass transition is characterized by a sharp drop in the storage modulus (a measure of elasticity) over this temperature range. Figure 9 shows the relationship between storage modulus and temperature for polymers. Qualitatively, the glass-rubber transition

region can be interpreted as the onset of long range, coordinated molecular motion (Sperling, 1986).

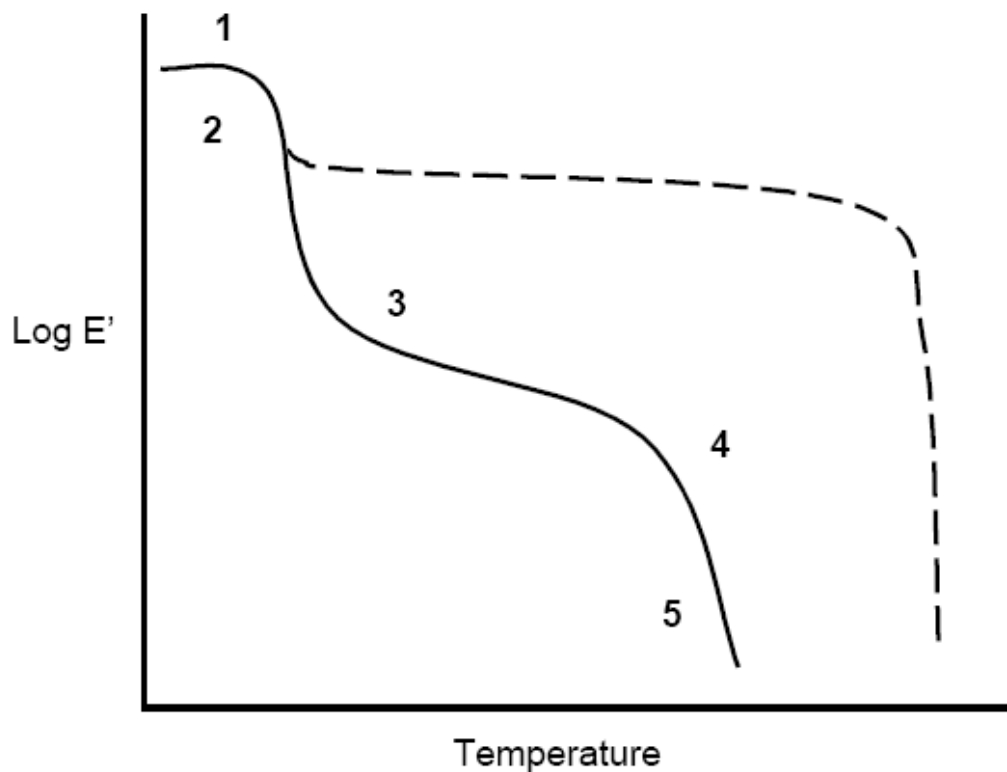


Figure 9 Five regions of viscoelastic behavior for a linear, amorphous polymer. Also illustrated are effects of crystallinity (dashed line) (Sperling, 1986). (1) Glassy region, (2) Glass transition region, (3) Rubbery plateau region, (4) Rubbery flow region, (5) Liquid flow region

The effect of molecular weight of polymers on their glass transition has been studied for a long time. It was shown that the glass transition temperature increases with increasing molecular weight (Roos, 1995b). With increasing chain length of the polymer, the free volume decreases and consequently, the glass transition temperature increases. On the other hand, addition of low molecular weight components would increase the free volume, and hence reduce the glass transition temperature (Slade and Levine, 1991).

Glass transition can be observed experimentally by measuring physical, mechanical, electrical, or thermodynamic properties of the polymer as a function of temperature. As has been mentioned earlier, a discontinuity in several physical properties is seen during glass transition (Sperling, 1986, White and Cakebread, 1966). Among the methods commonly used,

thermal methods that measure the change in enthalpy seem most popular. Differential Thermal Analysis (DTA) and Differential Scanning Calorimetry (DSC) dominate this field. DSC has been used by several researchers to determine T_g of several food powders and other food products (Chuy and Labuza, 1994, Jouppila and Roos, 1994). Figure 10 shows a typical thermogram obtained from DSC.

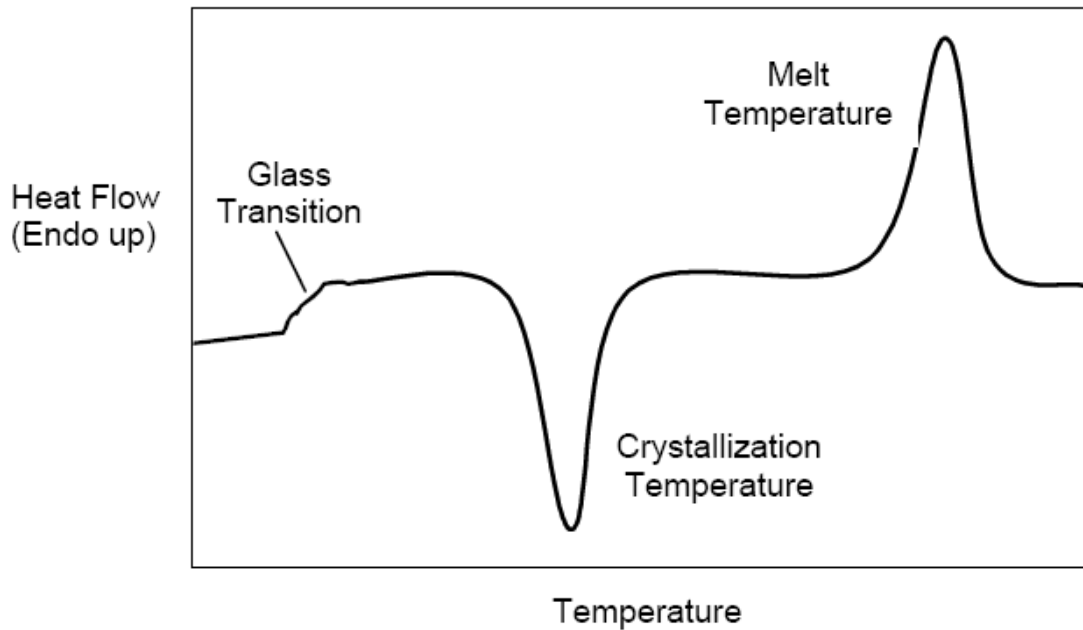


Figure 10 Typical DSC thermogram of a partially crystalline material

Infant foods are the mixture of different components thus: it is very difficult to determine the T_g of this mixture. There are a number of relationships that describe the T_g of mixed systems. For example, Couchman and Karasz, (1978) proposed a relationship for binary systems:

$$T_g = \frac{w_1 \Delta C_{p1} T_{g1} + w_2 \Delta C_{p2} T_{g2}}{w_1 \Delta C_{p1} + w_2 \Delta C_{p2}} \quad (1)$$

The glass transition of the mixture, T_g is related to the glass transition temperatures of the individual components and heat capacity increment at glass transition, ΔC_{pi} , where w_i is the mass fraction of the i^{th} component.

Chapter I: Literature Review

A ΔC_p of $1.94 \text{ J g}^{-1} \text{ }^\circ\text{C}^{-1}$ and $0.52 \text{ J g}^{-1} \text{ }^\circ\text{C}^{-1}$ is generally used for pure water and lactose respectively (Kedward *et al.*, 2000). Kalichevsky and Blanshard (1993) extended the Couchman-Karas equation for modeling plasticization of a three-component system (Equation 2) containing amylopectin, sugar and water. Similarly, it is possible to model plasticization of milk powders as a three components system of milk protein-lactose-water. However, T_g values for pure dry milk protein is required and no such data is available in the literature. It must be noted that the Couchman-Karas equation reduces to the empirical Gordon-Taylor equation when $k = \Delta C_{p2}/\Delta C_{p1}$.

$$T_g = \frac{w_1 \Delta C_{p1} T_{g1} + w_2 \Delta C_{p2} T_{g2} + w_3 \Delta C_{p3} T_{g3}}{w_1 \Delta C_{p1} + w_2 \Delta C_{p2}} \quad (2)$$

Also the Gordon-Taylor equation that was proposed by Gordon and Taylor (1952) for a binary mixture:

$$T_g = \frac{w_1 T_{g1} + K w_2 T_{g2}}{w_1 + K w_2} \quad (3)$$

Where T_g is the glass transition temperature of the mixture, T_{g1} and T_{g2} are the glass transition temperature of components 1 and 2, w_1 and w_2 are the weight fractions of components 1 and 2, and k is a constant.

The T_g of milk proteins are not typically measured in anhydrous state but they are determined for α -casein with 20% water to be 8°C and for β -casein at relative humidity of 0.11 to be 120°C (Mauer *et al.*, 2000, Mizuno *et al.*, 1999). Matveev *et al.* (1997, 2000) revealed that the T_g of proteins are based on the structure and additive contribution of proteins. They calculated the T_g of some dry proteins such as α -casein = 165°C , β -casein = 164°C , α -lac = 151°C , BLG = 155°C and κ -casein = 164°C .

The T_g Values could be used in state diagram for predicting the system state as it was explained for predicting the storage conditions.

I.6 Lactose crystallization during storage

The amorphous state of sugars and other components in foods is important for their stability and functionality. Amorphous foods can pick up moisture and go from the metastable glassy state to the rubbery state followed by crystallization (Saltmarch and Labuza, 1980, Slade and Levine, 1991, White and Cakebread, 1966). The crystalline state is thermodynamically favored as it has a lower free energy due to the structured arrangement of the molecules. Crystallization of sugars in food products leads to several undesirable effects such as graining in confectionery and hardening of soft cookies (sucrose crystallization), poor reconstituability of milk powders and sandiness in ice cream (lactose crystallization), staling of bread (starch recrystallization) etc. Several researchers have studied the occurrence of crystallization with respect to glass transition (Roos and Karel, 1992, Slade and Levine, 1991). Though the overall mechanism of crystallization is known, not much work has been done pertaining to nucleation and crystallization kinetics of sugars in foods. Several theories have been developed to understand the mechanism of crystallization and predict the extent of crystallinity. It must be kept in mind that most of these theories were developed for crystallization in bulk polymers and have been adapted by food scientists to sugars and other carbohydrates.

During storage of powder containing lactose, lactose has an important role for physicochemical changed and stability. In our study, the model infant formulations were prepared by freeze-drying method to obtained and study the amorphous state of lactose and its effect on physicochemical properties of formulations. In this part, the theories of crystallization in general, crystalline form of lactose and crystallization process are explained.

I.6.1 Crystallization Models

I.6.1.1 Avrami model

In all phase transformations, the new phase is nucleated by tiny “germ-nuclei” which are already present in the old phase and whose effective number can be altered by temperature and the duration of supercooling. Germ nuclei of different sizes will have different rates of

transition into the new phase. Earlier theories of crystallization kinetics such as Mehl-Johnson assumed a temperature dependent constant rate of spontaneous nucleation of the new phase proportional to volume of the untransformed region. Avrami, (1939) found the assumption of constant rate of nucleation contrary to experimental data. As transformation proceeds, the rate of nucleation decreases much faster than in proportion to the volume of the unchanged phase. He developed a model that quantitatively accounted for many phenomena seen in phase changes (Equation 4). A special case of the model was a constant rate of nucleation, which accounted for the Johnson-Mehl and other similar models.

$$\theta = 1 - e^{-kt^n} \quad (4)$$

Where θ is crystallinity, t is time, k is a rate constant, and n is the Avrami exponent.

Sperling (1986) presented a simple analogy to visualize the Avrami model. He considered the whole process similar to rain drops falling in a puddle. This produces expanding circles of waves that intersect and cover the whole surface. The drops could fall either sporadically or all at once, striking the puddle at random points. The expanding waves are the growth fronts of the crystal and the points of impact are the nuclei. If two drops fall too close to each other, then the waves that originate from them will intersect and will be prevented from further expansion.

I.6.1.2 Hoffman equation

The Avrami equation provides useful data on the overall kinetics of crystallization. However, it fails to give any insights on the molecular organization of the crystalline regions and the structure of the growing crystallites. Hoffman's theory of nucleation deals with the kinetics and thermodynamics of growth mechanisms as well as the morphology of crystalline regions (Hoffman, 1983). This theory accounts for the different temperature dependence associated with transport of the molecules to the crystallization interface and their adhesion to the crystal surface. The equation is most applicable at temperatures close to but below the melting point.

I.6.1.3 Williams-Landel-Ferry (WLF) model

Although the WLF model was not developed for crystallization, several scientists (Roos and Karel, 1990, Slade and Levine, 1988) have applied WLF kinetics to crystallization. It is based on the assumption that crystallization depends on the viscosity of the polymer, which in turn can be described by the WLF equation. Glass transition is a second order phase change where a solid goes from a metastable amorphous glassy state (characterized by viscosity $> 10^1$ Pa.s and hence low molecular mobility) to the rubbery state characterized by lower viscosity and increased molecular mobility (Sperling, 1986). A single empirical function can be used to describe the temperature dependence of all mechanical relaxation processes above the glass transition temperature (Williams *et al.*, 1955). Earlier work had suggested that the ratio a_T of any mechanical relaxation time at temperature T to its value at a reference temperature T_0 appeared to be identical over a wide range of time (Ferry and Fitzgerald, 1953). Essentially, a_T reflects the temperature dependence of segmental mobility in a polymer on which the rates of all configurational rearrangements depend. If a suitable reference temperature T_s is chosen for different polymer systems, and a_T is expressed as a function of $T-T_s$, the function was found identical for several systems. It was found that this temperature dependence might also be extended to organic low-molecular weight glass-forming liquids such as sugars. Since T_s was chosen arbitrarily for each polymer, Williams *et al.* (1955) suggested the use of T_g , the glass transition temperature which was an independent physical measurement. However, T_g data depends on thermal history and is subjective depending on the measurement technique. In addition, determination of relaxation properties such as viscosity may be difficult near T_g . Hence, the authors decided to retain T_s as a reference temperature. T_s is usually chosen as $T_g + 50$ °C.

The empirical formula used in the WLF model using T_g as a reference temperature is:

$$\log a_T = \frac{17.44(T - T_g)}{51.6 + (T - T_g)} \quad (5)$$

Peleg, (1992) studied the validity of the WLF model with fixed universal constants and T_g and compared it to the original form with variable coefficients and T_s as reference temperature. Particularly in the literature found on food components and sugars, the two versions were

used interchangeably although they are not mathematically identical. It was concluded that there was considerable discrepancy in prediction using the two versions of the equation. Due to the hyperbolic form of the equation, Peleg (1992) stated that the WLF model should be used only when the temperature range being studied is large enough for the curvature in the function to be noticed. For the narrow temperature range that is of interest in foods, especially around the reference temperature (T_g or T_s), linear regression gave good fit for the data.

The WLF model was found to hold only within the temperature range between T_g and $T_g + 100$. Below T_g , $\log a_T$ increases less rapidly with decreasing temperature than as indicated by the equation. Above $T_g + 100$, the temperature dependence of viscosity for ordinary liquids varies widely due to differences in molecular structure. Supercooled liquids, within 100°C above their vitrification point show abnormally high temperature dependence of relaxation properties independent of their molecular structures and hence make the WLF equation universal in this temperature range.

Figure 11 illustrates the classical theory of crystallization kinetics applied to partially crystalline polymers (Wunderlich, 1976). Slade and Levine (1991) found that this theory also described the recrystallization of starch. The temperature dependence of both nucleation and crystal growth and the overall effect of temperature on the rate of crystallization can be seen. As it was explained, rates of crystallization are negligible below T_g . This is due to the high local viscosity, which does not allow translational or orientational mobility for nucleation to take place. Crystal growth also does not occur below the T_g because it is diffusion limited.

Above the melting temperature T_m , it should be obvious that rates of nucleation and propagation would be zero. Though the maximum overall growth rate curve appears symmetric, the maximum is usually closer to T_g than T_m . This indicates that the crystallization processes in these cases are limited by nucleation. The maximum is usually reached at about $T_g + 50$ for sugars. WLF kinetics may be applied to crystallization processes in the temperature range between T_g and T_m . A striking difference in the behavior of simple sugar mixtures and semicrystalline polymers lies in the effect of supercooling ($T - T_g$). While sugars crystallize at high rates close to T_m , the rate of isothermal crystallization is high close to the T_g in polymers (Arvanitoyannis and Blanshard, 1994). This may indicate the nature of the overall crystallization process in the two systems as being limited by the rate of crystal growth and rate of nucleation respectively. Further work is warranted on the crystallization of natural and synthetic polymers in order to understand the similarities or differences in crystallization mechanisms.

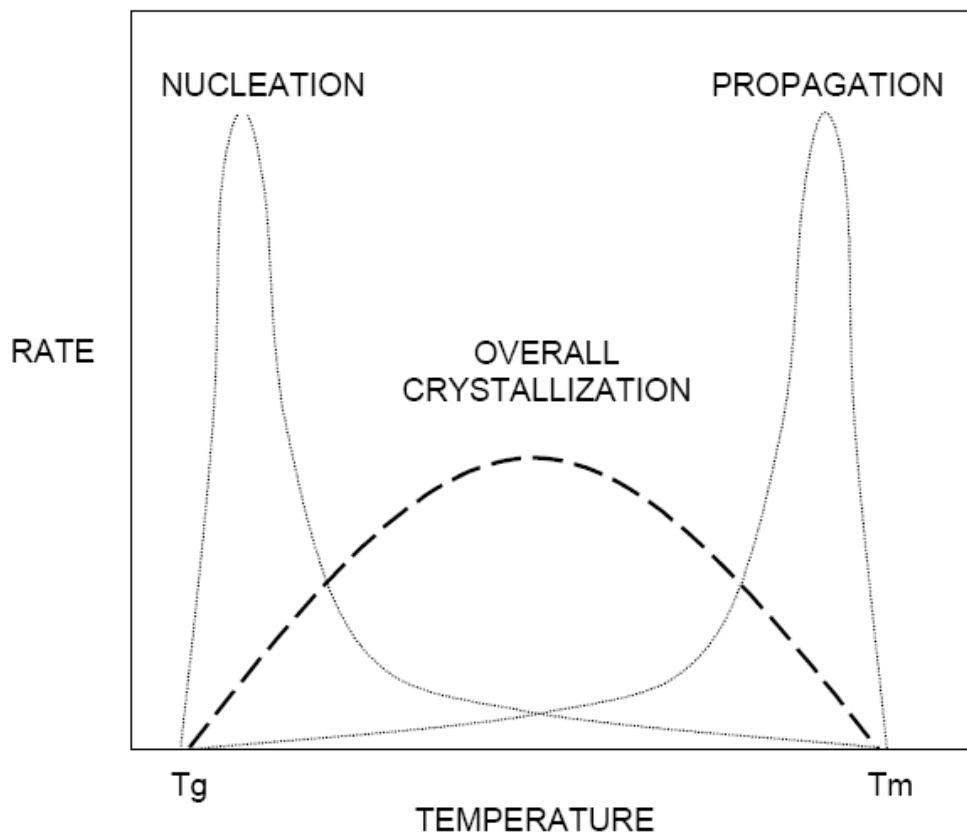


Figure 11 Effect of temperature on crystallization (Slade and Levine, 1991)

Several researchers have modeled time to crystallization of lactose based on WLF kinetics (Arvanitoyannis and Blanshard, 1994, Roos and Karel, 1992). The WLF model is a useful tool for predicting time to crystallization. However, the model is empirical in nature and does not reveal anything about the mechanism of nucleation or crystal growth. Therefore other models that offer insights into the crystallization mechanism are often more useful.

Roos and Karel (1992) stated that crystallization of sugars was independent of the moisture content, but depended on the temperature above the glass transition ($T - T_g$) which depended on the extent of plasticization. They stored lactose at constant relative humidities and constant moisture content. At constant moisture content (resembling a sealed can or package), crystallization of amorphous lactose released water, which was adsorbed by the remaining amorphous material. This further depressed the T_g and resulted in an autocatalytic reaction with more lactose being crystallized. When the samples were incubated at constant relative humidities, the moisture content in the amorphous matrix was constant and hence T_g was constant. The rate of crystallization was found to increase with increasing $T - T_g$ and was modeled by the WLF equation.

I.6.2 Crystalline forms of lactose

Different forms of crystalline lactose exist with different physicals and thermal properties. Many researchers studied the crystallization of lactose in solutions (Garnier *et al.*, 2002a, Garnier *et al.*, 2002b, Smart, 1988) and in powder state (Jouppila *et al.*, 1997, Vuataz, 1999, Vuataz, 2002). Crystalline forms of lactose depend on the preservation time and many other conditions such as humidity, storage temperature and preparation process. It is difficult to compare the results of different authors that they studied different forms of lactose crystals just after crystallization (Sebhatu *et al.*, 1994) or after some days of storage (Jouppila *et al.*, 1997). Angberg (1995) and Vuataz (2002) showed that mutarotation could change the ratios between α , β forms of lactose during storage, and it depends on the relative humidity. Figura (1993) also showed the mutarotation could appear in amorphous state too.

Numerous methods exist to determine the characteristic of lactose such as high performance liquid chromatography (HPLC) for quantitative measurement of lactose in liquid dairy products (Drapier-Bêche, 1997), polarimetry and gas chromatography (GC) to measure of α and β forms of lactose (Roetman and Van Schaick, 1975). Others techniques such as Differential Scanning Calorimetry (DSC), Infrared spectroscopy and X-ray diffraction are used by numerous authors (Hill *et al.*, 1998, Itoh *et al.*, 1977, Ottenhof *et al.*, 2003, Wolkers *et al.*, 2004).

I.6.2.1 Crystallization process

Amorphous regions in the crystals are generally thermodynamically unstable, meaning they are in a higher energy state than the crystalline form. Within amorphous regions, there is substantial absorption of water vapor, which can cause physical and chemical transitions to occur. If the powder surface is all amorphous, the interactions between the powder and other phases will be different to that which is observed when the materials are in the crystalline state (Buckton and Darcy, 1999). Crystallization in the solid glassy state below glassy transition temperature (T_g) is kinetically inhibited (Roos, 1995b) and this phenomenon causes the most drastic changes to the physical properties of food (Roos, 1995b, Roos and Karel, 1992). It may considerably affect food stability and it may cause impaired rehydration properties of food powders (Roos and Karel, 1992). Lactose crystallization in milk powders leads to increased free fat and flavor deterioration, and may promote non-enzymatic browning

(Kim, et al., 1981). In food formulation, high amounts of high molecular weight compounds are needed to increase T_g (Roos and Karel, 1991b) but crystallization can also be delayed by incorporation of high molecular weight substances (Levine and Slade, 1986) or other sugars (Herrington and Brandfield, 1984). Many factors affect crystallization such as temperature, relative humidity, storage time, and composition of the mixture (e.g. the proteins in milk powder or infant foods). The rate of crystallization increases as the relative humidity during storage increases (Nasirpour *et al.*, 2006). The rate of crystallization could be successfully modeled using the Avrami equation when relative humidity is $>66.2\%$ and the maximum crystallization occur at a relative humidity of 70 % (Jouppila *et al.*, 1997). As many researchers showed lactose crystallization, occurs above glassy transition temperature (T_g) (Kedward *et al.*, 1998, Roos, 1995b). If the storage temperature is higher than the T_g , crystallization may occur as molecular mobility and diffusion increase and as viscosity decreases exponentially above T_g (Slade and Levine, 1991). Crystallization temperature of lactose and various type of milk powder as a function of storage temperature, often described as difference between storage temperature and glass transition temperature ($T-T_g$). (Roos and Karel, 1992). Knudsen *et al.*, (2002) studied the induction and kinetics of crystallization lactose in infant formula and whole milk. They showed that induction time for infant formula and milk powder is longer than that of lactose and the difference is due to adsorption rate difference. Infant formula and milk powder contain protein; crystallization of lactose is retarded due to a competitive absorption of water (Knudsen *et al.*, 2002, Thomas *et al.*, 2004a). Crystallization of lactose impairs the solubility of the powder and often occurs concurrently with various deteriorative changes such as caking, discoloration, loss of lysine and lipid oxidation (Jouppila *et al.*, 1997, Knudsen *et al.*, 2002). Hydrophobic molecules can inhibit crystallization of sugar with the forming of strong hydrogen bonds (Sarciaux and Hageman, 1997). As a result, crystal nucleation and growth could be retarded.

I.7 Justification of the study

Crystallization of amorphous carbohydrates and sugars may occur in a number of low and frozen foods, resulting in serious quality defects. Well-known examples of such foods are dairy powders and ice cream, in which crystallization of amorphous lactose has been found to accelerate chemical reactions and sandiness, respectively. Previous studies have suggested the importance of glass transition temperature in controlling crystallization of amorphous

components and importance of adding protein on lactose crystallization. However, only a few studies have related crystallization delay to protein and polysaccharide in model foods using mixture design.

Several factors affect lactose crystallization such as RH, storage temperature and composition of mixture. In the present study, effect of different storage RH on lactose crystallization and non-enzymatic browning was investigated. This study puts forward the presence of interactions and nature of interactions that influence physico-chemical properties of model foods. In this study, we considered that dairy powders are stored in room temperature. Therefore, it was important to determine all physico-chemical changes at room temperature.

Infant foods were chosen because it contains a large quantity of lactose. BLG was chosen as protein and gelatinized starch as polysaccharide. It is important to note that BLG is the most studied protein in food science. Investigation of crystallization behavior of amorphous lactose has a great practical importance, because this carbohydrate can crystallize in numerous dairy products, affecting their storage stability and quality attributes.

Chapter II: Materials and Methods

II.1 Characterization of the powder and formulations preparation

Powders used in this study were alpha monohydrate lactose (Prolabo France), β -lactoglobulin (BLG) (Davisco, Eden Prairie, MN, USA) and wheat starch (Sigma-Aldrich, St Louis, USA).

II.1.1 Physicochemical properties determination

Several physicochemical experiments were carried out on the commercial powder samples in order to characterize their purities.

Water content

Water content of samples was measured by AOAC 927.05 standard (AOAC, 19995). Approximately 1 g of powder was mixed with Fontainebleau sand that was dried before and placed in the metal cups with 5 cm diameter. The cups were placed in the oven. They were dried at 103°C until weight stabilization of the samples, i.e. 5h for BLG and starch and 6h30 for lactose. After drying, the samples were placed on P₂O₅ for cooling and they were weighed. Water content measurement of lactose is very difficult because of caramelization.

Water activity

The water activity of the powders was measured by putting approximately 1 g of powders at different RH ranging from 11 to 54 at 20°C according to microclimates method (Landrock and Proctor, 1951).

Ash content

The ash quantities of samples were measured by official standard AACC 08-01. 1-1.5 g of samples was placed in an oven (Nagat Trignac, France) at 550°C until C-free samples were obtained. Then samples were cooled in desiccators and weighed.

Fat content

Fat content of samples were measured according to Roese-Gottlieb method using a butirometer.

Mineral content

Sodium, calcium, potassium and magnesium quantities were measured by atomic absorption spectroscopy.

II.1.1.2 BLG purity

Protein content

Total protein of samples was measured using Kjeldahl method. Approximately 1 g of the samples was placed on a digestion tube with 2 ml of concentrated sulfuric acid (H_2SO_4), 1 ml of H_2O_2 and around 1 g of metallic catalyst. The digestion tube is placed into a digestion block where it is heated to the boiling temperature of the mixture. Digestion is usually completed after 2 hours at 370 to 400 °C. Distillation was carried out using a semi automatic instrument (Gerhardt, Paris, France) then protein quantity was determined by titration of ammonia with sulfuric acid 0.01 N. Result was calculated as g nitrogen per 100 g dried sample. Protein quantity of BLG used in this study was around 93.2 %.

BLG content

The BLG used in this study was analyzed at Nestlé research center in Lausanne by HPLC method. The results showed that commercial BLG powder contained around 98.2 % BLG type A and B and a small quantity of α -la (1.6 %).

II.2 Model infant formulation preparation

Model formulations were prepared by mixing 3 major components of infant formulations (lactose, BLG, and starch) according to the European standard for infant formulation (Guidance Note on the Implementation of European Communities (Infant Formulae and Follow-on Formulae Regulations, 1998 to 200), 2001). A mixture design was used to cover the ratios of components in the ranges indicated in the standard (see Table 9).

Chapter II: Materials and Methods

Table 9 Initial experimental area for formulations

Components	Percentage (%w/w)	
	Minimum	Maximum
Lactose	50	80
Starch	10	25
BLG	10	25

Starch is not required in the European standard for infant formulation but is interesting to study, as its nutritional and functional properties have led to its use in some formulations on the market. When starch is present in formulations, precooked or cooked starch must be used; thus, in this study gelatinized starch was used. Gelatinized wheat starch (Sigma Chemical Co., St. Louis, MO) was produced using 5% (wt/wt) solids suspensions. The suspensions were heated to boiling under continuous stirring to obtain gelatinized starch. Full gelatinization was confirmed by polarized light microscopy. After cooling, this solution was mixed with a BLG (Davisco Foods International, Inc., Eden Prairie, MN) solution (10%) and a lactose (Prolabo, Fontenay-sous-Bois, France) solution (15%) at different ratios to obtain the formulations presented in Table 10. After 30 min of mixing, 70 mL of the final mixture was placed into a 500-mL round-bottomed flask and frozen at -40°C for 45 min in a refrigerating bath (Polystat cc241; Peter Huber Kaltemaschinenbau GmbH, Offenburg, Germany). The samples were then freeze-dried (Christ Alpha 1–2; Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 48 h at -60°C and 10 Pa. The water content of all samples was measured after freeze-drying, and all contained less than 2% water (dry basis). The samples were stored for 2 weeks on P2O₅ to complete the drying process. Three samples of each formulation were kept on P2O₅ for 3 months to determine the initial water content of the samples. The amorphous state of powders was checked by differential scanning calorimetry (DSC).

In this study, the co-lyophilized formulations contained 3 components and will be referred to as F-lactose/starch/ BLG (by wt). For example, F80/10/10 means the formulation contained 80% lactose, 10% gelatinized starch, and 10% BLG (by weight).

Chapter II: Materials and Methods

Table 10 Composition of model infant formulations

Formulations	Lactose	Starch	BLG
F80/10/10	80	10	10
F65/10/25	65	10	25
F65/25/10	65	25	10
F50/25/25	50	25	25
F72.5/10/17.5	72.5	10	17.5
F72.5/17.5/10	72.5	17.5	10
F57.5/17.5/25	57.5	17.5	25
F57.5/25/17.5	57.5	25	17.5
F65/17.5/17.5	65	17.5	17.5
F72.5/13.75/13.75	72.5	13.75	13.75
F65/13.75/21.25	65	13.75	21.25
F65/21.25/13.75	65	21.25	13.75
F57.5/21.25/21.25	57.5	21.25	21.25

II.3 Characterization of amorphous lactose

II.3.1 Water sorption

Water sorption properties of freeze-dried materials were determined gravimetrically after storage under various RH condition (Table 11) at 20 °C.

Table 11 Saturated Salts solutions and their corresponding a_w according to Greenspan 1977

Salt	RH %	Salt	RH %
P ₂ O ₅	~ 0	NaBr	59
LiCl	11	KI	69
CH ₃ COOK	23	NaCl	75
MgCl ₂	33	KCl	85
K ₂ CO ₃	43	KNO ₃	95
Mg(NO ₃) ₂	54		

Various RH were achieved in desiccators by using saturated salt solutions. RH was used in the present study to refer to the relative vapor pressure in a salt solution and that of pure water ($a_w = p/p_0$). The relative vapor pressure of water in freeze-dried formulations and in the surrounding atmosphere in the desiccators was assumed to be equal to a steady-state corresponding to a_w of freeze-dried formulations. The RH was considered as being equal to $p/p_0 * 100\%$ (Labuza, 1968).

Powder formulations obtained after freeze-drying were stored for 2 weeks on P_2O_5 to complete drying. Approximately 0.5 g of each formulation was placed at different RHs ranging from 0 to 95% and mass changes was measured after 1, 3, 7 days and each week during 3 months.

II.3.2 Moisture sorption prediction of formulations

There are different models that explain moisture sorption properties of different products such as BET, GAB etc. However, in this research, some mathematical models were developed to predict the moisture sorption of formulations at different RH. Lactose, starch and BLG quantities, and RH were the principal parameters used in these models to predict moisture sorption of formulations. A series of linear models describing the effect of each component on moisture sorption at each RH were found. Effect of interactions on moisture sorption was not significant and this phenomenon will be explained in the results section. A quadratic model predicted the effect of RH and composition of each formulation on moisture sorption properties.

Equation 6 shows the linear*quadratic model which was used to predict moisture sorption of formulation. This model is valid for RH at which lactose was crystallized.

$$Y = AX_{lac} + BX_{starch} + CX_{BLG} + ADX_{lac}X_{RH} + BDX_{starch}X_{RH} + CDX_{BLG}X_{RH} + AD^2X_{lac}X_{RH}^2 + BD^2X_{starch}X_{RH}^2 + CD^2X_{BLG}X_{RH}^2 \quad (6)$$

Where X_{lac} , X_{starch} , X_{BLG} and X_{RH} are lactose, starch, BLG quantities and RH respectively and A, B, C and D are the coefficients.

II.3.2.1 Procedure

Water sorption properties of formulations were determined by gravimetrically methods. The samples were stored on P_2O_5 for 2 weeks to complete the drying process. Triplicate samples with an average sample size of 0.5 g were placed in desiccators containing saturated salt solutions to obtain different RH ranging from 0 to 0.95. The samples were stored at different RH and 20°C. Experiments were carried out one and three days after storage and each week during 3 months.

II.4 Evaluation of crystallization

The occurrence and extent of crystallization was assessed by gravimetric and electronic scanning microscopy methods.

II.4.1 Gravimetric methods

The extent of crystallization was evaluated by monitoring the weight of samples under various RH conditions. The change in sample weight resulting from loss of water can be used as an estimation of the degree of crystallization. Therefore, it is a typical water sorption study, in which sample is stored in desiccators over a salt solution and removed at time intervals, and weighed. The decrease in the amount of sorbed water is an indication of crystallization, and the extent of crystallization can be derived from water loss.

II.4.2 Scanning Electron Microscopy (SEM)

II.4.2.1 Principle

Scanning electron microscope is in principle a microscope generating an electron beam scanning forth and back over a sample. Due to the interaction between the beam and sample, several different signals are produced providing the user with detailed information about the surface structure, differences of atomic number within sample or information about elemental content. Electrons are generated in the electron gun. The most commonly used gun is a tungsten-hairpin gun in which a tungsten filament serves as the source of electrons. By

applying a current through the filament, the tungsten wire will heat up and emission of electrons can be achieved. Generated electrons will be focused in front of an anode. To move the electrons down the column, a voltage difference between the tungsten filament and the anode will be applied. This voltage difference is called the accelerating voltage and can be varied between 0.2 and 40 KV determining the energy and wavelength of the electrons within the beam. The beam of electrons will be condensed and focused as a fine spot on the sample by 2 to 3 electromagnetic lenses located in the microscope column. Two main functions of the first two lenses, condenser lens 1 (C1) and condenser lens 2 (C2), are to control the beam current (number of electrons striking the specimen) and the final size of the area illuminated on the sample (spot size). The third condenser lens (C3) or also called the final lens, will be used primarily to focus the beam of electrons on the surface of the sample. Upon interaction of the electron beam with the sample, various signals (secondary electrons, back scattered electrons and x-rays) are produced and can be detected. The secondary electron detector will detect low energy electrons produced near the surface of the sample providing a predominantly topographical image. The back scattered electron detector will detect high energy electrons that have been scattered backward and providing information about the presence of differences in atomic number of a sample.

II.4.2.2 Procedure

The samples were mounted on double-sided adhesive carbon tabs and were coated with gold using a metal sprayer (Biorad, E-5200, auto sputter coater) and were examined with a scanning electron microscope (Hitachi S2500, Hitachi, Tokyo, Japan).

II.4.3 Melting behavior: Differential Scanning Calorimetry (DSC)

II.4.3.1 Principle

Differential scanning calorimetry is a thermoanalytical technique in which the difference in amount of heat required to increase the temperature of a sample and reference are measured as a function of temperature. Both the sample and reference are maintained at very nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function

of time. The reference sample should have a well-defined heat capacity over the range of temperatures to be scanned. The basic principle underlying this technique is that, when the sample undergoes a physical transformation such as phase transitions more (or less) heat will need to flow to it than the reference to maintain both at the same temperature.

Whether more or less heat must flow to the sample depends on whether the process is exothermic or endothermic. For example, as a solid sample melts to a liquid it will require more heat flowing to the sample to increase its temperature at the same rate as the reference. This is due to the absorption of heat by the sample as it undergoes the endothermic phase transition from solid to liquid. Likewise, as the sample undergoes exothermic processes (such as crystallization) less heat is required to raise the sample temperature. By observing the difference in heat flow between the sample and reference, differential scanning calorimeters are able to measure the amount of energy absorbed or released during such transitions.

Figure 12 shows the thermogram of amorphous lactose obtained by DSC.

As it was explained, it can be seen from Figure 12, the T_g of lactose is at around 100 °C. This was obtained by changing the baseline of DSC lactose curve. When lactose was heated, an exothermic peak was observed at around 170 °C, thus indicating crystallization of amorphous lactose. Melting peak can be seen around 210 and 215 °C as an endothermic peak.

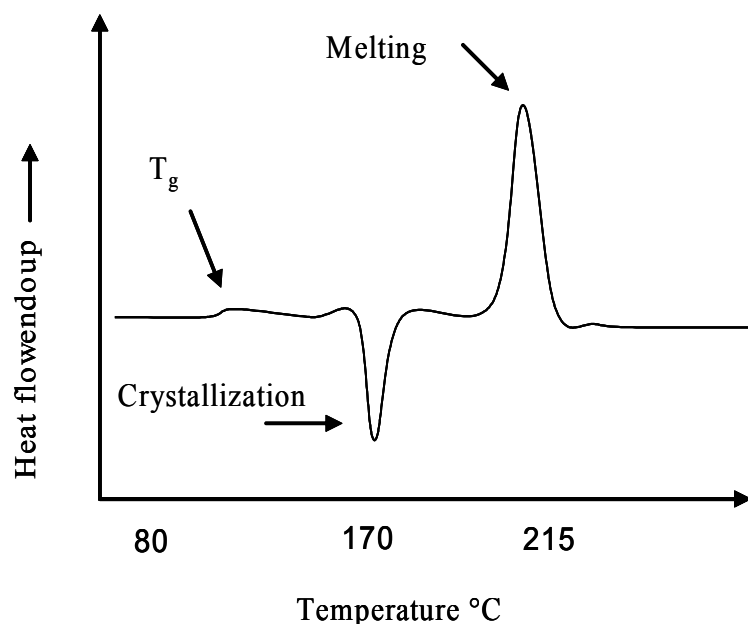


Figure 12 DSC curve of amorphous lactose (from Sebhatu et al 1994)

Figure 13 shows the DSC curve of α -monohydrate crystallized lactose. This thermogram shows an endothermic peak at around 220 °C corresponding to the melting point of α -lactose crystals. The other endothermic peak at around 150 °C corresponded to structured-water, liberated from lactose crystals.

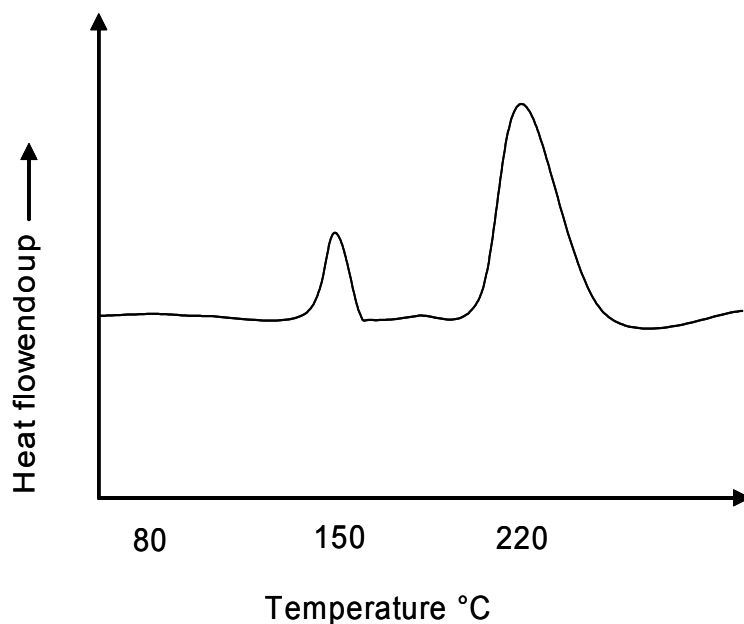


Figure 13 DSC curve of α -monohydrate crystallized lactose from (Sebhatu *et al.*, 1994)

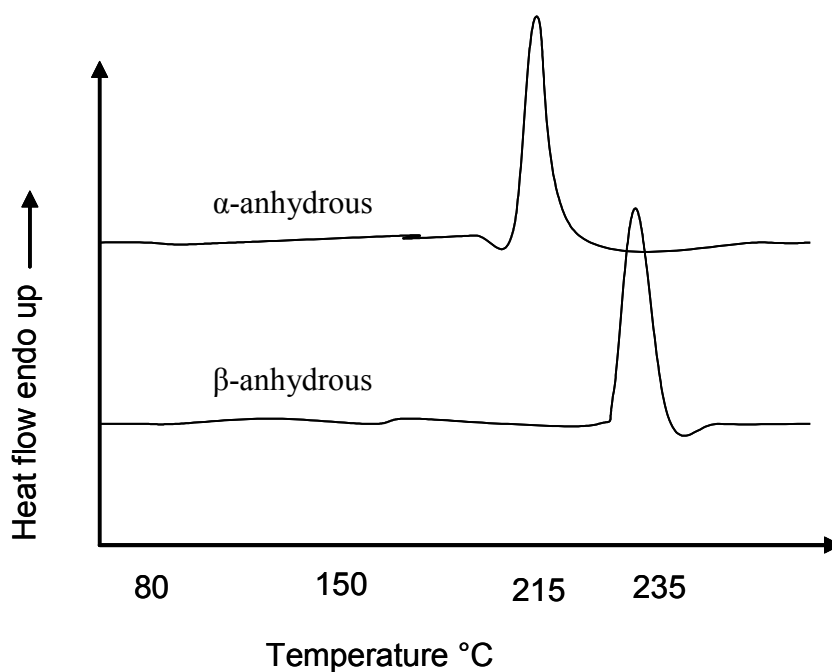


Figure 14 DSC curve of α and β anhydrous of lactose from (Sebhatu *et al.*, 1994)

Figure 14 shows the DSC curve of α and β anhydrous of lactose. In the case of α -anhydrous, there is an endothermic peak at around 215 °C (Figure 14) and for β -anhydrous an endothermic peak at around 235 °C showing the melting points of α and β anhydrous lactose.

Figure 15 shows principle parts of a DSC. There are two pans. In one pan, the sample pan and the other one is the reference pan. Each pan sits on top of a heater.

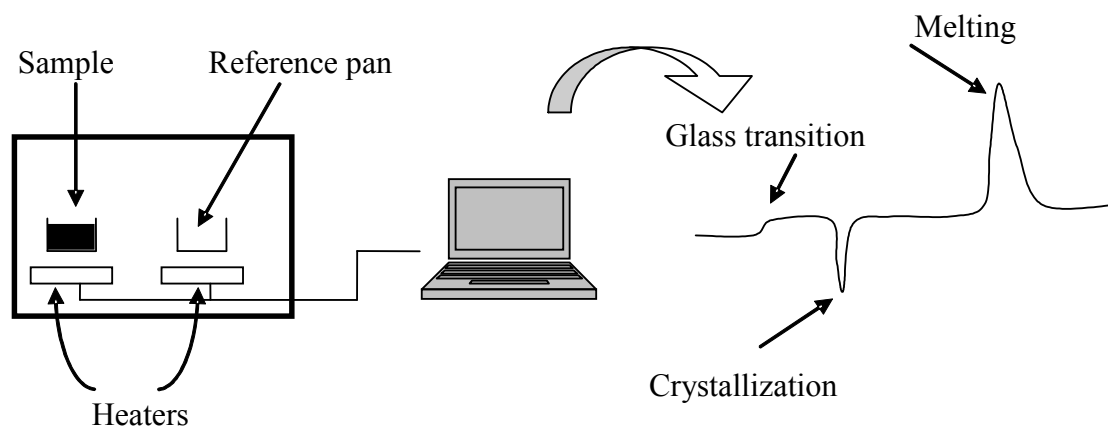


Figure 15 principle parts of DSC and an example of result can be obtained

II.4.3.2 Procedure

DSC measurements were carried out with a Perkin Elmer Pyris 1 (Perkin Elmer Corp., Norwalk, USA) for the formulations stored at various relative humidities (RH) ranging from 0 to 85%. The DSC was calibrated by indium and an empty capsule was used as reference. The states of lactose were determined by scanning of samples (~ 5mg) in open DSC aluminum pan (50 μ L) at temperatures ranging from 25 to 250°C with 10 °C/min under nitrogen atmosphere.

II.5 Kinetic of lactose crystallization by Dynamic Vapor Sorption (DVS)

II.5.1 Principle

Dynamic Vapor Sorption (DVS) is a new technique developed to collect continuous weight change data over time at any desired RH between 0 and 98% in a short amount of time. A schematic of a typical DVS system configuration is shown in Figure 16. At the heart of the DVS system is an ultra-sensitive recording microbalance capable of measuring changes in sample mass lower than 1 part in 10 million.

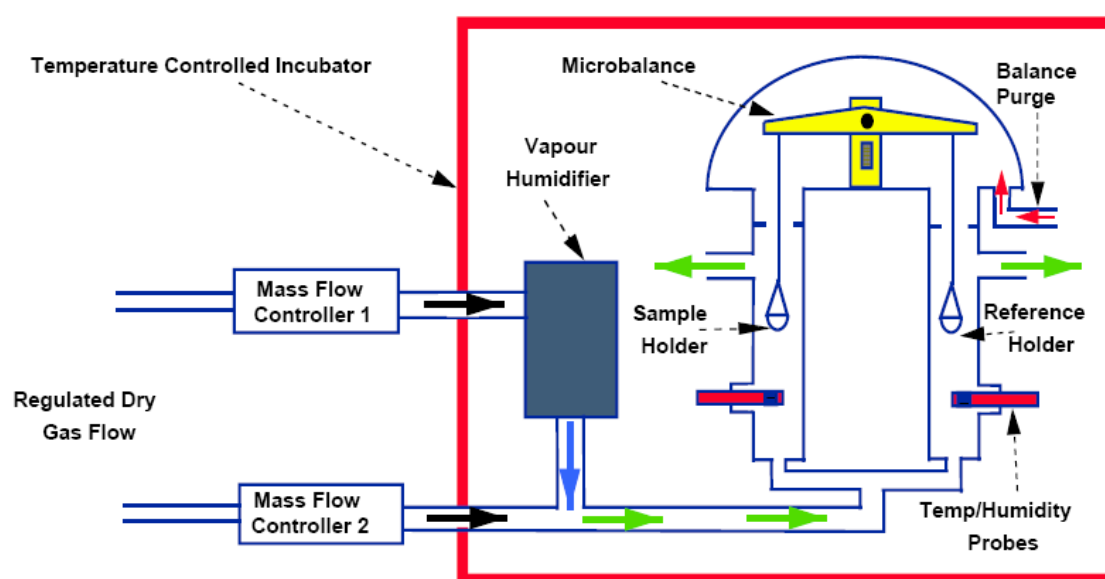


Figure 16 Schematic of a Dynamic Vapor Sorption

The main instrument is housed in a precisely controlled constant temperature incubator. This ensures very high instrument baseline stability as well as accurate control of the relative humidity generation. Microbalance is housed in a separate temperature zone from the sample region due to the wide temperature span of the incubator (5-85°C).

The required humidity's are generated by mixing dry and saturated vapor gas flows in the correct proportions using mass flow controllers. Humidity and temperature probes are situated just below the sample and reference holders to give independent verification of system performance. The sample and reference compartments of the DVS contain a sample pan that hangs on a loop connected to the microbalance (Figure 16).

II.5.2 Procedure

Samples of about 15-20 mg were loaded on one side of the pan balance and the program set to control the humidity at 0% for 5 h (drying phase). This step allowed the sample humidity to decrease to zero and internally equilibrate prior to humidification. Then RH was increased to 70% for 15 to 100 h (to allow crystallization) depending on the lactose quantity in formulations. Mass changes (m) and the rate of mass changes (dm/dt) was plot against time to compare lactose crystallization delay in the formulations.

II.6 Infrared spectroscopy

II.6.1 Principle

The infrared portion of the electromagnetic spectrum is divided into three regions: the near-, mid- and far- infrared, named for their relation to the visible spectrum. The far-infrared, (approx. 400-10 cm^{-1}) lying adjacent to the microwave region, has low energy and may be used for rotational spectroscopy. The mid-infrared (approx. 4000-400 cm^{-1}) may be used to study the fundamental vibrations and associated rotational-vibational structure, whilst the higher energy near-IR (14000-4000 cm^{-1}) can excite overtone or harmonic vibrations.

Infrared spectroscopy works because chemical bonds have specific frequencies at which they vibrate corresponding to energy levels. The resonant frequencies or vibrational frequencies are determined by the shape of the molecular potential energy surfaces, the masses of the atoms and, eventually by the associated vibronic coupling.

Simple diatomic molecules have only one bond, which may stretch. Molecules that are more complex may have many bonds, and vibrations can be conjugated, leading to infrared absorptions at characteristic frequencies that may be related to chemical groups. The atoms in a CH_2 group, commonly found in organic compounds can vibrate in six different ways, symmetrical and asymmetrical stretching, scissoring, rocking, wagging, and twisting as shown in Figure 17:

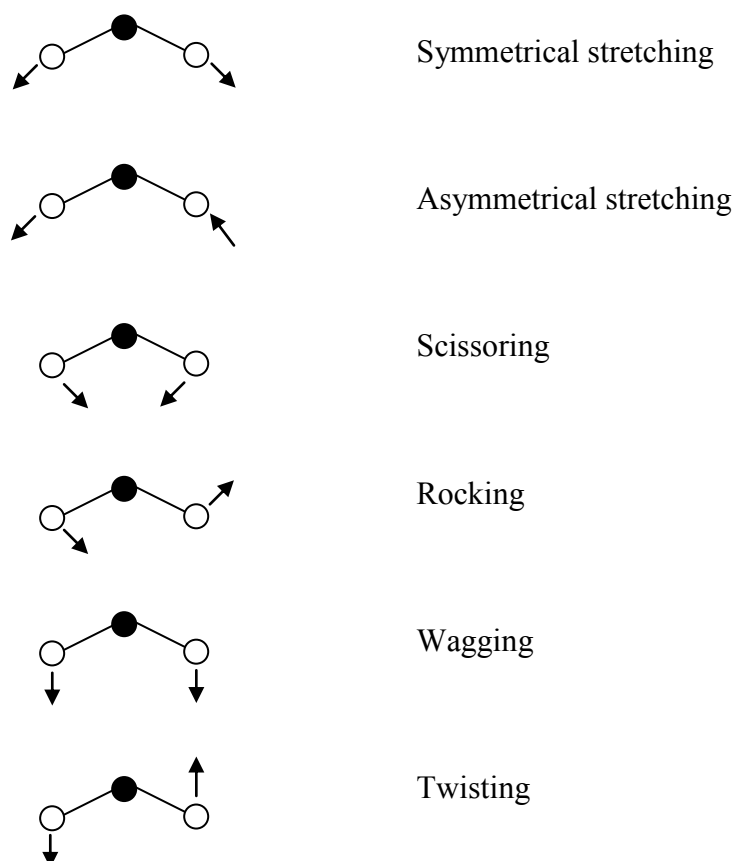


Figure 17 Vibration modes of a CH₂ molecule

Detailed information about the infrared absorptions observed for various bonded atoms and groups is presented in Figure 18.

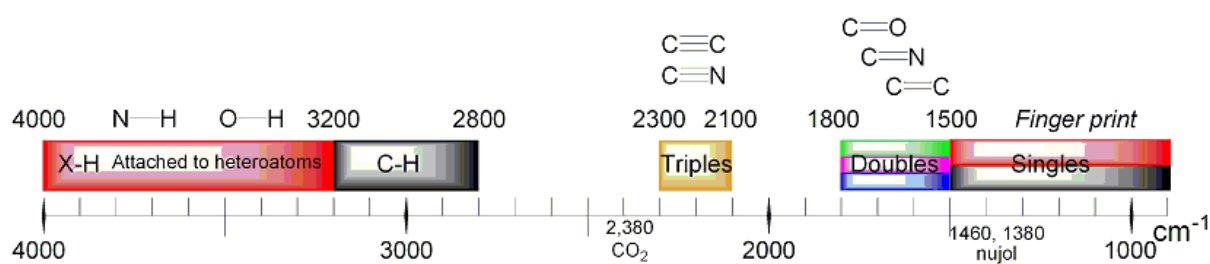


Figure 18 Summary of absorptions of bonds in organic molecules

II.6.2 Fourier transform infrared (FTIR) spectroscopy

FTIR is a measurement technique for collecting infrared spectra. Instead of recording the amount of energy absorbed when the frequency of the infrared light is varied (monochromator), the IR light is guided through an interferometer. After passing the sample, the measured signal is the interferogram. Performing a mathematical Fourier Transform on this signal, results in a spectrum identical to that from conventional (dispersive) infrared spectroscopy.

II.6.3 Procedure

Samples of lactose, BLG, gelatinized starch and the formulations stored at different RH were prepared as KBr (potassium bromide) pellets by grinding of 25mg samples and 475 mg KBr. KBr is transparent to infrared waves. This ratio was chosen to achieve absorption between 1 and 2. This powder mixture was then crushed in a mechanical die press to form a translucent pellet through which the beam of the spectrometer can pass. This salt does not absorb infrared light, so spectral lines will only appear from the analyte.

The absorbance spectra were recorded with a Tensor 27 (Bruker, Wissembourg, France) Fourier Transform Infrared (FTIR) in the region from 4000 to 400 cm^{-1} using 4 cm^{-1} resolution and an accumulation of 64 scans. The system was continuously purged with dry air. The spectra were treated with different method using OPUS/IR (Bruker, Wissembourg, France). Firstly, baseline of spectra was corrected then they were smoothed by 11 points using the Savitzky-Golay algorithm. For comparing, the spectra were normalized by two methods: min-max and vector normalization. In result section, the normalization method used for each spectrum is explained. In some regions of spectra, the second derivative was carried out to show more details about these regions. Mathematically treating the data by use of the second derivative function will result in the exclusion of the upward baseline shift. By this method a peak of negative displacement in the second derivative spectrum corresponds directly to a positively displaced peak in the original spectrum.

II.7 Experimental design

II.7.1 What is a mixture design?

In a mixture experiment, the independent factors are proportions of different components of a blend. For example, if we want to optimize color changes of our model formulations, the factors of interest might be the proportions of each component and the storage condition. The fact that the proportions of the different factors must sum to 100% complicates the design as well as the analysis of mixture experiments.

When the mixture components are subjected to the constraint that they must sum to one, there are standard mixture designs for fitting standard models, such as Simplex-Lattice designs, Simplex-Centroid designs and D-optimal designs. When mixture components are subject to additional constraints, such as a maximum and/or minimum value for each component, designs other than the standard mixture designs, referred to as constrained mixture designs or Extreme-Vertices designs, are appropriate.

In mixture experiments, the measured response is assumed to depend only on the relative proportions of the ingredients or components in the mixture and not on the amount of the mixture. The amount of the mixture could also be studied as an additional factor in the experiment; however, this would be an example of mixture and process variables being treated together.

In mixture problems, the purpose of the experiment is to model the blending surface with some form of mathematical equation so that:

1. Predictions of the response for any mixture or combination of the ingredients can be made empirically, or
2. Some measure of the influence on the response of each component singly and in combination with other components can be obtained.

When working with three components, the experimental domain corresponds to an equilateral triangle. All the possible mixtures can be identified by a point in this space, with the vertices corresponding to the pure components. In our case, it was clear that the region of interest was only a small part of the triangle, but the range of variation of each component had to be defined. This can be a very crucial step, since if the range is too large, only "obvious" effects can be obtained, while, if the range is too small, it is possible that the response does not change significantly.

II.7.2 Model

The main difference between the models of the experimental designs for independent variables and the models of the mixture design is that in the latter, no constant term is present.

The linear model for a three-component mixture is therefore the following:

$$Y=b_1X_1+b_2X_2+b_3X_3 \quad (7)$$

Where X_i is the fraction of component i . The resulting response surface is planar, and it can be easily understood that the coefficients correspond to the responses obtained with the pure components. This also means that the magnitude of a coefficient has no relation at all with the effect of the same component, and therefore, its interpretation is not as easy as it is with the experimental designs for independent variables.

The quadratic model for a three-component mixture is the following:

$$Y=b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3 \quad (8)$$

The coefficients b_i correspond to the response obtained with the pure component i , while the coefficients b_{ij} are contrasts comparing the response at the midpoint of the edge connecting the vertices of components i and j with the responses at the vertices i and j . These three terms are said to represent measures of departures from the plane of the second-degree surface resulting from the nonadditive blending of the components (Cornell 1990). With this model, synergic or antagonistic effects of binary mixtures can therefore be taken into account.

To consider also the effect of ternary blending among the components, a term $b_{123}X_1X_2X_3$ must be added, by that obtaining what is called a "special cubic model":

$$Y=b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{123}X_1X_2X_3 \quad (9)$$

To correlate both the composition of the mixture and the level of the process variable with the characteristics of the final product, we postulated a special cubic model, to which a linear term for the process variable has been added. With four components in the mixture and one process variable, the model was the following:

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{123}X_1X_2X_3 + b_{124}X_1X_2X_4 + b_{134}X_1X_3X_4 + b_{234}X_2X_3X_4 \quad (10)$$

Where X_1 , X_2 and X_3 were the fractions of each component in the mixture, corresponding to lactose, gelatinized starch and BLG, respectively. X_4 was the process variable. The response, Y , was the moisture sorption and color changes of the formulation measured at different RH.

II.7.3 The experimental matrix

Since there are no "standard" designs for the postulated model, the D-optimality criterion had to be applied. Being $X_{n,p}$ the model matrix of an experimental design made by n experiments by which a model with p coefficients has to be estimated, the quality of the experimental design can be evaluated by looking at the determinant of its dispersion matrix: the lower its value, the better the design and therefore the higher the quality of the information that can be obtained. Minimizing the determinant of the dispersion matrix corresponds to maximizing the determinant of the information matrix.

Given a set of candidate experiments, and having defined the number n of experiments to perform, the D-optimal design is composed by the subset of n experiments having the highest determinant of its information matrix. This subset can be quite easily found by applying appropriate algorithms.

Since the determinants of information matrices with different number of experiments cannot be directly compared, the normalized determinant is often used: $\det(X'X)/np$. It is therefore possible to directly compare the gain in information and the increase in the experimental effort. The best solution will therefore be the one producing the best compromise between the quality of the obtainable information and the number of experiments to be performed.

Now the different step of preparing a mixture design is explain.

II.7.4 Entering the mixture components

Firstly, the names of the components is entered, their allowed low and high levels, the total amount of the mixture, and the units the total is measured in. The low and high entries are constraints on a single component (For example, there are legal requirements on the level of starch in infant food).

The limits on any component will range from 0%, not in the mixture, to 100%, only this ingredient is present, a pure component. The total of all of the ingredients must be 100% or less. The total may be less than 100% if the components form a constant portion in a larger mix.

II.7.4.1 Mixture model terms

The degree of a mixture model indicates the presence of certain terms. The following are in the Scheffe model form.

Degree	Description
1	Linear – $b_1, b_2, b_3, \dots$
2	Quadratic – $b_{12}, b_{13}, b_{23}, \dots$
3	Special Cubic – $b_{123}, \dots$
4	Cubic – $b_{12}(b_1 - b_2), b_{13}(b_1 - b_3), \dots$

Higher order models require more points. Selecting the highest degree for the model in the experiment will ensure that there are enough design points to evaluate that model.

II.7.4.2 Scheffe model form

A special polynomial for fitting data was used from mixture experiments. These polynomials can be recognized by their lack of an intercept term. They can be derived from the usual Response Surface Methodology (RSM) models by substitution of the overall constraint that all components add to one.

II.7.4.3 Crossed model

A crossed design is a combination of mixture components and process factors. As an example, you may be blending ingredients together and then processing them in an oven.

You might want to study the ingredients and how they interact with the oven temperature and time in the oven. In color changes part, a cross model was used. RH and storage time were considered as two numerical process parameters. There are two design types that are used to create crossed designs. These are the D-optimal design and the user defined design.

Chapter III: Results and Discussion

III.1 Moisture sorption properties of model infant formulations

Water sorption properties of food have been reviewed (Labuza *et al.*, 1970) and it is well recognized that moisture sorption affects the stability and quality attributes of all dehydrated foods. The control of moisture sorption and water activity is a basic food processing technique and influences chemical deterioration, Maillard browning, lactose crystallization, gradual loss of solubility and rate of rehydration (Morrissey, 1985).

All formulations were stored at different RH ranging from ~ 0 to 95 % to study the moisture sorption properties of formulations. First, moisture sorption isotherms of all formulations were obtained by desiccator method. Second, these results were analyzed using a mixture design to determine the effect of each component on moisture sorption properties of formulations. Moreover, mixture design could put forward the presence of interactions between components and it can suggest the mathematical models predicting moisture sorption properties of model formulations. Finally, the kinetic of crystallization was studied by two methods: desiccator method and Dynamic Vapor Sorption. Moisture sorption results obtained were afterward compared to that of SEM and DSC.

III.1.1 Moisture sorption isotherm of model infant formulations

After two-week storage of samples on P₂O₅, their moisture sorption isotherms were measured by placing the samples at different RH ranging from ~ 0 to 95 %. Figure 19 shows the moisture sorption isotherms of lactose, gelatinized starch, BLG, as well as two model infant formulations at 20 °C.

Moisture sorption isotherms of BLG and gelatinized starch were sigmoid shape and they adsorbed a large quantity of water at high RH, like most hydrophilic proteins and polysaccharides (Mathlouti and Rogé, 2003). Above 75 % RH, BLG adsorbed more water than gelatinized starch. Amorphous lactose, on the contrary, adsorbed less water than gelatinized starch and BLG due to crystallization phenomenon occurring above 43 % RH. That observation (break point on the lactose curve) was in agreement with that noted by numerous authors (Darcy and Buckton, 1997, Jouppila *et al.*, 1997, Mazzobre *et al.*, 2001, Thomas *et al.*, 2004a).

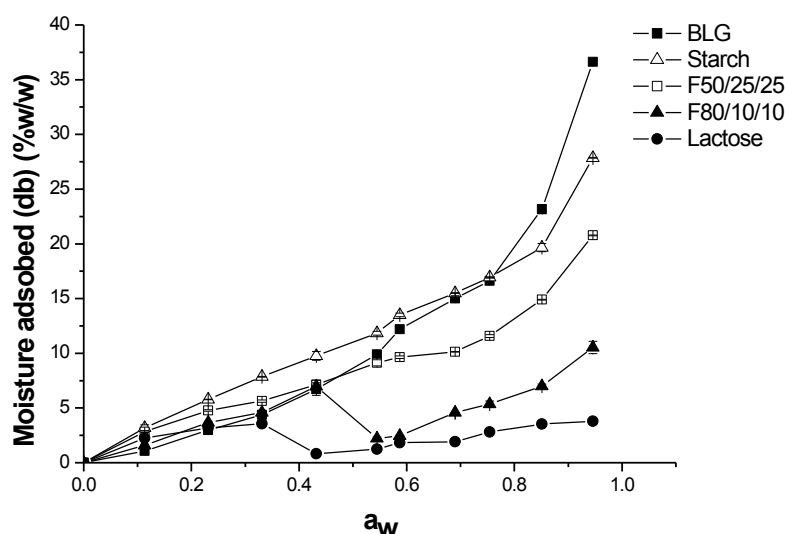


Figure 19 Moisture sorption isotherms of BLG, gelatinized starch, lactose and two formulations after 3 months storage at 20 °C

When lactose is present in its amorphous state, its exposure to increasing humidity level, results in water uptake that reach sorption levels far higher than that of its crystalline form. A larger area available for water sorption in amorphous, less regular structure of lactose, accounts for the effect of lactose state on amount of water uptake. Some hydrogen bonds are disrupted and at some given water activities, sugar molecules are somewhat diluted and acquire sufficient mobility to undergo transformation from the metastable amorphous state to the more stable crystalline form. During crystallization process, lactose released water and that could be observed as a break point on moisture sorption curve. Moisture sorption isotherms of two formulations (F80/10/10 and F50/25/25) were situated between lactose and gelatinized starch and BLG curves. The sorption properties of formulations thus varied with their components sorption properties.

Figure 20 shows moisture sorption isotherms of all formulations studied, after three months storage at different RH.

This figure clearly shows that by increasing lactose quantity in the formulations, the amount of adsorbed moisture decreased, in particular after lactose crystallization. Formulation containing 50 % lactose, 25 % gelatinized starch and 25 % BLG thus adsorbed more water than the four other formulations with higher lactose content (Figure 20a).

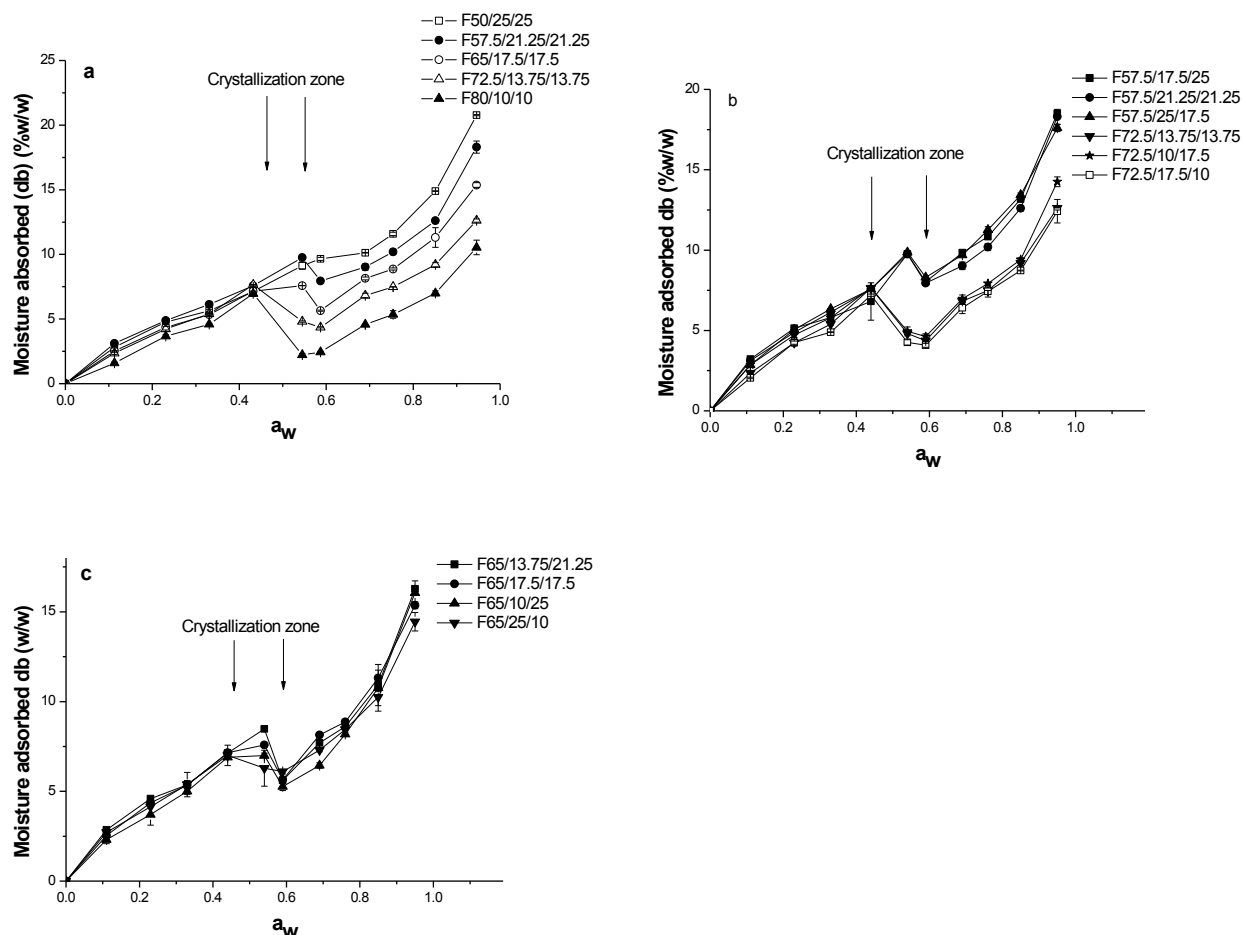


Figure 20 Moisture sorption isotherms of formulations after three months storage at 20 °C; a: formulations containing different lactose quantity, b: formulations containing 57.5 and 72.5 % lactose and c: formulations containing 65 % lactose

Moreover, formulations containing 57.5 % lactose adsorbed more or less the same amount of moisture whatever the gelatinized or BLG content. This is however not the case for sample F72.5/10/17.5. At 95 % RH, it can be seen on Figure 20b, that formulation adsorbed more water than other formulations containing the same lactose quantity. This is because BLG adsorbs more water at high RH, and sample F72.5/10/17.5 had higher protein content than the other formulations with 72.5 % lactose. Similar result was found for formulations containing 65 % lactose (Figure 20c). The amount of water adsorbed at the break point and the magnitude of the drop in the isotherm are inversely related to the amount of lactose present in the formulation (Figure 20a, b and c). Figure 20a indicates that the isotherm of the sample with 80 % lactose content had a sharp drop at 43 % RH. The magnitude of this fall varied greatly with lactose content. A decrease in the lactose quantity entailed a smoother drop.

When the sample contained only 50 % lactose, the break point is not clearly marked. In addition, crystallization occurred at higher RH for this formulation.

As percentage of lactose increased, the moisture adsorption and crystallization RH decreased. The amount of water adsorbed by formulation increased with increasing amount of BLG and gelatinized starch. Moreover, the value of RH at which the break in the isotherm was observed varies with the composition of the sample. All formulations (containing 20 to 50 % gelatinized starch and BLG) showed identical isotherms up to 43 % but above, these formulations, containing higher quantity of gelatinized starch and BLG adsorbed more water.

III.1.2 Lactose Crystallization

In our experiments, lactose crystallization occurred at higher RH values. For all formulations, water liberation (indicating crystallization) occurred at higher RH than lactose crystallization RH. This confirmed that the effect of each component, on the crystallization RH of the resulting mixture, was not additive. Table 12 shows crystallization RH for each formulation.

For all formulations, crystallization occurred at different RH over time. The first crystallization RH could be observed after one day, and the second and third later on. Crystallization RH decreased with increasing lactose quantity. Jouppila and Roos (1994) studied crystallization phenomenon in whole and skim milk and they compared their results with pure lactose. Those authors believed that milk protein could retard lactose crystallization process. In addition, carboxy-methyl cellulose and some proteins namely rh-GH (growth hormone) could also delay lactose crystallization (Costantino *et al.*, 1998, Karmas *et al.*, 1992). Haque and Roos (2006) obtained similar results for mixtures of lactose and proteins but the delaying effect was greater in freeze-dried than in spray-dried materials. Knudsen *et al.* (2002) explained that lactose crystallization was delayed due to competitive adsorption of water between lactose and milk proteins. Moreover, Thomas *et al.* (2004a) showed that if mixture components are mixed after individual component drying, there was no change in crystallization a_w .

Chapter III: Results and Discussion

Table 12 Composition of infant formulations, minimum RH that lactose is crystallized and moisture adsorption (% w/w, db) at 95 % RH

Formulations	Lactose	Starch	BLG	Crystallization RH (%)	Moisture Sorption (%) RH=95%
F80/10/10	80	10	10	59, 54	10.54
F65/10/25	65	10	25	69, 59	16.06
F65/25/10	65	25	10	69, 59, 54	14.45
F50/25/25	50	25	25	75, 69	20.77
F72.5/10/17.5	72.5	10	17.5	69, 59, 54	14.25
F72.5/17.5/10	72.5	17.5	10	69, 59, 54	12.42
F57.5/17.5/25	57.5	17.5	25	69, 59	18.51
F57.5/25/17.5	57.5	25	17.5	69, 59	17.60
F65/17.5/17.5	65	17.5	17.5	69, 59	15.36
F72.5/13.75/13.75	72.5	13.75	13.75	69, 59, 54	12.62
F65/13.75/21.25	65	13.75	21.25	69, 59	16.28
F65/21.25/13.75	65	21.25	13.75	69, 59, 54	16.06
F57.5/21.25/21.25	57.5	21.25	21.25	69, 59	18.30

Lactose crystallization occurred over time and it was a time dependent phenomenon. Indeed, for some formulations it was noted at different RH over time. The difference in induction time indicates the difference in tendency for amorphous lactose to adsorb water.

Crystallization of amorphous lactose is related to its T_g and occurs above T_g (Jouppila and Roos, 1994). Generally, T_g increases by the addition of high molecular weight proteins and carbohydrates. Thus, in presence of these compounds, it is necessary to increase the RH to reach the T_g at which lactose crystallization occurs. However, other authors showed that crystallization delay in a system containing sugar that was co-lyophilized with polymers and proteins, such as growth hormone, does not depend on the increase of the system's T_g (Mazzobre *et al.*, 2001). In addition, crystallization is attributed to thermodynamic, geometric or kinetics factors, associated to the modification of molecular environment in the combined systems that probably affect nucleation and/or crystal growth. According to the hypothesis that sugars could replace water in a dried protein, it is possible that there was a solid-state protein-sugar interaction or sugar-sugar interaction, which masked water monolayer sites from the humidified atmosphere. Thus, interactions between these components had an important effect on crystallization RH, but had no effect on moisture sorption of samples.

III.1.3 Comparison between calculated and experimental results

For any mixture in equilibrium, a_w of all components are equal. If only water is transferred between components during the process of equilibrium, each component will assume an equilibrium moisture content (EMC) at a given a_w equal to what can be obtained from its individual sorption isotherm. Expressing the EMC on a dry basis, the composite EMC for the mixture can be calculated from the weighted-average hypothesis using this equation:

$$M_{\text{cal}} = \frac{\sum W_i M_i}{\sum W_i} \quad (11)$$

Where M_{cal} is the calculated total moisture content at a specific water activity (a_w), W_i is the individual component weight expressed on a dry basis, of each component at the specified a_w . This equation was tested over a_w ranging from 0.33 to 0.9 for binary and multi components mixtures containing both macromolecules and solutes (Lang and Steinberg, 1980).

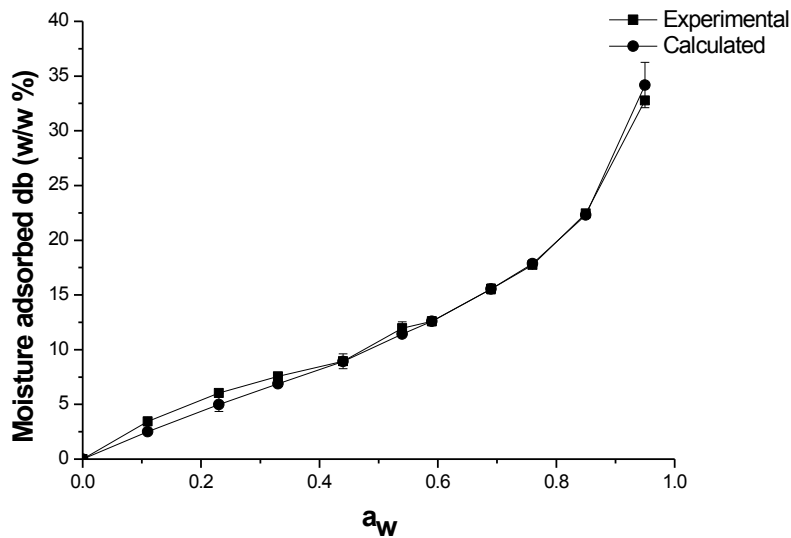


Figure 21 Moisture sorption isotherms of mixture containing 50 % BLG and 50 % gelatinized starch. Experimental moisture sorption obtained from mixing the two components after gelatinization of starch in liquid state and the mixture was co-lyophilized

III.1.3.1 Binary mixtures

Moisture sorption isotherms of lactose, gelatinized starch and BLG and two binary mixtures were measured. The moisture sorption isotherms of lactose, gelatinized starch and BLG were presented in Figure 19. Figure 21 shows moisture sorption isotherms of a mixture containing 50 % BLG, 50 % gelatinized starch and Figure 22 shows moisture sorption isotherms of mixture containing 50 % lactose, 50 % gelatinized starch.

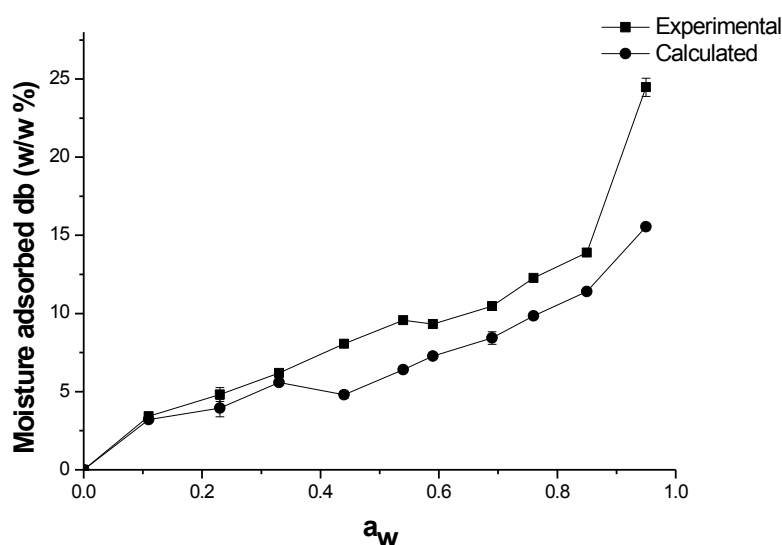


Figure 22 Moisture sorption isotherms of mixture containing 50 % lactose and 50 % gelatinized starch. Experimental moisture sorption obtained from mixing the two components after gelatinization of starch in liquid state and the mixture was co-lyophilized

The experimental moisture sorption isotherm of mixture containing 50 % gelatinized starch and 50 % BLG was the same as the moisture sorption isotherm obtained by calculating the moisture sorption isotherm of each component (Figure 21). Nevertheless, the moisture sorption isotherm obtained for a mixture with 50 % lactose and 50 % gelatinized starch, was not the same as the moisture sorption isotherm deduced from the moisture sorption isotherm of each component.

These results therefore show that the moisture sorption isotherm of mixtures containing lactose could not be calculated from moisture sorption isotherm of each component. More details are presented in SEM part about this phenomenon.

III.1.3.2 Ternary mixture

The binary mixture explained above was modified and the three components were mixed at different ratios to obtain 13 formulations. Figure 23a and b shows experimental and calculated moisture sorption isotherms of these formulations.

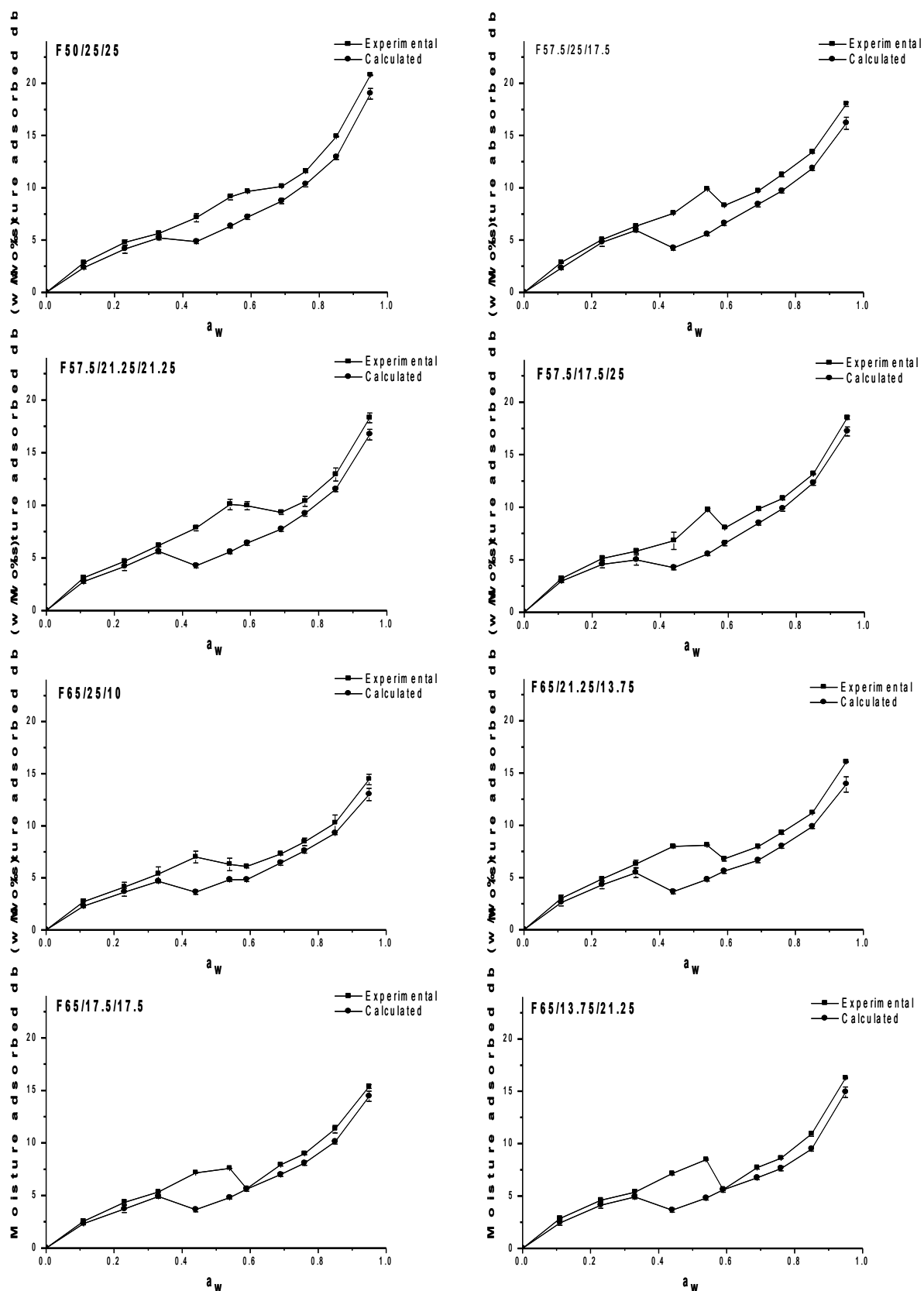


Figure 23a experimental and calculated moisture sorption isotherms of different formulations. Samples stored 3 months at different RH (0-95%) and 20 °C.

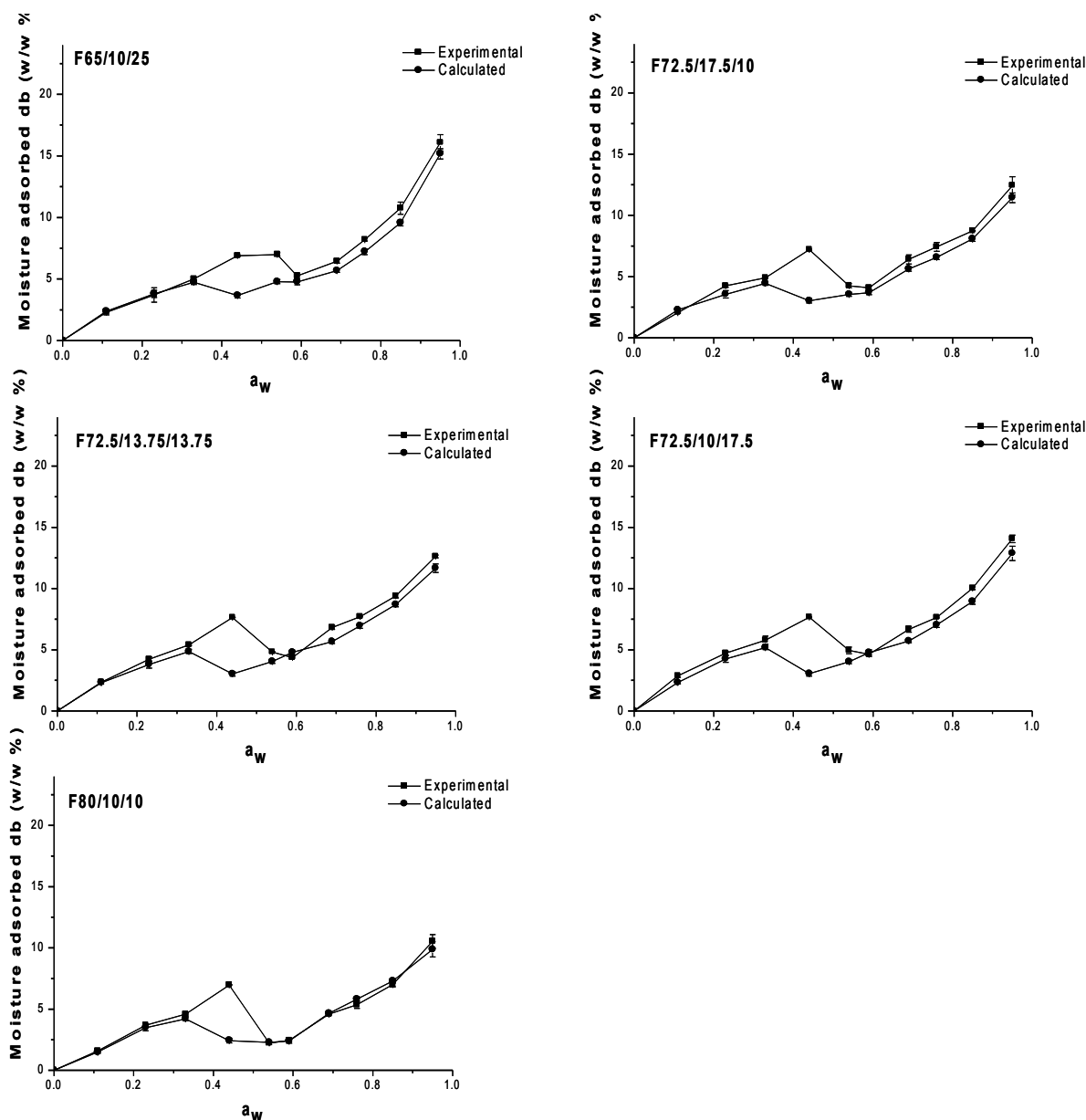


Figure 23b experimental and calculated moisture sorption isotherms of different formulations. Samples stored 3 months at different RH (0-95 %) and 20 °C.

In all formulations, the calculated isotherms were situated below the experimental curve except, at some RHs and for some formulations. In all formulations, the calculated and experimental results were the same at RHs below 33 %. Pure lactose crystallized at 43 % RH but at this RH, the formulations did not crystallize. Thus, a big difference between calculated and experimental results could be observed in all formulations due to lactose crystallization. Crystallized lactose adsorbs less water than amorphous lactose. For example, formulations containing large quantity of lactose (80 and 72.5 %) moisture sorption quantity at the

crystallization zone (54 and 59 % RH) for experimental formulations were the same or sometimes less than the calculated points. The difference between calculated and experimental curve was more important in the formulations containing 50 and 57.5 % lactose. The maximum differences were observed between calculated and experimental curves at crystallization point of lactose.

III.1.4 Onset and Kinetic of Crystallization

Formulations containing 50 to 80 % lactose, 10 to 25 % gelatinized starch and 10 to 25 % BLG were stored during 3 months at different RH ranging from ~ 0 to 95%. The quantity of adsorbed water for each formulation was determined after 1, 3, 7 days and each week during 3 months. The moisture sorption isotherms of formulations are presented in Figure 24 and Figure 25a and b. Development of crystallinity during storage was studied at 20 °C and at different RH as shows in Figure 24. This combination was chosen, because the effects of RH on lactose crystallization in formulations with high lactose content were of main interest. Figure 24a shows the moisture sorption isotherms of F50/25/25 during storage at 20 °C. Crystallization of this formulation occurred after one day storage at 75% RH. It can be seen from this figure that water liberation, indicating lactose crystallization, occurred during storage at 75% RH and it takes around 2 weeks for equilibrium to be reached. The break point for F50/25/25 was not as clear as for sample F80/10/10 after equilibrium since water liberated was adsorbed by BLG and gelatinized starch. Moisture sorption isotherm curve was sigmoid in shape. For sample F50/25/25 water liberation occurred at 69 % and at 59, 54 and 43 % RHs, water liberation was not observed. In the case of sample F80/10/10 water liberation occurred at 54 % and at 59 and 69 %, lactose was in a crystallized state. Moisture content of sample F80/10/10 at 43 % was more than that of samples stored at 54, 59, 69 %, because lactose was amorphous at 43 % RH and not at the other RH values. As it has been explained before, amorphous materials have a greater adsorption surface than crystallized materials. This is why adsorption of water of amorphous lactose was higher than that of crystallized lactose.

Crystallization delay was observed for products containing mixtures of proteins and/or polysaccharides with lactose or other sugars and it was attributed to higher T_g or interactions between components. Figure 24b shows that for sample F80/10/10, crystallization occurred at RH greater or equal to 54 %. Equilibrium time for all formulations inferior to 43 % RH was

less than one week and not until 12 weeks when RH is superior to 43 %. For example, equilibrium time for F80/10/10 was 21 days at 54 % RH and for F72.5/13.75/13.75 after 60 days at 54 % RH.

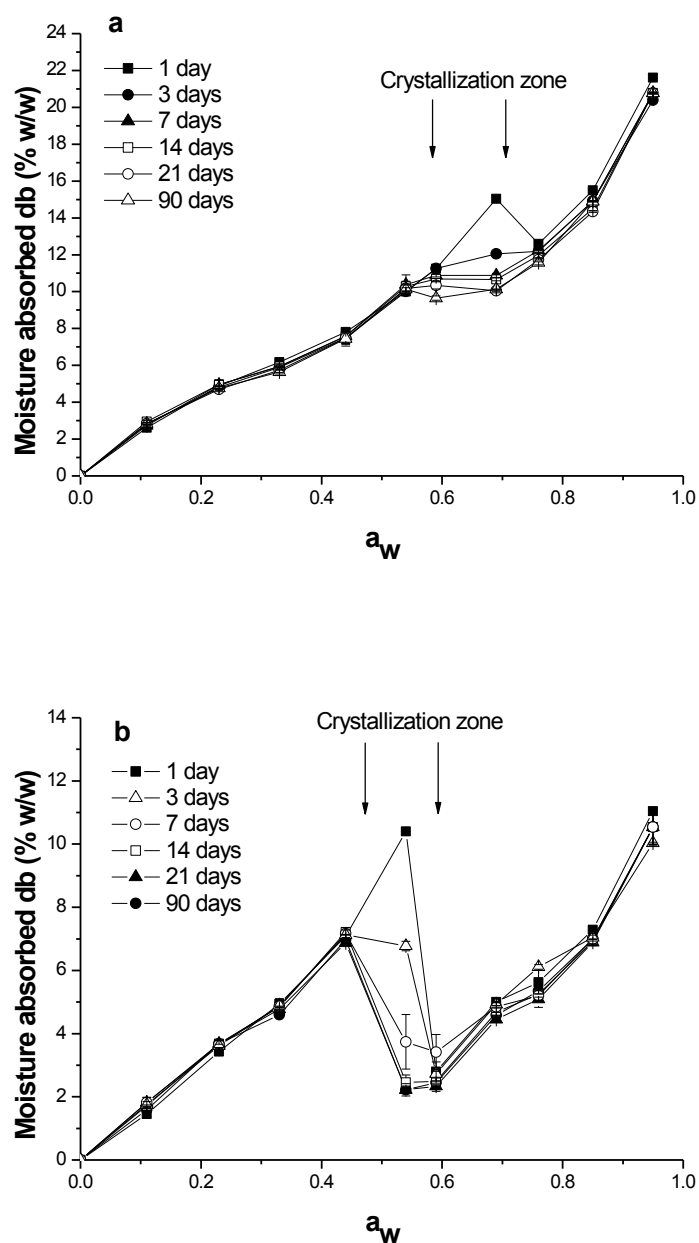


Figure 24 Moisture sorption isotherms changes during storage at 20 °C; a: F50/25/25, b: F80/10/10

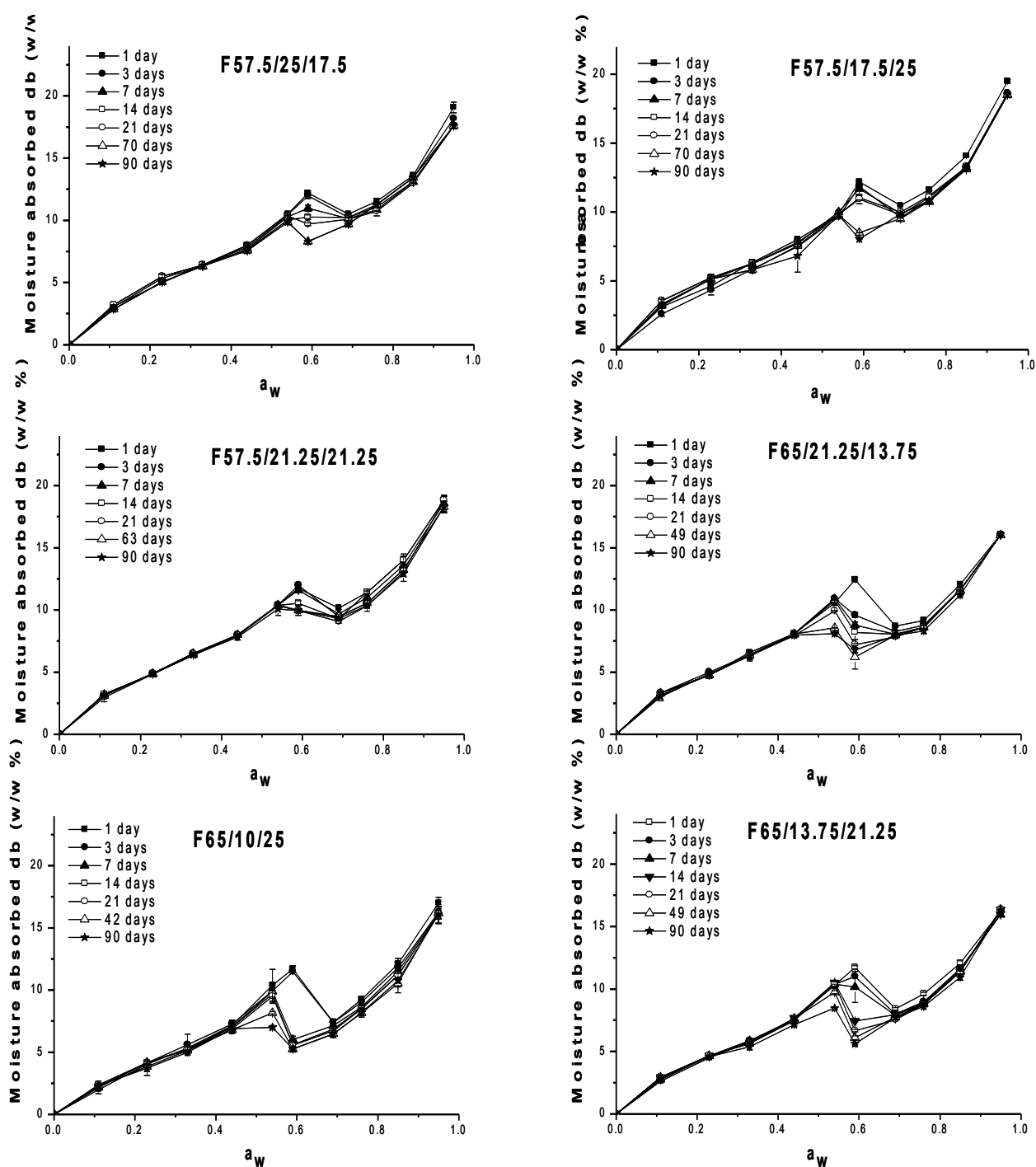


Figure 25a Moisture sorption changes of formulations during storage at 20 °C.

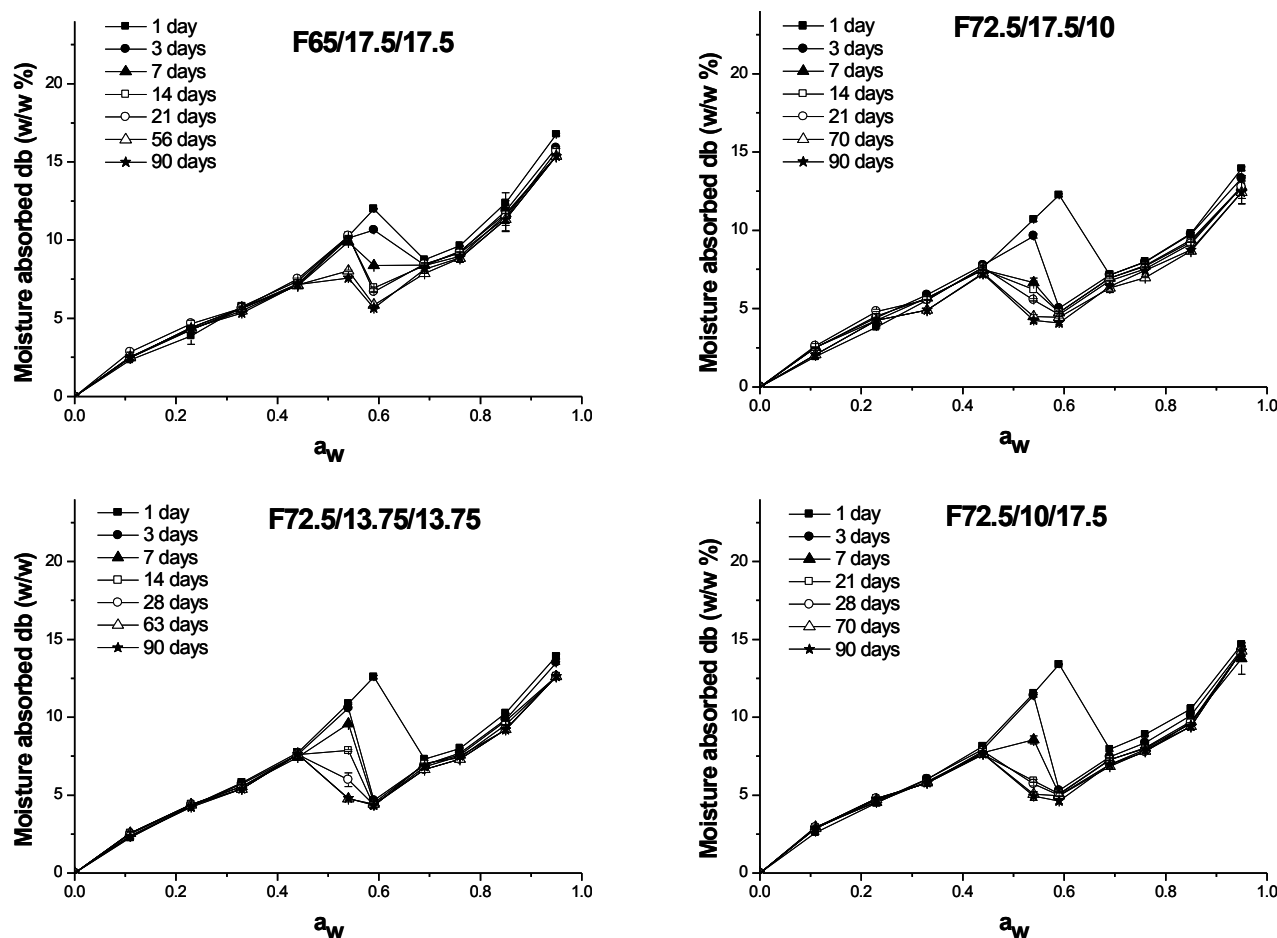


Figure 25b Moisture sorption changes of formulations during storage at 20 °C.

All formulations adsorbed more or less the same quantity of water at 59 % RH before crystallization. This is a critical RH value for crystallization in our samples because at this RH water liberation could be observed in all formulations.

In the case of sample F72.5/13.75/13.75, the first crystallization RH, which could be observed after one day storage, was at 69 %, the second occurring after 3 days storage was at 59 % and the third taking place after 56 days storage was at 54 %. As the percentage of gelatinized starch and BLG increased, equilibrium time and crystallization RH increased. That maybe attributed to the change in lactose environment, induced by gelatinized starch and BLG, which prevented lactose moisture adsorption. The molecular weight of BLG is 18300 Da and that of gelatinized starch is even higher. They therefore increased the viscosity of the system and limited diffusion phenomenon. Numerous authors (Knudsen *et al.*, 2002, Mazzobre *et al.*, 2001) noted crystallization delay, but until now, no clear explanations for this phenomenon is

available. Lactose crystallization attributes to increasing the mixture T_g and change in lactose environment.

In this study, gelatinized starch was used. During the crystallization process, hydrogen bonds between glucose chains in the crystallites were primarily disrupted and perhaps some of those in the amorphous regions as well. When the gelatinized starch was then mixed with lactose and BLG, hydrogen bonds were formed again, thus retarding lactose crystallization.

Results obtained indicated that the addition of BLG and gelatinized starch in formulations changed the lactose crystallization kinetic. Several parameters, such as molecular environment changes, interactions between lactose/BLG/starch in solid state that replaced protein or polysaccharide by water in powder state, affect lactose crystallization. As BLG and gelatinized starch content in formulations increased, crystallization RH or the delay time (which is the time necessary for lactose crystallization at the same RH to occur) increased. Many parameters played important roles in delay time, namely moisture sorption rate of lactose, viscosity changes brought by the addition of BLG and gelatinized starch, and moisture sorption inhibited by BLG and gelatinized starch. Moreover, the competition among these three components for moisture adsorption was an important factor. In the same conditions, BLG and gelatinized starch adsorbed more water than lactose (Figure 19).

III.1.5 Effect of gelatinized starch and BLG on moisture sorption properties of formulations

Results obtained from moisture sorption isotherms were analyzed. A mixture design was used to study the moisture sorption properties of samples during 3 months storage at different RH and at 20 °C. A cubic model explained the effect of each components and RH on moisture sorption of samples, taking into account all possible interactions between components. Linear and cubic models were used to correlate the effect of formulations compositions at 95 % RH on moisture sorption properties of samples.

Chapter III: Results and Discussion

Table 13 Estimation of coefficient of model and probability of models (equation 1)

Response	Name	Coefficient	Probability>F
Moisture sorption at 95.6 %	Model		<0.0001 Significant
	b ₁	3.77	<0.0001
	b ₂	31.77	<0.0001
	b ₃	43.56	<0.0001
Moisture sorption at 85 %	Model		<0.0001 Significant
	b ₁	1.72	<0.0001
	b ₂	26.46	<0.0001
	b ₃	30.28	<0.0001
Moisture sorption at 75 %	Model		<0.0001 Significant
	b ₁	1.37	<0.0001
	b ₂	26.46	<0.0001
	b ₃	23.09	<0.0001
Moisture sorption at 69 %	Model		<0.0001 Significant
	b ₁	1.43	<0.0001
	b ₂	20.39	<0.0001
	b ₃	19.06	<0.0001
Moisture sorption at 59 %	Model		<0.0006 Significant
	b ₁	-2.75	<0.0006
	b ₂	25.45	<0.0006
	b ₃	21.70	<0.0006

The value of probability less than 0.0500 indicate model terms are significant

The value greater than 0.1000 indicate the model terms are not significant

First series models were suggested to clarify the effect of components on moisture sorption at constant RH. In addition, second series models were suggested by adding RH as a process parameter to find a mathematical model to predict moisture sorption of formulations at different RH. The coefficients of the cubic model were estimated by the least square method from the results given by the experiments. Table 13 shows these coefficients for each factor according to the following equation for moisture sorption at different RH after 3 months storage at 20°C:

$$Y = b_1 X_{lac} + b_2 X_{starch} + b_3 X_{BLG} + b_{12} X_{lac} X_{starch} + b_{13} X_{lac} X_{BLG} + b_{23} X_{starch} X_{BLG} + b_{123} X_{lac} X_{starch} X_{BLG} \quad (11)$$

Chapter III: Results and Discussion

Where X_{lac} , X_{starch} and X_{BLG} are lactose, gelatinized starch and BLG quantity respectively and b_1 , b_2 and b_3 are the coefficients. The response Y is the moisture sorption at 95, 85, 75, 69 and 59 % RH.

A linear model was found to explain the effect of each components on moisture sorption at all RH above lactose crystallization RH. Thus, by eliminating the interactions from the previous equation, we have the effect of each component on moisture sorption:

$$Y(95) = 3.77X_{lac} + 31.77X_{starch} + 43.56X_{BLG} \quad (12)$$

Where Y (95%) is moisture sorption of samples at 95 % RH. This equation shows that moisture sorption properties of formulations depended on moisture sorption behaviors of each component. The interactions between the components had no significant effect on Y .

Equations 13, 14 and 15 show moisture sorption properties of the formulations at RHs of 85, 75 and 69 %, respectively.

$$Y(85) = 1.72X_{lac} + 26.46X_{starch} + 30.28X_{BLG} \quad (13)$$

$$Y(75) = 1.37X_{lac} + 22.27X_{starch} + 23.09X_{BLG} \quad (14)$$

$$Y(69) = 1.43X_{lac} + 20.39X_{starch} + 19.06X_{BLG} \quad (15)$$

Equations 2-5 agree with the data in Figure 26. BLG and gelatinized starch had more effect on moisture sorption properties of formulations. Moreover, BLG had more effect compared to gelatinized starch on moisture sorption at RHs above 69 % but below the 69 % RHs, gelatinized starch had more effect on moisture sorption properties of formulations. These models confirm the result shown in Figure 19.

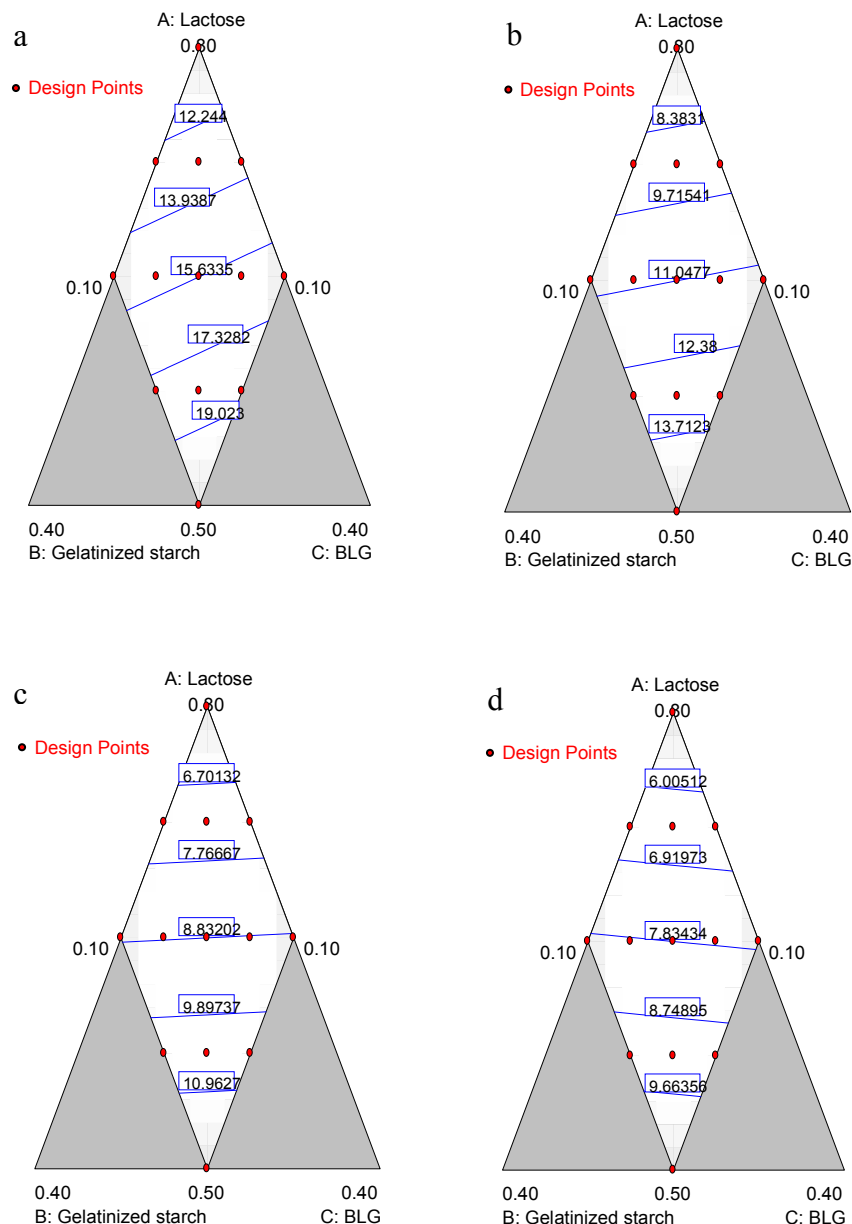


Figure 26 Experimental domain and design points. The lines between the design points shows changes in the moisture adsorbed in experimental domain at 95 (a), 75 (b), 69(c) and 59 % RH (d)

A quadratic model (equation 16) was found to predict the effect of RH's and composition of each formulation on moisture sorption properties. In this model, effect of each component was linear as shown in Equations 12-15 and RHs had a quadratic effect on moisture sorption properties. Equation 16 and Table 14 shows this model and probability of its elements. Effect of each component and all possible interactions between the components and RH were significant.

$$Y = 37.901X_{lac} + 35.133X_{starch} + 101.577X_{BLG} - 97.374X_{lac}X_{RH} - 69.275X_{starch}X_{RH} - 273.949X_{BLG}X_{RH} + 64.643X_{lac}X_{RH}^2 + 69.249X_{starch}X_{RH}^2 + 223.995X_{BLG}X_{RH}^2 \quad (16)$$

The statistical analysis (all interactions between components and RHs were significant) and R^2 results ($R^2=0.98$) showed that there was a strong agreement between calculated and experimental values (Figure 27).

Table 14 Estimation of coefficients and probability for crossed model (equation 6)

Response	Name	Coefficient	Probability
Moisture sorption	Model		<0.0001
	b _{lac}	37.901	<0.0001
	b _{starch}	35.133	<0.0001
	b _{BLG}	101.577	<0.0001
	b _{lac} *RH	-97.374	< 0.0001
	b _{starch} *RH	-69.275	<0.0001
	b _{BLG} *RH	-273.949	< 0.0001
	b _{lac} *RH ²	64.643	<0.0001
	b _{starch} *RH ²	69.249	0.032
	b _{BLG} *RH ²	223.995	0.0012

The value of probability less than 0.0500 indicate model terms are significant

The value greater than 0.1000 indicate the model terms are not significant

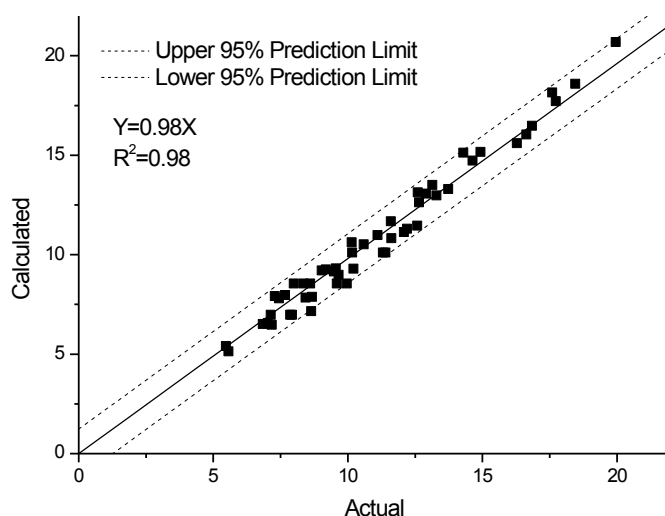


Figure 27 Correlation between actual and calculated data for moisture sorption. Calculated data were calculated from equation 6.

III.1.6 Conclusion

Lactose crystallization RH changed by addition of proteins or saccharides. This phenomenon was observed previously. Some researchers believed that T_g changes in lactose mixtures is responsible for this phenomenon. However, in some systems involving human growth hormone and trehalose, lactose crystallization RH changed without any change in the system T_g . In this condition, other parameters such as molecule environment changes and the interactions between the components affect lactose crystallization.

Stabilization of lactose against crystallization could also be attributed to preferential exclusion, surface activity, steric hindrance of lactose-protein and lactose-polysaccharide and/or increased systems viscosity limiting structural movement. During lyophilization, water is replaced by lactose and starch in BLG structure and a long chain length may interfere with intermolecular hydrogen bonding between lactose and BLG. Therefore, these bonds prevent or retard the lactose crystallization.

Mixture design used in this part is an interesting method for analyzing results of moisture sorption. Results show that interactions between lactose, BLG and gelatinized starch had no significant effect on moisture sorption properties of formulations. The mathematical models proposed in this study are interesting tools for analyzing the results of moisture sorption in other mixtures containing these components.

III.2 Kinetic of crystallization studied by Dynamic Vapor Sorption (DVS)

After 2 weeks storage of samples on P_2O_5 , the crystallization kinetic was measured by placing the samples at 70 % RH. Figure 28 shows adsorbed water of samples vs. time for lactose, F80/10/10, F 72.5/10/17.5, F65/21.25/13.75, F57.5/25/17.5 and F50/25/25 at 70 % RH. Pure lactose adsorbed about 7.6 % (w/w) and crystallized. A sharp drop in this graph shows water liberation (last step in crystallization). By increasing BLG and starch quantity in formulations, adsorbed water increased. After equilibrium ($dm/dt \approx 0$), as it was expected, the formulation containing 50 % lactose adsorbed more water than other formulations (around 15 % w/w). In addition, pure lactose adsorbed around 1.8 % water after crystallization, indicating that crystallized lactose is not hygroscopic.

Crystallization phenomenon occurs in two steps: nucleation and then growth of these nuclei, followed by water liberation. The quantity of water liberated during crystallization depends on many parameters such as lactose crystals form, size, and arrangement of crystals. Crystallization of pure lactose was more rapid compared to other formulations. However, when crystallization phenomenon is rapid, different steps of crystallization occur at the same time. Water liberation occurred at the same time as nucleation and crystals growth. This is why maximum water adsorbed before crystallization in the case of pure lactose, was less than the other formulations. In the case of F80/10/10, this formulation adsorbed around 18.3 % water at 70 % RH before crystallization and liberated a large quantity of water. This is also the case for F72.5/10/17.5. Vuataz, (1988) found that lactose in skim milk powder crystallized at 39.1% RH at 25 °C after 200 h of storage. However, crystallization occurred more slowly than in the present study for lactose/protein mixtures.

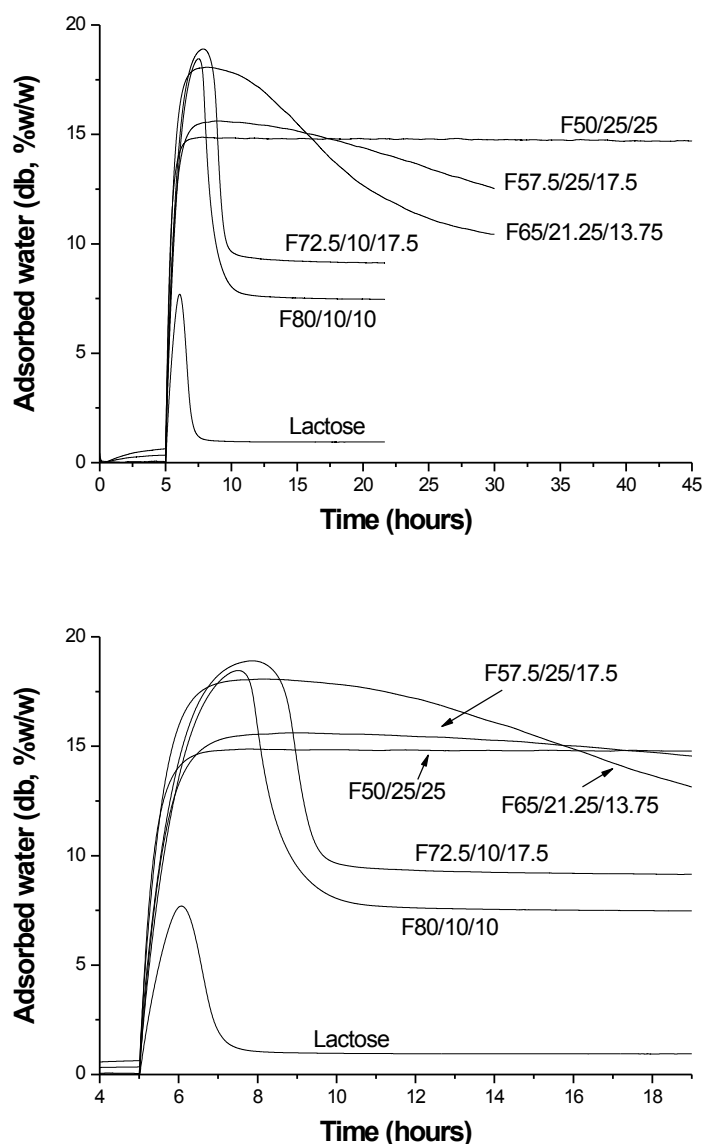


Figure 28 Moisture sorption of several formulations and lactose at 70 % RH and 20 °C.

In the case of F50/25/25, water liberation (drop in moisture sorption curve) was not clear in this figure. Because this formulation was not crystallized at 70 %, RH after 42 hours or only the first step of crystallization (nucleation) occurred but the second step (crystals growth) required a longer storage period at this RH. However, for this sample, by increasing the RH to 80 % RH, water liberation was observed after about 3h 40min (Figure 29). Lactose state in samples, after experiment, was checked by scanning electronic microscopy.

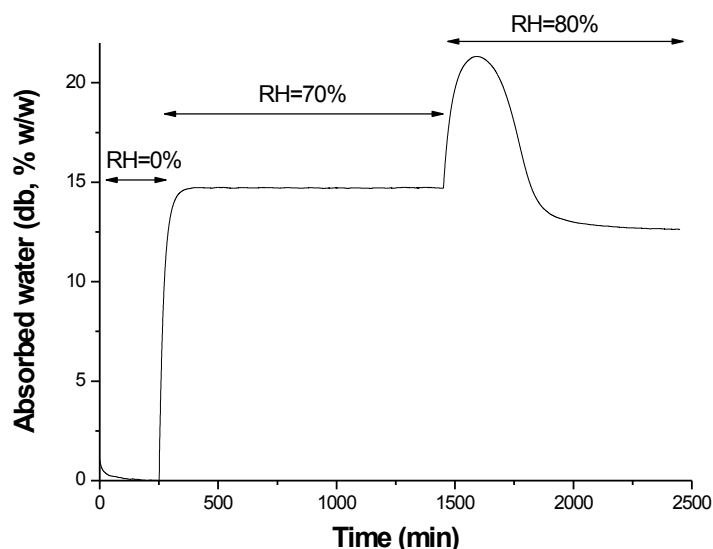


Figure 29 Moisture sorption of F50/25/25. This formulation was stored for 250 minutes at $\sim 0\%$ RH then RH was increased to 70 % for 1250 minutes and finally RH was increased to 80 %.

F80/10/10 and F72.5/10/17.5 adsorbed more water than F50/25/25 before water liberation, and crystallized. Water liberation is clear in Figure 28 for these formulations and after crystallization, they adsorbed less water than F50/25/25. As numerous authors showed, moisture sorption properties of formulations depend on the moisture sorption properties of each component (Labuza *et al.*, 1970, Thomas *et al.*, 2004a). Lactose, BLG and starch adsorb about 1.72, 16.85 and 15.27 % (w/w) at 70 % RH respectively. By considering these values, adsorbed water quantity can be calculated for all formulations. For example, in the case of F50/25/25, calculated value (the value is calculated from moisture sorption of each component) of adsorbed water is around 8.4 % (w/w). There is a big difference between calculated and experimental value (14.7 % w/w). This difference is due to absence of crystallization in these formulations. Table 15 shows the experimental and calculated values of adsorbed water of formulations at 70 % RH. This difference exists for all samples except for F80/10/10 and if formulations contain lactose, adsorbed water cannot be calculated from each component.

Chapter III: Results and Discussion

Table 15 comparison between calculated and experimental data for adsorbed moisture (% w/w) at 70 % RH measured by desiccators and DVS methods. The calculated data were obtained from the moisture sorption of each component at 70 % RH.

Formulation	Calculated	Experimental ^a	Experimental ^b
F80/10/10	4.60	4.58	7.35
F65/10/25	5.69	6.44	9.01
F65/25/10	6.63	7.30	8.78
F50/25/25	8.90	10.13	14.70
F72.5/10/17.5	5.73	6.77	9.14
F72.5/17.5/10	5.61	6.42	7.70
F57.5/17.5/25	8.49	9.83	NE
F57.5/25/17.5	8.40	9.69	12.36
F65/17.5/17.5	6.99	7.90	8.96
F72.5/13.75/13.75	5.67	6.82	8.25
F65/13.75/21.25	6.80	7.70	10.48
F65/21.25/13.75	6.69	7.96	8
F57.5/21.25/21.25	7.82	9.33	NE

^a by desiccators method

^b by DVS method

NE; Not Equilibrated

Recently, Bronlund and Paterson (2004) used this approach (calculating the moisture sorption isotherm from each component) for predicting the isotherm of a crystalline lactose powder containing 9 % amorphous lactose. This method predicted the measured isotherm well up to the breakpoint in the isotherm, which was due to amorphous lactose crystallization. It was suggested that this simple additive isotherm approach could be used for measuring the amorphous lactose content of lactose powders. It was shown in previous part when formulations contain lactose, calculated and experimental results are not the same. The tightly packed molecules in pure lactose adsorb very little water, and only on crystal surfaces. By adding the gelatinized starch and BLG, form and arrangement of lactose crystals changed. Thus, lactose in formulations adsorbed more water compared to pure lactose in the same condition. Therefore, the difference between calculated and experimental result is due to the larger area available for water sorption for lactose that co-lyophilized with gelatinized starch and BLG comparing to pure lactose. By increasing the lactose crystals size, the crystals surface decrease thus they adsorb less water.

Figure 30a and b show the micrograph of five formulations at 69 % RH with different storage time. These micrographs are different compared to spray-dried milk powder or whey powder in which the particles have spherical forms (Miriam and Labuza 1980).

Lactose crystallization occurred in all samples except F50/25/25 in the conditions described in Figure 30a and b. Formulation F50/25/25 had a smooth surface, and with rough and irregular edges. In this case, the particles looked like plate or sheet and lactose crystals could not be observed on the surface of particles. It was noted that water liberation was not observed in this case (Figure 28).

In the case of F57.5/25/17.5, lactose crystals appeared on the particles surfaces after 25 hours storage at 70 % RH and water liberation was observed on Figure 28 in these conditions. In this formulation, lactose crystals were smaller than in other formulations presented in Figure 30a and b. Moreover, lactose crystals were not packed together compared to formulations containing more lactose. By increasing lactose quantity in formulations, lactose crystals were bigger and more compact and consequently they liberated more water.

Formulations containing a large quantity of lactose, for example F80/10/10 had big crystals compared to formulations containing less lactose, for example F57.5/25/17.5. In addition, lactose crystals in the case of F80/10/10 were more compact. Size, form and arrangement of lactose crystals are very important for moisture sorption properties of formulations. Results of microscopy described why the moisture sorption of formulations could not be calculated from moisture sorption properties of each component.

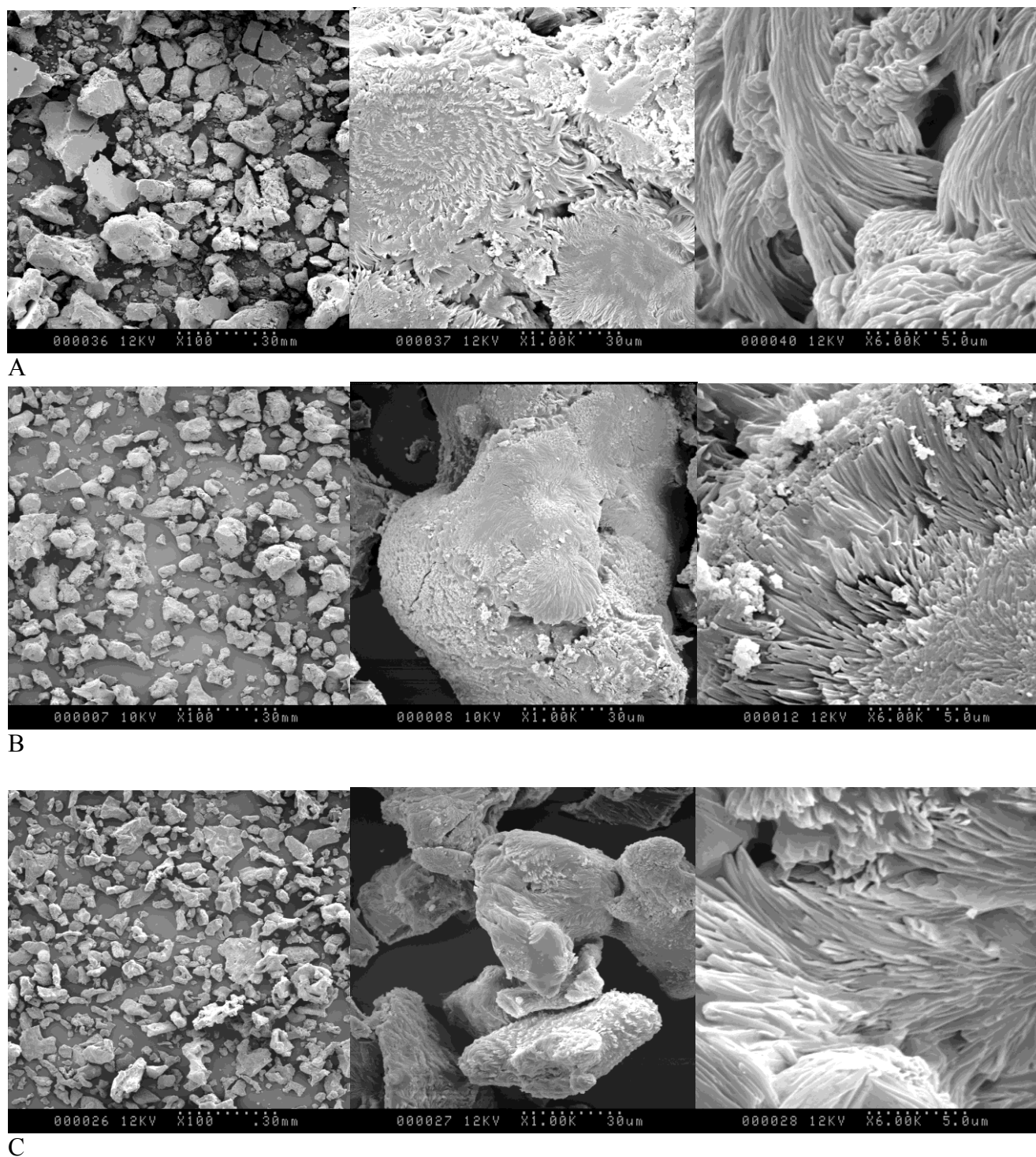
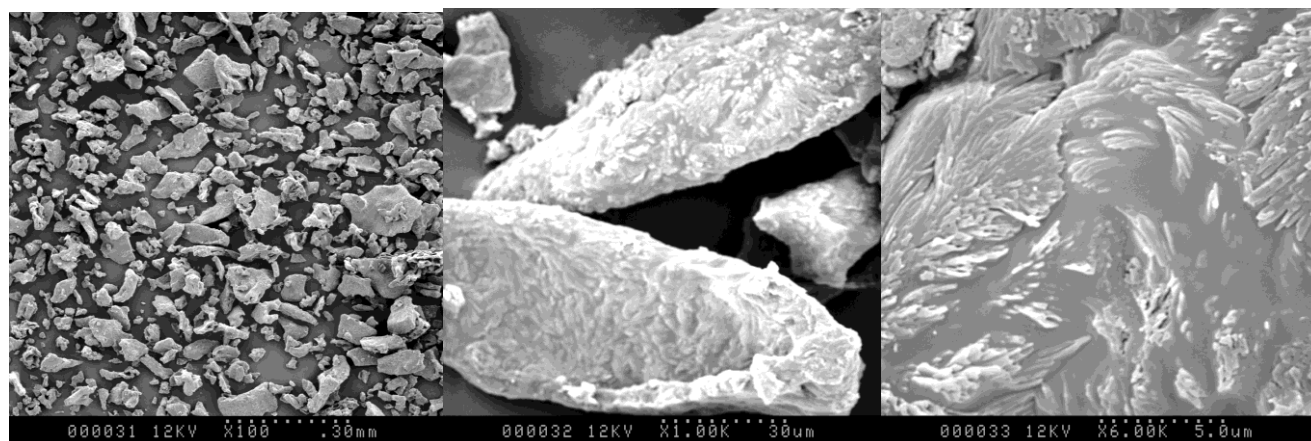
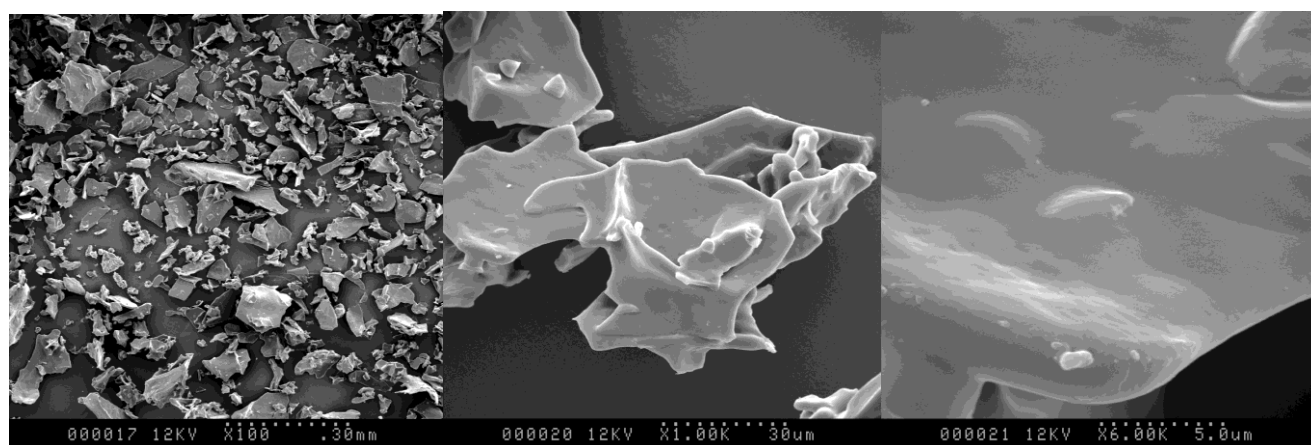


Figure 30a SEM micrographs obtained from different formulations: A: F80/10/10, B: F72.5/10/17.5, C: F65/21.25/13.75 these formulations were stored 17, 17, 25 hours at 69 % RH respectively



D



E

Figure 30b SEM micrographs obtained from different formulations: D: F57.5/25/17.5, E: F50/25/25 these formulations were stored 25 and 42 hours at 69 % RH respectively

In order to compare results, rate of mass changes (dm/dt) was plotted against time and the results are shown in Figure 31.

The minimum values in this figure show the maximum rate of water liberation during crystallization. This time was around 98 minutes for pure lactose and increased by increasing BLG and starch quantity in formulations. For example, in the case of F80/10/10, this time was around 183 minutes. Moreover, by increasing BLG and starch to 50 % (w/w) in formulations, water liberation was not observed until 2500 minutes ($\sim 42h$) storage at 70 % RH.

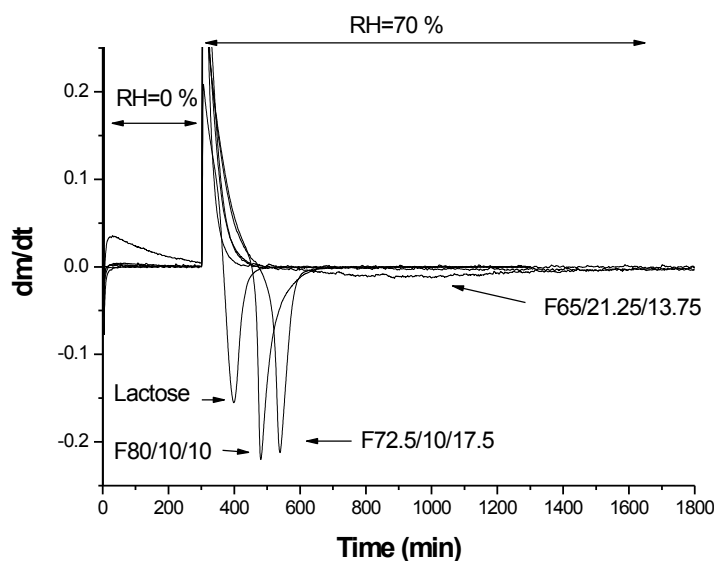


Figure 31 Mass changes rate of lactose and four formulations during experiment. The minimum values in this figure show the maximum rate of water liberation during crystallization

Results of crystallization time were analyzed by mixture design and the results of analysis of variance and contour plot of predicted results are presented in Table 16 and Figure 32.

Table 16 Analysis of variance of crystallization time at 70 % RH

Source	DF	F value	Probability>F	
Model (quadratic)	5	194.44	<0.0001	Significant
Linear Mixture	2	463.53	<0.0001	
Lactose-starch	1	20.10	0.0029	
Lactose- β -lg	1	21.40	0.0024	
Starch- β -lg	1	3.62	0.0990	

Values of "Probability > F" less than 0.0500 indicate model terms are significant. In this case Linear Mixture Components, Lactose-starch, Lactose-BLG are significant model terms

Interactions between lactose-starch and lactose-BLG had significant effect on crystallization time and interactions between starch-BLG had no significant effect on crystallization time at 70 % RH.

The coefficients of the quadratic model were estimated by the least square method from the results given by the experiment and are presented in equation 17. The statistical analysis and

Chapter III: Results and Discussion

R^2 results ($R^2=0.99$) showed that there was a strong agreement between calculated and experimental values.

$$\begin{aligned} \text{Crystallization time (min)} = & 2105 \times \text{lactose} + 26577 \times \text{starch} + 29011 \times \text{BLG} \\ & - 41785 \times \text{lactose} \times \text{starch} - 43118 \times \text{lactose} \times \text{BLG} - 26132 \times \text{starch} \times \text{BLG} \end{aligned} \quad (17)$$

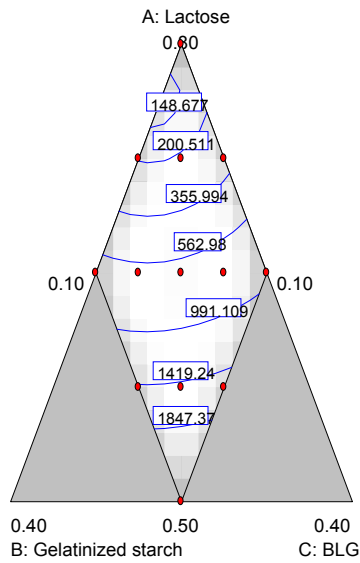


Figure 32 Contour plots of predicted response of crystallization time of formulations at 70 % RH and 20 °C

This model shows the importance of each component and the interactions between the components. Based on this equation, the influence of BLG and gelatinized starch on crystallization time is more important. In addition, interactions between components had a negative effect on crystallization time.

If this model is reduced to a linear one, the effect of each component is clearer as shown in equation 18.

$$\text{Crystallization time (min)} = -2037 \times \text{lactose} + 5558 \times \text{starch} + 7126 \times \text{BLG} \quad (18)$$

Equation 18 elucidated that BLG had more effect on crystallization time compared to starch. Figure shows the contour plots of predicted results of crystallization time at 70 % RH and 20 °C.

III.2.1 Conclusion

Dynamic Vapor Sorption (DVS) is a rapid and efficient method to determine kinetic and moisture sorption properties of samples especially in pharmaceutical and food researches. However, the time necessary for time-dependent phenomena (such as crystallization) to take place, should be taken into consideration when using DVS.

Moisture sorption kinetic of the samples depends on the composition and the interactions between the components. Water adsorption values were found to be higher in samples analyzed by DVS than in those analyzed by dessicator methods. This is because lactose crystals in samples analyzed by DVS were smaller compared to samples stored for 3 months at the same RH.

Interactions between lactose-BLG and lactose-gelatinized starch had significant effect on lactose crystallization kinetic. Therefore, thermal properties of formulations are studied in order to understand better the effect of interactions. Moreover, the nature of these interactions is studied by infrared spectroscopy.

III.3 Lactose state in model infant formulation

Several techniques were used to study crystallization phenomenon in lactose and model infant foods: moisture sorption isotherm, differential scanning calorimetry, Fourier transform infrared spectroscopy and scanning electronic microscopy. Information regarding lactose crystallization or lactose state obtained from these methods can be compared to water liberation in moisture sorption isotherms.

III.3.1 Differential Scanning Calorimetry (DSC)

DSC analysis of lactose at several RH was carried out by Thomas *et al.* 2004. A brief summary of results obtained with pure lactose is presented here.

The DSC data of different form of lactose are presented at Figure 33. For amorphous lactose, two peaks were noted: the first, exothermic, at around 175°C and the second, endothermic, at around 218°C, corresponded to crystallization and melting of α -lactose respectively.

Before storage and after 12 weeks storage at 11, 23 and 33 % RH, an exothermic peak noted at around 175 °C indicated the amorphous state of lactose. Lactose stored at 39 % RH showed a melting peak of α -lactose at around 222 °C and an endothermic peak at around 85 °C. The thermogram of lactose stored at 43 % RH showed a shoulder at around 150 °C and a melting peak at around 242 °C corresponding to β -anhydrous crystals form. This shoulder showed the presence of α -monohydrate crystal that liberated water at 150 °C.

Samples stored at RH between 54 to 95 % contained α -monohydrate. Their thermograms thus showed an endothermic peak at 150 °C. These samples contained different percentage of α and β form of lactose crystals.

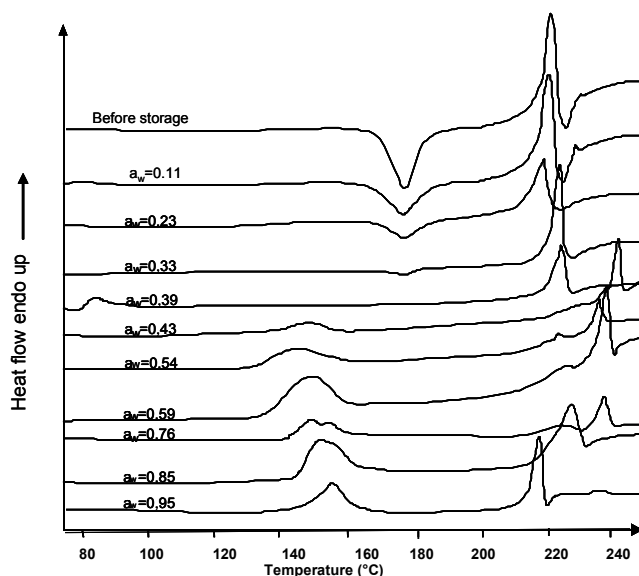


Figure 33 DSC thermograms of lactose before storage and after 12 weeks storage at different RH ranging from 11 to 95 % and 20 °C, data from Thomas (2004)

DSC thermograms of BLG and gelatinized starch showed no peak when analyzed in open pan (results not shown). The DSC profile of F80/10/10 is presented in Figure 34.

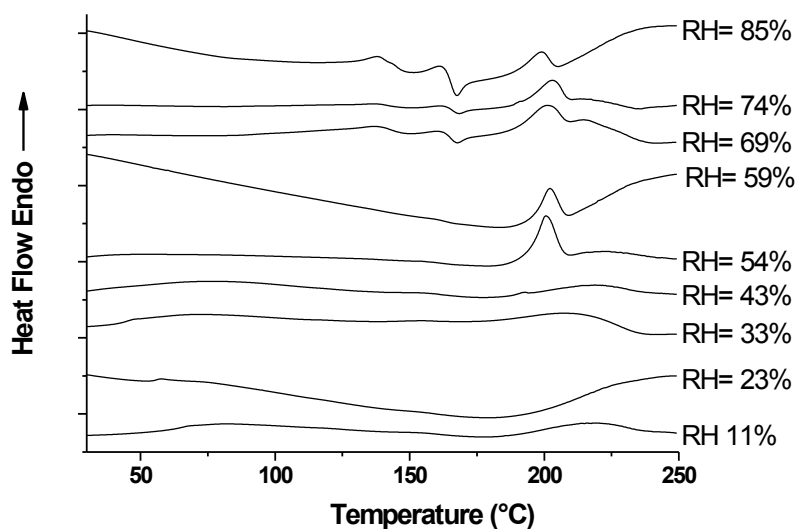


Figure 34 DSC thermograms of F80/10/10 after 3 months storage at different RH ranging from 11 to 85 % and 20 °C

This formulation, stored between 11 to 43 % RH, showed no clear peak. As explained from results obtained from SEM, this formulation was in amorphous state in this condition. Crystallization heat is not large enough to be detected when there were 20 % BLG and

gelatinized starch in the formulation. Endothermic peak due to water loss at around 140 °C, observed for samples stored at 69, 75, 85 % RH, showed the presence of α -monohydrate lactose. Temperature at which endothermic peak appears changes in different conditions such as heating rate (Itoh *et al.*, 1977) and mixture composition. For example, Chidavaenzia *et al.* (2001) showed water losses or dehydration at around 115 °C and Angberg (1995) reported that dehydration of different forms of lactose occurs between 132 to 147 °C.

Furthermore, the intensity of this peak increased by increasing the RH in our samples. The peak at 201 °C was attributed to melting of anhydrous α -lactose. The absence of melting in the 230–240°C temperature region indicated the absence of crystalline β -lactose in the sample. Generally, melting peak of α -lactose is around 216 °C but in this study, this peak shifted to 201 °C. Chidavaenzi *et al.* (1997) showed that melting of monohydrate lactose occurred at 204 °C as an endothermic peak at this temperature. Moreover, Erdogdu *et al.* (1995) showed an increase in the endothermic peak in starch-lactose mixtures. They observed that by increasing the lactose quantity in starch-lactose mixture, the endothermic peak increased. Thermogram of starch containing 13 % moisture shows two endothermic peaks at around 175 and 190 °C (Yu and Christie, 2001). First peak was less intense and sometimes it looked like a shoulder.

Moreover, the presence of only one peak at around 201 °C indicated strong interactions between these components or maybe covalent bonds between lactose and BLG. It will be explained later from results obtained by infrared spectroscopy, that these bonds were hydrogen bonds also the covalent bonds. By increasing gelatinized starch and BLG quantity in formulations, melting temperature decreased. F80/10/10 showed an endothermic peak in samples stored at higher than 54 % RH corresponding to melting of mixture of α and β lactose. Formulation containing 80 % lactose (F80/10/10) above 54 % RH became crystallized. The DSC profile of this formulation, which was stored at 85 % RH shows two endothermic peak at around 145°C and 200°C corresponding to water liberation and melting of lactose respectively and an exothermic peak at around 168 °C.

Figure 35 shows the DSC thermogram of F50/25/25 after 3 months storage at different RH and 20 °C.

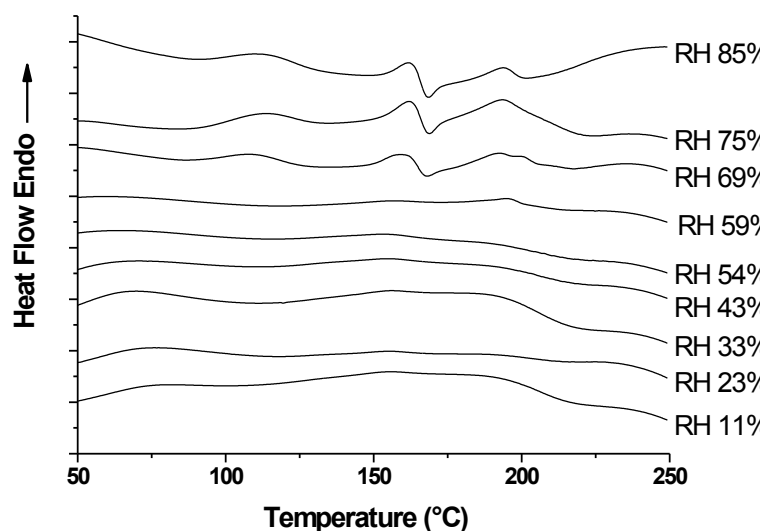


Figure 35 DSC thermograms of F50/25/25 after 3 months storage at different RH ranging from 11 to 85 % and 20 °C

Melting temperature in this formulation was observed at around 195 °C and it can be seen in the formulations stored at high RH, from 59 to 85 %. These results agreed with that found by moisture sorption isotherms. For example, water liberation for this formulation was observed at 59 % RH. An endothermic peak can be observed in this figure (Figure 35) at around 160 °C, showing water losses or dehydration of α -monohydrate lactose.

Figure 36 shows DSC thermograms of formulations after three months storage at different RH and 20 °C. Melting temperature varied from 191 to 201 °C. DSC thermograms of formulations were not as clear as thermograms of lactose. Thus, interpretation of results was complicated.

In all formulations stored at 75 and 69 % RH, an endothermic peak was observed at around 150 to 160 °C (Figure 36a and b). This peak corresponded to water loss of α -monohydrate lactose. The intensity of this peak decreased by decreasing the RH and it disappeared at and below 54 % RH (Figure 36c) in all formulations. Formulations stored at 59 % RH showed only an endothermic peak at around 200 °C corresponding to melting point of formulations. In all formulations, one melting peak was observed, showing the presence of strong interactions between components.

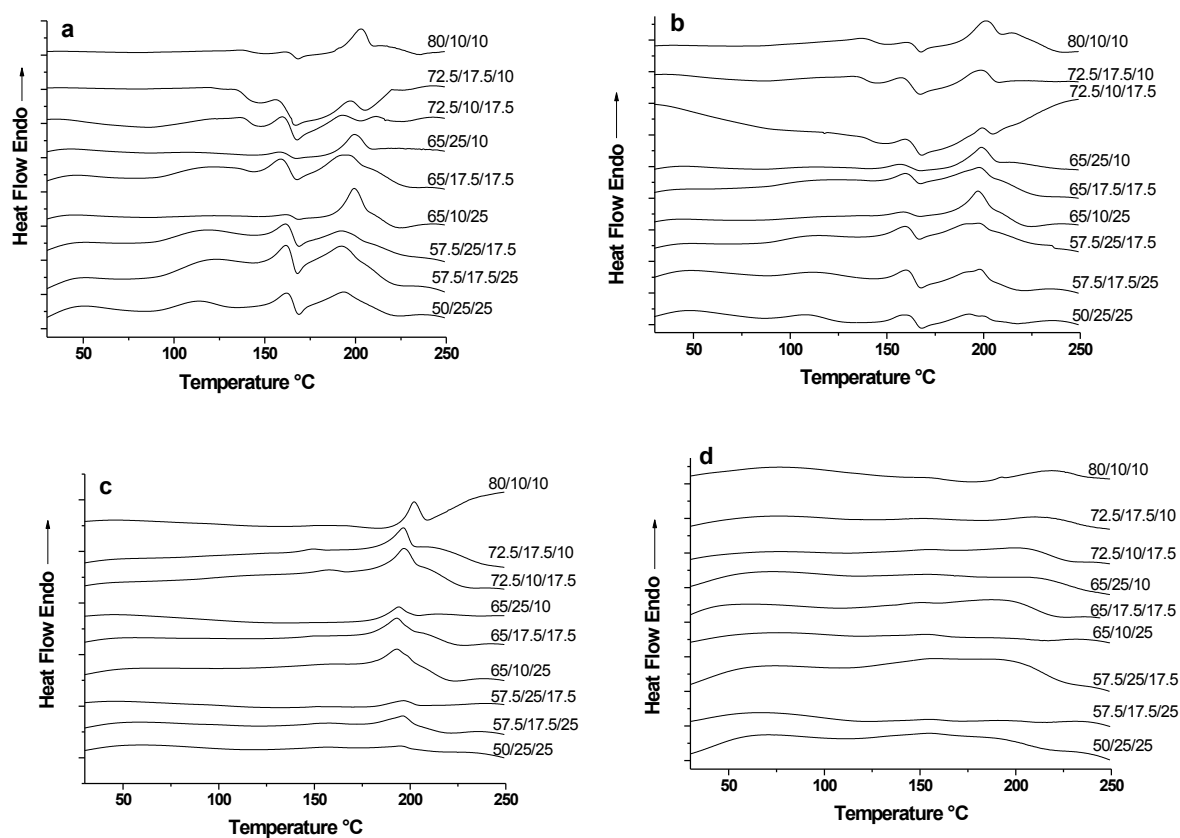


Figure 36 DSC thermograms of different formulation stored at 20 °C and 75 % (a), 69 % (b), 59 % (c) and 43 % (d) RH

Formulations stored at and below 43 % RH, showed no identical peak. At these RHs the crystallization peak was masked by BLG and starch.

III.3.2 Scanning Electronic Microscopy (SEM) of formulations

Thomas (2004) showed micrographs of lactose and BLG, thus in this part the result of the formulations containing lactose, BLG and gelatinized starch stored at several RHs for three months will be explained.

Figure 37 and Figure 38 show the micrographs of F50/25/25 and F80/10/10 after 3 months storage at ~ 0, 43, 54 and 59 % RH and 20 °C.

Freeze-dried formulations containing amorphous lactose resembled broken glass or flake-like structure similar to that of lactose. Similar type of structure was also observed by Millqvist-Fureby *et al.* (1999) in other freeze-dried carbohydrate/protein mixtures. An important change in particle structure was observed when formulations were stored at high RH where crystallization occurred. Powders became agglomerated in some cases and had a rough surface structure and nearly rod-like structure. Moreover, lactose crystals appeared on the surface of the particles.

In the case of F50/25/25, the particle had irregular forms with sharp edges and different size. The irregular particle form increased by increasing the lactose quantity in formulations. It has been shown by numerous authors that milk powder contains spherical particles with the smoothed surface (Al Mahdi *et al.*, 2006, Saltmarch and Labuza, 1980). Nevertheless, in our formulation, surface of sample was not smooth and the particles were not spherical. Moreover, small particles could be observed on the large surface of particles. This formulation crystallized after three months at 59 % and lactose crystals were observed on the surface of the particles (Figure 37e). Moreover, lactose crystallization did not occur at 54, 43, ~ 0 % RH. These results agreed with results found from moisture sorption isotherms.

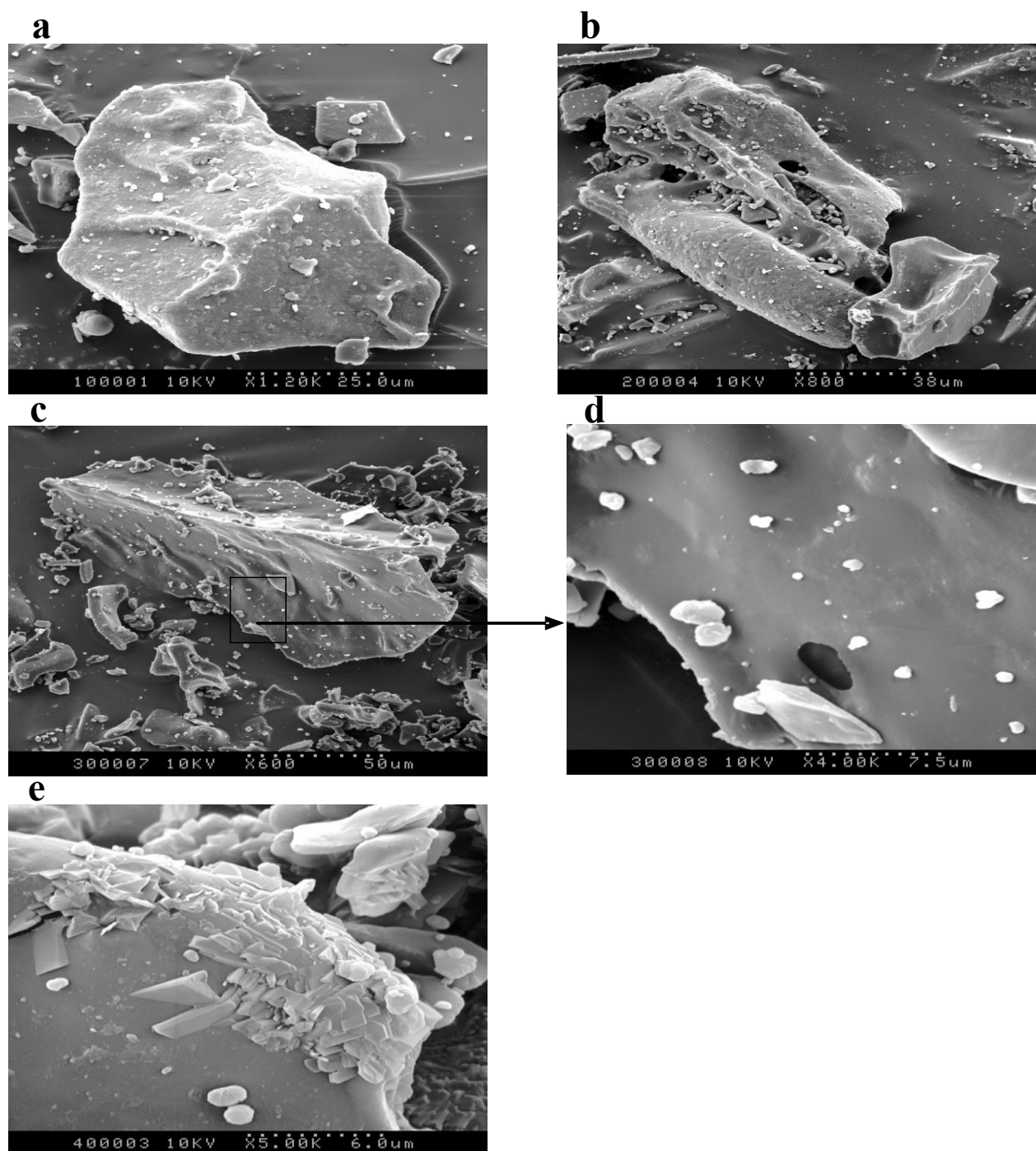


Figure 37 SEM micrographs of F50/25/25 stored at ~ 0 (a), 43 (b), 54 (c, d), and 59 (e) and 20 °C for 3 months

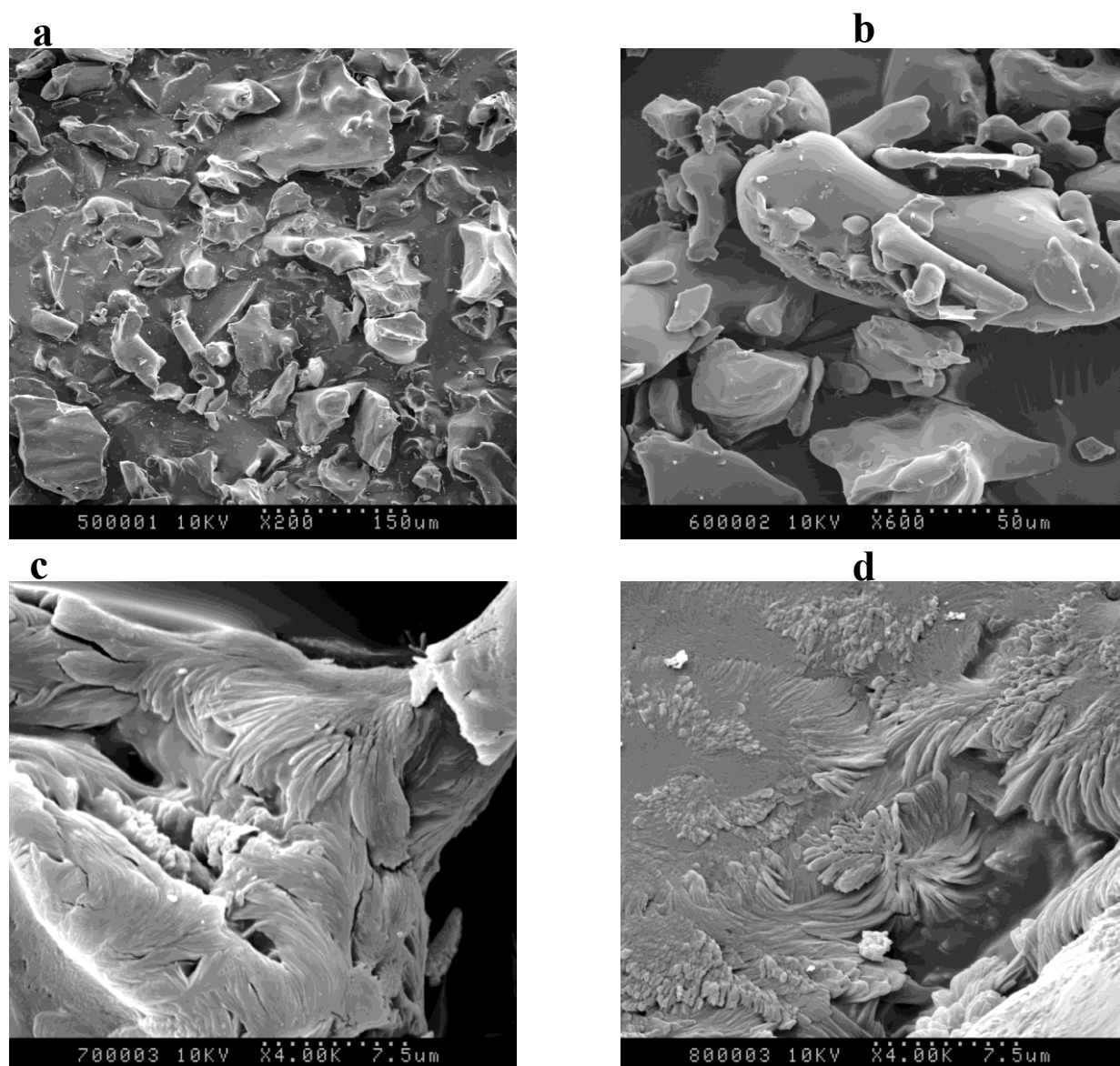


Figure 38 SEM micrographs of F80/10/10 stored at 20 °C and different RHs; ~ 0 (a), 43 (b), 54 (c) and 59 % (d) for 3 months

F80/10/10 (Figure 38) had a broken glass or flake-like structure. The particle surfaces were smooth and less small particle could be observed on the surface of particles. Lactose crystallization could be observed at 54 and 59 % RH (Figure 38c and d). Lactose crystals in this case are big and very compact compared to F50/25/25 where the crystals were smaller and less compact.

It was explained that moisture sorption properties of samples containing lactose could not be calculated because of lactose crystals size and therefore of the internal surface of particles. Now, to show the difference in lactose crystals size, the micrographs of lactose and five

formulations are presented in Figure 39. In the case of pure lactose, after 3 months storage at 69 % RH, a compact structure could be observed (Figure 39a). The big and very compact crystals with rounded edges could be observed in this figure. The lactose crystals had a smooth surface and different sizes.

In the case of F80/10/10, lactose crystals appeared on the surface of particles (Figure 39b). Lactose crystals are big and they have a nearly compact structure resembling big plates. The difference between this formulation and pure lactose, is that the crystals are more voluminous compared to F80/10/10. By increasing BLG and gelatinized starch quantity in formulations, lactose crystals are smaller and less compact. For example, in the case of F50/25/25, some small crystals are observed on the surface of particles.

It is interesting to know the chemical composition of particle surface. This can put forward, our hypothesis about the calculation of moisture content of samples containing lactose. If all lactose present in the formulation is found on the surface of particles, then we can expect having big crystals on the surface of particles. Numerous authors studied the surface composition of powder containing proteins and saccharides by Electron Spectroscopy for Chemical Analysis (ESCA) (Fäldt and Bergenståhl, 1994, Millqvist-Fureby *et al.*, 1999).

Fäldt and Bergenståhl (1994) showed that the protein appears on the surface of spray-dried mixture of protein-lactose particles. In another study, Fäldt and Bergenståhl (1996) showed, by SEM, that when crystallization occurs, lactose crystals appear on the surface of particles but by analyzing the surface of particles, an intact protein film was found on the surface of lactose crystals. Gaiani *et al.*, (2006) reported similar results for mixtures of spray-dried dairy powders containing high quantity of proteins.

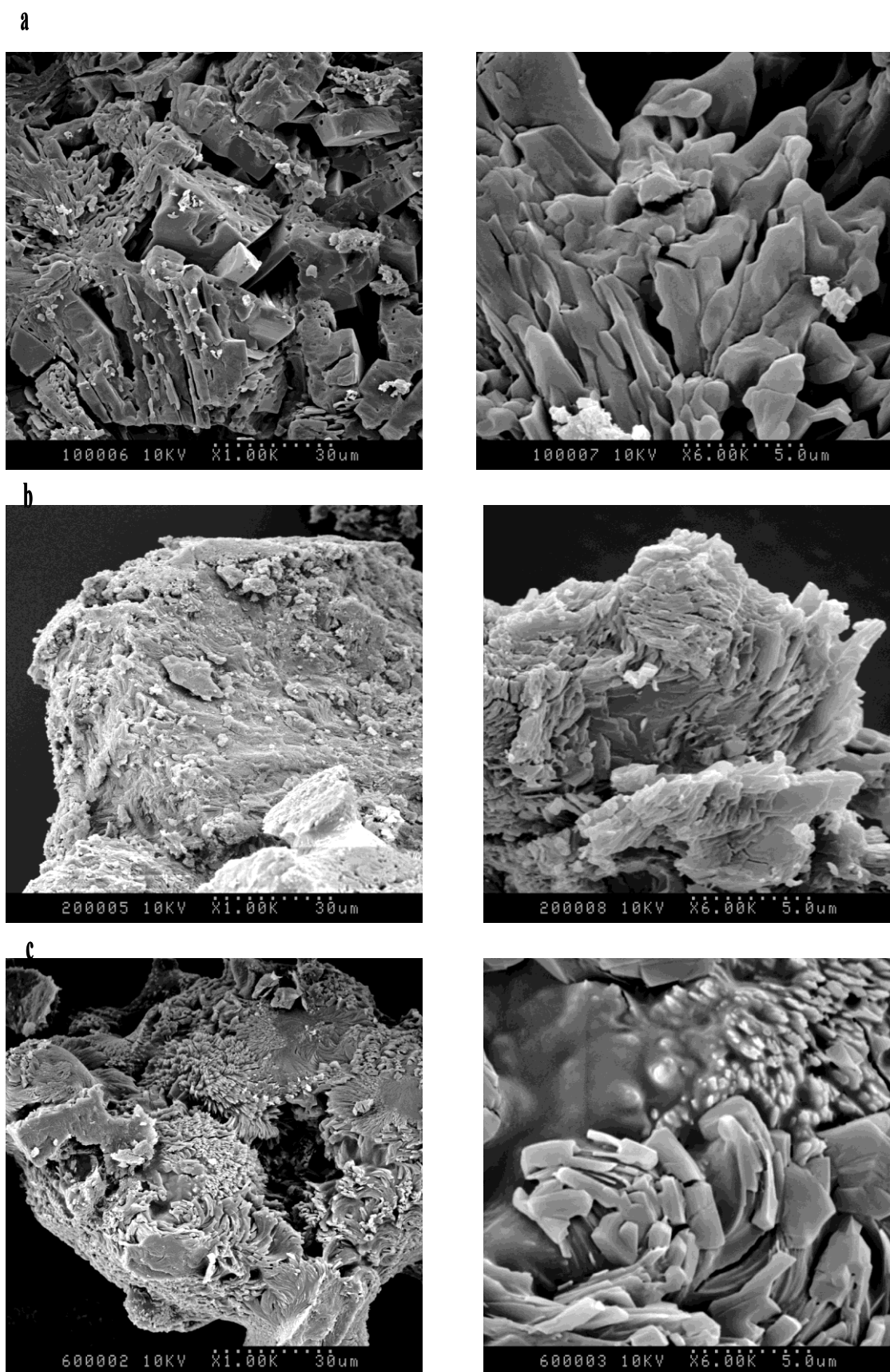


Figure 39a SEM micrographs of lactose (a), F80/10/10 (b), F72.5/10/17.5 (c), stored at 69 % RH and 20 °C for 3 months

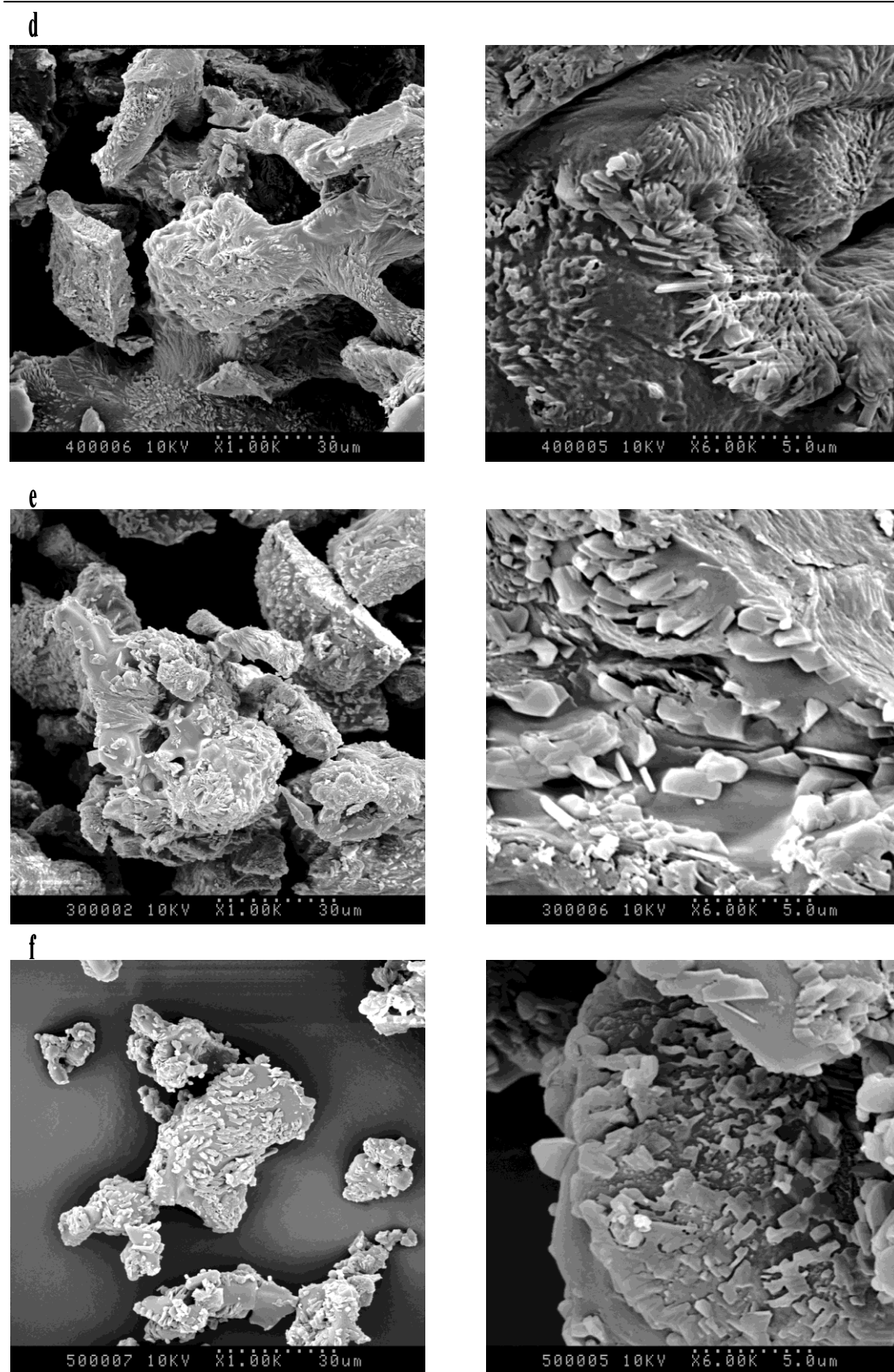


Figure 39b SEM micrographs of F65/21.25/13.75 (d), F57.5/25/17.5 (e), F50/25/25 (f) stored at 69 % RH and 20 °C for 3 months

For freeze-drying, the mechanism is more uncertain, but it is possible that also for this process the adsorption mechanism is valid. If this is the case, the protein would adsorb on ice crystal surfaces in the frozen material prior to drying, which could be expected to result in an increase in the protein content on the surface of the freeze-dried powder compared to the bulk composition. Millqvist-fureby *et al.* (1999) showed that proteins accumulated at the surface of freeze-dried protein/carbohydrate powders and the mechanism of this adsorption is different compared to spray-drying method.

The result of Millqvist-fureby *et al.* (1999) showed the protein composition of particle surface reached until 80 % of protein quantity is present. Therefore, this protein quantity on the particle surface may prevent enlargement and compact structure of lactose in the formulations that was observed for pure lactose.

It is interesting to compare these results with those presented in kinetic of lactose crystallization. The difference between two series of micrographs is the storage time. Micrographs that was shown in lactose crystallization kinetic part, was obtained after several hours (between 17 to 42 hours) depending on the lactose quantity at 69 % RH but the micrographs that was explained in Figure 39 was obtained after three months storage at 69 % RH.

For example, crystallization occurred in F50/25/25 after three months storage at 69 % RH (Figure 39 f) but this formulation was amorphous after around 42 hours at 69 %.

The more interesting comparison between two series of micrographs is the surface of particles. It can be seen that the surface of formulations stored several hours at 69 % RH is smoother and lactose crystals seem to be inside the particles and some parts of crystals on the surface of particles. Nevertheless, after 3 months storage at 69 % RH, lactose migrated on particles surface. For examples, in the case of F57.5/25/17.5, lactose crystals can be observed after 25 hours at 69 %, but these crystals are not completely on the particle surfaces whereas, after 3 months storage of this formulation at the same RH, lactose crystals are observed on the surface of particles. The same results were found for other formulations.

These results showed that, during storage of formulations at high RH, lactose migrated to the surface of particles. Fäldt and Bergenståhl (1994) showed that the composition of particles surfaces did not change and the proteins were on the particles surfaces. They studied this phenomenon after 4 days storage of samples at 76 %. It is possible in long-term storage, that an important migration of lactose could occur and particle surface composition could change.

III.3.3 Infrared spectroscopy of lactose crystallization

III.3.3.1 IR spectroscopy of pure lactose

It is important to know the structure changes of lactose at different RH. Amorphous lactose was stored at several RH ranging from 0 to 85 % for 3 months and the different spectra bands were studied. Figure 40 shows spectra of amorphous and crystallized lactose.

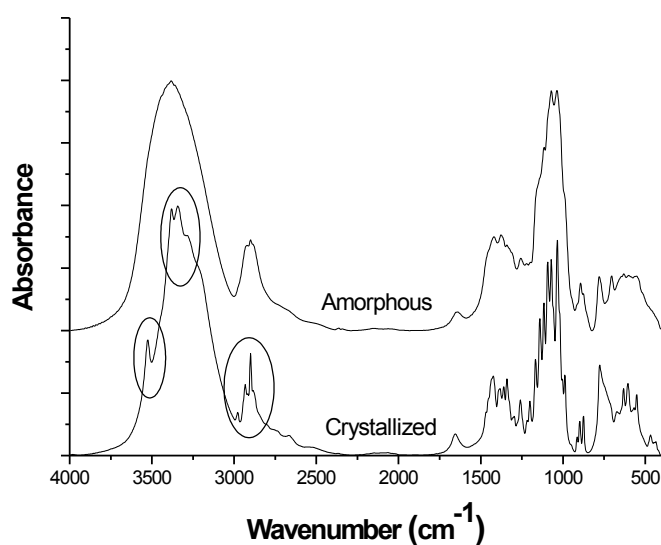


Figure 40 Infrared spectra of pure lactose in amorphous state (0 %) and crystallized state (85 %) at 20 °C. Baselines of spectra were corrected and then all spectra were smoothed by 9 points and were normalized by min-max method

Crystallized lactose showed the presence of one sharp band around 3380 cm^{-1} and a few shoulder peaks in the O-H stretching region between 3050 and 3600 cm^{-1} showing the presence of hydrogen bonds. The broad form of O-H band of amorphous lactose indicated a wide range of hydrogen bond lengths and orientations. The main reason for sharper peaks in the crystalline state is the higher degree of homogeneity of intramolecular interactions (Wolkers *et al.*, 2004). This leads to less dispersion of vibrational levels and a higher conformational selectivity. Crystallization caused a decrease in stretching vibrations of OH bonds (ν_{OH}), suggesting an increase in hydrogen bond density and strength (Ottenhof *et al.*, 2003). Numerous researchers (Maalouly and Jaillais, 2005, Ottenhof *et al.*, 2003) interpreted changes in wavenumber of the stretching vibration of OH group, in the region 3000 to 3600 cm^{-1} , as being mainly due to hydrogen bonds. Indeed, the stretching vibration of an OH group involved in a hydrogen bond is expected to have a lower wavenumber than its non-hydrogen

bonded counterpart as it will have a lower bond force constant due to electron delocalisation in hydrogen bonded structures.

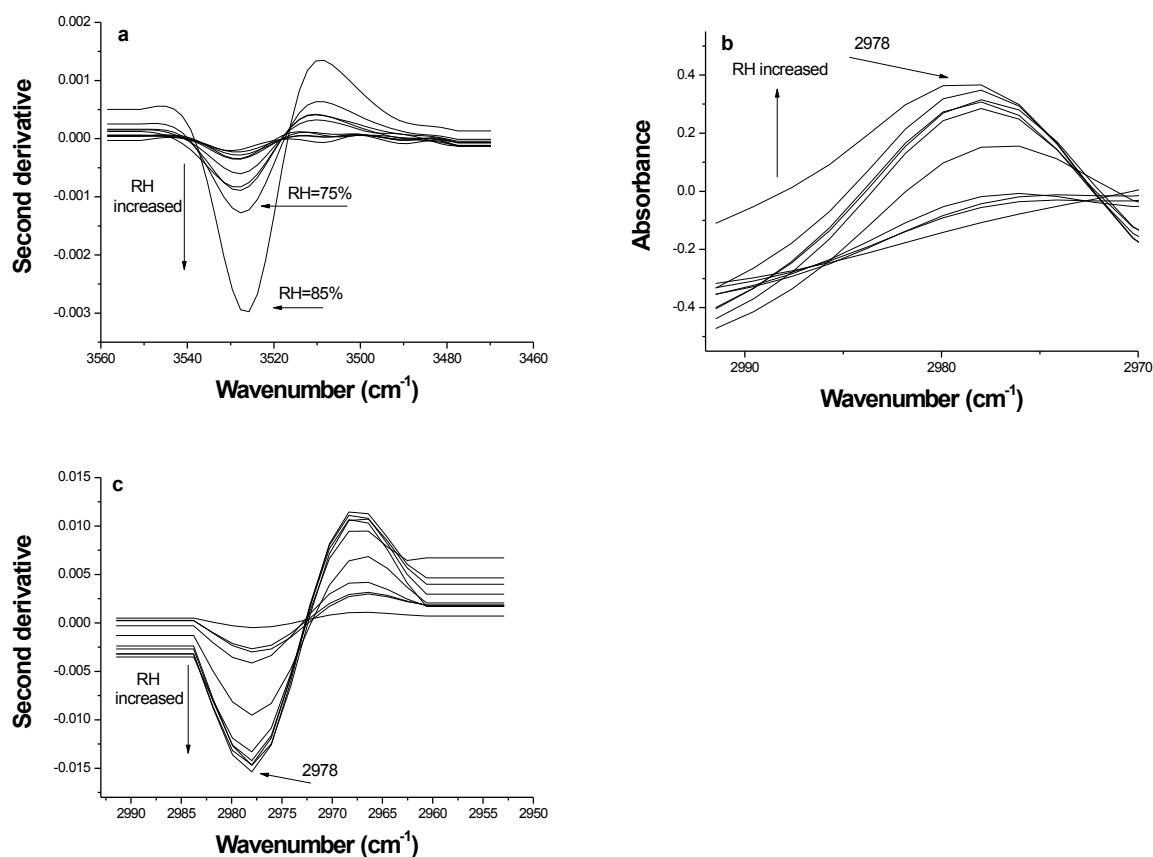


Figure 41 a: second derivative of spectral region of 3460 to 3560 (cm^{-1}), b and c absorbance and second derivative of spectral region of 2950 to 2990 (cm^{-1}), RH varies between 0 and 85 % and all spectra were smoothed by 9 points and were normalized by vector method

By increasing the RH, a new peak was observed at 3525 (cm^{-1}) indicating the hydration of lactose at high RH. The second derivative of this region gave more details which are presented in Figure 41a. Figure 41a shows that by increasing the RH, the intensity of the peak at 3527 cm^{-1} increased. Moreover, the peak at 3509 cm^{-1} was observed in crystallized state samples (high RH). This region is interesting for the study of lactose crystallization.

The second region of the infrared spectra of pure lactose spectra which was studied lies between 2800 and 3000 cm^{-1} , corresponding to C–H bonds. The analysis of crystallized lactose resulted in the presence of four peaks in this region, whereas that of amorphous lactose showed one broad and one weak peak in this region.

Moreover, between the two broad peaks (2900 and 3370 cm^{-1}), a weak peak was observed at 2978 cm^{-1} . This peak was sensitive to RH and appeared as RH increased. Its intensity also increased as RH increased (Figure 41b and c). For example, in the case of the sample stored at 0% RH, there was no peak in this region but by increasing the RH to more than 43% this peak was clearly observed.

III.3.3.2 Influence of BLG and starch on lactose spectra

Infrared spectra of crystalline sugars are complex (Tipson and Parker, 1980), therefore, in order to investigate the presence of hydrogen bonding between lactose, starch and BLG that have been freeze-dried together and the effect of storage conditions, infrared spectra of these components were compared.

Figure 42 shows the infrared spectra of crystallized lactose, F65/10/25 before storage and at two RH after 3 months storage. This formulation was chosen because the maximum yellow color changes occurred in this formulation. It can be seen that the spectrum of lactose alone differ from the formulations at different storage conditions.

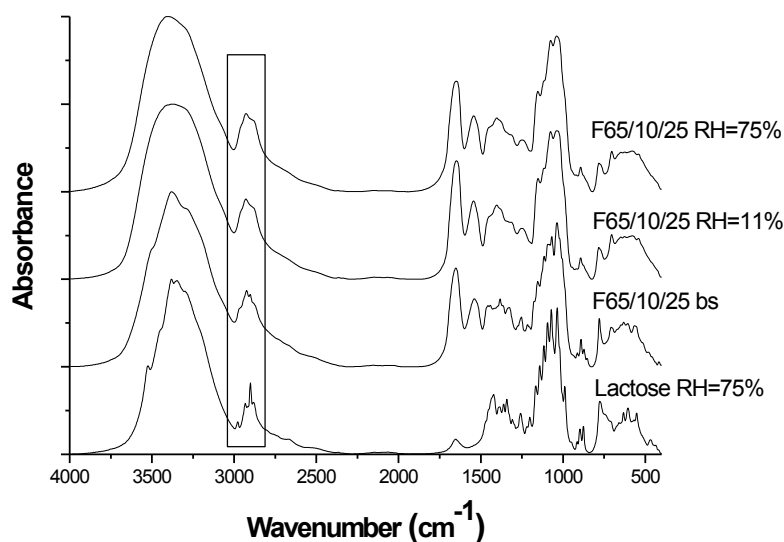


Figure 42 Infrared spectra of F65/10/25 before storage (bs), after three months storage at 11 and 75 % RH and lactose after three month storage at 75 % RH. Baselines of spectra were corrected, then all spectra were smoothed by 9 points and were normalized by min-max method

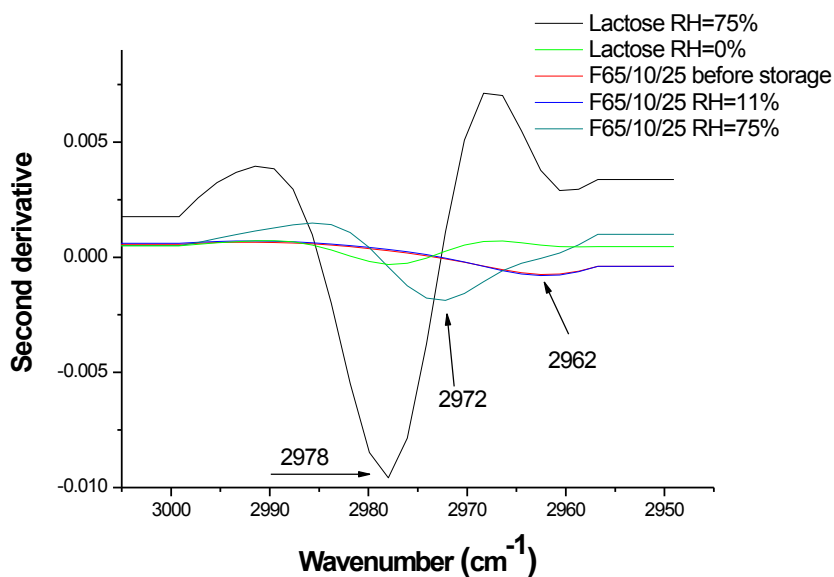


Figure 43 Second derivative of crystallized lactose, F65/10/25 before and after 3 months storage at 11 and 75 % RH; the spectra were normalized by vector method. The contribution of BLG and starch to the absorbance in this region were subtracted from the spectra

In C–H stretching mode (2800 to 3000 cm^{-1}), there was fusion of 4 bands (around 2970 cm^{-1}) into a one broad band and a shoulder band after 3 months storage in the presence of starch and BLG.

As explained for the C–H stretching band of lactose at 2978 (cm^{-1}), changing the RH, changed the spectra intensity. Nevertheless, when BLG and gelatinized starch were added to formulations, this peak shifted to 2972 cm^{-1} (Figure 43). Moreover, in the case of formulations F65/10/25 before storage and after 3 months storage at 11 % RH, this peak shifted to 2962 cm^{-1} and occurred at the same wavenumber, before and after storage. By increasing the RH from 11 % to 75 % this peak was found between lactose and the formulation before storage and that stored at 11 %. This may be due to some hydrogen bonds that had been formed between lactose, BLG and starch being replaced by water at high RH in the formulation. These results were observed for other formulations containing different quantity of BLG and starch.

III.4 Non-covalent interactions by infrared spectroscopy

Non-covalent interactions between lactose, BLG and gelatinized starch were studied using mid infrared spectroscopy. Firstly, spectrum of each component was obtained at different RH and secondly, model infant formulations were analyzed at different RH ranging from ~ 0 to 85 %. In this part, the infrared spectra of each component will be discussed and then the spectra of formulations and effect of each component on infrared spectra of formulation will be explained.

III.4.1 General spectra

Figure 44 and Figure 45 show the infrared spectra of lactose, BLG and gelatinized starch at two RH (0 and 75 %) after 3 months storage.

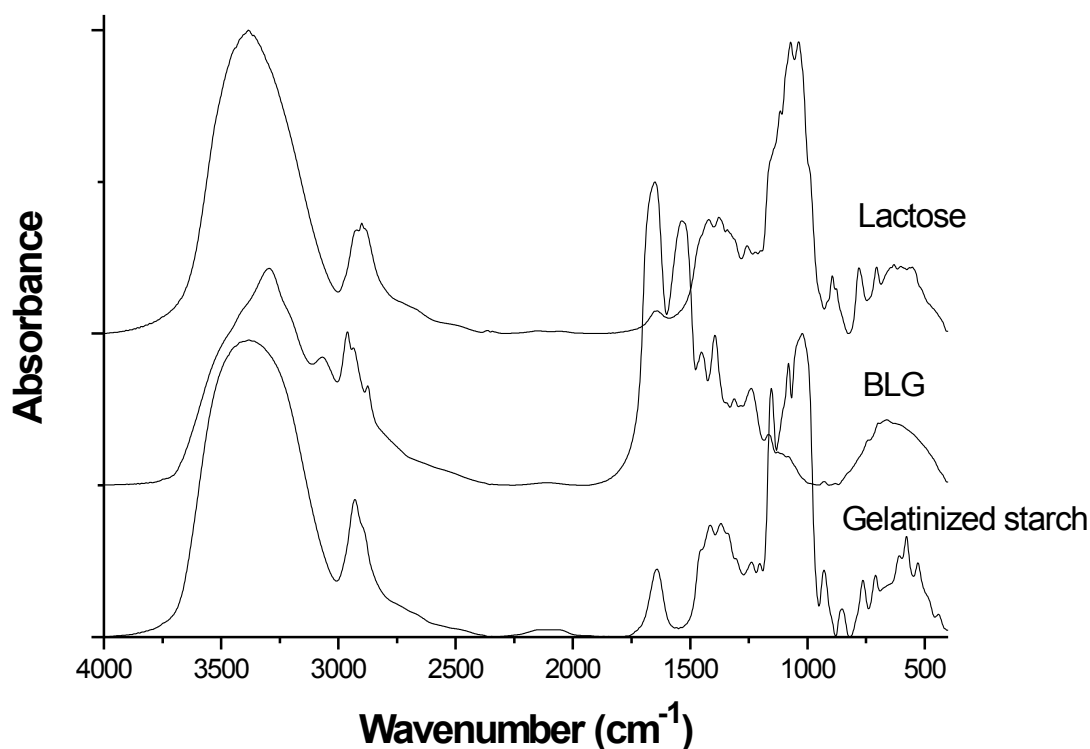


Figure 44 Infrared spectra of lactose, BLG and starch, stored at 20 °C and ~ 0 % RH for 3 months. Baselines of spectra were corrected, then the spectra were smoothed by 9 points and normalized by min-max method

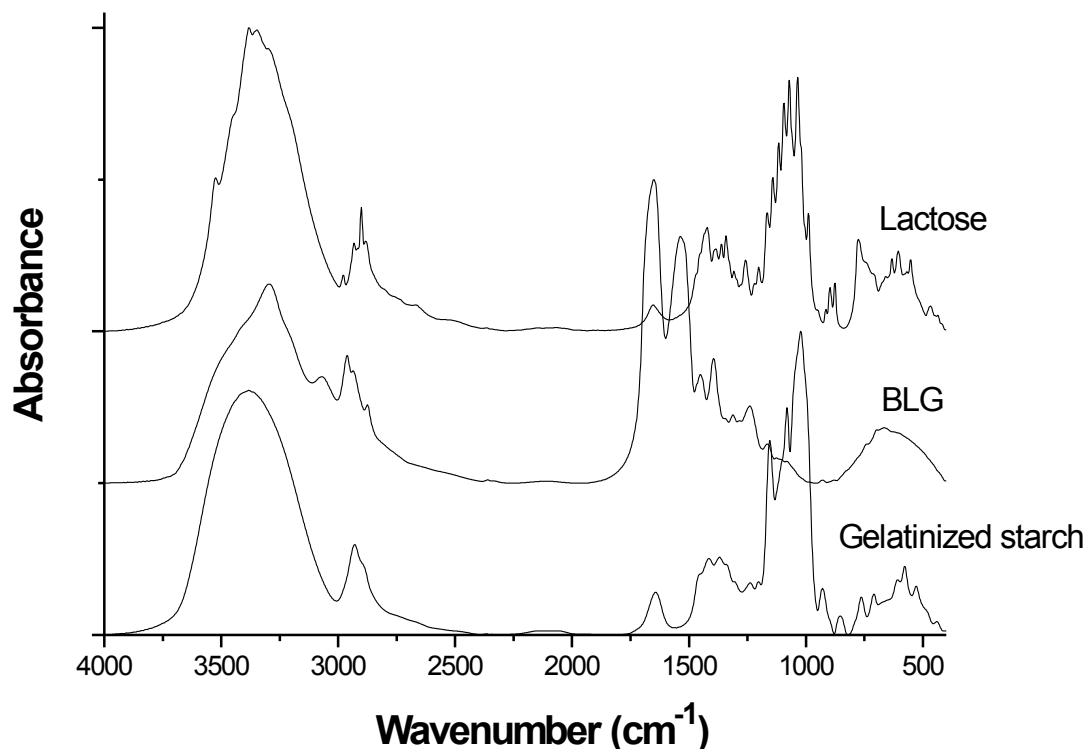


Figure 45 Infrared spectra of lactose, BLG and starch, stored at 20 °C and 75 % RH for 3 months. The base lines of spectra were corrected, then the spectra were smoothed by 9 points and normalized by min-max method

The major difference between these spectra can be observed at 3600-2800 and 1800-700 cm^{-1} .

III.4.2 Spectral region of 3600 to 2800 cm^{-1}

The region spectral of 3600 to 2800 cm^{-1} can be divided into two parts. First part corresponds to O-H bonds (around 3600 to 3000 cm^{-1}) and the second (3000 to 2800 cm^{-1}) corresponds to C-H bonds of carbohydrates. This region (3600 to 2800 cm^{-1}) is known as amide A of proteins corresponding to N-H bonds. The first part located between 3600 and 3000 cm^{-1} and characterized by an intense and large band is attributed to stretching vibration of O-H. If hydrogen bonds (inter and intra molecular) exist between molecules, it can be observed by a decrease in wavenumber of O-H stretching vibration peak. The second part that can be observed as a small peak is attributed to different stretching vibration mode of C-H bonds (Longhi *et al.*, 1986). In amorphous state (Figure 44) the spectrum of lactose is smooth in this

region and after 3 months storage of lactose at high RH, some small peaks appeared showing the crystallization of lactose.

Amide A is the designation for the band in the region near 3300 cm^{-1} that is a Fermi resonance pair (the other being amide B in the region near 3050 cm^{-1}) between essentially 100 % N-H stretch (s), which predominates in amide A, and combination and/or overtones from $1500\text{-}1700\text{ cm}^{-1}$ region. Analysis of this resonance pair permits recovery of the unperturbed N-H s frequency $\nu(\text{N-H})$.

It was thought that, since N-Hs is in such a localized mode, it should not depend on conformation and that its frequency would be affected only by external interactions such as hydrogen bonding. Thus a lower $\nu(\text{N-H})$ would be associated with a stronger hydrogen bond, and vice versa. Result shows that the spectra of BLG in the region of $3100\text{ to }3600\text{ cm}^{-1}$ at two RH (0 and 75 %), had the same shape, showing that an increase in the RH or water content, did not change the BLG spectra in this region.

Figure 46 shows more details about this region. This figure shows the bands position of each component and effect of water quantity on infra spectra of components. For example, BLG stored at $\sim 0\%$ and 75% RH had the same peak at around 3297 cm^{-1} .

The form and position of the peak of stretching vibration of O-H (νOH) shows the amorphous and crystalline state of lactose. Amorphous lactose stored at $\sim 0\%$ RH had a smooth peak at νOH region. This is because of random hydrogen bonds between amorphous lactose. Crystallized lactose (RH=75 %) had not the same form as amorphous lactose in this region and it was previously explained in lactose crystallization section.

Gelatinized starch stored at $\sim 0\%$ RH, showed a sharp smooth band around 3385 cm^{-1} by increasing the RH to 75 %, this peak was sharper than sample stored at 0 % RH. In addition, some shoulders appeared in this region and they were similar in shape to amorphous lactose peak.

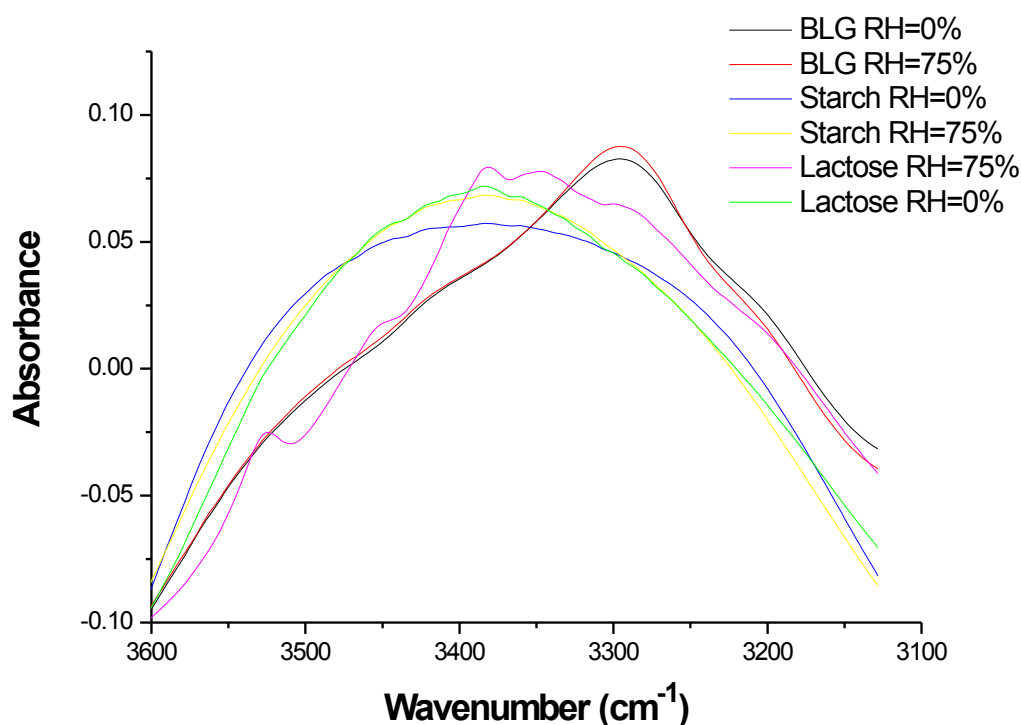


Figure 46 Infrared spectra of lactose, BLG and gelatinized starch after 3 months storage at ~ 0 and 75 % RH. The baseline of spectra were adjusted then the spectra were cut and normalized by vector method

III.4.3 Region spectral of 1700 to 1600 cm^{-1}

For proteins, infrared signal is principally due to the vibrations of the amide groups of the polypeptide backbone. An amide group gives rise to specific vibrational modes (Bandeekar, 1992), that so-called amide modes, among which the amide I band is the most intense and the most studied. It originates from the C=O stretching vibration of the amide group weakly coupled to the N-H bending and the C-N stretching vibrations. The amide I mode is very sensitive to the geometry, local environment, and hydrogen bond strength of the amide groups. Each type of secondary structure is thought to absorb at a specific frequency (Jackson and Mantsch, 1995). Moreover, this region corresponds to O-H scissoring mode of water that corresponds to lactose crystallization water. There is a wealth of information that can be used to derive structural information by analyzing the shape and position of bands in the Amide I region of the spectrum. The presence of a number of amide I band frequencies have been

correlated with the presence of α -helical, antiparallel and parallel β -sheets and random coil structures (Byler and Susi, 1986). Figure 47 shows absorbance bands of lactose, BLG and gelatinized starch in this region.

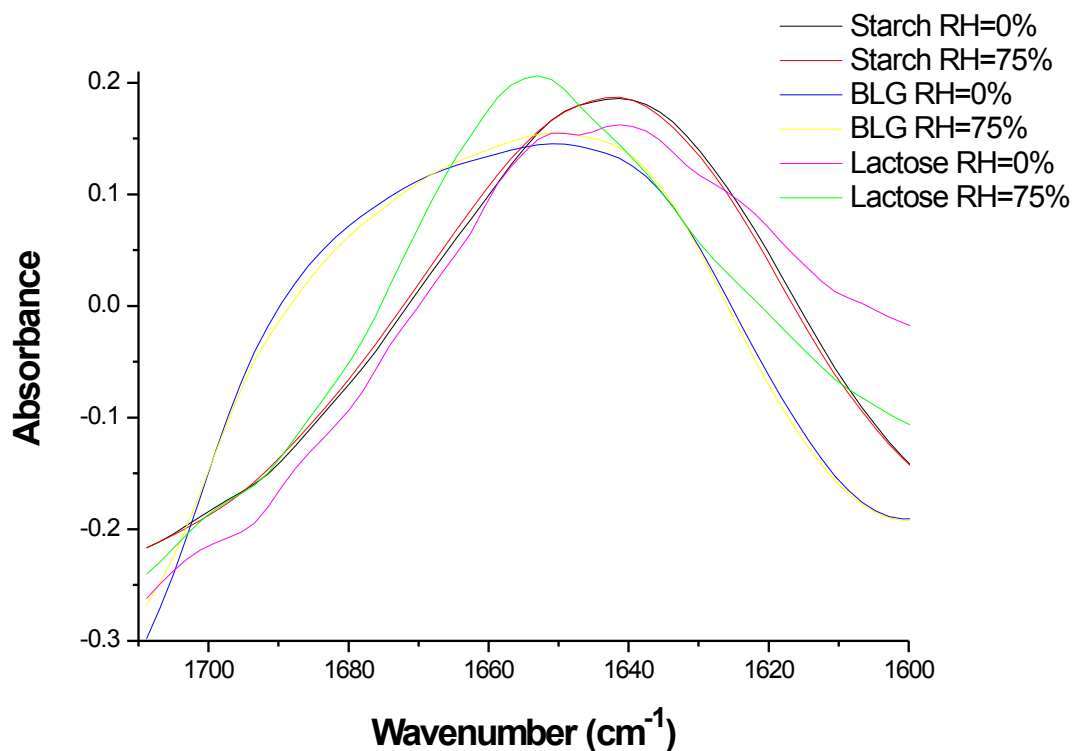


Figure 47 Infrared spectra of gelatinized starch, BLG and lactose at ~ 0 and 75 % RH and 20 °C after 3 months storage. The baseline of spectra were corrected, then the spectra were smoothed by 9 points and the region of 1600-1700 cm^{-1} were cut and normalized by vector method

Amorphous and crystallized lactose were different in this region and H_2O scissoring mode of residual water shifted from 1653 cm^{-1} in the crystalline form to 1639 cm^{-1} in the amorphous form, indicating that the water molecules in crystalline lactose are more strongly hydrogen bonded to the sugar molecules compared to residual water in amorphous lactose. Wolkers *et al.* (2004) found the same results for trehalose. Moreover, amorphous lactose had one sharp peak and one shoulder in this region compared to crystallized lactose that had only one sharp peak.

Gelatinized starch stored at ~ 0 and 75 % RH had the same peak in this region showing that high RH did not change the gelatinized starch spectra.

The comparison between BLG stored at two RH (~ 0 and 75 %) showed the effect of hydration on BLG structure. The result of second derivative (result not shown) shows that there was no change in wavenumber of BLG stored at high RH and low RH.

III.4.4 Infrared Spectra of formulations

Figure 48 and Figure 49 show the infrared spectra of F65/10/25 at four RHs and 20 °C after 3 months storage. The formulations stored at ~ 0 and 43 % are amorphous and it can be seen in the stretching vibration of O-H at around 3200 to 3600 cm^{-1} (Figure 49). These formulations had a sharp, strong peak without shoulder at around 3410 cm^{-1} .

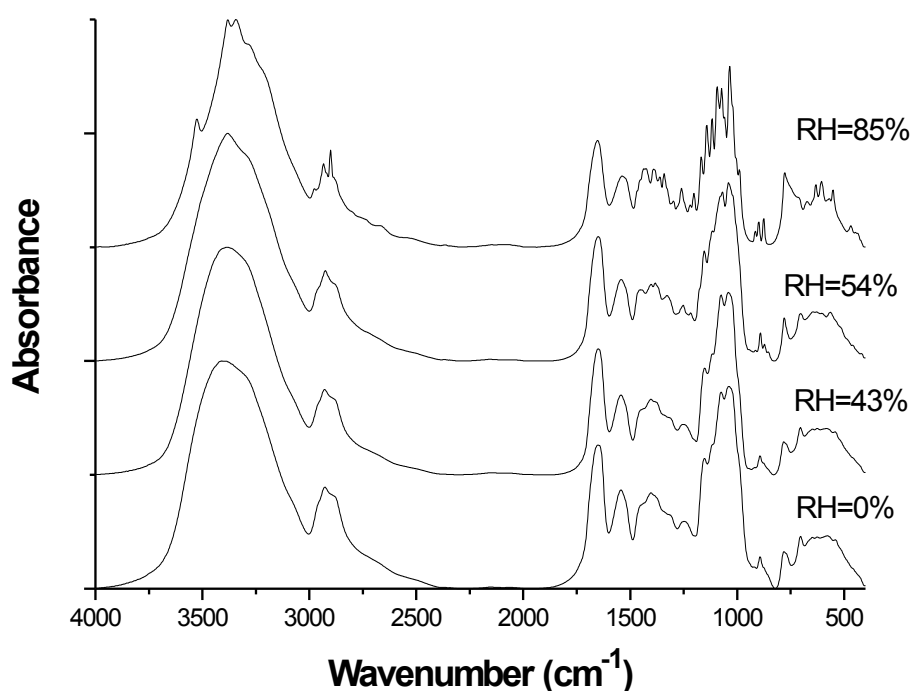


Figure 48 Infrared spectra of F65/10/25 at different RH and 20 °C after 3 months storage. The baselines were corrected, then the spectra were smoothed by 9 points and were normalized by min-max method

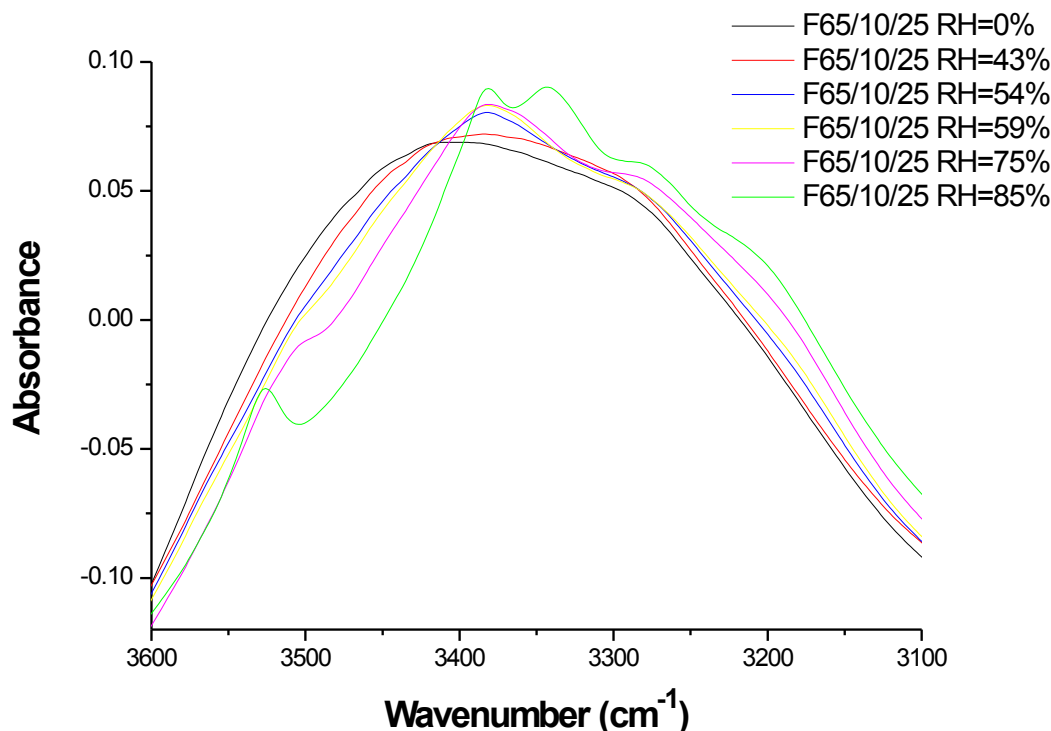


Figure 49 Infrared spectra of F65/10/25 at different RH and 20 °C after 3 months storage. The baselines were corrected and the spectra were smoothed by 9 points and were cut and were normalized by vector method

By increasing the RH to 54 %, a small shoulder appeared at around 3300 cm^{-1} and it was clearer in formulation stored at higher RH for example, 59 and 75 % RH. As explained in the case of F65/10/25, water liberation was not observed at 54 % RH, thus by analyzing this region, it is possible to know the crystallized or amorphous state of lactose in formulation. For example, F65/10/25 was amorphous after 3 months storage at 43 % RH and in the stretching vibration of O-H (3200-3600 cm^{-1}), a sharp peak could be observed, and as it is clearly seen in Figure 48 and Figure 49, by increasing the RH to 54 % a small shoulder could be seen. By increasing the RH to 59 and 75 %, these shoulders became clearer. It means that crystallization started for this formulation at 54 % RH after 3 months storage but it was not complete. These results agreed with result found for crystallization by moisture sorption isotherm.

Figure 50 shows the second derivative of F65/10/25 stored at different RH. The sample stored at 0 % RH showed a peak at 1632 cm^{-1} and by increasing the RH, the wavenumber increased. When crystallization occurred in samples, for example in the case of F65/10/25 stored at 59 % or higher RH, the increase in wavenumber was more important.

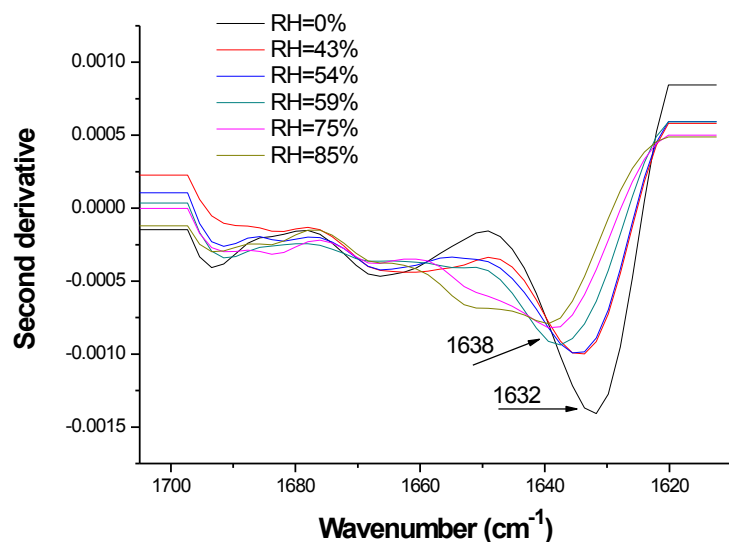


Figure 50 Second derivative of F65/10/25 stored 3 months at different RH. The baselines were corrected then the spectra were smoothed by 9 points and the amide I band was cut and normalized by vector method

III.4.4.1 Influence of lactose and starch on BLG spectrum

Figure 51 shows the infrared spectra of freeze-dried BLG and BLG without treatment. The amide I of BLG and formulations were compared to show the influence of lactose and starch on BLG structure. The spectra of BLG, before and after freeze-drying, were first compared. The sharp peak at 1638 cm^{-1} denotes β -sheet structure of BLG, which is a predominant structure in this protein (Figure 51). This peak shifted to 1635 cm^{-1} after freeze-drying and as it was shown by numerous authors, freeze-drying induced changes in secondary structure (Allison *et al.*, 1999, Anchordoquy *et al.*, 2001). The intensity of peak at 1638 cm^{-1} decreased after freeze-drying of BLG, showing the decrease in β -sheet structure. Moreover, reduction in intensity of β -sheet band in freeze-dried BLG indicates perturbation of the protein secondary structure (Izutsu and Kojima, 2002). In addition, the second peak around 1693 cm^{-1} shows the antiparallel β -sheet structure or protein aggregation (Jackson and Mantsch, 1995). The intensity of this peak was more important in the case of freeze-dried BLG. This because after freeze-drying, aggregation of BLG occurred, indicated by a small shoulder in Figure 51a.

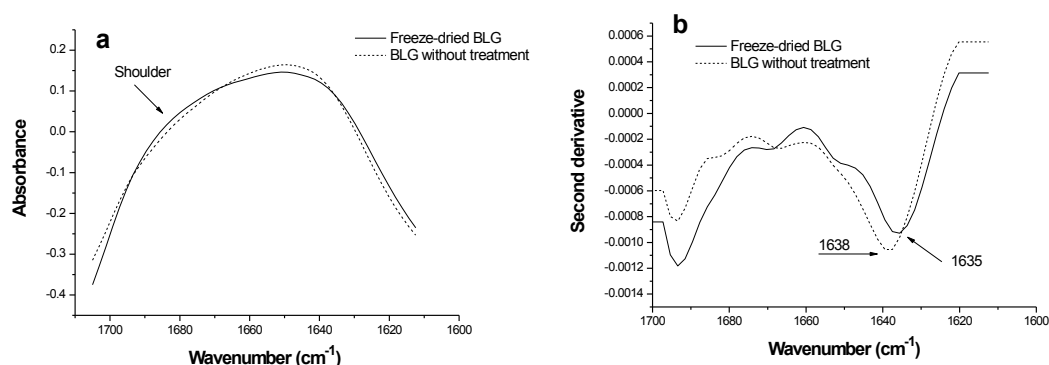


Figure 51 Amide I band of freeze-dried and without treatment BLG, a; absorbance and b ; second derivative. The baselines were corrected and the spectra were smoothed by 9 points and were cut and normalized by vector method

Figure 52 shows the infrared spectra of formulations containing 65 % lactose and different quantity of BLG and gelatinized starch after 3 months storage at 11 % RH. These spectra had the same peak position and shape. To show more details, the second derivative of amide I of these formulations is presented in Figure 53. All formulations containing 65 % lactose had a peak at 1631 cm^{-1} corresponding to β -sheet structure of BLG. Freeze-dried BLG had a peak at 1635 cm^{-1} (Figure 53). The decrease in wavenumber in formulation containing 65 % lactose shows the presence of hydrogen bonds between BLG, lactose and gelatinized starch. This phenomenon was observed by numerous authors (Carpenter *et al.*, 2002, Jovanović *et al.*, 2006, Kreilgaard *et al.*, 1998) and indicated that carbohydrate especially lactose can replace water in solid state. In addition, lactose and gelatinized starch protect BLG against aggregation and in all formulations containing gelatinized starch and lactose, the small shoulder that was shown in freeze-dried BLG, was not observed. In all processes including freeze-drying, spray drying or other process, stresses associated with freezing and drying may cause irreversible damage to the protein, seen as structural denaturation and aggregation (Heller *et al.*, 1999).

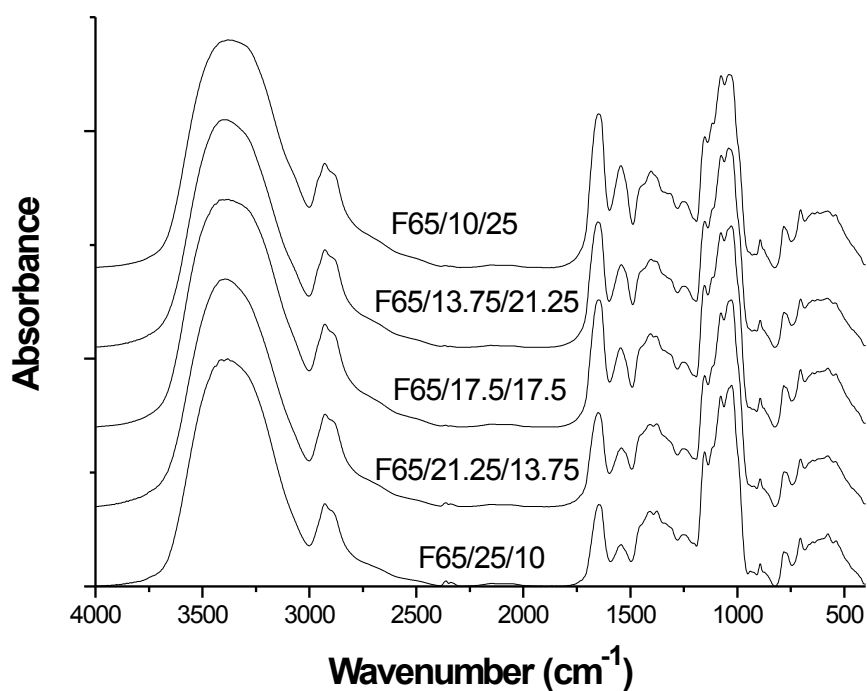


Figure 52 Infrared spectra of formulations containing the same quantity of lactose (65 %) obtained after 3 months storage at 11 % RH. The baselines were corrected then the spectra were smoothed by 9 points and normalized by min-max method

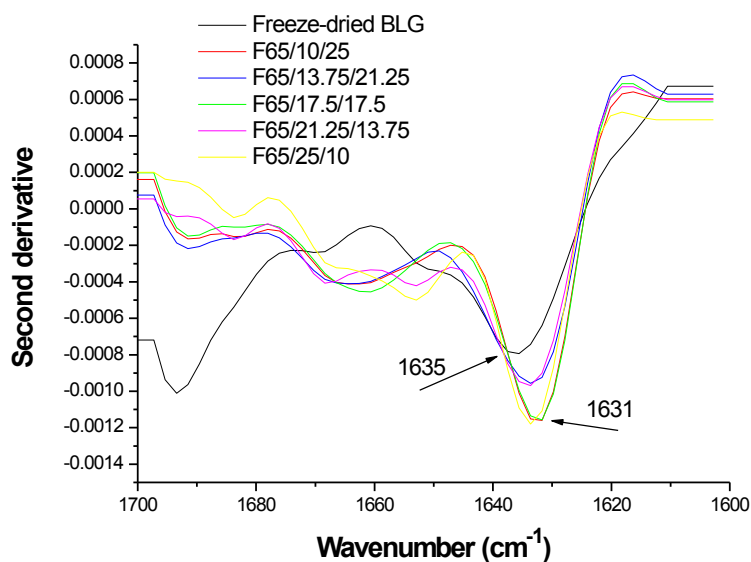


Figure 53 Second derivative of formulations containing 65 % lactose and BLG stored 3 months at 11 % RH. The baselines were corrected then the spectra were smoothed by 9 points and the amide I band was cut and normalized by vector method. Lactose and gelatinized starch spectra were subtracted from formulation spectra

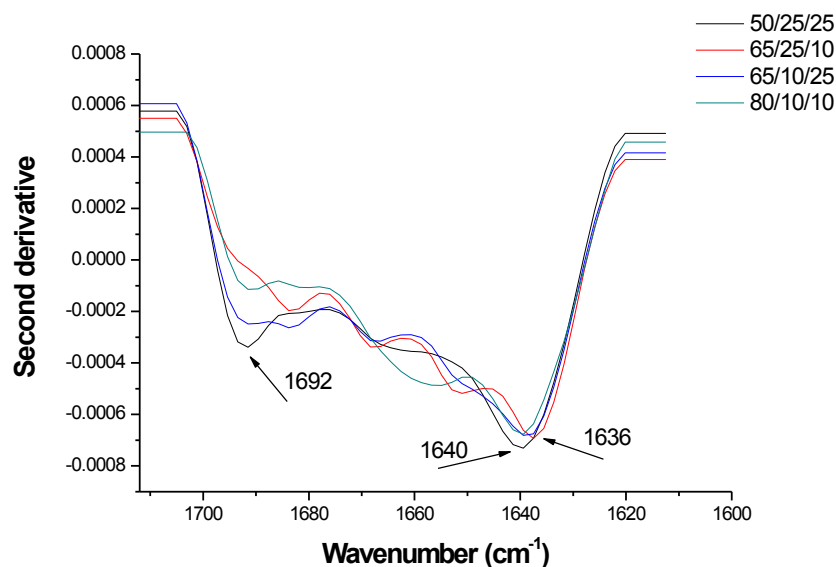


Figure 54 Second derivatives of four formulations after 3 months storage at 75 % RH. The baselines were adjusted then all spectra were smoothed by 9 points and the amide I band was cut and normalized by vector method. Lactose and gelatinized starch spectra were subtracted from formulation spectra

Figure 54 shows the second derivative of different formulations stored at 75 % RH. There was a little shift in amide I of all formulations compared to freeze-dried BLG, but the peak at 1638 cm^{-1} in BLG without treatment remained more or less the same when lactose and gelatinized starch were added. Moreover, the intensity of this peak decreased after freeze-drying in all samples compared to BLG without treatment. As, explained in the spectra of BLG, freeze-dried BLG had a more intense peak at 1693 cm^{-1} . This peak was less intense after three months storage at 75 % RH and even disappeared in the case of 65/10/25. In addition, this antiparallel β -sheet structure was not observed in the samples stored at 11 % RH.

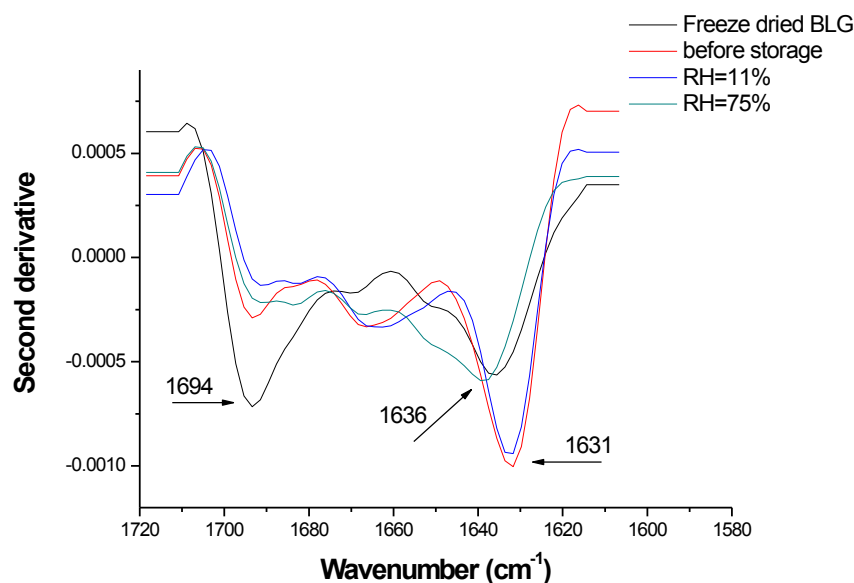


Figure 55 Second derivatives of F65/10/25 and freeze-dried BLG. The baselines were adjusted then all spectra were smoothed by 9 points and the amide I band was cut and normalized by vector method. Lactose and gelatinized starch spectra were subtracted from formulation spectra

Figure 55 shows BLG structure changes in formulation F65/10/25 before storage and after storage at 11 % and 75 % RH. The peak at 1635 cm^{-1} for BLG after freeze-drying shifted to 1631 cm^{-1} for sample after freeze-drying and after 3 months storage at 11 % RH. This is because of the formation of hydrogen bonds between BLG and gelatinized starch and lactose. Therefore, direct sugar-protein interactions are responsible for the observed hydrogen bonding to BLG in the dried preparation and storage at 11 % RH. However, by increasing storage RH to 75 %, the peak at 1636 cm^{-1} was nearly the same as that for freeze-dried BLG. The intensity of this peak increased in the formulations after freeze-drying and stored at 11 % RH, but for freeze-dried BLG and sample stored at 75 % RH, the peak had the same intensity. By adding gelatinized starch and lactose to formulations, after freeze-drying and storage at 11 % RH, the intensity of the peak at around 1635 cm^{-1} increased. Moreover, antiparallel β -sheet structure of BLG (peak at around 1694 cm^{-1}) nearly did not exist in these formulations.

III.4.5 Conclusion

Results obtained from infrared spectroscopy showed that lactose crystallization can be identified by changes in different infrared spectra such as stretching vibration of O-H bonds at around 3600 to 3200 cm^{-1} and stretching vibration of C-H bonds at around 3000 to 2800 cm^{-1} . In addition, crystallized lactose showed some shoulders in stretching vibration of O-H bonds at around 3600 to 3200 cm^{-1} and stretching vibration of C-H bonds, showing rearrangement of lactose crystals.

Freeze-drying caused aggregation of BLG and it can be seen in amide I region of proteins as a shoulder in infrared spectra of BLG. By increasing the RH from ~ 0 to 75 %, changes in wavenumber, were not observed.

In formulation containing lactose, BLG and gelatinized starch several changes were observed before and after storage at different RHs. In all formulations, the amide I band of BLG shifted to smaller wavenumbers showing the presence of hydrogen bonds between these components. By increasing the RH from ~ 0 or 11 % to 75 or 85 % the amide I band of BLG shifted to wavenumber greater than BLG. In this condition, maybe the hydrogen bonds that existed between BLG and lactose, gelatinized starch were replaced by water. In high RH conditions, lactose is in crystallized state and it is not hydrophilic. Thus, the number of hydrogen bonds present between BLG and lactose decreased. Another hypothesis that can be suggested for this phenomenon is that in high RH, nonenzymatic browning occurred between lactose and BLG and may influence the changes in wavenumber of amide I band of BLG.

III.5 Non-enzymatic browning in model infant formulations

The degree of non-enzymatic browning (NEB) is measured by different methods such as content of hydroxymethyl furfural (HMF) or other Maillard products such as Furosine (Van Renterghem and De Block, 1996), color and pigments formation (Burin *et al.*, 2000, Morales and Van Boekel, 1998) and free monosaccharide (Villamiel and Corzo, 2000). Effect of non-enzymatic browning on nutritional and physico-chemical properties of infant formulation and dried milk was presented in bibliography section. The objective of this part was to determine non-enzymatic browning kinetics and the effects of different storage conditions and formulation compositions on the non-enzymatic browning behavior of models baby foods. According to the study presented, application of the results to real food systems should take into account differences in physical properties of dried and low-moisture foods.

Non-enzymatic browning was studied by measuring the samples color at four RHs (11, 43, 54 and 75 %) during three months of storage. These RHs were chosen because, the color changes can be studied in amorphous (11 and 43 % RH) and crystallized lactose (54 and 75 % RH). Results of color changes during storage for each formulation are presented in Figure 56a and b. Kinetic of non-enzymatic browning is different for each formulation and RH.

A sharp increase in color b was observed during the first week of storage for all samples stored at 54 and 75 % RH. In addition, color b was developed slowly in the second week until the end of storage. Rate of color b changes was extremely slow at 11 and 43 % RH. At these RHs, lactose crystallization did not occur significantly. If formulations T_g is above 20 °C as far as 11 and 43 % RH, rate of non-enzymatic browning is very slow as many authors have shown (Acevedo *et al.*, 2006, Miao and Roos, 2005).

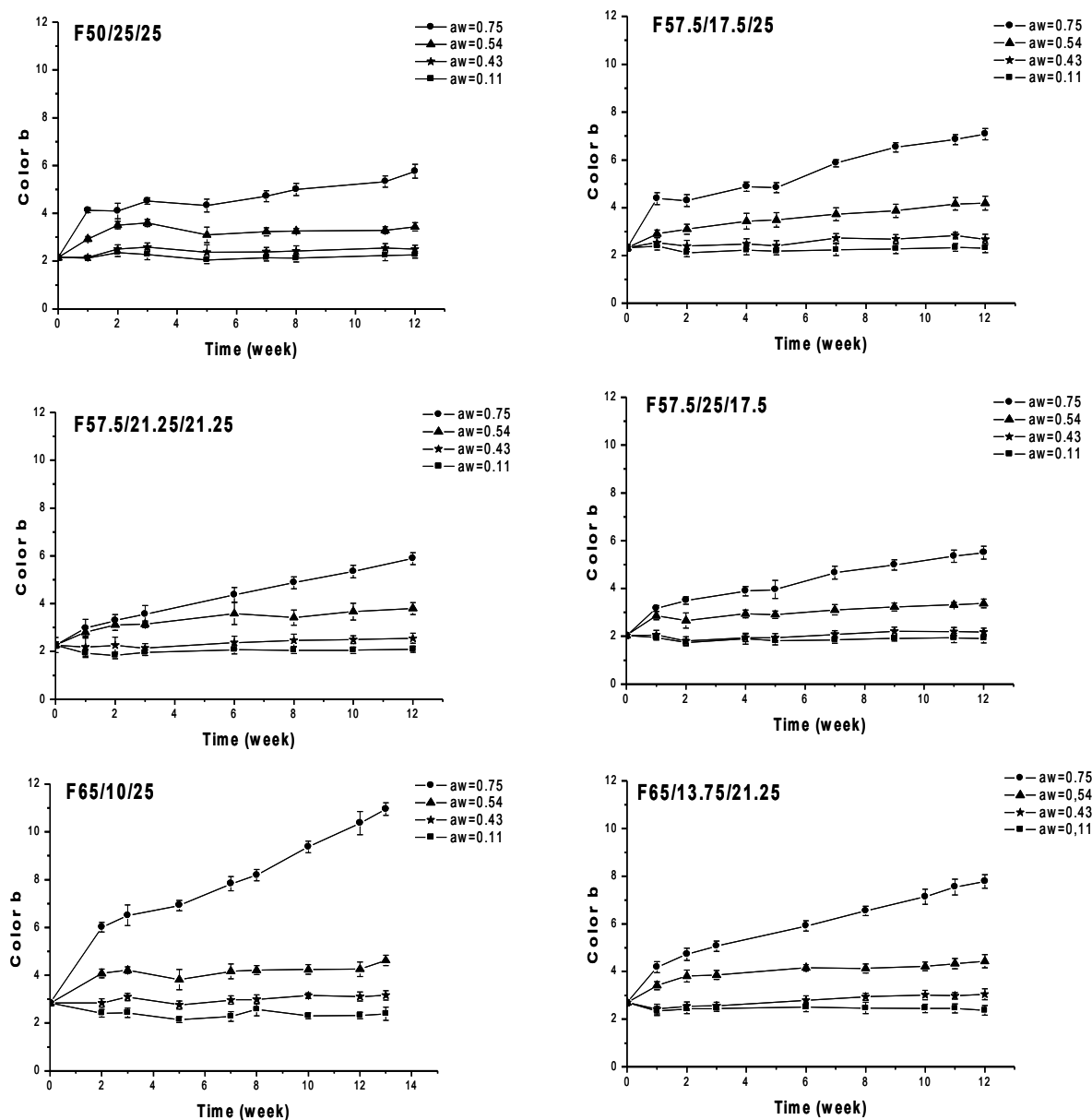


Figure 56a color b changes of model formulations containing different quantity of lactose, BLG and starch stored 3 months at 11, 43, 54 and 75 % RH

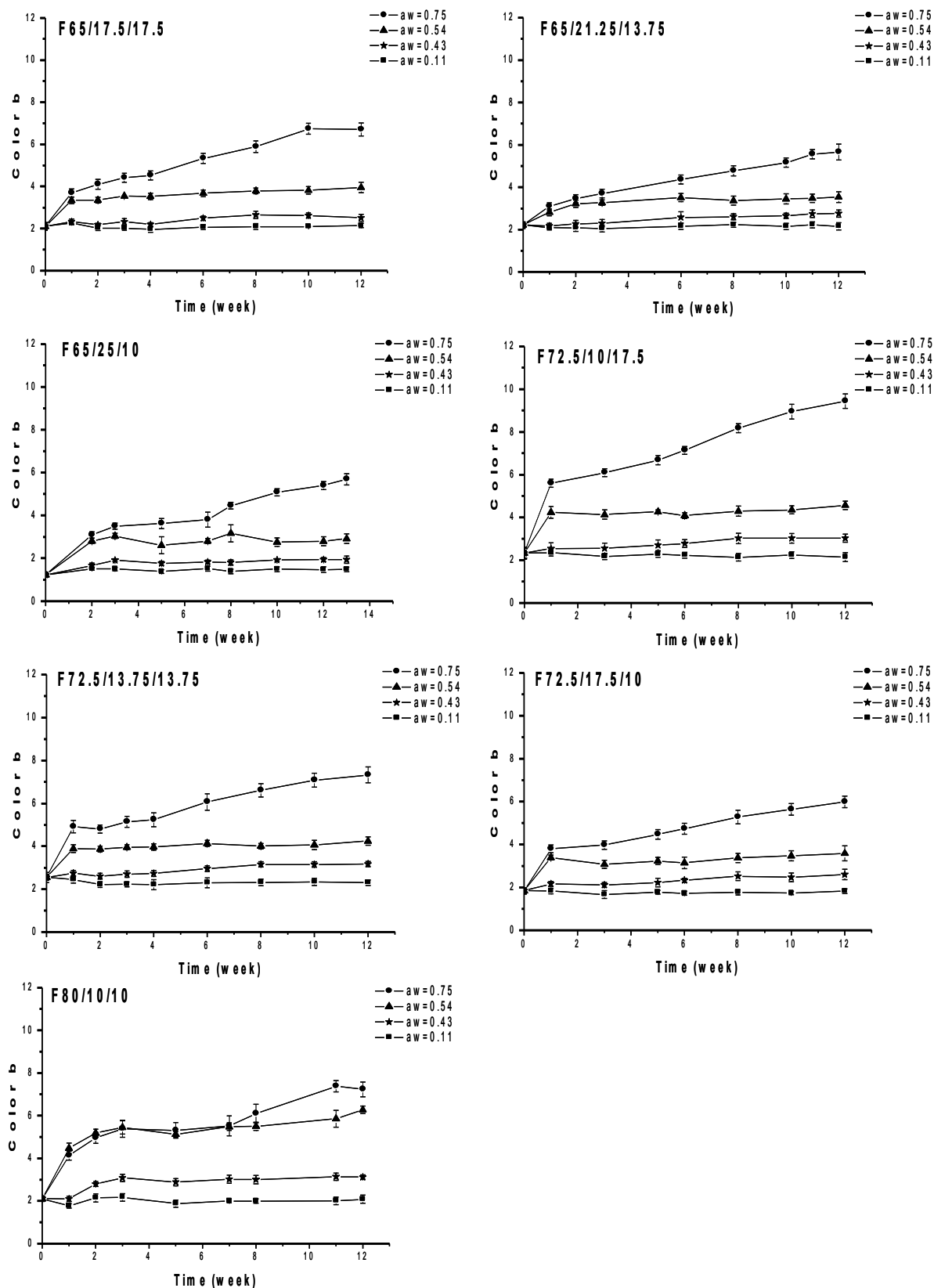


Figure 56b color b changes of model formulations containing different quantity of lactose, BLG and starch stored 3 months at 11, 43, 54 and 75 % RH

This was attributed to the fact that non-enzymatic browning is diffusion-controlled and it is favored by water plasticization of the matrix where the reaction takes place. Karmas *et al.*, (1992) related glass transition temperature with non-enzymatic browning kinetics and Karmas and Karel, (1994) suggested that non-enzymatic browning was affected by water activity, glass transition, and crystallization of the matrix. By adding BLG and gelatinized starch, the RH at which lactose crystallization occur, increased. Lactose crystallization had an important effect on color changes. For example, in the case of F80/10/10, color b changes were more or less the same until the 8th weeks for the samples stored at 75 and 54% RH. Nevertheless, after 12 weeks storage, the sample stored at 75 % RH had color b greater than the sample stored at 54 % RH. It was explained in moisture sorption isotherm section that lactose crystallization occurred after a few days in this formulation stored at 54 % RH.

Maximum color b development occurred in the samples stored at 75 % RH. A big difference in color b was observed in all samples except F80/10/10 between 75 and 54 % RH. For example, in the case of F50/25/25, a big difference was observed between color b measured at 75 and 54 % RH. These results show that color b changes depended on the RH and composition of formulations as many authors showed (Labuza *et al.*, 1970, Miao and Roos, 2005, Miao and Roos, 2006).

Maximum of color b was noted for formulation containing maximum of BLG and minimum of gelatinized starch (i.e. F65/10/25). In this case, color b increased to around 12 at 75 % RH that shows maximum interactions and reactions occurring in these conditions. By increasing the gelatinized starch quantity in formulations, color b changes decreased. This can be seen in formulations containing 25 and 21.25 % gelatinized starch (F50/25/25 and F57.5/21.25/21.25). In these formulations, color b at 75 % RH was around 6. This can be explained by the dilution of the reactant (lactose and BLG) in the presence of gelatinized starch, but also by mobility restrictions promoted by the presence of the polymer. Burin *et al.*, (2004) showed that the lower browning rate when maltodextrin was present in lactose-containing systems was mainly related to the delay of lactose crystallization.

Our results show that non-enzymatic browning or color b changes depended on lactose crystallization, RH and composition. If lactose crystallization occurred in the formulations color b changes increased. The maximum of non-enzymatic browning in milk powder was located between 43 % and 75 % RH (Acevedo *et al.*, 2006). The presence of protein and polysaccharides can be the cause for the higher RH at which the maximum non-enzymatic browning was observed in our formulations. Franzen *et al.* (1990) showed that the maximum non-enzymatic browning rate for milk powder was at 33 % RH. In Franzen's experiments,

however, the humidification of the systems was performed with ice and this procedure could lead to systems with a higher degree of lactose crystallization. Roos and Jouppila (1996) found that the rate of non-enzymatic browning reaction in dairy powders increased significantly when the materials were stored above 44 % RH that was sufficient to depress T_g to below storage temperature and promote lactose crystallization.

An important increase in color b was observed in first week of storage at 75 % RH. This was followed by a linear increase in color b during storage, indicating zero-order kinetics. This was shown by Miao and Roos (2005) for freeze-dried and spray-dried model samples.

Results of color b changes were analyzed by mixture design. First series of models were suggested to calculate the color b in the end of storage at each RH. Second series of models were developed to predict the color changes at different RH ranging from 11 to 75 % during storage.

Figure 57 shows the counter plot of color b after 3 months storage at 75 % RH and 20 °C. This figure shows that maximum of color b could be observed in the formulations containing the maximum of BLG and the minimum of gelatinized starch. At 75 % RH, all formulations were in crystallized state thus crystallization effect was the same for all formulations. When crystallization occurs, as it was explained, maximum of color b was observed in the formulations. Color b increased by increasing BLG quantity or decreasing gelatinized starch in the formulations. Combinations between these components affect the color b changes after three months storage at 75 % RH. For example, F57.5/17.5/25 had nearly the same value of color b as F80/10/10.

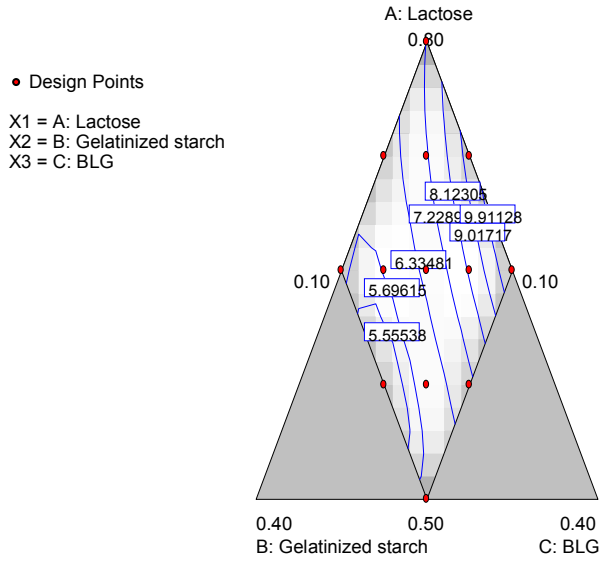


Figure 57 Contour plot of predicted response of color changes after 3 months storage at 75 % RH and 20 °C

Equation 19 shows the mathematical model predicting the color b after 3 months storage at 75 % RH and 20 °C.

$$\begin{aligned} \text{Color } b = & 0.8 \times \text{Lactose} + 33.7 \times \text{Starch} - 39.4 \times \text{BLG} - 9.4 \times \text{Lactose} \times \text{Starch} \\ & + 155 \times \text{Lactose} \times \text{BLG} + 249 \times \text{Starch} \times \text{BLG} - 861 \times \text{Lactose} \times \text{Starch} \times \text{BLG} \end{aligned} \quad (19)$$

Mass ratio of each component must be put in this equation. For example, for F80/10/10, these values must be used 0.8, 0.1 and 0.1 for lactose, gelatinized starch and BLG, respectively. Analysis of these results shows that the interactions between the components had a significant effect on color b changes after three months storage at 75 % RH and 20 °C. To show more details about effect of each component on color b the cubic model (equation 19) was reduced to a linear one. This equation is presented in equation 20.

$$\text{Color } b = 9.4 \times \text{Lactose} - 14.3 \times \text{Starch} + 19.4 \times \text{BLG} \quad (20)$$

This equation shows that lactose and BLG had a positive effect on color changes and the coefficient of BLG was two times bigger than lactose. Moreover, starch had a negative effect on color b changes.

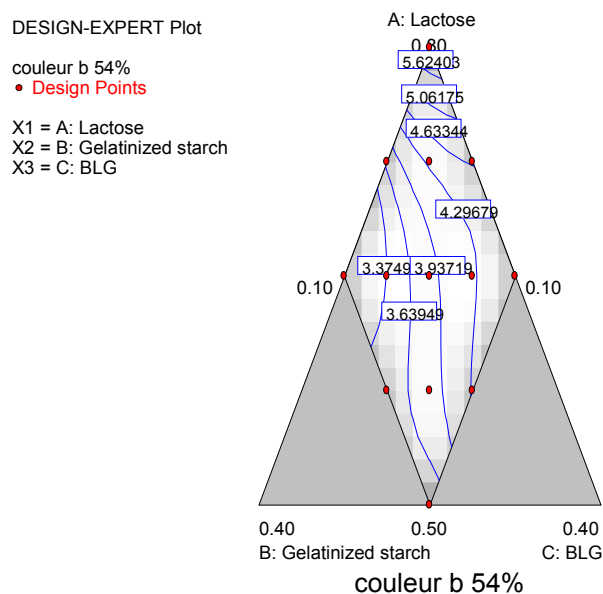


Figure 58 Contour plot of predicted response of color b changes after 3 months storage at 54 % RH and 20 °C

Figure 58 shows the contour plot of color b after 3 months storage at 54 % RH and 20 °C. By increasing the lactose quantity in formulations, color b increased in the end of storage. This is because, formulations containing a large quantity of lactose, for example, F80/10/10 crystallized at 54 % RH. Lactose crystallization had an important role in non-enzymatic browning, because lactose crystallization results in the concentration of non-enzymatic browning reactants in the non-crystalline volume and enhances non-enzymatic browning (Karmas and Karel, 1994, Roos and Himberg, 1994).

Another parameter influencing color b changes at 54 % RH is the composition of each formulation as it was explained for color changes at 75 % RH. For example, three formulations containing 72.5 % lactose (F72.5/10/17.5, F72.5/13.75/13.75 and F72.5/17.5/10) had the difference color b at the end of storage. The formulation containing maximum of BLG and minimum of gelatinized starch quantity had a bigger color b, the formulation containing the minimum of BLG, and maximum of gelatinized starch had a smaller color b.

Results of color b at 54 % RH were analyzed; a cubic model (equation 21) was used to estimate the color b changes after 3 months storage at 54 % RH. Interactions between the components had significant effect on color b.

$$\begin{aligned} \text{Colorb} = & 24 \times \text{Lactose} + 141 \times \text{Starch} + 143 \times \text{BLG} - 281 \times \text{Lactose} \times \text{Starch} \\ & - 266 \times \text{Lactose} \times \text{BLG} - 863 \times \text{Starch} \times \text{BLG} + 1358 \times \text{Lactose} \times \text{Starch} \times \text{BLG} \end{aligned} \quad (21)$$

By reducing this model to a linear one, effect of each component is clearer. Equation 22 shows linear model of color b changes at 54 % RH and 20 °C.

$$\text{Colorb} = 6.3 \times \text{Lactose} - 5.9 \times \text{Starch} + 5.7 \times \text{BLG} \quad (22)$$

Lactose and BLG had a positive effect on color b changes of formulations after 3 months storage at 54 % RH and they had more or less the same coefficient. This shows that lactose crystallization had an important effect on color b changes. By comparing equation 12 with 10, two linear models for predicting the color b changes at 75 and 54 % RH, it can be noted that when lactose crystallization occurred (equation 20), BLG had more effect than lactose on color b. In the second case (equation 22) where lactose crystallization did not occur in all samples, BLG and lactose had nearly the same effect on color b changes.

Figure 59 shows the contour plot of color b changes after three months storage of formulations at 43 % RH and 20 °C. This figure shows that the color b changes of formulations depended on the percentage of each component in the formulations. By increasing the gelatinized starch quantity in formulation color b decreased. At 43 % RH, all formulations were in amorphous state and as it was explained in, Figure 56 there was little or no change in color b. It is interesting to know that pure BLG was more yellow than lactose and gelatinized starch (result not shown). Moreover, gelatinized starch was less yellow than other components.

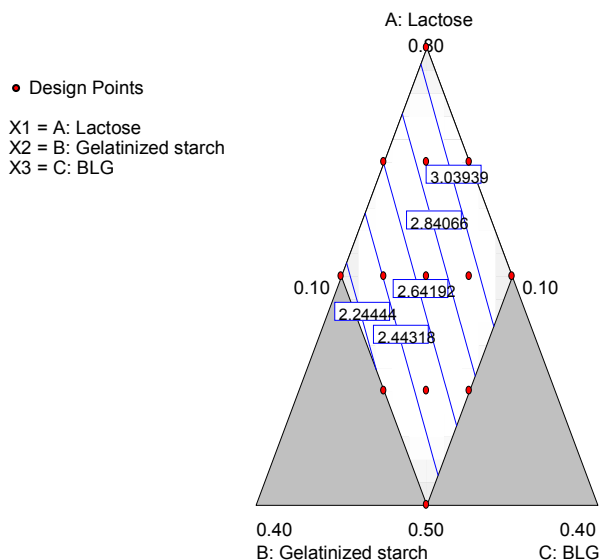


Figure 59 Contour plot of predicted response of color changes after 3 months storage at 43 % RH and 20 °C

Interactions between the components had no significant effect on color b at 43 % RH and 20 °C. Therefore, a linear model was used to predict color b of formulations after three months storage at 43 % RH and 20 °C, as it is presented in equation 23.

$$Colorb = 3.6 \times Lactose - 2.5 \times Starch + 4.5 \times BLG \quad (22)$$

BLG lactose had a positive and gelatinized starch had a negative effect (color less yellow) on color b changes in formulations stored at 43 % RH.

The models that were found for color b changes at 11 % RH were not significant, thus these results are not presented.

It is interesting to find a model to predict the color b changes of formulations based on different RH, storage time, and composition. Results of the formulations stored at different RHs were analyzed. A linear*cubic model (crossed model) was used to predict the formulation colors b* during storage by considering the RH and time as two numerical processes parameters.

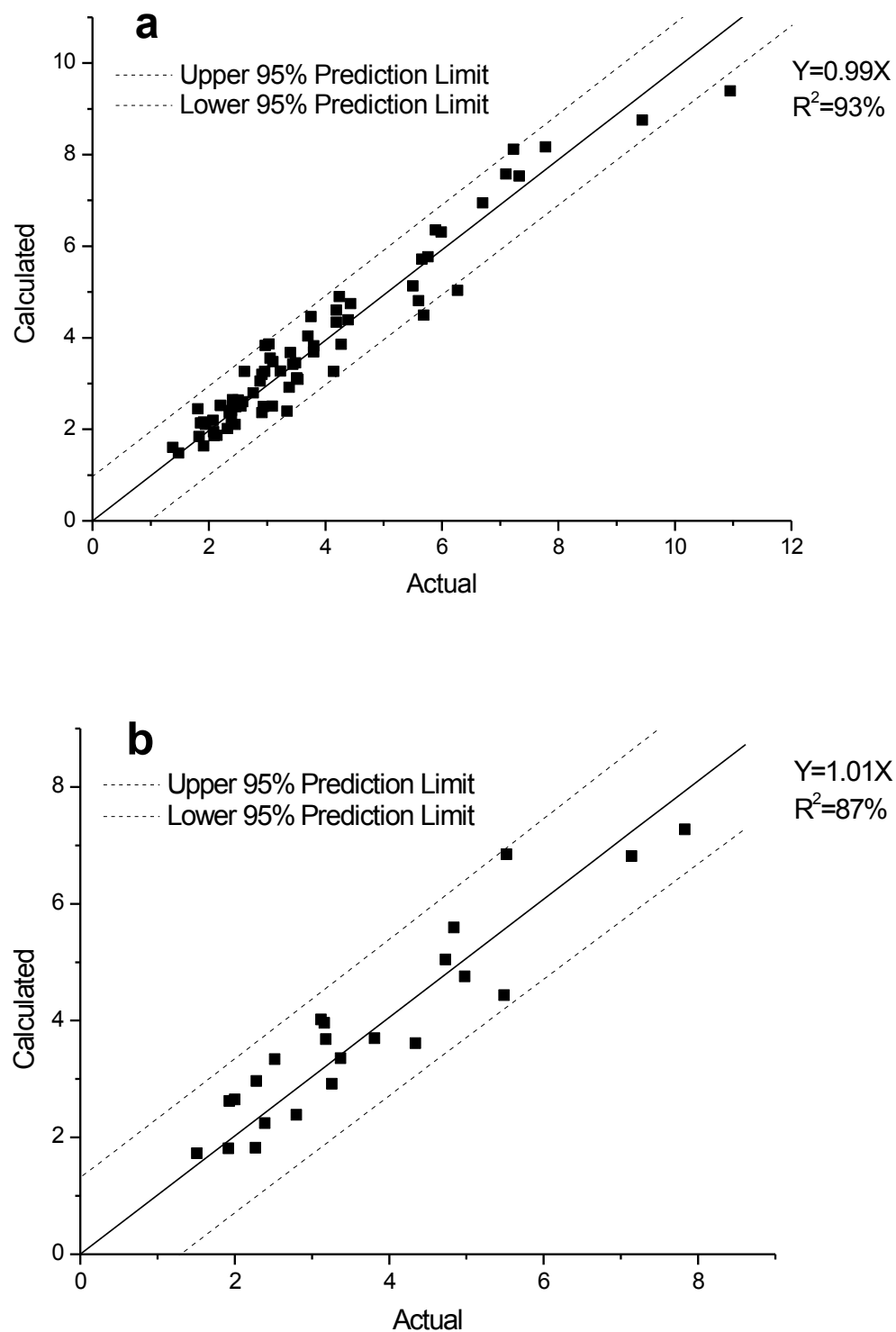


Figure 60 Correlation between actual and calculated data; data was used for model (a) and validation of model (b)

Equation 24 presents this model.

$$\begin{aligned} Y(b) = & 2.126X_{lac} - 3.33X_{starch} + 10.456X_{BLG} - 5.074X_{lac}X_t + 0.043X_{lac}X_{RH} + 29.657X_{sta} \quad (24) \\ & - 0.082X_{starch}X_{RH} - 48.552X_{BLG}X_t - 0.03X_{BLG}X_{RH} + 10.434X_{lac}X_t^2 - 5.3E - 4X_{lac}X_{RH}^2 \\ & - 39.94X_{starch}X_t^2 + 1.036E - 3X_{starch}X_{RH}^2 + 61.275X_{BLG}X_t^2 - 9.731E - 5X_{BLG}X_{RH}^2 + 0.084X_{lac}X_tX_{RH} \\ & - 0.169X_{starch}X_tX_{RH} + 0.183X_{BLG}X_tX_{RH} \end{aligned}$$

Where $Y(b)$ is color b at defined RH and time, X_{lac} , X_{starch} and X_{BLG} , X_t and X_{RH} are percentage of lactose, gelatinized starch, BLG, time (day) and RH respectively. By this model, color b^* could be calculated over time (from 7 to 92 days) and at different RH (11 to 75 %). The probability and R^2 results ($R^2=0.93$) provided proof that there was a good agreement between calculated and experimental values. Figure 60 a and b show the position of the points that was chosen for model and validation of model respectively.

Non-enzymatic browning occurs in samples containing protein and reducing sugar. This reaction is important in different aspect such as changes in nutritional quality of proteins, organoleptic quality and allergenic compounds production. This model (7) could predict the yellow color changes in formulation containing lactose, BLG (protein) and gelatinized starch during storage. Color changes and non-enzymatic browning could be controlled by changing storage parameter (Ottenhof *et al.*, 2003) and formulation components (lactose, BLG and gelatinized starch). In this model RH, time had positive effect on yellow color development while gelatinized starch had negative effect.

III.5.1 Conclusion

Results of color b changes of model infant foods showed that RH had an important effect on color b changes during storage. Lactose crystallization was also very important on color b changes and a significant difference was observed between formulations in which lactose crystallization occurred and the formulations in which lactose was in amorphous state. An important increase in Color b occurred in the first storage week and then color b changes increased linearly at high RH. Mixture design is an interesting method to study and explain the effect of each component on color changes during storage.

Conclusion

In the present study, water sorption properties, kinetic of lactose crystallization, lactose state, and the interactions between lactose, BLG and starch were determined for model infant foods. Results found in this study were analyzed by mixture design to know the effects of each component on the measured properties of model infant food. This method could put forward the presence of interactions between these components. For example, interactions had no significant effect on moisture sorption properties of formulations. Moreover, moisture sorption of the formulation can be calculated from moisture sorption properties of each component, when lactose crystallization does not occur. Therefore, calculation of moisture sorption in food containing lactose is not possible. This is because, lactose crystals size decreases with the addition of proteins and polysaccharides to the formulations and consequently the internal surface increase or more sites are available for adsorbing water.

Lactose crystallization RH changes by adding proteins or saccharides. This phenomenon was observed previously. Stabilization of lactose against crystallization could also be attributed to preferential exclusion, surface activity, steric hindrance of lactose-protein and lactose-polysaccharide and/or increased of viscosity of systems limiting structural movement.

During storage of formulations at high RH, lactose crystals migrated to the surface of particles. This phenomenon was observed by comparing the SEM photo of sample after crystallization and after 3 months storage at the same condition (temperature and RH). Lactose crystals size increased after 3 months storage compared to samples stored for a few hours at the same RH. It is possible in long-term storage, that an important migration of lactose to the surface could occur and particle surface composition could change.

Freeze-drying caused aggregation of BLG and it can be seen in amide I region of proteins as a shoulder in infrared spectra of BLG.

In formulations containing lactose, BLG and gelatinized starch, several changes were observed before and after storage at different RHs. In all formulations, the amide I band of BLG shifted to smaller wavenumbers showing the presence of hydrogen bonds between these components. By increasing the RH from ~ 0 or 11 % to 75 or 85 % the amide I band of BLG shifted to higher wavenumber. In this condition, hydrogen bonds that existed between BLG

Conclusion

and lactose, gelatinized starch were replaced by water. In high RH conditions, lactose is in crystallized state and it is not hydrophilic. Thus, the number of hydrogen bonds present between BLG and lactose decreased. Another hypothesis that can be suggested for this phenomenon is that in high RH, non-enzymatic browning occurred between lactose and BLG and may influence the changes in wavenumber of amide I band of BLG.

Results of color b changes of model infant foods stored at different RHs showed that RH had an important effect on color b changes during storage. Results showed that lactose crystallization was very important on color b changes and a big difference was observed between formulations at which lactose crystallization occurred and the formulations in which lactose was in amorphous state. An important increase in color b occurred in the first storage week and then color b changes increased linearly at high RH. Mixture design was an interesting method to study and explain the effects of each component on color changes during storage. As perspective work, it would be interesting to produce the same model powders by spray drying and to check if lactose/BLG/starch interactions are similar and if the consequences are the same on stabilization with respect to crystallization. It is interesting to measure the T_g of mixture containing lactose, protein and polysaccharide and compare it to the result obtained by models like Gordon-Taylor, Couchman-Karas. It will also be interesting to hydrolyzing proteins for reducing the allergies to cow proteins.

Moreover, it will be interesting to study the composition changes of particles by Electron Spectroscopy for Chemical Analysis (ESCA) to put forward the migration of lactose to particle surface during storage at high RH. In addition, this study can be completed by coupling the dynamic vapor sorption (DVS) with infrared spectroscopy to study the structure changes of samples at different RH and time.

Lactose crystallization is a complex phenomenon, and researches investigating this phenomenon were conducted by dessicator method. This method is not accurate. When dessicators with samples are opened, RH changes occur, and this triggers lactose crystallization. When DVS analysis is used, RH changes problem does not occur and crystallization RH of lactose can thus be accurately measured.

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AUTORISATION DE SOUTENANCE DE THESE
DU DOCTORAT DE L'INSTITUT NATIONAL
POLYTECHNIQUE DE LORRAINE

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VU LES RAPPORTS ETABLIS PAR :

Monsieur Bernard CUQ, Professeur, ENSAM, INRA, Montpellier

Monsieur Cyrille ANDRES, Professeur, Université de Bourgogne, Dijon

Le Président de l'Institut National Polytechnique de Lorraine, autorise :

Monsieur NASIRPOUR Ali

à soutenir devant un jury de l'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE,
une thèse intitulée :

**"Effet de l'hydratation d'une formulation infantile modèle (lactose B-lactoglobuline,
amidon) sur les changements d'état et les interactions entre constituants"**

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en vue de l'obtention du titre de :

DOCTEUR DE L'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

Spécialité : « **Procédés biotechnologiques et alimentaires** »

Fait à Vandoeuvre, le 03 octobre 2006

Le Président de l'I.N.P.L.,

L. SCHUFFENECKER

