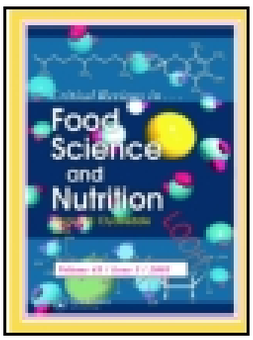

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Chemical methods and techniques to monitor early Maillard reaction in milk products; a review

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Abstract

Maillard reaction is an extensively studied, yet unresolved chemical reaction that occurs as a result of application of the heat and during the storage of foods. The formation of advanced glycation end products (AGEs) has been the focus of several investigations recently. These molecules which are formed at the advanced stage of the Maillard reaction, are suspected to be involved in autoimmune diseases in humans. Therefore, understanding to which extent this reaction occurs in foods, is of vital significance. Because of their composition, milk products are ideal media for this reaction, especially when application of heat and prolonged storage are considered. Thus, in this work several chemical approaches to monitor this reaction in an early stage are reviewed. This is mostly done regarding available lysine blockage which takes place in the very beginning of the reaction. The most popular methods and their applications to various products are reviewed. The methods including their modifications are described in detail and

their findings are discussed. The present paper provides an insight into the history of the most frequently-used methods and provides an overview on the indicators of the Maillard reaction in the early stage with its focus on milk products and especially milk powders.

Keywords

Maillard reaction, AGEs, available lysine, milk powder

1. Introduction

The application of heat to food products to assure microbiological safety and to guarantee the promised shelf-life has become a central part of food processing. Perhaps one of the most thoroughly studied yet not fully understood chemical reactions that occurs as a result of heat treatment is non-enzymatic browning reaction ; the Maillard reaction. While the benefits of heat processing and Maillard reaction cannot be disregarded, the negative consequences must be taken into consideration as well. The extent of impaired nutritional characteristics is defined by the processing type and conditions; whether pasteurization, sterilization, roasting, evaporation or drying is the method of choice. The important point is that once the reaction is started during processing, it continues throughout the product's shelf life. Therefore, manufacturing and storage circumstances, are the two determining factors in this regard.

Milk powders hold special position among all other food products due to the sensitivity of the main target group (infants) and also the vast range of applications they have in the food industry. They are widely used in the formulations of chocolate, bakery products, dairy, meat, dried, frozen and ready to eat meals. Furthermore, because of its composition, milk is an ideal media for the Maillard reaction. Thus, understanding this reaction and characterizing its products is of vital significance.

Among all different Maillard products known so far, advanced glycation end products (AGEs) are the molecules suspected for imposing serious health risks on humans. The issue of AGEs' contribution to autoimmune diseases has been discussed in several papers including (Goldberg et

al., 2004); (Henle, 2005); (Bengmark, 2007); (Nguyen et al., 2013); (Siciliano et al., 2015);(Vlassara and Palace, 2002); (Poulsen et al., 2013, Birlouez-Aragon et al., 2010). N^{ε}-carboxymethyllysine (CML), pyrraline, pentosidine are a few of AGE molecules detected in milk products (Siciliano et al., 2015, Pischetsrieder and Henle, 2012, Melnik, 2009, Nguyen et al., 2013).

In brief, high amounts of glycated proteins in the body leads to the activation of RAGE (a special receptor for AGEs) and eventually induce a pro-inflammatory status in the body which might lead to chronic diseases such as diabetes, renal disease, allergy and coronary heart disease. The point to be noted here is that Maillard is not a straightforward reaction to follow and there are great deal of parallel pathways occur simultaneously which are controlled by both intrinsic and extrinsic factors. The challenge is to find the markers associated to different stages, and to quantify their concentrations as precisely as possible and finally to estimate the state of the reaction for that particular circumstance and matrix. In other words, product composition, processing conditions, storage quality and further transportation and handling must be taken into consideration in order to obtain a clearer picture.

Due to the significance of the issue for both consumers and industry, obtaining a full understanding of the Maillard reaction is of vital importance; the knowledge which would be a great asset to optimize the processing in order to have a safer product at the end. Therefore, the aim of the present paper is to review the chemical methods that have been applied to milk products, specifically milk powders for monitoring the Maillard reaction directly or indirectly; to

measure the indicators that can help us determine the status of the reaction and to minimize the formation of harmful molecules by adjusting the process.

2. Maillard Reaction Pathways

Reducing sugars and amino acids are the molecules which initiate this complex set of reactions. Specifically as for milk, the main reactants are lactose and lysine in both caseins and whey proteins. One molecule of water is subsequently eliminated and the reaction continues with the formation of a Schiff's base which goes through Amadori rearrangement to constitute Amadori compound which is lactulosyllysine with respect to milk. Up to this step, what happened is referred to as *early Maillard reaction* (Figure 1) and perhaps its main consequence is that, lysine as one of the essential amino acids in milk loses its availability, i.e., at the very beginning of the Maillard reaction (O'Brien, 2009).

Advanced Maillard reaction starts when the Amadori compound breaks down into a range of different products according to the pH of the system (Figure 2).

In systems with pH less than 7, the main molecules formed as a result of degradation are Hydroxymethyl furfural (HMF), furfuryl alcohol and pyrraline. This pathway is called 1,2 enolization route or sometimes 3-deoxyosone-pathway. Hence, for milk and milk products 1,2 enolization is not the main pathway. Therefore, for the second group of foods with neutral and alkaline pH there is another way called 2,3 enolization route or 1-deoxyosone-pathway and leads to the formation of β-pyranone, 3-furanone, reductones, α-dicarbonyls, cyclopentenone, galactosylisomaltol and acetylpyrrole . It should be mentioned here that under normal conditions stability of the Amadori compound in milk (lactulosyllysine) is rather high, unless the product is

exposed to high temperature or stored for a significant period of time. After extensive research being done in this field, now there are a few methods make it possible to follow each of 1, 2 enolization or 2, 3 enolization pathways which will be discussed later (Van Boekel, 1998), (Tomas and Jiri, 2003).

One group of compounds that are formed during the Maillard reaction and have been the focus of the scientific debates in the recent years are AGEs; the molecules with negative consequences on health. One of them is carboxymethyl lysine (CML) which is a product of Amadori compound's degradation. Lysylpyrraline is the other AGE molecule that has been identified in heated milk and it is formed through the 3-deoxyosone pathway. It has recently been shown that detection of this molecule in heated milk is a marker for advanced stage of Maillard reaction. Pentosidine is another example. Although AGEs can be found in foods in very low amounts (usually a few μmol/l), this cannot obviate their role as possible health risks (Van Boekel, 1998).

Most of the compounds responsible for the flavor and odor of thermally treated foods are produced in the Maillard reaction and during Strecker degradation from amino acids. In short, amino acids are decarboxylated and converted into Strecker aldehydes and consequently, carbon dioxide is released (Tomas and Jiri, 2003). These compounds have been identified in different milk products; 3-methylbutanal in pasteurized and sterilized milk, 2-methylbutanal and isobutanal in UHT milk and 2-methylbutanal and isobutanal in milk powder (O'Brien, 2009).

Finally the Maillard reaction pathway approaches its end when molecules from degradation of Amadori compound start to polymerize and generate the brown pigments known as melanoidins which have high molecular weight. It has been shown that ratio of the sugars and amino acids

has a large influence on the formation of brown pigments; while sugars have a promotional effect, amino acids seem to have a preventive role in this regard (O'Brien, 2009).

3. Quantification of Maillard indicators

In order to improve our understanding of the initiation and extent of the Maillard reaction there are a number of chemical methods which will be reviewed in the following parts.

3.1. Determination of available lysine

Among all possibilities to monitor the Maillard reaction, a conventional approach is to estimate the amount of amino acids that are still unbound or have free reactive groups in the food system. Availability of lysine as the most frequent essential amino acid in milk products has been the focus of several studies for a long time. In other words, free ε-NH_2 groups in lysine can interact with other reactive compounds and therefore become unavailable. By going through literature, one can find a wide variety of methods for determination of available lysine. A summary of these methods as well as their advantages and disadvantages is discussed in the following.

3.1.1. 1-fluoro-2,4-dinitrobenzene (FDNB)

A number of methods that chemically measure available lysine are based on the fact that acid and high temperature hydrolyze that part of lysine that is bound to reactants and make it free in order to be determined by chemical methods and FDNB is one of them.

The reagent, fluorodinitrobenzene reacts with the ε-NH_2 groups of lysine and forms a lysine derivative called dinitrophenyl-lysine (DNP-lysine). This reaction is sometimes referred to as Sanger reaction. Following acid hydrolysis by hydrochloric acid, the DNP-lysine can be

measured by different techniques and the amount is considered to be equivalent to available lysine. Spectrophotometric methods have been used for a long time for the quantification of DNP-lysine (a yellow substance). However, in the more recent method modifications, spectrophotometric methods are replaced by the application of liquid chromatography which is more specific and accurate (Blom et al., 1967).

One of the most mentioned drawbacks of this method is that during acid hydrolysis some of the DNP-lysine is lost; therefore a correction factor of 1.09 is used for the compensation which is only an estimation and might not be a true representative of the amount of loss. This is more problematic regarding carbohydrate-rich foods. Therefore, application of this method for carbohydrate-rich foods is not recommended because of the large amounts of by products produced during the procedure which might interfere with the determination of DNP-lysine (Blom et al., 1967).

Walker mentioned in his paper that FDNB method usually underestimate the amount of available lysine in foods. Leaf protein concentrates that were analyzed, always had significantly lower values measured by this method. Moreover, in this paper FDNB is criticized as a very time-consuming method which limited the number of samples that can be run simultaneously (Walker, 1979).

In a study by Hurrel and Finot in 1983 nutritional changes in heat-damaged milk powders were investigated by a few methods including FDNB. They concluded that even if modified FDNB method is recommended by the Association of Analytical Chemists in 1980, it is not suitable for measurement of lysine in milk powder. Another conclusion in that paper worth to mention is that

one part of lysine (Deoxyketosyl-lysine compounds) that is measured by chemical methods as "available" lysine, cannot be utilized by the body, thus it is not bioavailable (Hurrell et al., 1983).

In 1984, International Association of Fish Meal Manufacturers (IAFMM) organized an extensive research among 16 labs in different countries in order to find a suitable method for determination of lysine in different fish meals to be comparable with the biological rat assay. They concluded that none of the applied chemical methods could be a true representative of the extent of lysine damage in the samples and these methods are better to be used for determining the quality loss in the food items, rather than looking at their absolute values. In other words, the values obtained by the chemical methods were significantly different from the values by the biological rat assay and FDNB was recognized as a chemical method which underestimated the available lysine in fish samples and had a high variation considering within lab and between lab results (Barlow et al., 1984).

In a similar study by Carpenter and colleagues in 1989 they analyzed 17 food samples regarding available lysine content by 4 different chemical methods (reaction with FDNB, dye, OPA and total amino acids). The samples belonged to both animal and vegetable foods and ranged from non-fat milk and salami to chick peas and soy proteins. In this paper, FDNB was presented as the first reagent used for the determination of available lysine in foods and the correlation coefficient of this method with rat assay was 0.90 for non-fat milk. However, the values measured by FDNB were significantly lower in comparison with other chemical methods (Carpenter et al., 1989).

In 1991, Vigo et al. in an attempt to find a more suitable method to analyze available lysine in milk products made a comparison between FDNB and o-phthaldialdehyde (OPA). The samples included pasteurized milk, sweetened condensed milk, milk powder and a model system comprised of casein, lactose and sucrose. As an improvement to FDNB method and in order to decrease the interferences, the authors suggested that if samples are dialyzed before reaction with FDNB, then the results are comparable with the results from OPA method. Otherwise, the values for FDNB (without dialysis) were considerably lower than the OPA values. Although this suggestion could improve the results, it increased the analysis time to 3 days which is considered a major weak point for daily use of a method (Vigo et al., 1992).

Soybean meals and peas were evaluated for their available lysine contents in one study to determine if extrusion temperature and water content can impose any negative effect. The authors stated that FDNB is a reasonably good option in this respect, even if it might not indicate a good correlation with rat assay. Being time-consuming and laborious, loss of lysine during acid hydrolysis were mentioned as some of the disadvantages. Furthermore, it was concluded that parts of lysine which have not gone through any structural changes during processing and storage cannot be detected by this technique, one possible reason for underestimation of available lysine by FDNB method (Hendriks et al., 1994).

In 1997 a group of researchers tried to modify the method and shorten the analysis time and loss of lysine in two types of infant milk formulae. As a result of their work, a modified version of FDNB was created which decreased the hydrolysis time from usually 12 h to 2.30 h by using an oil bath that was equipped with vacuum. They could also minimize the interfering materials and

optimized the resolution by using liquid chromatography instead of colorimetric methods. The mobile phase for C18 column was 35% methanol and 65% sodium acetate buffer. The modified method was successfully applied to powdered and liquid infant formulae in terms of linearity, precision, sensitivity and recovery (Albalá-Hurtado et al., 1997).

In order to determine the effects of processing (toasting and roller-drying) on available lysine in infant cereals, FDNB followed by HPLC was successfully applied. The detection was done with UV diode array detector and the absorption was carried out at 360 nm without any interference (Fernandez-Artigas et al., 1999).

In another study in 2004 by the same group of researchers, the progress of Maillard reaction in infant cereals during storage was monitored by measuring the available lysine. The samples were categorized according to their composition and ingredients; they were either wheat-based or rice-based, with or without milk and they had been stored under various temperatures and water activities. FDNB was the method of choice for the analysis and the results showed that samples which contain milk in the formulation lose their lysine during storage two times faster than the samples without milk (Ramírez-Jiménez et al., 2004a).

Later in 2009 the same researchers in Granada University tried to monitor Maillard reaction in infant formulas this time by using four indicators. Available lysine (FDNB), furosine, pyrraline and intensity of fluorescence were measured at predetermined points during processing. Samples were taken from mixing, oil addition and drying steps for further analysis. Results showed that there is a correlation between loss of lysine and formation of furosine during manufacturing.

However, regarding sensitivity furosine could be a better indicator of thermal damage in industrial scale (Contreras-Calderón et al., 2009).

3.1.2. OPA (O-phthalaldehyde)

In 1981, a group of researchers at Carolina State University developed a method for measurement of available lysine. Their primary aim, was to establish a procedure for routine analysis of available lysine which is fast and accurate at the same time.

The principle of the method is based on the reaction of ε-NH_2 groups of proteins with O-phthalaldehyde which produces a fluorescent substance in a very short time (around 1 min) that its fluorescence intensity is measured with a fluorimeter. A wide range of proteins with various concentrations of lysine were chosen for this purpose including Lysozyme, Ovalbumin, k-Casein, Trypsin inhibitor, β-Lactoglobulin, α-Lactalbumin, Bovine serum albumin (BSA).

The experiments were reasonably successful and a linear relationship was observed between the lysine content and the fluorescence intensity. The main strength of the procedure was that neither prior hydrolysis and amino acid analysis, nor heating and solvent extraction was needed unlike the methods existed at that time. Furthermore, the reagent contained sodium dodecyl sulfate (SDS) which made the solubility of the proteins incredibly easier and unlike the previous methods a very small amount of the sample was enough for the experiment (1-40μg). On the other hand, the fluorescent molecule was not very stable and it must be measured after 2 minutes when the sample and the reagent are mixed (Goodno et al., 1981).

Later in a collaborative research which was done among three universities in USA in 1989, total and available lysine contents of a number of animal and plant foods were measured and

compared with the results from a rat assay. The goal was to find a chemical method which could give closer results to the biological rat assay. The methods were FDNB, OPA and dye binding. Besides, total lysine was determined by amino acid analysis.

The details of the OPA procedure in this work were a little different from the original OPA procedure described previously and there were some modifications. In general, 17 foods were analyzed in this study including tuna fish, casein, beef salami, beef stew, chicken frankfurter, pea protein concentrate, soy protein isolated, peanut butter, oats, rice, wheat and a non-fat dried milk sample which was deliberately heat damaged.

The authors define OPA as a method with great sensitivity, despite showing less correlation with the rat assay among all methods studied in this experiment. In other words, the correlation coefficient for OPA was 0.85 and for the non-fat dried milk which was heat damaged 2.60 g available lysine per 100g protein was reported which was lower than the value obtained by the rat assay, 3.57 g available lysine per 100 g protein (Carpenter et al., 1989).

Since fluorimeter is not readily available in all the labs, in a work done in Argentina, the researchers modified the method by using spectrophotometer instead of fluorimeter for dairy model systems and milk products. The model system was composed of casein, lactose, sucrose and phosphate buffer solution. The milk products included pasteurized milk, sweetened condensed milk and milk powder. In the next step, they exposed the samples to different heat treatments; 60°C for 20 h, 100°C for 1 h, 120°C for 45 min. Unheated product was used as the control in all the experiments. In order to make the calibration curve, purified casein in sodium tetraborate buffer was used in different concentrations (1-10 mg/ml) and the absorbance was

measured at 340 nm. FDNB method was applied once more to the samples to compare the results. The results showed that OPA is a reproducible and reliable method and has a lot of advantages over FDNB including shorter time of analysis, without any need for acid hydrolysis and extraction and also it needs a tiny amount of sample. Moreover, carbohydrates do not interfere and it can be used for dairy products (Vigo et al., 1992).

In one study by the same researchers who established the spectrophotometer version of OPA, in order to compare the effects of carbohydrates on the loss of available lysine 4 model systems were made with casein and 4 sugars including glucose, fructose, lactose and maltose. Subsequently, the samples were equilibrated for 5 days over saturated salt solutions to reach a_w 0.52 and then were sealed in glasses and put at three different temperatures (37°, 50°, 60°C) for different periods of time. Determination of available lysine in all the samples were done using OPA method. The results showed that the highest reaction rate at all the studied temperatures belongs to glucose and the lowest to fructose. In case of casein and lactose at 37°C, after almost 17 days there was 50% decrease in the available lysine contents, while at 50°C in the same model system, almost 60 hours (less than 3 days) was enough to reduce the available lysine content to 50% and at 60°C the value was only 12 hours. It was concluded that changes in the available lysine contents of all the model systems followed first order reaction. It should be mentioned that the model systems were prepared by mixing the dry powders with phosphate buffer and subsequently freezing and freeze-drying (Naranjo et al., 1998).

The effects of manufacturing and storage on infant formulas were studied by two universities in Spain in 2000. Two types of milk-based infant formulas were chosen; adapted (infants 4-6

months) and follow-up formulas (older than 6 months). The processing steps and the storage conditions were exactly the same. The only difference was in the formulation that the adapted formulas had only lactose as carbohydrate, while in the formulation of follow-up formulas maltodextrin and lactose were used together besides proteins and lipids. The samples were taken out in some predetermined points and analyzed for their available lysine (by fluorimetric OPA) as well as Hydroxymethyl furfural (HMF) by HPLC. The heat treatments applied during manufacturing were pasteurization, evaporation, sterilization and spray-drying. After manufacturing, storage of the samples was done at 20°C and 37°C for 6 months in airtight containers. Regarding available lysine, the results showed that 20% decrease was occurred during manufacturing, compared to the raw cow milk. Storage time did not affect adapted infant formulas, while there was 16% decrease in the available lysine contents in follow-up infant formulas during that time (Ferrer et al., 2000).

Later in 2002, the same researchers in Argentina, designed a study in order to investigate the effects of different water activities (a_w) and temperatures on the of available lysine loss in milk models. The models were composed of lactose, casein and phosphate buffer which were frozen and freeze-dried. Three temperatures (37°, 50° and 60°C) and six water activities (0.33, 0.43, 0.52, 0.69, 0.85 and 0.98) were selected as the storage variables. The samples were reached to the decided water activities over saturated salt solutions and then sealed into containers and stored at different temperatures. Available lysine was measured using spectrophotometric OPA procedure. The results indicated that at 37° and 50°C, a_w 0.52 had the most destructive effect on the available lysine in the samples. But at 60°C, there were not any significant difference among a_w 0.33, 0.43 and 0.52. However, at all the conditions tested in this experiment, after certain

period of time, available lysine was decreased to some extent. It was also concluded that, at low water activities (especially at a_w 0.33 and 0.43) the rate of the reaction is more dependent on the temperature because of higher activation energies (Malec et al., 2002).

In another research conducted in University of Valencia in 2003, changes the in available lysine contents during shelf life were studied in different milk products. The aim was to validate OPA method by defining analytical parameters as well as comparing them with the results from FDNB method. The conclusion was that there was no significant difference between results of OPA and FDNB method. OPA is a reliable method with good linearity and accuracy with 2.1-5.9% intra-day and 3.5-10.2% inter-day precision, suitable for milk matrices. Based on the findings of this study, available lysine content of the UHT whole milk was significantly different from in-bottle sterilized milk. It was observed that the available lysine loss during shelf life of the UHT milks (3 months) was between 2.7-29%. The milks were analyzed once the products were out on the market and then they were kept in closed packages for 3 months at room temperature and analyzed one more time just before expiration date. The authors also concluded that there was not any significant difference between name and store brand UHT products on the market with respect to available lysine content (Ferrer et al., 2003).

Infant formulas, which contained milk powder in their formulations were analyzed by this method in order to evaluate the storage stability. After four weeks of storage in the air oven at 28°C there was 0.6-3% decrease in the lysine content. However, storage of samples at a_w 0.65 and 25°C reduced the lysine contents in the samples 17.6-21.5% after the same time period (Ramírez-Jiménez et al., 2004b).

In a similar study which was previously done with milk model systems in Argentina using various temperatures and water activities, kinetics of the Maillard reaction was studied in commercial skim milk powders by the same group of researchers. The water activity range used in this study was 0.31-0.98 with 30, 50 and 60°C as storage temperature. The target sample was a dispersion of skim milk powder (20% w/w) which was freeze-dried afterwards. Portions of samples were put into vacuum desiccators over different saturated salt solutions at 25°C until constant weight was achieved. Subsequently, tiny amounts of the samples (200 mg) were put into the sealed glass flasks and stored at desired temperatures for several months. Available lysine was measured using OPA method at intervals and the weight changes were recorded. The authors demonstrated that rate of the lysine loss was slightly lower for milk powder materials compared to the previously studied milk model systems. Interestingly, while there was not any remarkable difference in the reaction rate of various water activities at 37°C, the rate constant was considerably lower at a_w 0.32 compared to the following water activity point (0.47). This was the water activity that had the highest effect on the lysine loss in milk powder and 20% of the lysine content was decreased only during 7 days of storage at a_w 0.47 and 37°C. The authors explained this by glass transition phenomena in milk powder which occurs at 33°C at this water activity (0.32) and the fact that the reaction rate is noticeably lower around glass transition temperature due to the lower molecular mobility. The conclusion was that the kinetics of the available lysine loss in milk powder follows first order reaction at all the temperatures and water activities studied in this work (Pereyra Gonzales et al., 2010).

In a comprehensive research carried out in 2011, kinetics of available lysine reduction was studied regarding a concentrated model infant formula. The model was composed of skim milk

powder, lactose and whey protein isolate in an attempt to make it similar to the composition of typical milk based infant formulas on the market. The researchers tried to simulate the conditions of spray-drying with respect to temperature and water activity. Available lysine was quantified by OPA method at temperatures 60-90 °C and water activities 0.11-0.43 after 30 min heating in a water bath. The results indicated that there was around 14%, 29%, 54% and 78% decrease in the available lysine contents in the model systems heated at 60-70-80 and 90°C respectively. In order to understand the correlation of available lysine loss to the physical state of lactose, glass transition and molecular mobility was also investigated by using DSC (Differential Scanning Calorimetry) and NMR spectrometer in this study (Schmitz et al., 2011).

OPA was once again the method applied to model systems of whey proteins and lactose or glucose in order to determine the available lysine loss during storage at 37°C for 500 h. The model systems were first stored over saturated salt solutions to reach the a_w 0.52. The authors' conclusion was that available lysine is a sensitive indicator to study early stage of the Maillard reaction and it is possible to monitor the loss of available lysine in the model systems of whey protein with lactose and glucose during storage. Loss of the available lysine in the both model systems followed first-order reaction kinetics (Leiva et al., 2017).

3.1.3. Dye binding method

This method is quite an old method which was primarily introduced for the determination of protein content in food materials especially for milk proteins as an alternative for Kjeldahl method (Udy, 1956, Ashworth and Chaudry, 1962, Ashworth, 1966, Goh and Clandinin, 1978,

Sherbon, 1978, Moore et al., 2010). In those studies Orange G, Amido black and Acid Orange 12 were the dyes which were frequently employed.

In 1979 some researchers from Cambridge and California Universities in collaboration with Nestle applied the method for the first time for the quantification of available lysine to a wide range of food products including skim milk powder. Acid Orange 12 was the dye of choice and bovine plasma albumin, fish and meat meal, pea and soya flour, barley and corn were among the materials tested for their available lysine content. The principle of the method is to make a complex between the anionic dye and the free basic groups of the protein including imidazole group of histidine, guanidine group of arginine and ε-NH_2 group of lysine. In the next stage, the basicity of lysine is neutralized by a blocking substance, propionic anhydride in this case and from the difference between the two mentioned steps available lysine is estimated, after centrifugation and measuring the absorbance at 475 nm by spectrophotometer. It should be mentioned that the automated version of the method (Pro-Meter MK II) which was commercially available at that time was used in this work. All the results obtained by this method were compared with the results from FDNB method and also with the total lysine contents quantified by ion-exchange chromatography. The skim milk powder was heated at 37°C for 5, 11, 32 days while adjusted to 15% water content before heat treatment. Based on the findings of this study, there was a strong correlation between the available lysine results by the two methods. With respect to the skim milk powder, there was 15% decrease in the available lysine content after 5 days of heating at 37°C, 24% decrease after 11 days and 49% decrease after 32 days when analyzed by the dye method. It was also concluded that starch, cellulose, pectin, sugars, minerals, lipids and free amino acids do not interfere with the analysis. This explains the fact that this

method cannot be validated by spiking free lysine. Instead the method can be controlled by trying various weights of the sample material for the procedure. This needs to be carried out each time a new sample material gets into the procedure considering the matrix effects. The results of this work indicated that dye binding method is a reliable and accurate replacement for the FDNB method. However, it might overestimate the available lysine content especially for severely processed and damaged food materials (Hurrell et al., 1979).

In a study by University of Reading, 28 samples of leaf protein concentrates were analyzed by dye binding method to understand the effects of various drying methods on the available lysine contents. The investigated drying methods were forced-air oven, freeze-drying, vacuum oven and drying at room temperature. The samples were also analyzed by FDNB method afterwards as well as total amino acid quantification by ion-exchange chromatography. The results showed that there is a strong correlation between the two methods (correlation coefficient 0.90) with the higher values always belonging to the dye method. The final conclusion was that dye binding method is a simple and reproducible method that can be used as a quality control tool in food processing (Walker, 1979).

In another research conducted by Nestle, spray-dried whole milk powders containing 2.5% water content were investigated with respect to elevated temperatures during storage for 2 months. The temperatures examined were 60° and 70°C. There was also another sample of whole milk powder with 10% water content stored at 37°C for the same period of time. The aim was to expose milk powder to the conditions which might occur during storage and transportation in hot countries. In this study chemical methods such as dye-binding, FDNB and furosine were

compared with a few microbiological assays like Pediococcus and Tetrahymena for instance. The chemical methods all failed to fully estimate the extent of lysine damage and gave dissimilar results. The authors believe that dye-binding is generally a suitable technique. However, it might overestimate the lysine perceived as available especially for the products that have gone partially through Maillard and are considered heat-damaged. It was also shown that there is a huge difference between heating at 60° and 70°C regarding the amino acids loss (Hurrell et al., 1983).

Samples of cottonseed flour and spray-dried egg white were investigated for their available lysine contents by dye-binding and FDNB methods. The aim was to find a chemical method that gives the closest results to the rat assay. The conclusion was that dye method can give closer values to the rat assay (Anderson et al., 1984).

In another work in Hungary different types of soya beans were studied for their available lysine contents by using the dye method. They were exposed to different acid and heat treatments before analysis. Defining the experimental factors such as the optimum reaction time, the ratio between the dye and the sample and stoichiometry of the reaction were the main aims of the study. Dye binding that was applied to soya proteins for the first time in this study, was considered a useful and sensitive method for the aim of the study which was able to detect minor changes in the available lysine contents of soya beans (Perl et al., 1985).

The same group of researchers in Hungary, designed a study with three types of dyes to determine available lysine this time in spray-dried skim milk powder, meat, fish and milk proteins (previously treated with enzyme and acid). Their results indicated that for the skim milk powder the optimum reaction time after addition of the dye is achieved in 30 min, but it does not

impair if the mixing is continued for 120 min and the amount of dye bound to g protein for skim milk powder was in the range of 214-247 mg. The calculated RSD (relative standard deviation) for skim milk powder investigated in this study with Acid Orange 12 was 2.2%. The authors found this method to be accurate for the measurements of available lysine in the studied sample materials (Molnár-Perl et al., 1986).

In the study with 17 food samples explained before which aimed at finding the most comparable chemical method with rat studies, dye binding method indicated a good correlation for the non-fat dried milk. The correlation coefficient was 1.03 which gave a higher value for available lysine content of milk powder (9.26 g/100 g protein) compared to the result of rat experiment which was 8.95 g available lysine per 100 g protein (Carpenter et al., 1989).

Dye binding method was employed to measure available lysine in soya beans and peas after being processed with extruder. In this study, there were some modifications in the dye procedure including increasing the propionic anhydride amount from 0.2 to 0.4 ml and also optimizing the reaction time for soya bean and bovine serum albumin samples. The conclusion of this study was that dye-binding is a method with over-estimated values. However, there was a strong correlation of 0.97 between the dye method and FDNB method (Hendriks et al., 1994).

In another study, dye-binding method was successfully applied to commercial skim milk powders in order to understand the effects of spray-drying and storage during shelf-life at room temperature on the available lysine contents. To do so, available lysine content of raw milk (8.76 ± 0.26 g / 100g protein) was compared with the corresponding values for skim milk powder before storage (7.51 ± 0.23 g /100 g protein) and skim milk powder after 6 and 18 months of

storage (6.35 ± 0.13 and 6.20 ± 0.16 g / 100 g protein) at room temperature (20-30 °C). Loss of available lysine in skim milk powder before storage was determined to be 14.27% compared to the raw milk and there was 27.51% decrease in available lysine content after 6 months and 29.22% after 18 months of storage. It must be mentioned that the available lysine loss in this study was always compared to raw milk. Furthermore, the skim milk powders were kept at room temperature in closed packages during the storage (El and Kavas, 1997).

In studies recently conducted in Lund University, dye binding method was employed to investigate the initiation of the Maillard reaction in a variety of skim milk powders. The method was successfully validated regarding casein, BSA and skim milk powders (Aalaei et al., 2016b). The method was investigated critically regarding skim milk powders 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 (Aalaei et al., 2016a). This investigation demonstrated that the method is suitable and reliable for the determination of available lysine in skim milk powders with high precision and reasonable variation, 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 ± 0.07% in dry matter, followed by the spray-dried powder, 3.23 ± 0.08%, and the drum-dried sample, which had the lowest available lysine content of 3.04 ± 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%. The powders produced using the three different drying techniques were then stored for approximately 6 months (200 days) under different combinations of temperature (20ºC and 30ºC) and RH (33% and 52%). 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 30ºC 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 20ºC 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 ºC after 6 months was 5.2 – 22.4%, while storage at this RH at 20ºC caused no significant decrease in the available lysine. A RH of 33% and a temperature of 20ºC therefore appeared to be ideal conditions for the storage of SMPs (Aalaei et al., 2016 b).

3.1.4. Guanidination

This method was established by Mauron and Bujard in 1963 as a replacement for FDNB method for quantification of available lysine. Its principle is to convert lysine into homoarginine by O-methylisourea throughout incubation at alkaline conditions and room temperature. The sample subsequently goes through precipitation with Trichloroacetic acid (TCA), centrifugation and hydrolysis with HCl and quantification by gas liquid chromatography. Lyophilized, spray-dried and drum-dried milk powder, peanut and soy-bean flour were the materials analyzed in that study. The authors stated that in the lyophilized and spray-dried milk powders around 95% of the lysine could be transformed into homoarginine and therefore considered to be available. The results were published in Proceedings of the 6th International Nutrition Congress and no more detailed information of the results is available (Mauron and Bujard, 1964).

Guanidination method was validated and compared with a rat assay in Lund University, by using spray-dried skim milk powders undergone various heat-treatments. Non-heat treated skim milk powder was used as control and there were 4 other samples of skim milk powders heated at 100°C for 12, 24, 48 and 96 hours. Butyl stearate was used as internal standard. Linearity, precision and reproducibility of the method was evaluated in this study and the results of the method showed that available lysine content of the samples decreased to 59% after the heat treatment for 96 hours, i.e. from 7.23 g to 5.32 g available lysine per 100 g protein. The chemically determined available lysine correlated well with the biological available lysine measured by the rat experiment (Nair et al., 1978).

In another study carried out at Lund University, protein nutritional value of the lactose-hydrolyzed milk powder was evaluated during storage at different water activities (0.11- 0.62) at 25°C. Ordinary dried milk was also evaluated at two water activities (0.11 and 0.62) at 25°C. The sample materials were stored over saturated salt solutions and available lysine was quantified by guanidination method at intervals during 12 months. Protein nutritional value was obtained by performing a rat assay. With regard to ordinary milk powder there was 25% decrease in the available lysine after 9 months of storage at a_w 0.62 while the lactose-hydrolyzed milk powder lost 50% of its available lysine after only 1 month of storage in the similar storage conditions. It was also shown that at that a_w (0.62) loss of the available lysine was maximum in both of the studied materials. The correlation coefficient between the guanidination method and rat experiment was 0.83. The authors recommended that lactose-hydrolyzed milk powder must be dried below the a_w 0.11 to assure its nutritional stability during storage (Burvall et al., 1978).

In a study in Colorado state university, factors affecting the guanidination reaction were investigated for 8 protein sources. The variables included incubation time (12-144 h), reagent (O-methylisourea) concentration (0.1- 0.6 M), guanidination temperature (10, 20, 25, 30°C), pH of incubation and interference of carbohydrates (glucose, sucrose, potato starch). The variables were optimized for the test materials which included raw whole milk, raw whole egg, cottonseed, wheat, rice, soy, peanut and corn. The results indicated that the above mentioned factors strongly affect the conversion of lysine to homoarginine and must be optimized for every sample material before the quantification of available lysine. With regard to milk in order to achieve the highest conversion rate for lysine, pH 10.8 with incubation at 20°C for 96 h and O-methylisourea concentration of 0.5 M was required (Maga, 1981).

Reactive lysine in heated skim milk powders was measured with guanidination method in Massey University, New Zealand. The aim of the study was to investigate the digestibility of the reactive lysine by performing the rat assay, therefore guanidination was used in combination with the rat experiment in this study and it was referred to biolysine assay. The rats were fed with skim milk powders which were autoclaved at 121°C for 1, 3, 5 and 10 min. There were also peas heated at 110, 135, 150, 165°C for 15 min in forced air incubator. The results showed that the reactive lysine (the chemically measured lysine) in skim milk powder decreased from 38.1 mg/g dry weight in unheated sample to 6.7 mg/g dry weight, i.e., 82% decrease after 10 min which was the longest heat treatment in this case. It must be mentioned that the total lysine content measured by conventional amino acid analysis was also decreased from 38.1 to 25.6 mg/g dry weight after 10 min heating at 121°C. The conclusion drawn about the combined analysis of chemically and biologically available lysine which in this study were referred to

reactive and digestible lysine was that the method was a sensitive method which could even differentiate the reactive lysine content of non-heated skim milk powder with the corresponding value for the sample heated only for 1 min at 121ºC (Rutherfurd and Moughan, 1997).

The same researchers who developed biolysine assay applied the method to commercial skim milk powder and commercial lactose hydrolyzed skim milk powder. The materials were stored at three temperatures (30, 35, 40°C) and they were analyzed during 18 months. As explained before, guanidination method was used to measure reactive lysine and it was accompanied by the rat experiment. In other words, they quantified the reactive lysine in the meals given to the rats and also in the digesta taken from their intestines afterwards in order to determine the extent of lysine digestibility. The results indicated that in the normal skim milk powders stored at 30 and 35°C there was 22% decrease in the chemically quantified lysine after 18 months of storage and this decrease was 36% when the samples were stored at 40°C for 12 months. However, regarding the lactose-hydrolyzed skim milk powder there was 36% and 55% decreases after 18 months of storage at 30 and 35°C respectively and 58% when stored at 40°C for only 6 months. The general conclusion was that storage temperature plays a critical role in the lysine content and based on the results there was a significant difference between storage at 30° and 40°C. Skim milk powders stored at 30°C had 34% more lysine after 12 months compared to the storage at 40°C and this difference was even more remarkable with respect to the lactose-hydrolyzed skim milk powder (Rutherfurd and Moughan, 2008).

To better understand how the methods for quantification of available lysine in milk products developed gradually, figure 3 is provided.

3.2. Determination of Amadori product

3.2.1. Direct measurement of lactulosyllysine

Enzymatic hydrolysis of the protein in combination with liquid chromatography and mass spectrometry (LC/MS) is said to be a promising method to detect tiny amounts of lactulosyllysine in milk and milk products. However, enzymatic hydrolysis step being time-consuming is considered as a weak point for this approach (O'Brien, 2009, Higgs and Boland, 2014).

3.2.2. Measurement of furosine

As an alternative for the time-consuming lactulosyllysine method, furosine is regarded as a method for indirect measurement of Amadori product. However, this method is sometimes seen in the list of the methods for the estimation of blocked lysine in foods. Whatever the purpose is, application of furosine analysis in milk proteins has been tremendously increased.

The principle of this method is acid hydrolysis of the Amadori compound to lysine (40%), furosine (32%) and a small amount of pyridosine followed by analysis with HPLC. Perhaps the major drawback of the method is uncertainty of the conversion factor of the Amadori product to furosine (Van Boekel, 1998). During years, furosine method has been improved. Modifications have changed this method from a not so specific and external standard demanded method to a method which is specific and its standard (furosine) is nowadays commercially available

(O'Brien, 2009), (Hurrell et al., 1983). In the early furosine methods arginine was the compound used as standard because of its elution behavior which was very similar to furosine (Erbersdobler and Hupe, 1991).

The development of Maillard reaction was studied by measuring the furosine content of raw cow milk, pasteurized and UHT milk and commercially produced milk powders. HPLC was utilized for the quantification and the results showed that the furosine can increase from 9.7 mg in raw milk to 138 mg per 100 g protein in UHT milk. Commercial milk powders were also investigated in this study once before storage (51.5 mg in 100 g protein) and after one year storage at 4 °C (56.3 mg in 100 g protein) and after one and two years of storage at room temperature (275.1 and 448.3 mg in 100 g protein respectively) (Baptista and Carvalho, 2004).

In a study by (François et al., 2005), they successfully used furosine method in order to monitor early maillard reaction in whey protein concentrates and skim milk powders heated at 60°C and various relative humidities by ion exchange chromatograph.

According to (Bosch et al., 2008), for estimation of blocked lysine from furosine content the following formula can be used:

% blockage = (3.1× furosine ×100) / chromatographed lysine + 1.86 furosine)

Contreras-Calderon, Guerra-Hernandez and Garcia-Villanova (2009) evaluated the progress of Maillard reaction at different stages of manufacturing in four types of infant formulas by furosine method. They concluded that formation of the furosine was significantly higher in products made

in the industrial plant, compared to the ones in pilot plant, even if all the processing parameters like heat treatments and even the formulations were the same (Contreras-Calderón et al., 2009).

Furosine contents of raw milk, condensed milk and skim milk powder were quantified in a study aimed at evaluating the effect of heat treatment on a number of pilot-plant made and commercial milk products. While non-treated raw milk had 5.2 mg furosine per 100 g protein, the corresponding value for skim milk powder was 211-489 mg furosine / 100 g protein. The samples were then reconstituted and heat treated at 80 and 130°C for 4s which increased the furosine content even further in most of the cases (Sakkas et al., 2014).

Furosine contents of several lactose hydrolyzed dairy products formulated specially for elderly were determined using ion-pair RP-HPLC. Concentrations of furosine were in the range of 235-820 mg/100 g protein. When the products were stored at room temperature (20°C) for 4 months, their furosine contents increased 74-90% during the storage (Montilla et al., 2015).

4. Final considerations

In this paper, the most common chemical methods to study the Maillard reaction in an early phase and their applications on different dairy systems especially milk powders are reviewed. The importance of research on milk powders is not only due to their role in the formulation of infant formulas, but also because of their widespread utilization in the formulation of numerous other products. This become more complicated in the formulation, when milk powders are mixed with other ingredients and go through multiple steps of further processing and storage. 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.

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 ºC, 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 and this should also be the focus of future studies.

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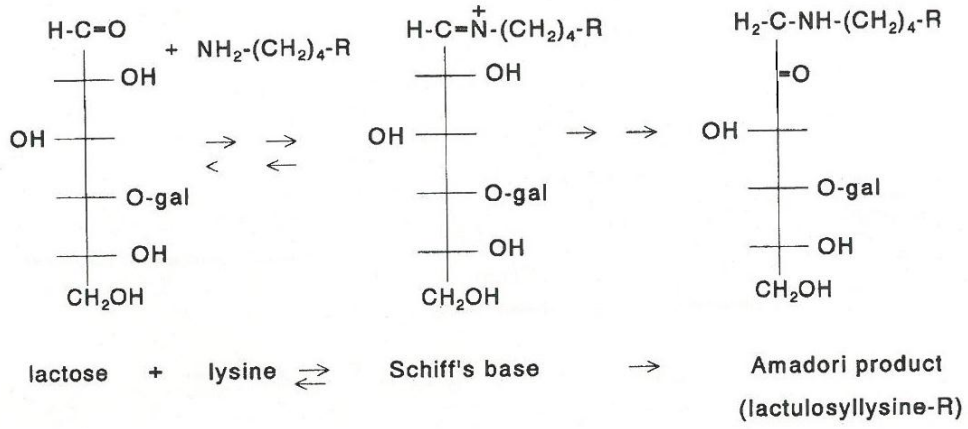

Figure 1. Early Maillard reaction in milk, adapted from (Van Boekel, 1998)

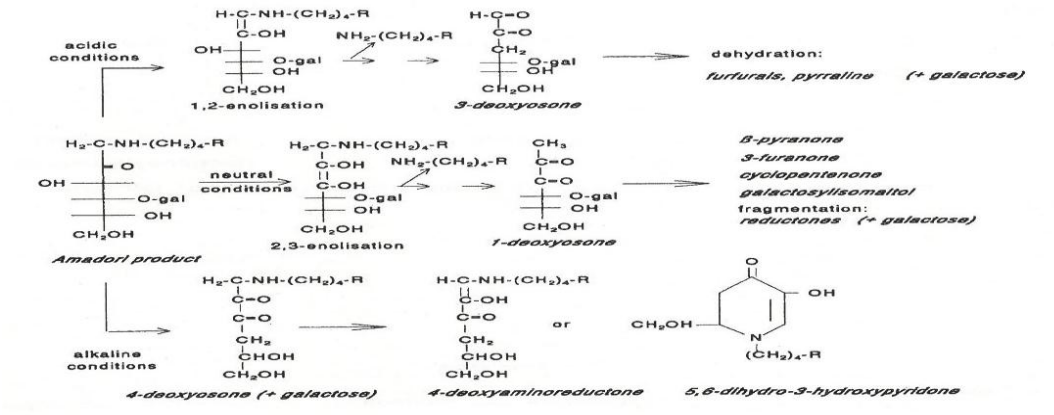

Figure 2. Degradation of Amadori compound under acidic, neutral and basic conditions, adapted from (Van Boekel, 1998)

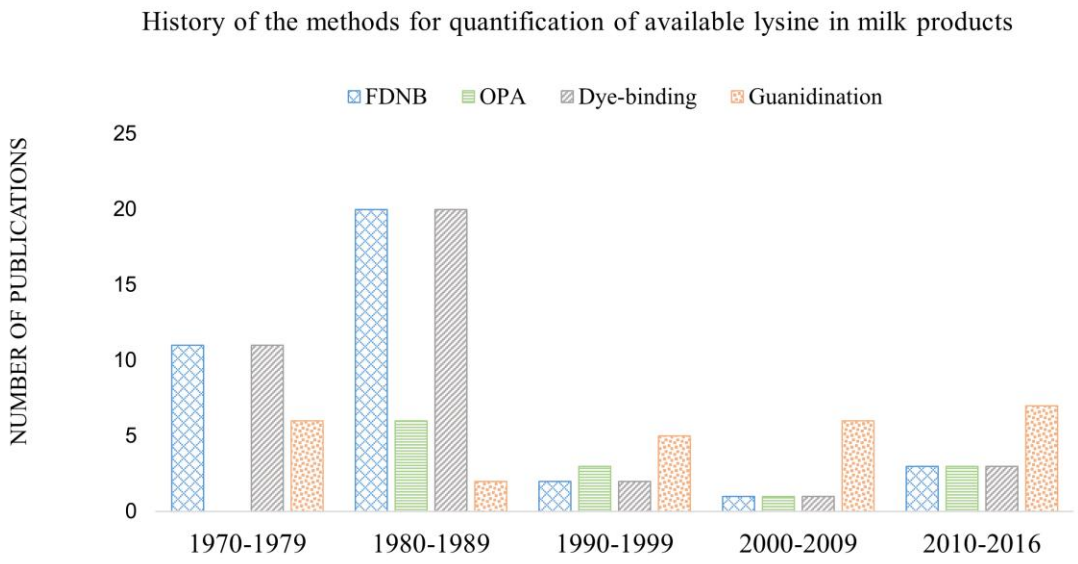

Figure 3. History of the publications regarding measurement of available lysine in milk products based on SciFinder database