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The Fluorescence of Advanced Maillard Products Is a Good Indicator of Lysine Damage during the Maillard Reaction

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The objective of this study was to determine whether in heat-treated milk-resembling models or milk there is a lag phase, before lactulosyllysine (LL) is converted into advanced Maillard products (AMP), and if there is a step during the heat treatment where LL is actively degraded into AMP. For that purpose, a low temperature (60−85 °C) and a long heat treatment (15−90 h) were chosen. We observe that the heat treatment first induces a parallel increase in furosine and AMP fluorescence, confirming that AMP are produced very early during the heat treatment. At this step, both indicators are correlated with each other and precisely reflect the lysine damage. After a time, however, furosine reaches a steady-state concentration, whereas AMP fluorescence still increases, remaining correlated with the lysine blockage. Nevertheless, heat treatment applied to milk does not reach this step so that AMP fluorescence appears as a rapid alternative to furosine quantification.

Keywords: *Maillard reaction; fluorescence; furosine; nutritional quality*

INTRODUCTION

The Maillard reaction is the main cause of nutritional damage in heat-treated milk. This reaction essentially affects the lysine residues because of the high reactivity of their free ϵ-NH_2 group in the presence of lactose. The resulting Amadori product ϵ-lactulosyllysine (LL) is no more bioavailable, explaining the decrease in the protein nutritional quality (*1*).

It is commonly assumed that the Amadori product is the main chemical form of lysine blockage in heat-treated milk, suggesting that only the early step of the Maillard reaction takes place (*2*, *3*). Furosine, the product of acid hydrolysis of the Amadori product, is therefore considered as a good indicator of lysine damage upon heating of milk (*4*). But it is now evident that the advanced Maillard reaction also takes place very early during the heat treatment, and even at low temperatures (*5*). Indeed, the FAST index, which measures the accumulation of fluorescent advanced Maillard products in the pH 4.6 soluble fraction of milk, previously appeared to be even more sensitive than furosine for detection of thermization of milk at 68 °C (*5*). Moreover, specific advanced Maillard products, such as carboxymethyllysine (*6*, *7*) and lysilpyrralines (*8*), were identified and their levels measured in UHT milk.

If we assume that advanced Maillard products are formed during milk heat treatment, it must be deduced that some lysine residues may be blocked under chemical forms other than LL. The question of the conversion factor to be applied in translating furosine as unavailable lysine then arises. A conversion factor of 3 was proposed by Bujart and Finot (*3*) for the production of furosine from LL. But more recently, Henle et al. (*9*) concluded on a conversion factor of 9, using a direct determination of the level of LL after enzymatic hydrolysis. In no case was the real lysine damage by the early and advanced Maillard reaction taken into account, possibly underestimating lysine unavailability. A discrepancy between both parameters is suggested by the data of Henle et al. (*9*), as the slope of the regression line between enzymatic LL and enzymatic lysine was shown to be <1 (0.671).

Estimation of the nutritional quality of heat-treated milk must involve the extent of advanced Maillard reaction because (i) it induces supplementary lysine blockage, (ii) at this step, other amino acids such as arginine [pentosidine (*10*)] and tryptophan (*11*) may be degraded, and (iii) some advanced Maillard products trigger antinutritional or mutagenic activity (*12*−*14*).

Different methods for achieving lysine loss are available; chromatographic quantification of lactulosyllysine or available lysine after enzymatic hydrolysis is a very accurate method for the estimation of the lysine damage (*9*) but is time-consuming and expensive; acid hydrolysis, however, releases 50% of the lysine initially bound as LL. Rapid colorimetric methods allow determination of the unreactive lysine by means of a dye reacting with the free NH_2 groups of peptidic lysine, such as methods based on *o*-phthalaldehyde (*15*), fluorescamine (*16*), and FDNB (*17*). But these methods have been criticized for their lack of sensitivity.

Our objective was to verify the accuracy of the FAST (fluorescence of advanced Maillard products and soluble tryptophan) method (*18*) in evaluating the lysine damage in systems, resembling heat-treated milk, composed of lactose and acetyllysine or protein mixtures (BSA and β-lactoglobulin) and milk samples. The FAST method evaluates the formation of fluorescent advanced Maillard products (AMP) in the pH 4.6 milk supernatant. A correction for the protein concentration in the supernatant is allowed by dividing the AMP fluorescence by that of Trp, an indicator of the protein concentration. The objective was first to determine whether there is a lag phase before LL is significantly converted into

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fluorescent AMP and second to verify if after a time, LL is not actively degraded in AMP. For that purpose, a low temperature of incubation was applied for a long period of time to precisely distinguish between the different steps of the Maillard reaction. The FAST method is compared to the furosine assay, and correlations with the extent of lysine damage, estimated by means of NH_2 reacting fluorescamine (*16*) in the simple models, and by chromatographic quantification after acid or enzymatic (Pronase) hydrolysis for the milk samples, are determined.

MATERIALS AND METHODS

Chemicals. N^{α}-Acetyl-L-lysine (AcL), β-lactose, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, and fluorescamine were from Sigma (Saint Quentin Fallavier, France). Bovine serum albumin (BSA) was from Euromedex (Souffelweyersheim, France), and anhydrous sodium tetraborate, sodium acetate trihydrate, Pronase E, and β-lactoglobulin (β-L) were from Fluka (Saint Quentin Fallavier, France). Hydrochloric acid, acetonitrile, and orthophosphoric acid were from Prolabo (Nogent sur Marne, France).

High-quality (HQ) pasteurized milk (peroxidase positive) was purchased in a French market.

Preparation of Model Samples. (1) Acetyllysine (1 g/L) with lactose (40 g/L), albumin (10 g/L) with lactose (40 g/L), and β-lactoglobulin (9.5 g/L) with lactose (40 g/L) were incubated in sodium phosphate buffer (0.1 M, pH 8.5) at 60 °C in an oil bath in stoppered tubes (Pyrex), in triplicate. At different incubation times (0, 3, 22, 30, 46, 53, and 70 h), a 1 mL aliquot was sampled and stored at −20 °C until analysis was carried out.

(2) HQ pasteurized milk (50 mL flask) was incubated for 10 h at 60 °C and then for 5 h at 85 °C in an oil bath. Each hour, aliquots were sampled and immediately prepared for analysis.

Fluorescamine Assay. This method for evaluating the ratio of blocked amino groups in glycated proteins by comparison with a control sample has been described by Yaylayan et al. (*16*). Two hundred microliters of the incubation mixtures was added to 2.2 mL of a 0.2 M sodium tetraborate buffer (pH 8.5) in 10 mL tubes. While the tubes were vigorously shaken on a vortex type mixer, 100 μL of a fluorescamine solution (15 mg/100 mL of acetone) was rapidly added and fluorescence measured after 5 min on a SPEX Fluoromax DM 3000 F apparatus (Jobin Yvon, France) in four-face acryl cuvettes (10 mm × 10 mm × 48 mm) (Sarstedt, France). The excitation and emission wavelengths were set at 390 and 475 nm, respectively. The fluorescence of the blank sample without fluorescamine was subtracted, because of interfering fluorescent AMP in the incubated samples.

The standard deviation of experimental repeatability was calculated from the results obtained from the three samples incubated independently and varied between 4 and 7%.

Fluorescence of Advanced Maillard Products (AMP). Samples (200 μL) were diluted in 2.3 mL of borate buffer (0.2 M, pH 8.5), and AMP fluorescence was quantified in counts of photons emitted per second (cps) (Spex, Jobin Yvon, France). The maximum excitation wavelength was 350 nm for AcLys and 360 nm for BSA, and the maximum emission wavelength was 430 nm for both AcL and BSA samples. For β-L and whey samples, the maximum excitation and emission wavelengths were 338 and 410 nm, respectively. The standard deviation of experimental repeatability (three independent incubations) was ~2.5% for AcLys and ~5% for BSA and β-L.

FAST (Fluorescence of Advanced Maillard Products and Soluble Tryptophan) Method Used with Milk Samples. This method is based on the simultaneous determination protein denaturation by Trp fluorescence (λ_{exc} = 290 nm; λ_{em} = 340 nm) and formation of fluorescent advanced Maillard products (λ_{exc} = 330 nm; λ_{em} = 420 nm) in the milk fraction that is soluble at pH 4.6 as described by Birlouez-Aragon et al. (*18*).

The FAST index is calculated as 100[AMP fluorescence (cps)/Trp fluorescence (cps)].

The standard deviation of experimental repeatability was 2.5% (each measure in duplicate).

Determination of the Level of Furosine. Aliquots (330 μL) of AcL, BSA, and β-L were hydrolyzed for 18 h with 670 μL of 11.65 N hydrochloric acid (7.8 N final normality) in stoppered tubes at 110 °C. Milk proteins were isolated by vigorously mixing 1 mL of milk with 1 mL of water, 2 mL of absolute ethanol, and 4 mL of dichloromethane to precipitate the lipid-free proteins. The mixture was centrifuged at 4000*g* for 10 min at 4 °C, and the proteic precipitate was hydrolyzed by adding 1 mL of 7.8 N HCl and heating at 110 °C for 18 h.

Furosine was quantified on a Waters 486 HPLC system equipped with a Hypersil C18 column (250 mm × 4.6 mm) and detected at 280 nm, as described previously (*19*). The standard deviation of experimental repeatability was ~7% for the model proteins (three independent incubations), and the standard deviation of analytical repeatability was 3% for milk samples (duplicate analysis).

Lysine Quantification. Lysine was quantified by HPLC fluorescence either after acid hydrolysis, as described previously, or after Pronase digestion for 4 h as described by Delhaye and Landry (*20*). After derivatization by (6-aminoquinolyl)-*N*-hydroxysuccinimidyl carbamate (AQC) using the method of Cohen et al. (*21*), lysine was separated by HPLC on a 4 μm AccQ-Tag C_{18} column (150 mm × 3.9 mm) (Waters) at 37 °C, using a gradient program (System 522, Kontron Instruments) from 100% eluant A [140 mM sodium acetate and 17 mM triethylamine (pH 5.05)] to 100% eluant B [60% acetonitrile in water (v/v)], at a flow rate of 1 mL/min.

The standard deviation of analytical repeatability was 4.5% (duplicate analysis).

Statistical Test. Linear regressions were calculated with GraphPad Prism 3 software. Comparison of means and slopes was carried out with nonparametrical tests (GraphPad Prism 3 software).

RESULTS

1. Model Systems. *1.1. Concentration of Unavailable Lysine as a Function of Incubation Time.* The specific reaction of fluorescamine with primary amino groups of proteins or amino acids allows detection and quantification of free amino groups in incubated samples (*16*). If a level of unavailable lysine equal to zero is assumed at the initial time, the concentration of unavailable lysine in the samples was determined as the relative difference between the concentration of reactive amines in the incubated sample and that in the nonincubated sample.

Unavailable lysine was not detected with accuracy before incubation for 30 h because of a high detection

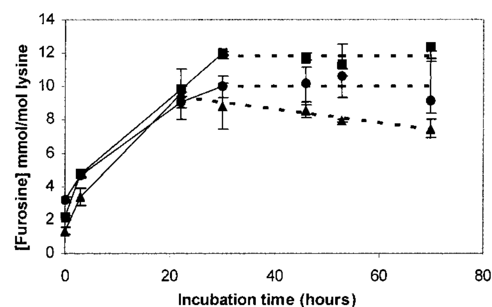

Figure 1. Evolution of furosine concentrations (millimoles per mole of lysine) in model systems as a function of incubation time: (▲) AcL (1 g/L) with lactose (40 g/L), (■) BSA (10 g/L) with lactose (40 g/L), and (●) β-L (9.5 g/L) with lactose (40 g/L).

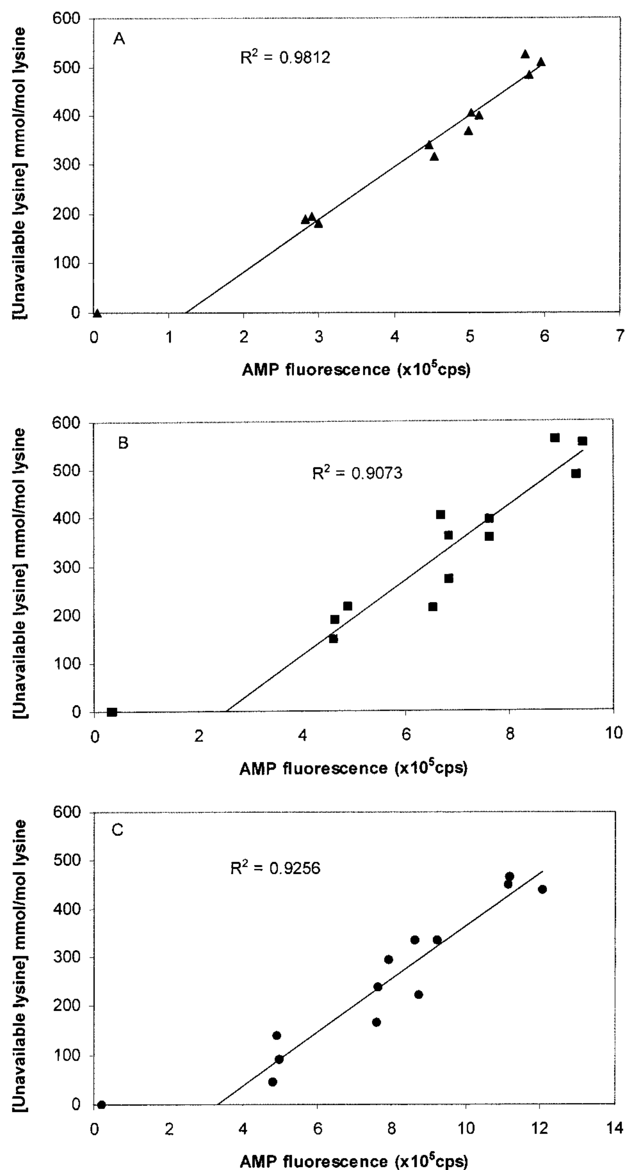

Figure 2. Regression lines between the concentration of unavailable lysine (millimoles per mole of lysine) and AMP fluorescence in (A) AcL (1 g/L), (B) BSA (10 g/L), and (C) β-L (9.5 g/L) incubated with lactose (40 g/L).

limit of 15−20%. After this time, the concentration of unavailable lysine increased linearly with incubation time ($R^2 = 0.99$, 0.93, and 0.89 for AcL, BSA, and β-L, respectively). The apparent zero-order rate constant (millimoles per mole of lysine per hour) for lysine loss was deduced from the slopes (a) of the regression lines. The slopes k_{lys} (millimoles per mole of lysine per hour) were found not to be significantly different in the three samples ($p = 0.2876$), i.e., 7.371 ± 0.2076 ($n = 14$) for AcL, 7.518 ± 0.5541 ($n = 15$) for BSA, and 6.456 ± 0.6356 ($n = 15$) for β-L.

1.2. Furosine Concentration as a Function of Time. Figure 1 shows the initial increase in furosine concentration per mole of lysine during the first 20−30 h of incubation. A steady-state concentration was then reached in the two protein models, whereas a slight but significant decrease ($p = 0.04$) was observed in AcL samples. The furosine steady-state concentration in the protein samples was calculated using all the data of the horizontal portion of the curve (slope not significantly different from 0), i.e., from 30 to 70 h. This steady-state

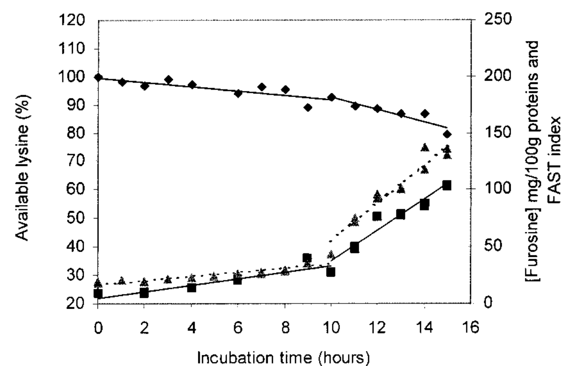

Figure 3. Evolution of the level of available lysine (%) (◆), the FAST index (▲), and furosine concentrations (milligrams per 100 g of proteins) (■) in milk as a function of incubation time. The temperature of the oil bath was set at 60 °C during the first 10 h and at 85 °C for the following 5 h. Analyses were carried out in duplicate, and the mean data are given.

concentration (millimoles per mole of lysine) was 11.8 ± 0.5 ($n = 10$) for BSA with lactose and 10.0 ± 1.0 ($n = 10$) for β-L with lactose.

In AcL samples, the highest level of furosine reached at 22 h was calculated to be 9.57 ± 0.13 mmol/mol of lysine ($n = 2$).

1.3. Accumulation of Fluorescent Advanced Maillard Products (AMP). AMP fluorescence increased linearly as a function of incubation time in the three samples (AcL, BSA, and β-L) ($R^2 > 0.98$). A significantly higher fluorescence was observed in both proteins than in AcL samples, and glycated β-L samples were more fluorescent than glycated BSA samples for similar incubation times. The apparent zero-order rate constant (cps per minute) of accumulation of fluorescent AMP was deduced from the slopes (a) of the regression lines: $a = 147.6 \pm 4.21$ ($n = 21$) for AcL, $a = 215.7 \pm 4.75$ ($n = 21$) for BSA, and $a = 272.3 \pm 3.45$ ($n = 21$) for β-L.

AMP fluorescence was linearly well correlated with the concentrations of unavailable lysine in the three model systems, as shown in Figure 2A−C. However, the regression line did not originate at zero, as a result of the concomitant increase in protein light scattering during protein denaturation and aggregation.

2. Milk Sample. Available lysine was evaluated by HPLC fluorescence after acid hydrolysis or Pronase digestion. A level of 556 ± 17.6 μmol of lysine/g ($n = 16$) was found in all the analyzed samples after acid hydrolysis so that no lysine blockage could be evidenced, despite the 10 h at 60 °C and 5 h at 85 °C. After Pronase digestion, lysine levels decreased linearly with incubation time, at a rate of 0.77 ± 0.18%/h ($R^2 = 0.70$, $n = 10$) at 60 °C and at a rate of 2.15 ± 0.46%/h ($R^2 = 0.84$, $n = 6$) at 85 °C. The lysine loss reached 21% at the end of the incubation time. Furosine concentrations and the FAST index could similarly be modelized as two regression lines depending on the temperature (Figure 3). The rate of formation of furosine increased 5 times between the two heat treatments: 2.82 ± 0.52 mg (100 g of proteins)$^{-1}$ h^{-1} ($R^2 = 0.81$, $n = 9$) at 60 °C and 13.41 ± 1.34 mg (100 g of proteins)$^{-1}$ h^{-1} ($R^2 = 0.92$, $n = 11$) at 85 °C; the FAST index increased 10 times faster at 85 °C (16.73 ± 1.50 h^{-1}, $R^2 = 0.93$, $n = 11$) than at 60 °C (1.75 ± 0.22 h^{-1}, $R^2 = 0.78$, $n = 18$). Moreover, a good linear correlation was found between the furosine concentration and the FAST index (slope of 1.27 ± 0.06, $R^2 = 0.96$, $n = 19$; Figure 4).

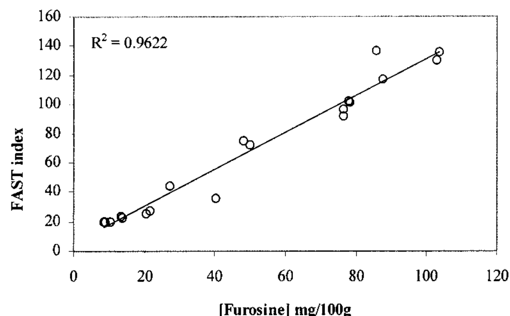

Figure 4. Linear correlation between the FAST index and furosine concentration (milligrams per 100 g of proteins) in incubated milk.

The FAST index and furosine concentrations were linearly correlated with the concentration of available lysine [R^2 = 0.83 (n = 15) and R^2 = 0.88 (n = 11), respectively]. The slopes of the regression lines allowed us to calculate the conversion factor between these two indicators of the Maillard reaction and the lysine loss under our conditions. A level of 10 mg of furosine/100 g was associated with 1.8% lysine blockage (i.e., 320 μmol/L in milk), and for the FAST index, each increment of 10 was related to a lysine blockage of 2% (i.e., 355 μmol/L in milk).

DISCUSSION

Heat treatment of milk is responsible for a loss of available lysine, because of the formation of the unavailable lactulosyllysine (LL), the first stable product of the Maillard reaction (*1*). The advanced step of the reaction further accelerates lysine blockage under brown and fluorescent heterocyclic compounds (*22*), but those products are not considered to accumulate significantly in heat-treated milk. Under such conditions, furosine, the acid product of ϵ-lactulosyllysine, seems to be a good indicator of lysine damage in milk (*2*, *23*−*25*). However, there is now evidence of accumulation of advanced Maillard products (AMP) fluorescence in milk very early during the heating process and at temperatures as mild as 68 °C (*5*).

In this study, we wanted to accurately determine the respective evolution of furosine and AMP fluorescence in heat-treated lactose/protein models and milk. A sufficiently low temperature was chosen (60−85 °C) to allow distinction of early and advanced steps of the Maillard reaction and determine the eventual existence of a lag phase before AMP accumulation. A long heat treatment was also applied to provide evidence of the step where the rate of transformation of LL in AMP becomes greater than the rate of production. At the same time, we wanted to determine which of these two indicators, furosine or AMP fluorescence, is better correlated with the total amount of lysine damage. The latter was determined in protein models by the simple indirect fluorescamine method, and by direct chromatographic quantification in milk samples.

In the lactose/protein samples, lysine blockage could only be estimated after incubation for 30 h at 60 °C, because of the high detection limit of the fluorescamine assay (~15% blockage). We had also to correct the fluorescamine signal for interference with endogenous AMP fluorescence accumulating during the heat treatment. These fluorophores have excitation and emission wavelengths near to those of NH_2-reacting fluorescamine, so they artificially increased the fluorescamine signal. A blank containing the sample without fluorescamine was therefore measured and systematically subtracted.

After 30 h, fluorescamine-reactive lysine concentration significantly decreased, following a zero-order rate, independent of the model, protein, or amino acid, despite different initial lysine concentrations. This observation is in agreement with the results of Baisier et al. (*26*) obtained on glycine/glucose mixtures, despite the observation of a first-order reaction probably due to limiting lysine in their system.

Furosine concentration increased during the first 24 h of heat treatment at 60 °C, but thereafter reached a steady-state concentration, indicating that the Amadori product is actively degraded into AMP, the rates of formation and degradation remaining constant. At the given pH (8.5) and temperature, the steady-state concentration was essentially dependent on the initial lactose and lysine concentrations. Consequently, in our model systems with the same concentration of lactose, furosine concentration was strictly related to the lysine content of the protein.

The formation of AMP fluorescence in those simple models described a zero-order reaction, confirming previous results (*26*, *27*). AMP fluorescence increased as rapidly as the furosine content during the first 24 h of incubation, and no lag phase was observed contrary to what was assumed by other authors (*28*). Moreover, a good correlation was observed between AMP fluorescence and the extent of lysine blockage over the total incubation time whatever the protein model. The intensity of AMP fluorescence was always more important in β-L and BSA than in AcLys samples and was not correlated with the lysine content of the protein. Maximal excitation wavelength fluorescence and emission wavelength fluorescence were also different for each protein. This can be explained by the complex mixture of fluorescent products which are formed during the Maillard reaction (*29*) involving numerous amino acids such as arginine and sulfur amino acids. Furthermore, the physicochemical environment of AMP may also differ between the proteins and influence their fluorescence quantum yield.

A more realistic model was studied, based on pasteurized milk heated first at 60 °C for 10 h and then at 85 °C for 5 h. Lysine blockage was evaluated by direct analysis after either acid hydrolysis or Pronase digestion. The very low level of blockage allowed by incubation at a mild temperature of 60 °C did not allow us to provide evidence of a lysine loss after acid hydrolysis, even after an additional 5 h at 85 °C. The error on lysine quantification (4.5%) must be in the range of the real decrease in the level of acid-released lysine concentration, knowing that 50% of the lysine residues initially bound to lactose are released upon hydrolysis. This method of lysine quantification is therefore only slightly sensitive and underestimates the amount of real damage. By contrast, Pronase digestion (associated with a very good standard deviation of repeatability of 3.5%) allowed determination of a significant lysine loss during the two steps of the heat treatment. A first linear decline at a mean rate of 0.78%/h at 60 °C was followed by a second one at a mean rate of 2.15%/h at 85 °C. Pronase digestion seems to be a good alternative for rapidly and directly evaluating the milk lysine damage upon heating, even if it surely does not take into account the loss of protein digestibility unlike the more complex and long

procedure proposed by Henle et al. (*9*). The furosine content and the relative AMP fluorescence accumulating in the pH 4.6 soluble proteins of milk (the FAST method) increased in a parallel fashion during the heat treatment. With their levels first increasing slowly but significantly, they then rapidly accumulated for the last 5 h at 85 °C, AMP fluorescence increasing less rapidly in the first step and more rapidly in the second step of heat treatment compared to furosine content. After 10 h at 60 °C, the furosine content of milk reached a value compatible with high pasteurized (peroxidase negative) milk [the initial milk was HQ pasteurized (peroxidase positive) with a furosine content of 8 mg/100 g]. After an additional 5 h at 85 °C, the furosine content was equivalent to the level expected for good indirect UHT milk (*5*, *19*). Similarly, the initial FAST index was that of HQ pasteurized milk (FAST index = 18) and reached a value of 44, equivalent to what is obtained for high pasteurized milk (*5*). But after an additional 5 h at 85 °C, the FAST index was very high, 130, corresponding to sterilized milk. It was already observed (*5*) that application of a low temperature for a long time is associated with a relatively high FAST index as compared to what would be expected from the furosine content. This probably results from a more rapid conversion of LL into AMP at higher temperatures. Both indicators of the Maillard reaction were however well correlated (as indicated by the zero-order rate of the two reactions). Furthermore, furosine and the FAST index were linearly correlated with the level of Pronase-digested lysine.

This study confirms that advanced Maillard products are formed as rapidly as LL (at the scale of the analytical methods that were used). The question of the extent of lysine residues blocked in the first and second steps of the reaction then arises. The regression lines observed between furosine content or the FAST index and the amount of available lysine allowed us to calculate the factor of conversion between those indicators of the Maillard reaction and lysine damage. The conversion factor for furosine was 5 times higher than that expected from the conversion of LL to furosine [taking into consideration the factor of 9 determined by Henle et al. (*9*)]. This allows us to provide evidence that lysine becomes unavailable not only under LL but also by formation of advanced Maillard products, and the latter are highly preponderant under our experimental conditions.

In conclusion, despite the lack of specificity of a global approach, we demonstrate that AMP fluorescence allows accurate evaluation of the total level of lysine damage upon heating. Moreover, AMP fluorescence is suitable even for very long heat treatments, what is not the case for furosine content, which after a time is no longer quantitatively associated with lysine damage. Such heat treatments have no application in the milk industry, but could be relevant for storage under high ambient temperatures. We demonstrate in incubated milk that the FAST method is a rapid alternative for measuring furosine concentration and is a good indicator of lysine damage.

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