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Processing and Storage stability of Skim Milk Powder Monitoring Early and Advanced Stages of the Maillard Reaction

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Processing and Storage Stability of Skim Milk Powder

Processing and Storage Stability of Skim Milk Powder

Monitoring Early and Advanced Stages of the Maillard Reaction

Kataneh Aalaei



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DOCTORAL DISSERTATION

Which, by due permission of the Faculty of Engineering, LTH,
Lund University, Sweden,
to be defended, in Lecture Hall C, at the Centre for Chemistry and Chemical
Engineering (Kemicentrum) on Friday 6th October, 2017 at 10.15

Faculty opponent

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Title and subtitle Processing and Storage Stability of Skim Milk powder Monitoring early and advanced stages of the Maillard reaction			
<p>Abstract The non-enzymatic browning known as the Maillard reaction has been the subject of extensive investigation for decades. Studies have focused on areas such as changes in flavour, colour, texture, and the nutritional properties of food materials as a result of this reaction. However, this complex reaction between the amino acids in proteins and reducing sugars in carbohydrates, is still not fully understood, especially the reaction mechanisms and potential impacts on health.</p> <p>Skim milk powder (SMP), is a multi-functional and extremely popular ingredient in the food industry, and is used in infant formulas, reconstituted and fermented dairy products, frozen desserts, bakery products, coffee whiteners, and even processed meat products. Despite the apparent good stability of SMP, it is prone to the Maillard reaction and its consequences, due to its composition, as well as the application of various kinds of thermal processing, and subsequent prolonged shelf life.</p> <p>The aims of this work were thus to improve our understanding of the occurrence of the Maillard reaction in SMP after the application of different drying techniques, and during subsequent storage under realistic conditions, in order to be able to predict and to control the reaction. Previous studies have mainly been conducted on model food systems at the conditions applicable to processing, i.e. at temperatures above 40 °C. In the present work, changes in two indicators of the early and advanced stages of the Maillard reaction, namely the available lysine and carboxymethyl lysine (CML), respectively, were monitored during storage.</p> <p>To obtain a better understanding of the impact of processing on the progression of the reaction, three different drying techniques were studied and compared on pilot scale: freeze-drying, spray-drying and drum-drying. The extent of the reaction during prolonged storage for 200 days was studied, considering three storage variables: temperature, relative humidity (RH) and time.</p> <p>The kinetics of the available lysine in a commercial, industrially produced SMP was subsequently established over 30 days (the maximum recommended period for the consumption of opened packages) under conditions normally encountered during domestic storage. Furthermore, the early and advanced stages of the reaction were studied in selected infant formulas available on the Swedish market.</p> <p>The results of these studies showed that the drying technique had a significant impact on the initiation of the Maillard reaction. Furthermore, the storage variables (temperature, RH and time) were also crucial factors in the gradual progression of the reaction during storage. The pattern of the decrease in the available lysine content during 200 days of storage was similar, regardless of the type of SMP. After storage at 52% RH and 30 °C, a 39.2 – 45.9% decrease in the available lysine content was seen after 200 days. The corresponding value following storage at 52% RH and 20 °C was 21.2 – 31.8%, indicating the importance of the storage temperature. Storage at 33% RH and 30 °C caused a 5.2 – 22.4% decrease in the available lysine content, while no significant decrease in the available lysine content was seen after storage at 33% RH and 20 °C, thus it was considered to be the ideal storage conditions for SMP.</p> <p>Studies of the advanced phase of the Maillard reaction using CML revealed that twice as much CML was formed in the spray-dried powders, than in the freeze-dried samples, after 200 days. The corresponding value in the drum-dried samples was 1.6 times that in the freeze-dried samples.</p> <p>The findings of this work have practical implications for SMP and SMP-based products in the food industry, and can be used to predict and control the Maillard reaction during storage, in order to ensure the safety of these products on the market.</p>			
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Processing and Storage Stability of Skim Milk Powder

Monitoring Early and Advanced Stages of the Maillard Reaction

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To Mum and Dad

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Abstract

The non-enzymatic browning known as the Maillard reaction has been the subject of extensive investigation for decades. Studies have focused on areas such as changes in flavour, colour, texture, and the nutritional properties of food materials as a result of this reaction. However, this complex reaction between the amino acids in proteins and reducing sugars in carbohydrates, is still not fully understood, especially the reaction mechanisms and potential impacts on health.

Skim milk powder (SMP), is a multi-functional and extremely popular ingredient in the food industry, and is used in infant formulas, reconstituted and fermented dairy products, frozen desserts, bakery products, coffee whiteners, and even processed meat products. Despite the apparent good stability of SMP, it is prone to the Maillard reaction and its consequences, due to its composition, as well as the application of various kinds of thermal processing, and subsequent prolonged shelf life.

The aims of this work were thus to improve our understanding of the occurrence of the Maillard reaction in SMP after the application of different drying techniques, and during subsequent storage under realistic conditions, in order to be able to predict and to control the reaction. Previous studies have mainly been conducted on model food systems at the conditions applicable to processing, i.e. at temperatures above 40 °C. In the present work, changes in two indicators of the early and advanced stages of the Maillard reaction, namely the available lysine and carboxymethyl lysine (CML), respectively, were monitored during storage.

To obtain a better understanding of the impact of processing on the progression of the reaction, three different drying techniques were studied and compared on pilot scale: freeze-drying, spray-drying and drum-drying. The extent of the reaction during prolonged storage for 200 days was studied, considering three storage variables: temperature, relative humidity (RH) and time.

The kinetics of the available lysine in a commercial, industrially produced SMP was subsequently established over 30 days (the maximum recommended period for the consumption of opened packages) under conditions normally encountered during domestic storage. Furthermore, the early and advanced stages of the reaction were studied in selected infant formulas available on the Swedish market.

The results of these studies showed that the drying technique had a significant impact on the initiation of the Maillard reaction. Furthermore, the storage variables (temperature, RH and time) were also crucial factors in the gradual progression of the reaction during storage. The pattern of the decrease in the available lysine content during 200 days of storage was similar, regardless of the type of SMP. After storage at 52% RH and 30 °C, a 39.2 – 45.9% decrease in the available lysine content was seen after 200 days. The corresponding value following storage at 52% RH and 20 °C was 21.2 – 31.8%, indicating the importance of the storage temperature. Storage at 33% RH and 30 °C caused a 5.2 – 22.4% decrease in the

available lysine content, while no significant decrease in the available lysine content was seen after storage at 33% RH and 20 °C, thus it was considered to be the ideal storage conditions for SMP.

Studies of the advanced phase of the Maillard reaction using CML revealed that twice as much CML was formed in the spray-dried powders, than in the freeze-dried samples, after 200 days. The corresponding value in the drum-dried samples was 1.6 times that in the freeze-dried samples.

The findings of this work have practical implications for SMP and SMP-based products in the food industry, and can be used to predict and control the Maillard reaction during storage, in order to ensure the safety of these products on the market.

Popular Scientific Summary

Milk is a sensitive food material and, due to its unique composition, may undergo physical, chemical and nutritional changes upon the removal of water during drying and during subsequent storage. What actually happens when a dry product like milk powder is stored for significant amount of time?

In recent years, the market and use of milk powders has increased enormously due to their long shelf life and various functional properties. They are used not only in the production of infant foods, but are also used extensively in the formulation of other products such as bakery and confectionary products, frozen and ready-to-eat meals, and even meat products.

Bearing in mind that milk powders may be stored for a long time before further processing, and that high temperatures are often used in the industry, leads to the question of whether milk powder changes during this time, or when it is kept at home for a long time after opening the package, does it have the same properties as the time the package was opened.

Milk powder is mainly composed of proteins and sugars. When the milk sugar (lactose) and the proteins interact with each other under certain conditions, a chemical reaction known as the Maillard reaction takes place. This reaction has attracted a great deal of attention, mostly due to its potential impact on health. However, this issue is still under extensive investigation.

Three different techniques can be used to produce milk powders. The most common technique is spray-drying. This technique benefits from rapid drying, as the milk is sprayed into a chamber at a high temperature, and the resulting droplets are transformed into powders in a few seconds. The second technique is freeze-drying. Here, the milk is first frozen, and the ice crystals are then converted into vapour under vacuum. Finally, the third technique is drum-drying, during which a very thin layer of milk is formed on the surface of a hot rotating drum which dries the milk. These three techniques were applied in this work to produce milk powders for storage experiments.

The effects of storage were also studied by creating different atmospheres in small enclosed spaces. The milk powders were exposed to various temperatures and humidities for several months and were then analysed.

The results of these experiments showed that the drying technique affects the progression of the Maillard reaction in milk powders. It was also shown that the progression of the reaction in milk powders is dependent on the temperature and humidity during storage. Some molecules suspected of being involved in the initiation or development of inflammatory diseases may be formed during the storage of milk powders. The findings of this work can be used to predict and to control the Maillard reaction in milk powders and other products based on milk powder.

List of Publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I. **Aalaei, K.** Rayner, M., Sjöholm, I. Chemical methods and techniques to monitor early Maillard reaction in milk products; a review. *Submitted to Critical Reviews in Food Science and Nutrition*
- II. **Aalaei, K.** Rayner, M. Tareke, E., Sjöholm, I. (2016). Application of a dye-binding method for the determination of available lysine in skim milk powders. *Food Chemistry*, 196, 815-820
- III. **Aalaei, K.** Rayner, M. and Sjöholm, I. (2016). Storage stability of freeze-dried, spray-dried and drum-dried skim milk powders evaluated by available lysine. *LWT - Food Science and Technology*, 73, 675-682
- IV. **Aalaei, K.** Rayner, M., Sjöholm, I. Kinetics of available lysine in stored commercial skim milk powder at moderate temperatures. *Accepted for publication. Food Research International*
- V. **Aalaei, K.** Sjöholm, I. Rayner, M. Tareke, E. (2017). The impact of different drying techniques and controlled storage on the development of advanced glycation end products in skim milk powders using isotope dilution ESI-LC-MS/MS. *Food and Bioprocess Technology*, 10(9), 1704-1714
- VI. **Aalaei, K.** Sjöholm, I. Rayner, M. Tareke, E. Monitoring of early and advanced stages of the Maillard reaction in infant formulas using available lysine and carboxymethyl-lysine. *Manuscript*

The Author's Contributions to the Papers

- I. The author collected all the relevant references, discussed the contents with the co-authors, and wrote the paper.
- II. The author designed the study together with the co-authors, performed all the experiments, evaluated the results together with the co-authors, and wrote the paper.
- III. The author designed the study together with the co-authors, performed all the experiments, evaluated the results together with the co-authors, and wrote the paper.
- IV. The author designed the study together with the co-authors, performed all the experiments, evaluated the results together with the co-authors, and wrote the paper.
- V. The author designed the study together with the co-authors, and performed all the experimental work for sample preparation. The LC-MS/MS measurements were made by S. Essén and E. Tareke. The author evaluated the results together with the co-authors, and wrote the paper.
- VI. The author designed the study together with the co-authors, and performed the experimental work, apart from the LC-MS/MS measurements. The author evaluated the results together with the co-authors, and wrote the paper.

Abbreviations

AGE	advanced glycation end product
CML	carboxymethyl lysine
DM	dry matter
ELISA	enzyme-linked immunosorbent assay
FDNB	1-fluoro-2, 4-dinitrobenzene
GC	gas chromatography
HMF	hydroxymethyl furfural
LC-MS/MS	liquid chromatography-tandem mass spectrometry
OPA	O-phthalaldehyde
RAGE	receptor for advanced glycation end product
RH	relative humidity
SMP	skim milk powder
SPE	solid-phase extraction
UHT	ultra-high temperature
WMP	whole milk powder

1. Introduction and Aims

Application of drying technologies on dairy products, specifically milk, is primarily to ensure microbiological safety and to extend the shelf life. However, drying is accompanied by both desirable and undesirable changes in the physical, chemical and, most importantly, nutritional characteristics of the product (Tomas & Jiri, 2003). Skim milk is obtained by centrifugation of full-fat milk, and contains only 0.05 – 0.08% fat (Walstra, Wouters, & Geurts, 2005). Skim milk powder (SMP) has become a staple ingredient in the food industry due to its multi-functional and nutritional properties. The application of SMP as an ingredient in various products, such as infant formulas, bakery products, reconstituted and fermented milk products, frozen desserts, coffee whiteners and even meat products, is extensive (Fox, 2001). According to the Food and Agriculture Organization of the United Nations, the worldwide production of SMP reached 2.3 million tons in 2016 (FAO, 2016).

When a food product composed of proteins and carbohydrates, such as milk powder, is exposed to heat treatment or extended storage, a complex series of reactions called non-enzymatic browning, or the Maillard reaction, may take place. This chemical reaction consists of several sequential and parallel pathways, and once it starts, it continues throughout the storage period. However, it can be controlled by optimizing the temperature, pH, water activity and composition of the product (Van Boekel, 2001).

Although SMPs belong to the rather stable group of food products, with an average shelf life of 2 years, they are liable to undergo the Maillard reaction because of their composition and the possibility of exposure to unfavourable conditions during thermal processing, transportation and storage (Arena, Renzone, D'Ambrosio, Salzano, & Scaloni, 2017).

One of the reasons why the Maillard reaction has recently attracted considerable attention is the formation of advanced glycation end products (AGEs). AGEs are a group of molecules formed during the advanced phase of the Maillard reaction, and are suspected of being involved in the initiation or development of inflammatory diseases such as diabetic complications (Bengmark, 2007).

The situation in some developing countries (with high consumption of milk powders) which have hot and humid climates, cause greater concern in this regard; where it can be expected to have less proper control over transportation and subsequent storage. 'Room temperature' in such environments is no longer around

20 °C, but rather 30 – 40 °C, which will cause the Maillard reaction to proceed at a higher rate.

Although the literature on the Maillard reaction in milk products is extensive, most studies have focused on model systems, and in only a few cases on real food systems. Furthermore, a large number of studies centre around temperatures exceeding 40 °C, which are relevant during industrial processing. In other words, little research has been carried out on the Maillard reaction during prolonged storage of real food systems under realistic storage conditions.

The work described in this thesis was conducted on a wide range of SMPs produced on pilot scale with different drying techniques. A commercial SMP, produced on an industrial scale, was also studied. The effects of extended storage of SMPs under the conditions that can be expected during domestic storage were investigated, in an attempt to understand the extent of the Maillard reaction when consumers store an opened package at room temperature, and consume the product intermittently over a long time.

In this work, the study of the Maillard reaction was divided into two parts: the early stage and the advanced stage of the reaction. This was achieved by selecting relevant markers, and monitoring their formation after manufacturing and during storage under different conditions. The available lysine is an indicator of the initiation of the Maillard reaction, and can be used to study the early stage of the reaction, while carboxymethyl lysine (CML) is formed in the advanced stage of the Maillard reaction, and was monitored during storage.

Therefore, the specific aims of this work were:

- to identify the existing methods and techniques that can be used to study the early phase of the Maillard reaction (quantification of available lysine) in SMP, and to select the appropriate method (Paper I),
- to validate the method for the determination of available lysine (dye-binding method) in regard to milk protein (casein) and SMPs (Paper II),
- to determine the extent of the early stage the Maillard reaction in SMPs after manufacturing and during prolonged storage (Paper III),
- to study the kinetics of available lysine during storage under realistic storage conditions (Paper IV),
- to understand the effects of manufacturing and storage on the advanced stage of the Maillard reaction in SMPs (Paper V), and
- to study the status of the early and advanced stages of the Maillard reaction in selected liquid and powder infant foods commercially available on the Swedish market (Paper VI).

1.1 Thesis scope

Since this thesis is a multidisciplinary work, an overview of the work described in this thesis is provided in Figure 1. Although health effects were outside the scope of the thesis, these are briefly touched upon by monitoring the CML formation during the storage of the SMPs. Other related areas, such as the physical state and crystallization of lactose, *in vivo* studies and human studies, were also outside the scope of this work.

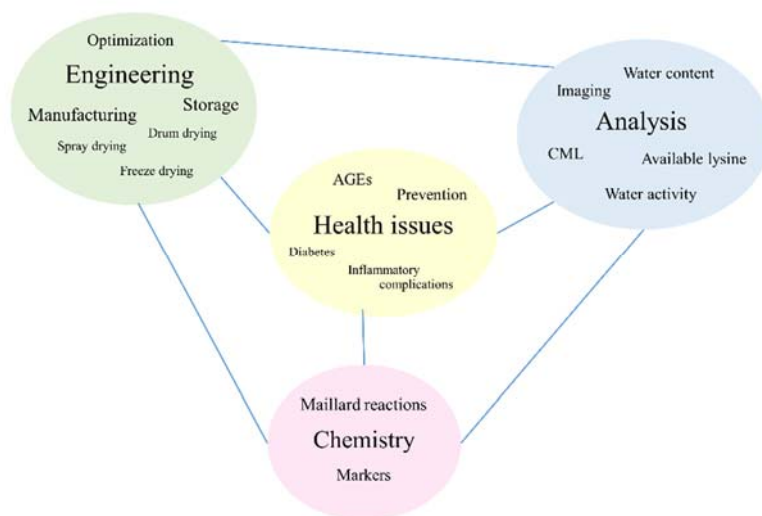


Figure 1. Overview of the multidisciplinary character of this work.

2. Background

2.1 The Maillard reaction

The concept of the Maillard reaction was first introduced in 1912 by the French physician and chemist Louis Camille Maillard, through his research on the reactions between amino acids and sugars (Maillard, 1912). Since then, extensive research has been carried out on the subject, leading to a large number of publications on this complex phenomenon in food science. Figure 2 *aims to provide a better understanding of the growing research on this reaction with regard to milk products.*

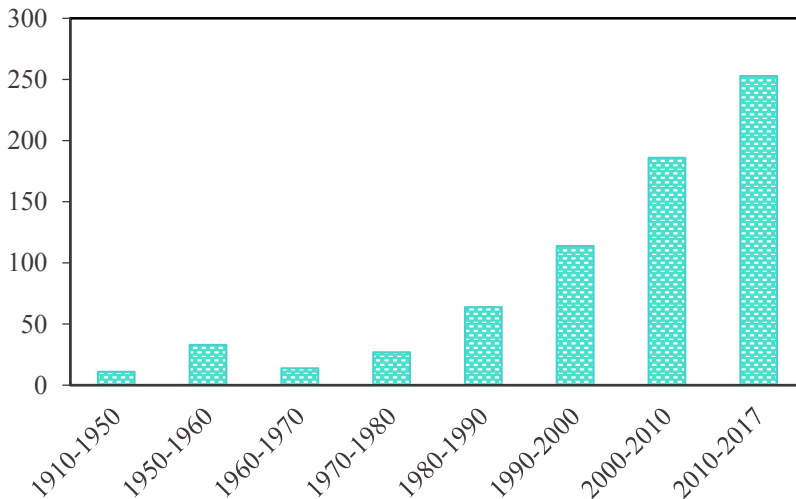


Figure 2. An overview of the number of publications on the Maillard reaction in milk products (1910-2017) using the search phrase “Maillard reactions in milk products”, based on bibliographic research in the SciFinder platform. Available at: <https://scifinder.cas.org>. Consulted on: April 24, 2017.

2.1.1 Reaction mechanisms

Reducing sugars and amino groups of proteins are the main reactants in the Maillard reaction. The reaction thus takes place when both components are present, for instance, in milk. In this case, the reducing sugar is lactose, a disaccharide of glucose and galactose, and the amino groups are ϵ -amino groups (shown in Figure 3), which are components of the essential amino acid lysine (Van Boekel, 1998). According to Porretta, foods containing these two components undergo the Maillard reaction even at refrigerated temperatures, although at a slower rate (Porretta, 1992). Due to the complexity of the reaction and the existence of several sequential and parallel pathways, a simplified illustration of the main steps in this reaction is presented in Figure 3.

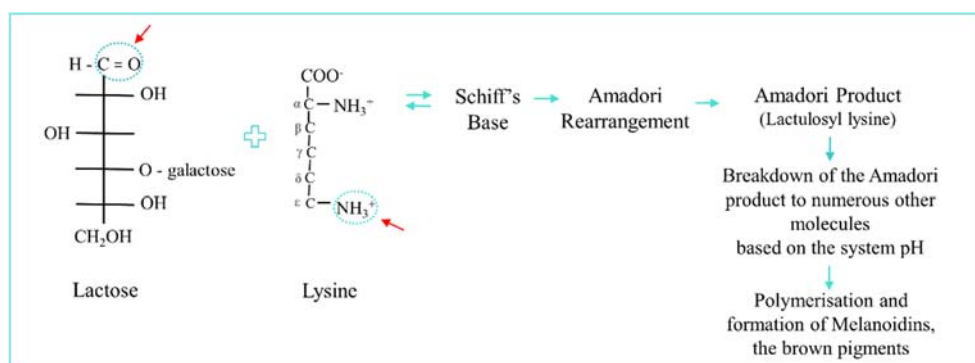


Figure 3. Simplified illustration of the Maillard reaction in milk products.

Traditionally, the Maillard reaction is divided into 3 steps. The interaction of lactose with lysine and the formation of the Amadori product is regarded as the *early* stage of the reaction. Degradation of the Amadori product and the formation of molecules such as CML, hydroxymethyl furfural (HMF), β -pyranone, 3-furanone, reductones, α -dicarbonyls, cyclopentenone, galactosylisomaltol and acetylpyrrole is considered as the *advanced* stage of the reaction and, finally, the generation of Melanoidins is the *final* stage (O'Brien, 2009). A point that can be concluded is that changes in colour and browning do not take place until the final steps, thereby the reaction has no visual indication until the late stage.

2.1.2 Consequences and health aspects

The consumption of thermally processed foods has increased over the past 30 years (Cordain et al., 2005). While the benefits of heat processing and the Maillard reaction cannot be disregarded, the possible negative consequences must also be

taken into consideration. In fact, several studies can be found in the literature on health-related issues of the Maillard reaction, and the potential risks of AGEs in particular (N. Ahmed & Thornalley, 2007; Bengmark, 2007; Chao, Huang, Hsu, Yin, & Guo, 2010; Henle, 2005; Kizer et al., 2014; Klenovics et al., 2013; Liu et al., 2016; Nass et al., 2007; Nin et al., 2011; Poulsen et al., 2013; Uribarri et al., 2005; Uribarri et al., 2010; Vlassara & Palace, 2002).

AGEs are not only formed in food materials as a result of processing or storage (exogenous), but they can also be generated under physiological conditions in the body (endogenous) (Uribarri et al., 2005). In both cases, they appear to contribute to the progression of inflammatory diseases, the most prevalent being type 2 diabetes and its complications (Van Nguyen, 2006). It has been reported that 422 million adults were living with diabetes in 2014, compared to 108 million in 1980 (WHO, 2016), i.e. the number has almost quadrupled in 30 years; the role of diet being indisputable. It is now clear that diabetics have higher plasma AGE levels than healthy individuals (Vlassara & Palace, 2002). A study of the relevant literature reveals that research on AGEs and their effects in the body has mainly focused on:

- the influence of dietary AGEs on circulating AGEs, mostly in animals, but also in diabetic and uremic patients and, to a lesser extent, in healthy individuals;
- the influence of AGEs, both exogenous and endogenous, on the progression of diabetes,
- the relationship between circulating AGEs and the plasma biomarkers associated with inflammatory diseases,
- The mechanisms of the formation of AGEs and how they exert their effects in the body; and
- the adsorption, metabolism and excretion or accumulation of AGEs in the body.

Briefly, glycated proteins (both from exogenous and endogenous sources) bind with the main receptor of AGEs in the body (RAGE), and activate a pro-inflammatory state which, in the long term, may lead to the development of chronic inflammatory diseases such as diabetes, renal disease and coronary heart disease (Poulsen et al., 2013). Most of the current knowledge in this area comes from animal studies, and only a few human studies have been carried out on the effects of high-AGE diets versus low-AGE diets (Inès Birlouez-Aragon et al., 2010; Chao et al., 2010; Klenovics et al., 2013). The results of these studies show that circulating AGEs are strongly correlated with food-derived AGEs, and that restricting the intake of AGEs in food is associated with a delay in the progression of diabetes and other age-related diseases, not only in diabetic patients, but also in

healthy individuals. Figure 4 shows how diet may be an important source of AGEs, contributing to the body's AGE pool.

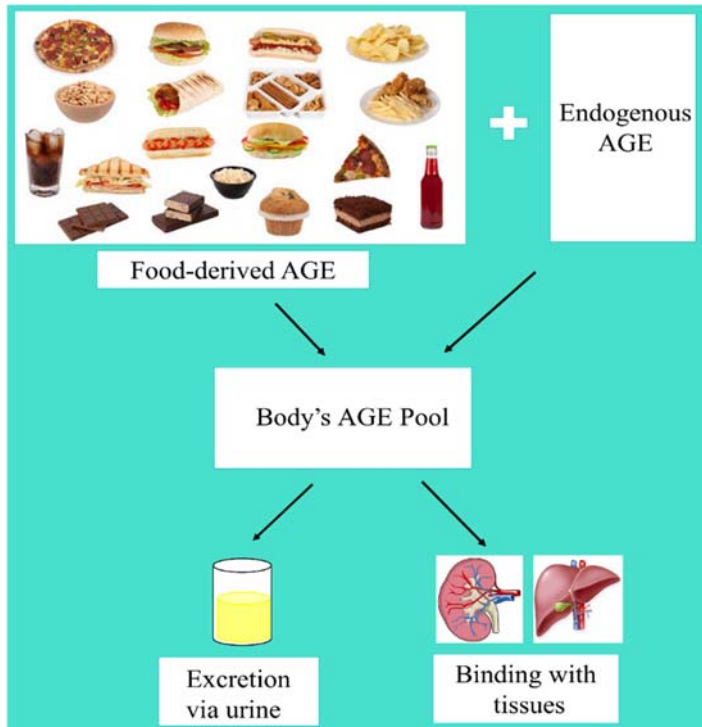


Figure 4. Dietary AGEs and their significant contributions to the body's AGE pool.

2.1.3 Markers used to follow the Maillard reaction

Available lysine

Milk protein, with its high proportion of essential or indispensable amino acids, is an important source of protein in the diet, as these amino acids are the vital building blocks of proteins that cannot be synthesized in the body. Bovine milk protein, which contains approximately 80% casein and 20% whey proteins, is one of the largest sources of the essential amino acid lysine in the body. It is reported that 8.2% of all the amino acids in casein and 8.7% of those in whey proteins consist of lysine (Pellegrino, Masotti, Cattaneo, Hogenboom, & de Noni, 2013). However, the high nutritional value of these proteins may be impaired as a result of technological processing and/or during storage. Several studies have focused on the aspect of impaired nutritional value of milk proteins through determination of the available lysine, i.e., by evaluating the quality of the milk protein as a dietary component (Anderson, Sneed, Skurray, & Carpenter, 1984; Carpenter et al., 1989;

El & Kavas, 1997; Erbersdobler & Hupe, 1991; Fernandez-Artigas, Garcia-Villanova, & Guerra-Hernandez, 1999).

In the present work, the available lysine was used as an indicator to understand whether the Maillard reaction, has started and if yes, to what extent. The terminology regarding lysine that has free reactive groups, and is available to react with other reactants, is not consistent in the literature. Throughout this thesis, the term “available lysine” is used to describe such lysine, while “bioavailable lysine” refers to lysine that can be utilized by the body (*in vivo*). In other words, the available lysine may not be completely bioavailable (Barlow et al., 1984).

In the studies reported in the literature, the available lysine has been determined as a marker to assess the effects of processing or to estimate the impact of storage on the initiation and development of the Maillard reaction. Chemically available lysine can be determined by means of a number of established techniques.

1-fluoro-2,4-dinitrobenzene (FDNB) is one of the oldest and most popular methods that has been modified several times by various researchers (Albalá-Hurtado, Bover-Cid, Izquierdo-Pulido, Veciana-Nogués, & Vidal-Carou, 1997; Barlow et al., 1984; Blom, Hendricks, & Caris, 1967; Carpenter et al., 1989; José Contreras-Calderón, Guerra-Hernández, & García-Villanova, 2009; Fernandez-Artigas et al., 1999; Hendriks, Moughan, Boer, & van der Poel, 1994; Hurrell, Finot, & Ford, 1983; Ramírez-Jiménez, García-Villanova, & Guerra-Hernández, 2004; Vigo, Malec, Gomez, & Llosa, 1992; Walker, 1979).

O-phthalaldehyde (OPA) is the other frequently used method, especially in milk products. It is based on the reaction of ϵ -NH₂ groups of proteins with O-phthalaldehyde, which produces a fluorescent substance in a very short time (around 1 min). The intensity of the fluorescence is then measured with a fluorimeter (Emilia Ferrer, Alegría, Farré, Abellán, & Romero, 2000; E. Ferrer, Alegría, Farré, Abellán, & Romero, 2003; Goodno, Swaisgood, & Catignani, 1981).

The guanidination method is another technique that has been quite popular. The principle is to convert lysine into homoarginine by O-methylisourea and to quantify this by gas chromatography (GC). This method has been applied by several groups (Burvall, Asp, Bosson, José, & Dahlqvist, 1978; Maga, 1981; Mauron & Bujard, 1964; Nair, Laser, Burvall, & Asp, 1978; Rutherford & Moughan, 2008). The methods mentioned above, including their modifications and applications to various sample materials, as well as their advantages and disadvantages, are described in detail in *Paper I*.

The *dye binding method* was applied for the determination of available lysine throughout this work (Hurrell, Lerman, & Carpenter, 1979). The method is fully described and validated in *Paper II*. Figure 5 illustrates the main steps in the determination of available lysine using this method.

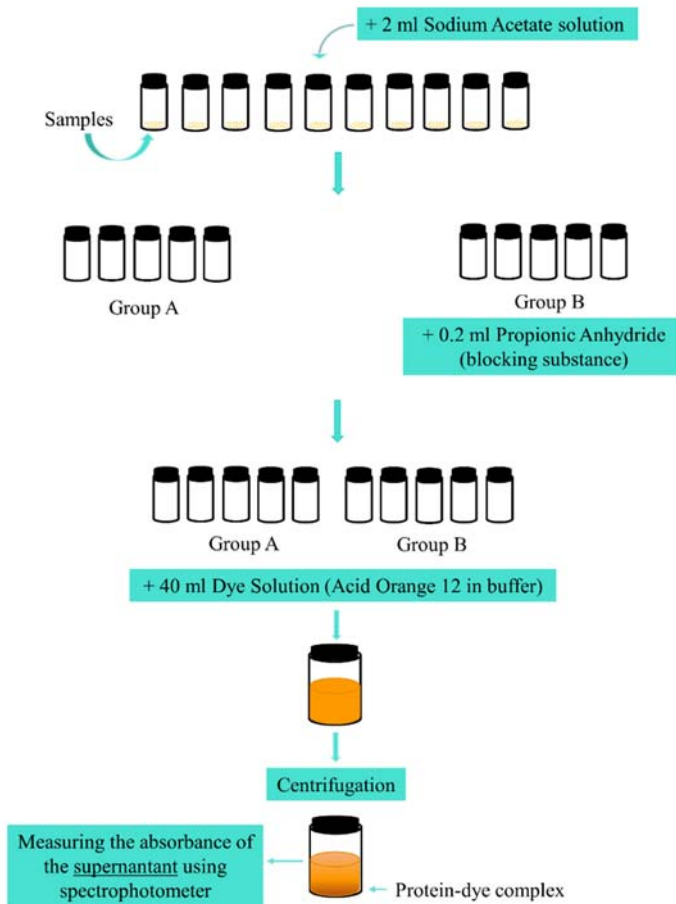


Figure 5. The dye-binding method applied in this work for the determination of available lysine using five replicates.

As is explained in *Paper II*, the dye solution, with its negative charges, binds rapidly with the positively charged amino acids (lysine, arginine, histidine). The basicity of the lysine group is then neutralized by the blocking substance, which is added only to half of the samples (group B). Therefore, in half of the samples (group A) the dye solution binds with lysine, arginine and histidine and in the other half (group B) only with arginine and histidine, since the reactive group of lysine is blocked. The difference in absorption between the two, gives the concentration of available lysine residues in the sample.

Carboxymethyl lysine (CML)

CML is the most frequently used indicator of the advanced stage of the Maillard reaction, and was the first AGE molecule detected in milk and milk products (Ames, 2008).

CML is not only formed via the reaction between lactose and lysine, followed by the degradation of the Amadori product, but also through the oxidation of sugars and peroxidation of lipids, as well as the reaction between ascorbic acid and lysine (H. T. Nguyen, van der Fels-Klerx, & van Boekel, 2013). This should be borne in mind with respect to food formulations, e.g. in case of products such as infant formulas. In other words, the composition of food together with the processing and storage conditions determine the formation of CML in a food product. Considering that the presence of poly-unsaturated fatty acids and ascorbic acid is negligible in skim milk, the main pathway for CML formation would be through the reaction of lactose and lysine, and subsequent degradation of the Amadori product, lactulosyllysine (Figure 6).

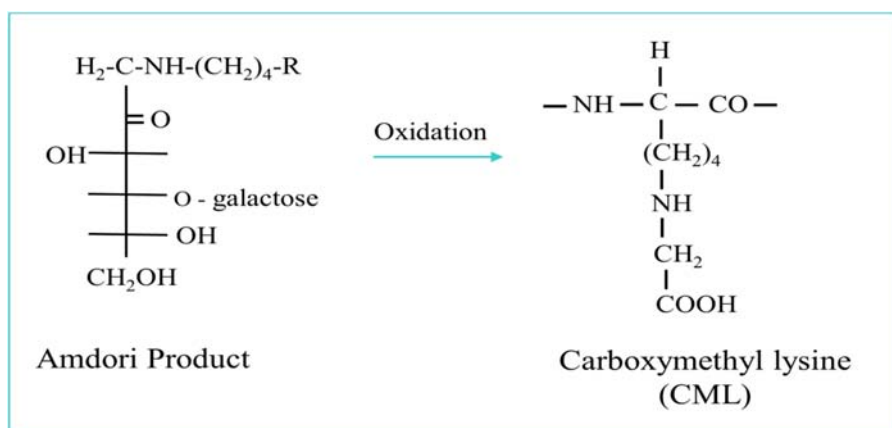


Figure 6. Formation of CML via degradation of the Amadori product; the main pathway in low-fat milk products such as SMP.

CML is an acid-stable compound that can be found in two forms in food materials: free CML and peptide-bound CML (Assar, Moloney, Lima, Magee, & Ames, 2008). In this work, the analysis of CML is conducted with respect to total CML, i.e. free CML + CML that is attached to lysine residues in peptides and proteins (*Paper V* and *Paper VI*).

There are two approaches for the quantification of CML: the immunochemical and the instrumental. Instrumental methods such as LC-MS/MS (liquid chromatography-tandem mass spectrometry) applied in this work, have better selectivity and sensitivity. Despite the fact that they are costly and require more sample clean-up and preparation, these techniques are preferable to methods such

as ELISA (enzyme-linked immunosorbent assay), which are based on anti-CML antibodies and whose reliability has been questioned (Ames, 2008; Tareke, Forslund, Lindh, Fahlgren, & Östman, 2013).

Food materials, depending on their composition, need to go through several steps before being analysed by means of LC-MS/MS. Figure 7 shows the main steps carried out in the CML analysis in the present work.

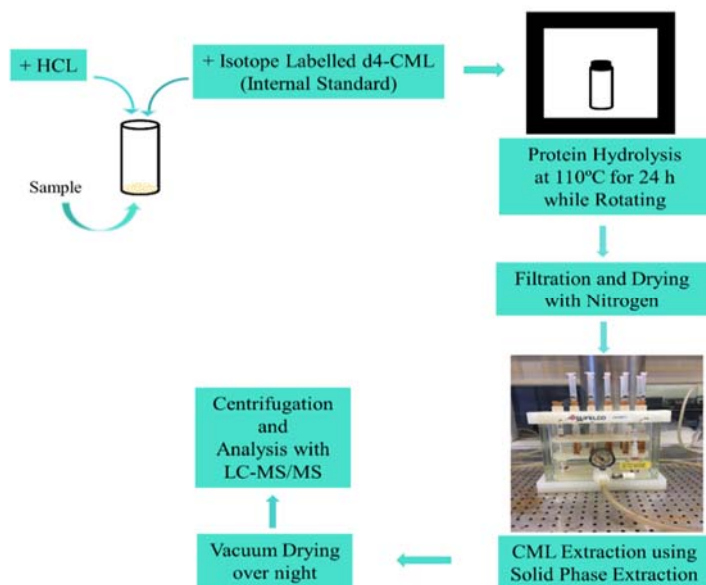


Figure 7. Sample preparation for CML analysis with two replicates.

As can be seen in Figure 7, samples were spiked with deuterated standards (d4-CML) to obtain isotopically labelled CML molecules. The internal standard was also added to the blank and the calibration standards. They were then hydrolysed using acid and a high temperature in order to detach the peptide and protein-bound CML.

CML was extracted using solid-phase extraction (SPE). This is a technique used for the extraction of the analyte of interest, in this case CML, based on the chemical characteristics of the compound. There are several different types of SPE. Cation-exchange SPE was employed in this work. The cartridges were conditioned with methanol and after being activated by nonafluoropentanoic acid (NFPA) and the addition of the samples, CML molecules were eluted using ammonia solution (*Papers V and VI*).

The methods of sample preparation and the subsequent quantification of CML using LC-MS/MS used throughout this work have been established and validated by Tareke et al. (Tareke et al., 2013).

2.2 Skim milk powder

SMP is obtained from full-fat milk after the partial removal of fat and water. SMP contains a maximum of 1% fat. The compositions of SMP and full-fat or whole milk powder (WMP) are given in Table 1.

Table 1.
Approximate composition of SMP and WMP (%w/w) (from Walstra, et al. 2005)

Composition (%)	SMP	WMP
Fat	1	26
Lactose	51	38
Casein	27	19.5
Whey proteins	6.6	5.3
Ash	8.5	6.3
Water	3-4	2.5

Based on the Food and Agriculture Organization of the United Nations, the production of SMP increased by 2.8% in 2016, compared with the previous year, and reached 2.3 million tons. The largest producer was the European Union, with an annual production of 700,000 tons (FAO, 2016).

Apart from its primary role as a source of infant nutrition, especially in the developing countries, the popularity of this product in industry is mainly due to its prolonged shelf life (1.5-2 years on average), reduced transportation and storage costs, as well as its numerous functional and nutritional properties. It is used extensively in dairy and non-dairy products to enhance the colour or flavour, or the emulsification, solubility and water-binding characteristics (Sharma, Jana, & Chavan, 2012).

Industrially produced milk powders are categorized into three groups according to the intensity of the heat treatment: low-heat, medium-heat and high-heat. The SMPs produced differ mainly in their content of denatured whey proteins, and thereby, tailored to be used in the formulation of different products (Skanderby, Westergaard, Partridge, & Muir, 2009).

The main applications of SMP in the food industry are illustrated in Figure 8. It should be noted that these are not the only industrial applications of this product. Other applications of SMP include the manufacture of reconstituted regular and UHT milk, and concentrated milk.

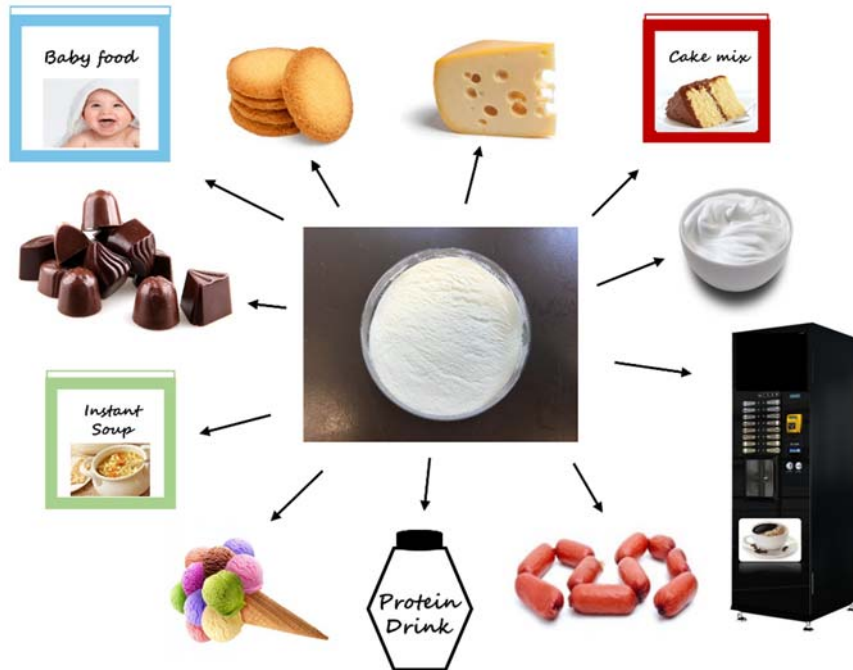


Figure 8. Applications of SMP as an ingredient in the food industry.

2.2.1 Processing

Up until 1960, drum-drying was the main technique used for the manufacture of SMP, after which spray-dryers dominated due to their energy efficiency, and today SMPs are almost entirely manufactured by spray-drying (Fox, 2001). The only application in which drum-drying is preferred over spray-drying is in the manufacture of chocolate, where the colour and caramelized taste of drum-dried milk powder is desirable (Sharma et al., 2012). The drying technologies applied in the present work were spray-drying, drum-drying and freeze-drying, on both lab and pilot scales.

Spray-drying is a process that converts liquid milk into powder by atomization, during which the milk is sprayed into a drying chamber using an atomizing device (nozzle). The droplets formed come into contact with hot air in the chamber and after only a few seconds they are converted to solid particles (Singh & Heldman, 2014). The spray-dryer used in this work is shown in Figure 9. The settings and details of the experiments are described in *Papers II* and *III*.



Figure 9. Lab-scale spray-dryer used in this work (Büchi mini spray-dryer B-290).

The industrial production of SMPs consists of several steps, including the separation of fat, pasteurization, evaporation, and concentration of the milk to 48-50% dry matter. Spray-drying is usually followed by fluidized-bed drying. Therefore, the resulting powders go through an agglomeration step, which leads to larger particles with better flow and solubility properties (Pisecky, 2012).

The second drying technique utilized in this work was drum-drying. During this process, the liquid milk was spread onto a rotating, steam-heated drum. The milk used was a 40% concentrated liquid obtained by mixing the previously produced freeze-dried SMP and liquid skim milk. The thin sheet formed on the drum surface is converted into dried flakes in around 40 seconds, which were subsequently collected and ground using a mortar and pestle (Figure 10).



Figure 10. The drum-dryer used in the present work (Goudsche Machinefabriek) and the sheets of SMP. The average surface temperature of the drum was 115°C, and the drying duration was 40 s.

Freeze-drying was the third technique used for the production of SMP, and especially to produce the control samples. This is a gentle drying technique that is mainly used for heat-sensitive food and pharmaceutical products.

The skim milk was frozen at -20 °C for 24 hours and then transferred to the freeze-dryer. Sublimation of ice crystals occurred by increasing the temperature according to the preset program (1 °C/h), and the milk was dried at a maximum temperature of 20 °C. The pilot-scale freeze-dryer is shown in Figure 11.



Figure 11. The freeze-dryer used (Labconco). The sample materials that had been frozen in metal containers were placed on the shelves inside the drying chamber.

In order to assess the effectiveness of drying and to characterize the dried products, two parameters were considered; *water activity* and *water (moisture) content*.

Water activity (a_w)

“The water activity of a product can be measured because a_w equals the relative humidity of air in equilibrium with the product. Accordingly, it can be determined by establishing the relative humidity at which the product neither absorbs nor releases water.” (Walstra et al., 2005)

Water activity is a parameter that is frequently used to determine the storage stability of products, and to ensure they are microbiologically safe. For the latter, the water activity of a product in the dry state must be below 0.7.

Water content (Dry Matter)

Determining the water content by drying a sample in an oven provides a measure of the “free” or unbound water in a food material, and this determines the shelf life and textural quality of the product (Rückold, Grobecker, & Isengard, 2000). The method applied in this work is a standard method established by the International Dairy Federation (IDF, 1993), which is drying at 102 °C for 2 h.

2.2.2 Storage

Storage must be well defined: Whether it is domestic, or industrial scale, if storage in closed packages is the target or if storage after opening a package is the matter, each may have a different scenario. The quality of dried food materials deteriorates upon storage due to chemical reactions which, in the case of milk powder, is mainly the Maillard reaction. The storage stability of SMP depends mainly on the water content, a_w (or RH of the air), the temperature and the lactose content (Higgs & Boland, 2008).

Guyomarc’h et al. showed that the interaction between lactose and proteins (lactosylation) occurs even in closed packages during storage at room temperature (20 °C) starting from week 8 (Guyomarc’h, Warin, Donald Muir, & Leaver, 2000). Considering that SMP has an average shelf life of 2 years, the reaction is expected to proceed considerably throughout the shelf life, even when the package has not been opened.

Although a number of studies have been carried out on the physical, chemical and nutritional changes in closed packages during storage, there can hardly be found studies focusing on the changes that take place in products after the package has been opened, and stored in the home, and the product is consumed over a significant period of time. This is considered to be a safe practice by consumers, as

they believe that a dried product is unlikely to become spoiled, and can thus be stored at 'room temperature' regardless of what this may be, until it is finished.

This is not only a concern in the domestic setting, but also in industry, where SMP is used as an ingredient in the formulation of other products. Commercial SMP is usually packaged in 25 kg sacks or bags and, since it is hygroscopic, it can easily absorb water, leading to the initiation of the Maillard reaction, especially after the sacks have been opened. This underlines the importance of storage variables, i.e. the temperature and the relative humidity of the air, studied in this work.

The effect of temperature has been studied previously. However, in most cases, the temperatures studied were 60 °C or higher (Brands & van Boekel, 2003; Charissou, Ait-Ameur, & Birlouez-Aragon, 2007; François Fenaille, Campos-Giménez, Guy, Schmitt, & Morgan, 2003; François, Corinne Appolonia, Robert, Gilles, & Alois, 2005; Franzen, Singh, & Okos, 1990; Ge Pan & Melton, 2007; Schmitz-Schug, Kulozik, & Foerst, 2014; Schmitz, Gianfrancesco, Kulozik, & Foerst, 2011). Only a few studies were found in which storage at moderate conditions was investigated (Malec, Pereyra Gonzales, Naranjo, & Vigo, 2002; Pereyra Gonzales, Naranjo, Leiva, & Malec, 2010).

Although the data obtained when applying relatively high temperatures are valuable and provide a better understanding of the effect of processing on the extent of the Maillard reaction, it is important to study the effects of lower temperatures as these are more common both in industry and in the home.

2.2.3 Safety concerns

As mentioned above, one source of concern is consumer attitudes towards, and behaviour regarding, dried products in general, i.e., consumers are not aware of the potential risks. This concern is greater in hot, humid conditions, often prevalent in less developed countries, where storage, handling and transportation may not be well-controlled.

Nguyen et al. studied the relationship between indoor and outdoor weather conditions (J. L. Nguyen, Schwartz, & Dockery, 2014). They found that indoor temperature and humidity correlated well with the corresponding outdoor conditions, specifically at higher outdoor temperatures or during the warmer months (May-September). The study was carried out in Massachusetts, USA, where the indoor temperature varied between 18 and 27 °C, and the indoor RH was 23-71%. It should be noted that this is only an average example, and does not reflect extreme weather conditions, even considering only the USA. Other regions of the world have higher temperatures and RH, which are of greater concern.

The other matter of concern is related to the manufacture of food products in which milk powder is part of the formulation. Storage in large quantities (usually in 25 kg sacks) before being mixed with other ingredients, fortification with other

provoking ingredients such as vitamin C, heat processing of various intensity and subsequent handling and storage depending on the product final physical state and shelf-life and final handling by the consumers, may add more complication and increase the AGE content.

3. Available Lysine as a Marker of Early Maillard Reaction

The content of available lysine may provide reliable information on whether the Maillard reaction has been initiated. The available lysine was used as an indicator to assess which drying method had the least effect on initiation of the Maillard reaction. The aim was to gain insight into the onset of the Maillard reaction and how fast it proceeds during storage, and to compare the impact of the storage variables. This is illustrated in Figure 12.

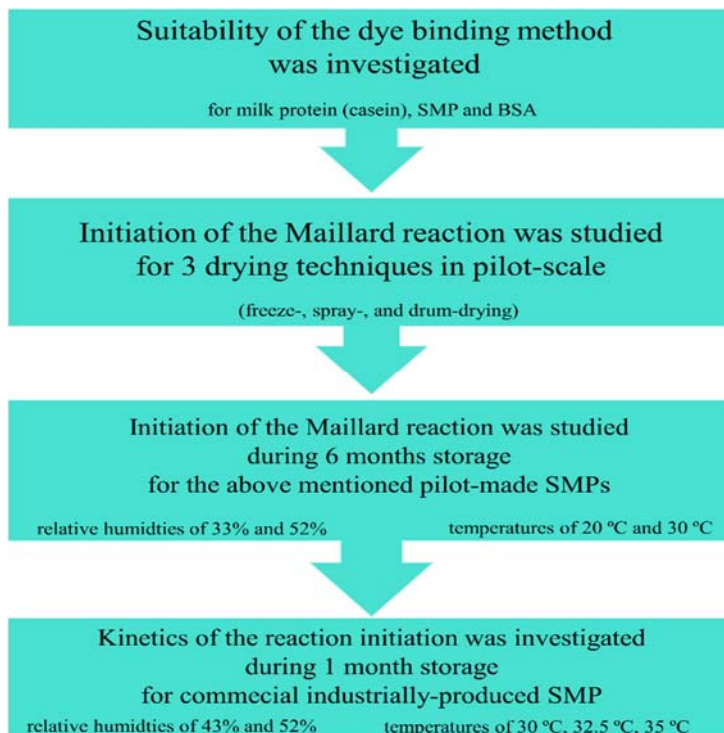


Figure 12. Successful employment of available lysine as a marker to monitor the progression of the Maillard reaction in the early stage (Papers II, III, IV).

3.1 Skim milk powder

As explained in detail in *Paper I*, there are several established methods for the quantification of available lysine. Selecting a method and reaching the point where the method can be used with reasonable certainty requires careful investigation and a significant amount of time. This was the focus of *Paper II*, in which the suitability of the dye-binding method was investigated specifically for SMP.

The method was investigated critically with a wide range of SMPs including 125 samples of freeze-dried, 80 samples of spray-dried and 100 samples of drum-dried powders; all made in the pilot plants and stored at various temperatures and humidities. Commercial SMPs (vacuum- and non-vacuum-packed), a non-heat-treated milk sample, which was collected directly after fat separation and freeze-dried (control), as well as purified casein and bovine serum albumin (BSA) with known lysine contents were also tested.

This investigation demonstrated that the method is suitable and reliable for the determination of available lysine in SMPs with high precision and reasonable variation (*Paper II*), and could be used to monitor the progression of the early Maillard reaction. The choice of drying technique was found to be significant: the freeze-dried powder had the highest available lysine content, $3.49 \pm 0.07\%$ in dry matter, followed by the spray-dried powder, $3.23 \pm 0.08\%$, and the drum-dried sample, which had the lowest available lysine content of $3.04 \pm 0.09\%$. In other words, spray-drying (in the context and conditions of this work) caused a 7.45% decrease in the available lysine content and the impact of the drum-drying was 12.89%.

It has previously been reported by Carpenter et al. that a 'non-fat dried milk' contained 9.26 g available lysine/100 g protein when the same dye-binding method was employed (Carpenter et al., 1989). Considering that the samples in the present work contained 36% protein, these findings are consistent with the results of their study. Similarly, Hurrell et al., who developed the dye-binding method, reported a value of 8.90 g available lysine/100 g protein for SMP (Hurrell et al., 1979).

The powders produced using the three different drying techniques were then stored for approximately 6 months (200 days) under different combinations of temperature (20 °C and 30 °C) and RH (33% and 52%) (*Paper III*). The results are summarized in Figures 13, 14 and 15.

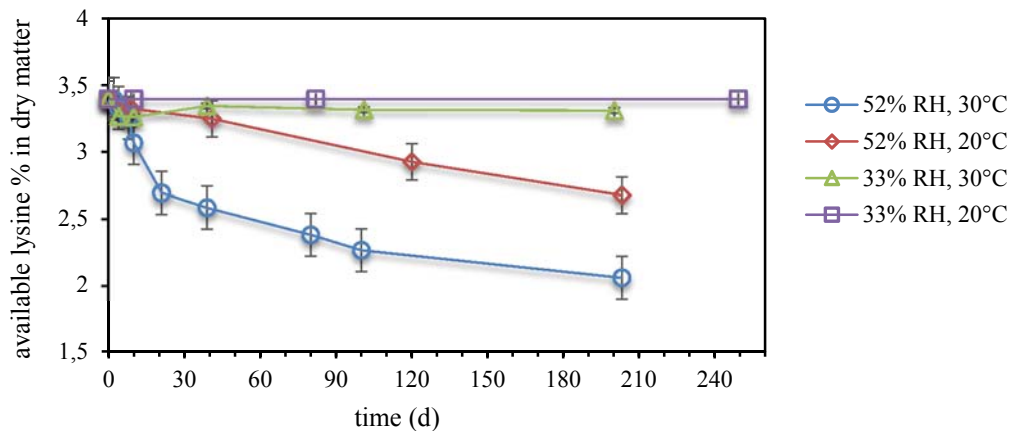


Figure 13. Monitoring of the early stage of the Maillard reaction using the amount of available lysine in *freeze-dried* SMP during 6 months' storage. The data points are the average of 5 replicates, and the error bars represent the standard deviation (Paper III).

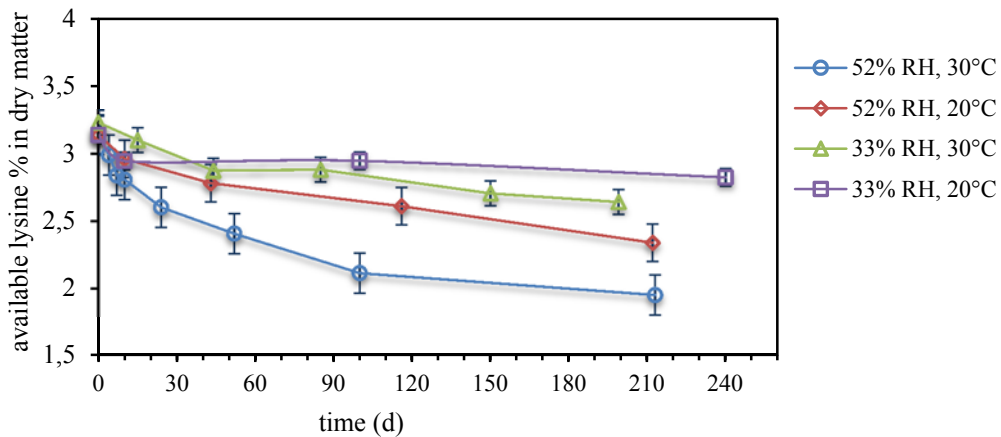


Figure 14. Monitoring the Maillard reaction in *spray-dried* SMP during 6 months storage. The data points are the average of 5 replicates and the error bars represent the standard deviations (Paper III).

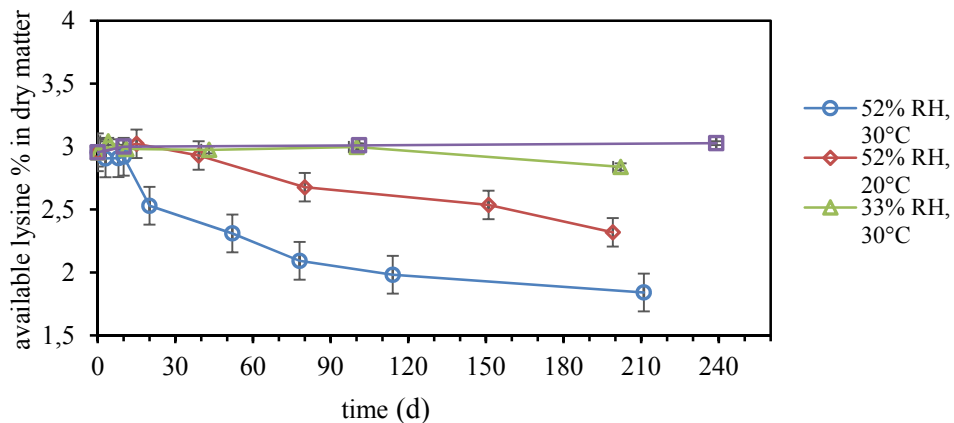


Figure 15. Monitoring the Maillard reaction in *drum-dried* SMP during 6 months storage. The data points are the average of 5 replicates and the error bars represent the standard deviations (Paper III).

From the storage experiments conducted with the freeze-dried (Figure 13), spray-dried (Figure 14) and drum-dried (Figure 15) powders it was concluded that both temperature and relative humidity are important parameters determining the decrease in the available lysine. After storage at 52% RH and 30 °C for 6 months, the SMPs exhibited a 39.2 – 45.9% decrease in the available lysine. The decrease in the available lysine after storage at the same RH but a lower temperature of 20 °C was 21.2 – 31.8%, demonstrating the important effect of storage temperature on the development of the initial phase of the Maillard reaction.

The corresponding decrease during storage at 33% RH and 30 °C after 6 months was 5.2 – 22.4%, while storage at this RH at 20 °C caused no significant decrease in the available lysine. A RH of 33% and a temperature of 20 °C therefore appear to be ideal conditions for the storage of SMPs.

Studies on the storage of milk powders under realistic conditions are scarce in the literature. Malec et al. conducted storage studies with model systems of lactose and casein and later on commercial SMP (see Table 2). In their study storage was carried out at temperatures of 37 °C, 50 °C and 60 °C, and water activity ranging from 0.33 – 0.98.

Despite the similar conditions to those used in the present work, there is a notable difference in the experimental set-up of the studies. In their study, the samples were stored in sealed containers in the incubators, after reaching the desired a_w in order to avoid the absorption or desorption of water. However, in the present work, the samples were stored in open containers, (Figure 16) first in desiccators with the desired RH, and then placed in the incubators at the pre-determined temperature. Therefore, the samples could absorb or desorb moisture

inside the desiccators at the fixed RH. The SMPs can therefore be expected to have different water contents and water activity at time 0, which could affect the reaction rate and its progression.

Table 2.

Previous storage studies carried out at mild temperatures

System studied	Storage temperature (°C)	Storage RH (%)	Storage duration (d)	Decrease in available lysine (%)	Reference
Casein lactose model system	37 °C	33 %	50	50 %	(Malec et al., 2002)
Casein lactose model system	37 °C	52 %	15	50 %	(Malec et al., 2002)
SMP	37 °C	47 %	40	60 %	(Pereyra Gonzales et al., 2010)
SMP	37 °C	32 %	200	10 %	(Pereyra Gonzales et al., 2010)

The difference in temperature between the studies can explain the slight difference between the present results and those from the studies carried out by Malec et al. The physical state of the lactose is the important factor determining the reaction rate, as discussed in *Paper III*.



Figure 16. Storage set-up used in the present work. The samples were placed in open Petri dishes inside the desiccators at pre-set relative humidities, and then in the incubator *to reach* the desired temperature.

Kinetics of the loss of available lysine in commercial SMP

After monitoring the changes in available lysine in the pilot-produced SMPs for 6 months, the dye-binding method was applied to a commercial SMP. The objective was to understand the reaction kinetics, specifically after opening the packages and during storage for 30 days, the period in which it is recommended that the products be consumed after being opened.

The sample material was a low-heat-treated, spray-dried industrially produced SMP, pasteurized at 73 °C for 15 s before drying. The commercial SMP was stored in the same way as the SMP samples described above, but at 6 combinations of storage conditions: 3 temperatures (30 °C, 32.5 °C, 35 °C) and 2 values of RH (43%, 52%) (*Paper IV*). To the best of the author's knowledge, no previous studies have been carried out on the kinetics of available lysine in SMP after opening the package, as the product is gradually consumed.

The results showed that the reaction proceeded linearly under all six storage conditions, but at a significantly higher rate at the higher storage temperature and RH (Figures 17, 18 and 19).

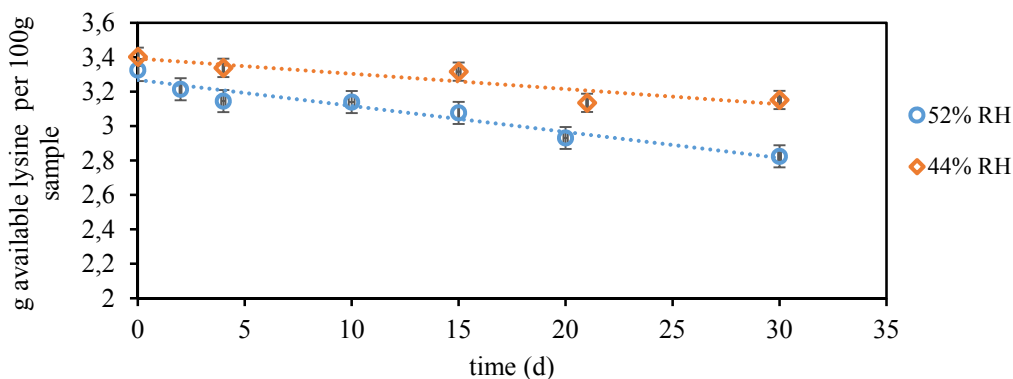


Figure 17. Kinetics of the available lysine in a commercial SMP stored at 30°C and two relative humidities for 30 days. The data points are the average of five replicates, and the error bars represent the standard deviation (*Paper IV*).

As can be seen in Figure 17, the RH had a significant impact on the reaction rate. After 30 days at 30 °C and 44% RH, there was a 5.93% decrease in the available lysine content, while the decrease was 13.74% at the higher RH of 52%. When the storage temperature was increased to 32.5 °C, the available lysine decreased to 11.86% after one month at 44% RH and to 22.22% at the higher RH of 52% (Figure 18).

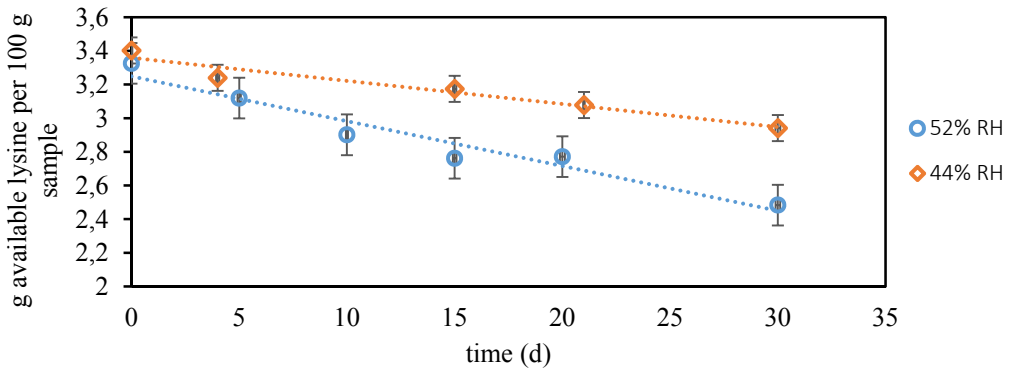


Figure 18. Storage of commercial SMP at 32.5 °C and two relative humidities for 30 days. The data points are the average of five replicates and the error bars represent the standard deviations (Paper IV).

Increasing the storage temperature for another 2.5 °C (35°C) induced a 15.82% decrease at 44% RH and 28.95% at 52% RH after 30 days (Figure 19).

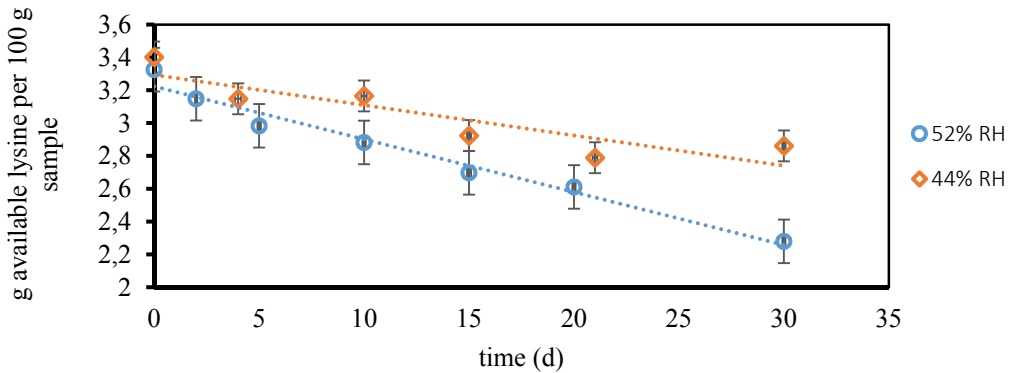


Figure 19. Storage of commercial SMP at 35 °C and two relative humidities for 30 days. The data points are the average of five replicates and the error bars represent the standard deviations (Paper IV).

An F-test was performed to evaluate whether a first-order or second-order model predicted the data most accurately. From the results of the kinetics study it was concluded that the kinetics of available lysine is more accurately modelled with a first-order model (*Paper IV*).

Several other studies found in the literature suggest that available lysine is best modelled with a first-order function (Emilia Ferrer et al., 2000; Labuza & Saltmarch, 1982; Malec et al., 2002; Naranjo, Malec, & Vigo, 1998; Naranjo,

Pereyra Gonzales, Leiva, & Malec, 2013). However, it has been reported in a few studies that a second-order model predicts the kinetics better (Schmitz-Schug et al., 2014; Schmitz et al., 2011). This indicates that the kinetics of the available lysine could be dependent on the experimental conditions.

The calculated first-order rate constants and the activation energies are presented in Table 3.

Table 3.

Reaction rate constants (k) and activation energies (E_a) with their confidence intervals for the decrease in lysine in SMPs stored for 30 days under six conditions

Relative humidity	First-order rate constant $k \times 10^3$ (h^{-1}) with 95% confidence intervals			E_a ($kJ\ mol^{-1}$)
	30°C	32.5°C	35°C	
44%	0.113 ± 0.021	0.181 ± 0.024	0.251 ± 0.029	170.8 ± 6.8
52%	0.206 ± 0.033	0.386 ± 0.027	0.486 ± 0.0171	142.5 ± 3.3

In the present work, the activation energies were calculated using the Arrhenius equation (*Paper IV*). The higher the activation energy, the more difficult it is for the reaction to take place. Activation energies for the available lysine in similar systems reported previously are around 100 $kJ\ mol^{-1}$ (Pereyra Gonzales et al., 2010; Schmitz-Schug et al., 2014; Thomsen et al., 2012).

Other important conclusions could be drawn from the kinetics study. Firstly, the impact of temperature was significant ($p < 0.05$), although the difference in the storage temperatures was small (2.5°C). Secondly, the RH affected the activation energy of the available lysine reaction, i.e. increasing the RH during storage from 43% to 52% decreased the activation energy of the reaction from 170 $kJ\ mol^{-1}$ to 142 $kJ\ mol^{-1}$. Miao and Roos also reported that the temperature dependency of the reaction decreased when the water content of the system was increased (Miao & Roos, 2004).

3.2 Infant formulas

Infant formulas are usually intended to mimic the composition of human milk. However, they are more liable to the Maillard reaction than cow's milk due to enrichment with provoking substances such as lactose, iron and vitamin C, and the fact that they have undergone several heat processing steps to ensure their safety (Pischetsrieder & Henle, 2012). In order to ensure their bacteriological safety, these products are exposed to pasteurization (72 °C, 15 s), sterilization (100 °C, 22 s) or spray-drying (inlet temperature of 175-185 °C). They are either in powder or

liquid form, and depending on the age of the infant for which they are intended, they are formulated either with lactose only, or with lactose and maltodextrin (Emilia Ferrer et al., 2000). Several powdered and liquid infant formulas available on the Swedish market were chosen from three manufacturers denoted A, B and C and the impact of processing and the physical state of the product on the development of the Maillard reaction was investigated. The results of the lysine analysis are presented in Table 4.

From the results presented in this table, it can be concluded that powder and liquid infant formulas have similar available lysine concentrations, regardless of the brand. Furthermore, considering that the concentrations of available lysine in these products are in the range 0.95 – 1.28 % in dry matter, the infant formulas show a 27.14 – 36.57% decrease in the available lysine, compared to the reference SMP sample (the reference sample was not exposed to heat treatment, and was collected after the fat separation step and freeze-dried. It contained 3.5% available lysine based on dry matter). Birlouez-Aragon et al. also found no significant difference between liquid and powder infant formulas with respect to available lysine. They reported a decrease of 20% in the available lysine compared to raw cow's milk (I. Birlouez-Aragon et al., 2004).

The present results are slightly higher than those reported by Contreras-Calderon. They found that the average decrease in available lysine in the infant formulas was 4032 – 6438 mg/100 g protein (J. Contreras-CalderÓN, Guerra-Hernández, & García-Villanova, 2015). In another study, the loss of available lysine in infant cereals as a result of processing ranged from 14 to 29% (Fernandez-Artigas et al., 1999). Similarly, Ferrer et al. reported a loss of 15.2 – 26.7% available lysine in infant formulas, compared with raw cow's milk (Emilia Ferrer et al., 2000).

Table 4.

Analysis of available lysine in commercial infant formulas using the dye-binding method. Available lysine is presented based on both dry matter and protein content. (The data are the average of three replicates \pm standard deviation (Paper VI))

Product	Protein base	Contains milk powder	Recommended age, from	Protein content% (n=2) \pm SD	Lysine (%DM) (n=3) \pm SD	Lysine (%protein) (n=3) \pm SD
Powder						
Manufacturer A	Oat	X	6 months	10.78 \pm 0.04	0.95 \pm 0.06	8.13 \pm 0.05
Manufacturer A	Maize	X	6 months	11.26 \pm 0.04	1.13 \pm 0.06	9.75 \pm 0.49
Manufacturer B	Oat	X	6 months	12.09 \pm 0.00	0.96 \pm 0.02	7.74 \pm 0.18
Manufacturer B	Oat	X	1 year	12.12 \pm 0.13	1.02 \pm 0.02	8.28 \pm 0.16
Liquid						
Manufacturer A	Oat	X	6 months	12.52 \pm 0.02	0.95 \pm 0.02	7.55 \pm 0.16
Manufacturer A	Maize	X	6 months	12.80 \pm 0.12	0.99 \pm 0.01	7.76 \pm 0.07
Manufacturer B	Oat	X	6 months	12.40 \pm 0.11	1.10 \pm 0.02	8.86 \pm 0.12
Manufacturer B	Oat	X	1 year	15.60 \pm 0.05	1.28 \pm 0.04	8.16 \pm 0.28
Manufacturer C	Oat	X	6 months	13.07 \pm 0.03	1.06 \pm 0.02	7.97 \pm 0.19
Manufacturer C	Oat	X	1 year	14.84 \pm 0.30	1.25 \pm 0.03	8.30 \pm 0.21

4. Carboxymethyl Lysine as a Marker of Advanced Maillard Reaction

After studying the early phase of the reaction using the available lysine, and investigating the governing parameters, attention was focused on the advanced stage of the Maillard reaction. The formation of CML, an established AGE molecule, was monitored in the pilot-scale-produced SMPs during storage under conditions similar to those studied previously when determining the amount of available lysine (*Paper V*). The importance of this part of the study was not only to gain insight into the advanced phase of the Maillard reaction, but also the role of CML as an identified AGE molecule, as AGEs have been associated with risk factors for diabetes complications and other autoimmune diseases.

4.1 Skim milk powder

Two kinds of techniques can be used for CML analysis: immunochemical and instrumental (Naila Ahmed et al., 2005; Bosch et al., 2007; Dittrich et al., 2006; Drusch, Faist, & Erbersdobler, 1999; Francis Fenaille et al., 2006; Hartkopf, Pahlke, Lüdemann, & Erbersdobler, 1994; Hull, Woodside, Ames, & Cuskelly, 2012; Plaza, Östman, & Tareke, 2016; Schwarzenbolz, Hofmann, Sparmann, & Henle, 2016; Takeuchi et al., 2015; Tareke et al., 2013; Troise, Fiore, Wiltafsky, & Fogliano, 2015). Instrumental methods, such as LC-MS/MS (liquid chromatography-tandem mass spectrometry) applied in this work, exhibit higher sensitivity and accuracy, as well as higher reproducibility.

To the best of the author's knowledge, no previous studies have been carried out on the gradual development of CML in SMP during prolonged storage under realistic storage conditions. There is thus a need for CML data from real complex food systems, obtained with a reliable instrumental technique.

The impact of the drying technique on the formation and development of CML during 200 days' storage was significant ($p < 0.05$). Before storage ($t=0$), the SMP produced with the drum-dryer had a higher CML concentration than the SMPs produced with the other two drying techniques. However, after 200 days of storage, the spray-dried SMP exhibited a significantly higher level of CML under

three of the storage conditions (Figure 20). Storage at 33% RH and 20 °C for 200 days caused no significant change in the concentration of CML in the samples, and are thus considered to be the best storage conditions among those investigated in this work.

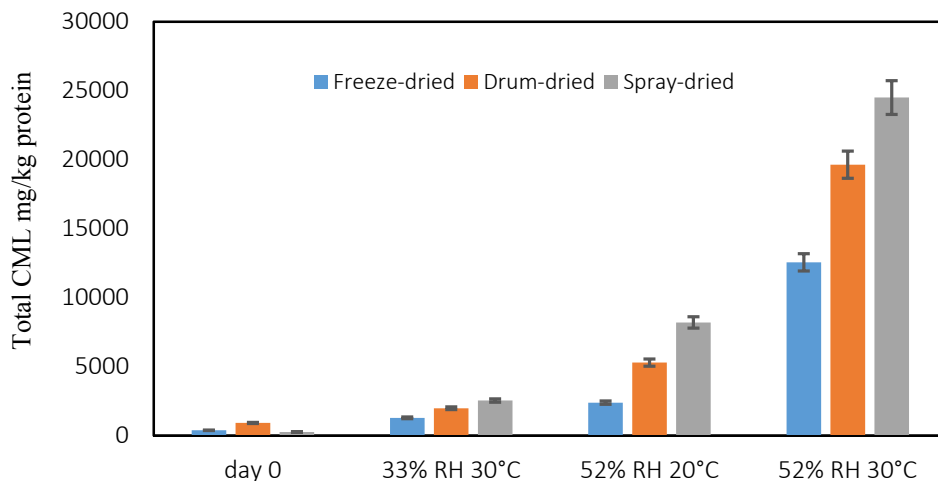


Figure 20. Effect of drying method and storage conditions on the development of CML before and after 200 days of storage. Data given are the average of two replicates, and the error bars represent the standard deviation (Paper V).

As was expected, freeze-drying had the least effect on the progression of the Maillard reaction, also in the advanced stage. The CML concentration in the spray-dried powder was unexpectedly high, being twice that in the freeze-dried powder (Figure 21). This could partly be explained by the different morphology and particle size of spray-dried powder. It has previously been reported that freeze-dried powders had 21 times larger particles ($410 \pm 9.41 \mu\text{m}$), than in a spray-dried powder produced by the lab-scale spray-dryer used in this work ($19.32 \pm 0.91 \mu\text{m}$) (Fyfe, Kravchuk, Nguyen, Deeth, & Bhandari, 2011; Langrish, Marquez, & Kota, 2006).

The reason why the spray-dried powder developed significantly higher levels of CML during and after storage is that smaller particles have a significantly higher surface area, and the reaction rate is thus higher.

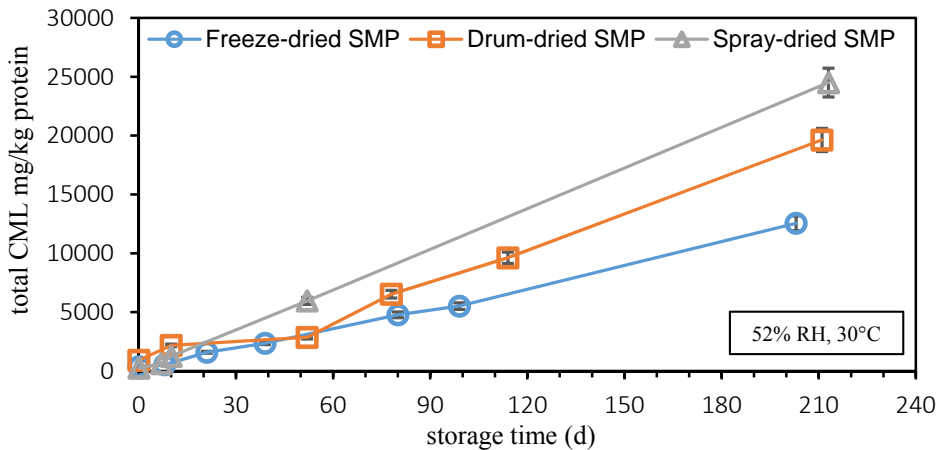


Figure 21. Change in total CML in freeze-dried, spray-dried, and drum-dried SMP during storage at 52% RH and 30°C. Data points are the average of two replicates, and the error bars represent the standard deviation (Paper V).

The impact of the storage temperature was significant ($p < 0.05$). The CML level in the freeze-dried powder after 200 days' storage was 5.3 times lower when the temperature was reduced from 30 °C to 20 °C as a RH of 52%. Similarly, the CML concentration in the spray-dried sample was 3 times lower, and in the drum-dried powder 3.7 times lower, at the lower storage temperature (Figure 22). This is an important finding of this work, which should be borne in mind regarding the long-term storage of SMP and SMP-based products.

The third storage condition, 33% RH and 30 °C, led to the production of significantly less CML in all three types of sample during storage (Figure 23).

Comparing the CML concentration after 200 days of storage under these conditions, and at the same temperature but higher RH (52% RH, 30 °C), reveals that the SMPs stored at the lower RH contained approximately 10 times less CML than those stored at the higher RH. Thus, the impact of relative humidity was also significant ($p < 0.05$). In fact, in the context of this study, the impact of RH was greater than the impact of the temperature. Therefore, it can be concluded that the relative humidity during storage is a very important parameter determining the AGE content and final safety status of SMPs.

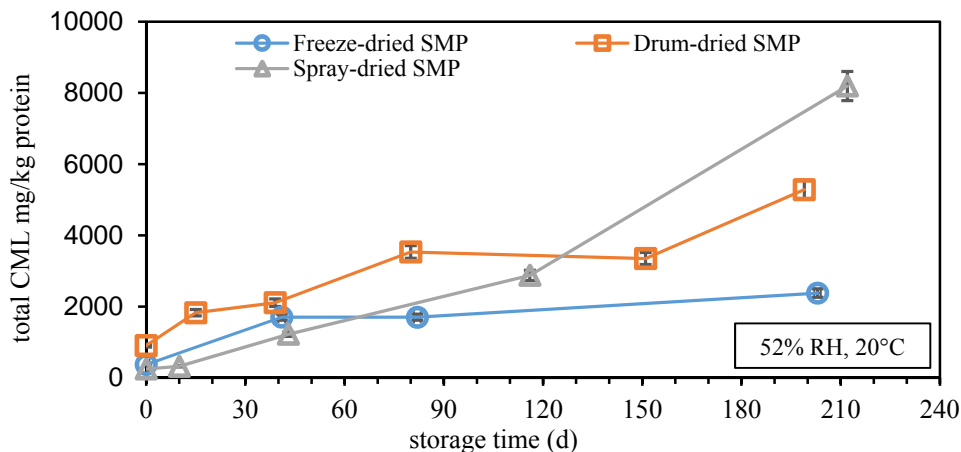


Figure 22. Change in total CML in freeze-dried, spray-dried, and drum-dried SMP during storage at 52% RH and 20 °C. Data points are the average of two replicates, and the error bars represent the standard deviation (Paper V).

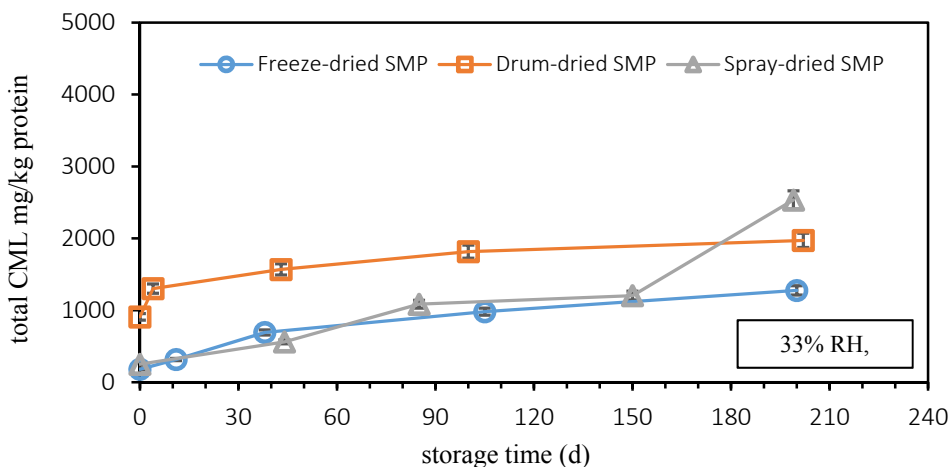


Figure 23. Change in total CML in freeze-dried, spray-dried, and drum-dried SMP during storage at 33% RH and 30 °C. Data points are the average of two replicates, and the error bars represent the standard deviation (Paper V).

4.2 Infant formulas

The same infant formulas evaluated regarding the early stage of the Maillard reaction (Table 4), were also studied by measuring the CML to determine the extent of the advanced stage of the reaction. Ten powder and liquid infant

formulas from three manufacturers available on the Swedish market were studied. Total CML was analysed using the procedure established by Tareke et al. 2013 (Figure 24).

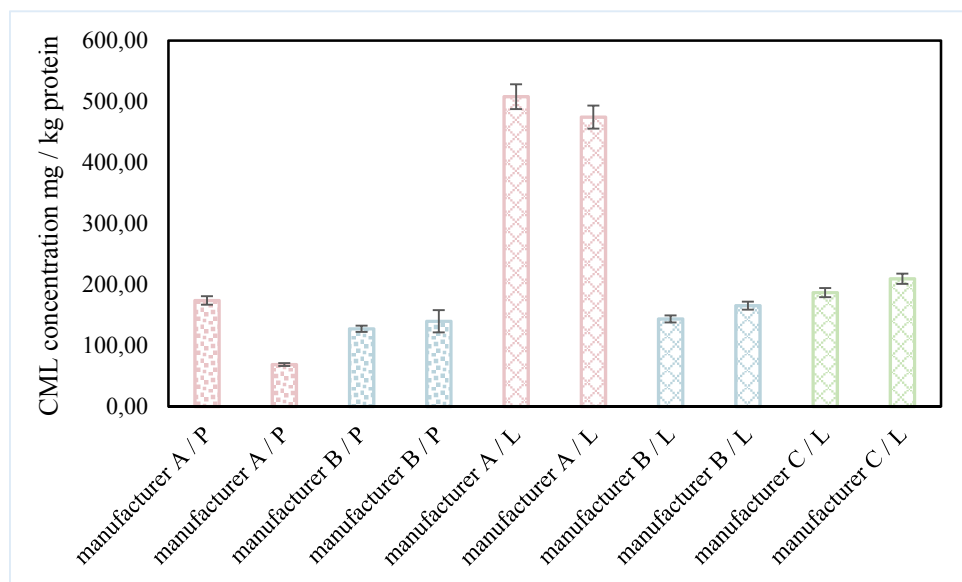


Figure 24. Values of total CML obtained when studying the advanced stage of the reaction in ten commercial infant formulas (four powders and six liquids). The CML concentration given is the average of two measurements, and the error bars show the standard deviation. The infant formulas are from 3 manufacturers A, B and C. P stands for powder and L means liquid (Paper VI).

Unlike the amounts of available lysine in the commercial infant formulas, which were quite similar (Section 3.2), the CML concentrations covered a broad range from 68.77 to 507.99 mg/kg protein. In the case of manufacturer A, the liquid infant formulas had significantly higher CML contents than the powder products.

The findings of this study are in agreement with those from previous studies. Hartkopf et al. reported CML concentrations in the range 50 – 200 mg/kg protein (Hartkopf et al., 1994). In another study, it was found that 16 powder infant formulas contained 5 – 70 mg CML per 100 g protein. The CML concentrations in these infant formulas were 28 – 389 times higher than in fresh human milk (Šebeková et al., 2008).

In another study by Dittrich et al. the CML content in 8 infant formulas available on the German market was reported to be 514 – 11372 ng/ml 35 times higher than the CML concentration in human milk (Dittrich et al., 2006).

5. Conclusions

The experiments described in this thesis and the results obtained reveal the importance of the choice of drying technique, as well as the influence of storage conditions during long-term storage on the progression of the Maillard reaction in SMPs, both in the early and advanced phases of the reaction. The most important findings are presented below.

- The impact of the drying technique was found to be significant. Freeze-drying had the least impact on the development of the Maillard reaction, and the powder obtained contained $3.49 \pm 0.07\%$ available lysine, based on the dry matter content. Spray-drying, in the context and conditions of this work, caused a 7.5% decrease in the available lysine, and the impact of drum-drying on the progression of the reaction in the early stage was 12.9%.
- The development of the Maillard reaction in the early stage is significantly influenced by both the temperature and relative humidity during storage. The pattern of decrease in the available lysine content during 200 days of storage was similar, regardless of the type of SMP. After storage at 52% RH and 30 °C, a 39.2 – 45.9% decrease in the available lysine content was seen after 200 days. The corresponding value for storage at 52% RH and 20 °C was 21.2 – 31.8%, indicating the importance of the storage temperature. Storage at 33% RH and 30 °C caused a 5.2 – 22.4% decrease in the available lysine content, while storage at the same RH and 20 °C resulted in no significant decrease in the available lysine content after 200 days. Thus, 33% RH and 20 °C were considered the ideal storage conditions for SMP.
- Under the conditions described in this work, the decrease in the available lysine in commercial SMP during 30 days of storage in an opened package under realistic storage conditions, followed a first-order reaction kinetics model. Storage experiments with commercial SMPs under six conditions confirmed once again that both temperature and relative humidity are important factors determining the progression of the Maillard reaction in the early phase. The RH influenced the

activation energy of the reaction; increasing the RH from 43% to 52% while the activation energy decreased from 170.8 ± 6.8 to 142.5 ± 3.3 kJ mol⁻¹.

- Progression of the Maillard reaction in the advanced phase during 200 days of storage, using CML as a marker, revealed that twice as much CML was produced in the spray-dried powders than in the freeze-dried samples after 200 days, while 1.6 times as much was found in the drum-dried samples. Storage at 52% RH led to about 10 times more CML in the samples, compared with 33% RH, at the same storage temperature. These findings demonstrate that AGEs may be formed during the storage of SMPs, after opening the packages, even at moderate RH and temperature.
- Under the storage conditions investigated in this work, storage at 33% RH and 20 °C was found to be the ideal environment for SMPs, as these had the least impact on the progression of the Maillard reaction, in both the early and advanced stages.
- The findings presented in this thesis provide a better understanding of the extent of the Maillard reaction and the development of advanced glycation end products in both SMPs as well as products based on SMPs, such as infant formulas. The results can be valuable in predicting the degree of the reaction during storage in order to control and to ensure the safety of similar products on the market.

6. Future Considerations

Due to the multi-disciplinary character of this work, there is still a considerable need for future studies, some suggestions for which are given below.

- Investigation of the physical state and lactose crystallization in SMPs may help to obtain a better understanding of the reason behind the progression of the Maillard reaction during the storage of SMPs and SMP-based products. These studies would provide deeper insight if combined with an electron microscopy imaging technique such as scanning electron microscopy.
- Progression of the Maillard reaction in infant formulas, both in the early and advanced stages, is another area that should be studied with regard to long-term storage under realistic storage conditions. No other studies in this area could be found in the current literature.
- Another important area of research concerns other commercial food products in which milk powder is used for various purposes. Storage of large quantities before being mixed with other ingredients, fortification with other provoking ingredients such as vitamin C, further heat processing and subsequent handling and storage, may increase the AGE content. This has not been investigated and should be given high priority in future studies.
- In this work, two markers of the early and advanced stages of the Maillard reaction were studied. To obtain a better understanding, other markers of the reaction, such as furosine and pyrrolidine, should be monitored during storage. Since each marker represents a different and specific phase of this complex reaction, their combination would provide more detailed information for the ultimate aim of controlling and preventing the reaction.

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