Effect of Malondialdehyde on the Determination of Furosine in Milk-Based Products

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For nutritional purposes the recombination of milk or milk proteins with polyunsaturated fatty acids had long been considered in newly designed products, such as functional foods. Four milk-resembling model systems were prepared having malondialdehyde content ups to 1 mM. Systems were heated between 110 and 150 °C for up to 30 min. The effect of the presence of bifunctional aldehydes on the determination of furosine as a heat-induced marker was investigated. Levels of 0.01 mM MAD in the reaction mixture could affect significantly the determination of furosine in a milk-based system. Impairment of lysyl residues through the analysis of furosine could be underestimated in the presence of malondialdehyde.

Keywords: *Malondialdehyde; furosine; lysine; Maillard reaction; milk*

INTRODUCTION

Furosine, ϵ-*N*-(2-furoylmethyl-L-lysine), is an artificial amino acid formed from the acid degradation under refluxing of lactulosyllysine (LALY) with concentrated HCl [see Finot et al. (1981)]. The Amadori product, LALY, is formed during the early stages of the reaction between reducing sugars and proteins, which is called the Maillard reaction. The formation of LALY has an important nutritional significance because it reduces the biological availability of lysine in milk and milk products (Erbersdobler, 1986). In this sense, furosine has been successfully used for the evaluation of heat treatment damage in several well-characterized foodstuffs, such as milk (Erbersdobler et al., 1987), pasta (Resmini et al., 1990b), Manchego cheese (Arias et al., 1998), eggs (Hidalgo et al., 1995), baby cereals (Guerra-Hernandez and Corzo, 1996), and tomato paste (Hidalgo et al., 1998). Lactose is the main sugar in milk, but in addition to lactose many other reducing species that could participate in the Maillard reaction can be found in newly developed milk-based products, such as infant formulas or liquid milk enriched with long-chain polyunsaturated fatty acids (PUFA) or vitamins. These reducing species include several sugars, vitamin C, and various small molecular weight compounds present, added, or produced from other reactions such as lipid oxidation or sugar breakdown products (Labuza and Schmidl, 1986). In addition to the classical negative impact on the flavor of milk, aldehydes (heptanal, octanal, or pentenal) are also known to react with amino groups in proteins, especially the ϵ-amino group of lysine (Kikugawa et al., 1988), causing the formation of both intramolecular and intermolecular cross-links that give rise to polymerization and fluorescence structures (Hidalgo and Kinsella, 1989).

It has been stated that protein−peroxidizing lipid interaction occurs mainly through two mechanisms: (a) protein−amino condensation reactions involving lipid peroxidation breakdown products such as malondialdehyde (MAD) and protein amino groups and (b) by reaction of proteins with lipid oxidation products with formation of protein-centered free radicals (Karel, 1977). MAD, a product of polyunsaturated lipid peroxidation, reacts under mild conditions with amino acids, free amino groups in proteins, and amino sugars, mainly N-substituted amino ketones, producing Schiff bases or dihydropyridines [e.g., Chio and Tappel (1969) and Gómez-Sánchez et al. (1992)]. It has also been stated that other factors determine the formation of MAD from peroxidized PUFA, such as the presence of transition metals, the degree of fatty acid unsaturation, pH, and temperature (Raharjo and Sofos, 1993). MAD is involved in the deterioration of foods and in biological processes such as cell aging and the cross-linking of proteins and enzymes, lipoproteins, DNA, amino phospholipids, and amino acids [e.g., Kanner et al. (1987)].

The nutritional benefits of the ingestion of higher amounts of PUFA, mainly the ω-3 group, have made them important in the development of new products; milk and milk proteins are widely versatile raw materials for these formulas. Furthermore, bivalent metals or vitamin C fortified milk-based products could catalyze the decomposition of hydroperoxides toward end-products of the lipid peroxidation (Frankel, 1991). Thus, it is important to characterize the effect of lipid peroxidation products on well-established heat-induced parameters, such as furosine. The aim of this work is to elucidate whether furosine analysis, as a standard index for heat damage in foods, could be affected by the presence of bifunctional aldehydes such as MAD in the food matrix.

MATERIALS AND METHODS

Samples. Sodium caseinate [spray-dried, 94% of total protein (N × 6.38) in the dry matter] was obtained from DMV-International (Veghel, Netherlands). Milk-resembling solutions (MRS systems) were made with 3% sodium caseinate and 5% lactose monohydrate dissolved in a simulated milk salt solution, designated SMUF (Jenness and Koops, 1962), and the final pH was adjusted to 6.65. Four model systems with

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different MAD contents were obtained [0 mM (MRS0 system), 0.01 mM (MRS1 system), 0.10 mM (MRS2 system), and 1.00 mM (MRS3 system)] by adding a certain amount from a stock solution of MAD (36.5 mM), and the final pH was readjusted at 6.65. The model systems were then heated, in duplicate, in an oil bath in tightly stoppered glass test tubes (160 mm × 120 mm length and diameter, respectively) at controlled temperatures between 110 and 150 °C for times up to 30 min.

Preparation of MAD Standard. Hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) to produce MAD was accomplished as in Kwon and Watts (1963) with some minor variations. A MAD stock solution of 36.5 mM was prepared by weighing 880 mg of TEP (Sigma, St. Louis, MO), dissolving it in ~90 mL of distilled water containing 1 mL of 1 N HCl, and diluting the mixture to 100 mL with distilled water. The solution was placed in a glass stopper, heated in a water bath at 50 °C for 60 min, and then cooled at room temperature. Several dilutions were carried out with acidified water and sodium−phosphate buffer (50 mM, pH 6.7). The optical density of the MAD stock solution was measured at 247 nm for the acidic solution and at 267 nm for the neutral solution at pH 6.7. Molar extinction coefficients of 13700 and 31506 were used for the acidic and neutral solutions, respectively, for calculation of MAD concentration in the standard solution (Esterbauer et al., 1984). A Shimadzu UV-1601 (Duisburg, Germany) recording spectrophotometer was used.

Furosine Determination. Sample (1.5 mL, containing ~45 mg of protein) was hydrolyzed in the presence of 8 mL of 8 M HCl under nitrogen at 110 °C for 23 h in a screw-cap Pyrex vial. Furosine was separated by ion-pairing HPLC with a C8 Alltech furosine-dedicated column held at 35 °C after solid-phase separation in a prewetted Sep-Pak C18 cartridge as described by Resmini et al. (1990a).

Available Lysyl Residue Determination. Available lysyl residues in casein were determined fluorometrically after derivatization with *o*-phthaldialdehyde (OPA) as described by Morales et al. (1995). Briefly, 1.5 mL of an SDS (12% w/v) solution was added to 1.5 mL of model system and refrigerated overnight. Then, 100 μL of sample was mixed with 3 mL of freshly prepared OPA reagent (80 mg in 2 mL of 95% methanol; 50 mL of 0.1 M, pH 10, sodium borate buffer, 5 mL of 20% SDS, and 0.2 mL of β-mercaptoethanol made up to 100 mL with distilled water) with constant stirring and incubated for 2 min at 25 °C. Relative fluorescence (RF) was measured against an unheated sample within 3 min, at emission and excitation wavelengths of 455 and 340 nm, respectively. A Kontron SMF-25 (Milan, Italy) recording spectrofluorometer was used.

HPLC Equipment. The chromatographic system consisted of an MD-420 pump, an MD-465 autosampler, an M-4322 spectrophotometer UV−VIS, and an MT-2 computing integrator connected to a PC, all from Kontron Instruments.

Statistical and Kinetic Analysis. Data obtained from the chemical analysis were evaluated statistically by descriptive parameters; multifactor analysis of variance (ANOVA) and Chi-square test for normality were performed by applying the Statgraphics v. 7.0 statistical package (Statistical Graphics Corp., Rockville, MD). Differences in means between model systems were resolved by Fisher's least significant difference (LSD) tests to provide the confidence intervals, and significant factors were identified by the *F* test (Snedecor and Cochran, 1980). All of the statistical procedures were performed at a significance level of 95% ($p < 0.05$). Lysine loss and furosine were addressed as dependent variables, with temperature, time, and MAD content as independent variables.

RESULTS AND DISCUSSION

Four different model systems were prepared with different MAD contents to study the effect of the presence of MAD on the determination of furosine in a lactose/casein model system resembling a milk-based product. After the addition of MAD and subsequent homogenization, visual differences in colors among the unheated model systems could not be observed. After heating, apart from the temperature/time effect on browning, we were not able to see visual differences between groups. Chemically, MAD (O═CHCH═CHOH, enol form) may react with an amine group ($R-NH_2$) to produce an initial ene-amine Schiff base (O═CHCH═CHNH−R), and then, by further condensation with another amine, an amino-inino-propene compound (RN═CHCH═CHNHR) is formed.

Figure 1 describes the relative losses of available lysine determined by the OPA fluorescent reagent in the systems without MAD addition (MRS0) and higher addition of MAD (MRS3). The fluorescence value of the sample, after reaction with OPA reagent, is expressed as a function of the relative fluorescence of an unprocessed sample by assuming 100% of available lysine content (~20 mM) in an unheated sodium caseinate plus lactose solution. The reaction of lysine with OPA is stoichiometric. Because OPA reacts far better with the ϵ-NH_2 groups in lysine than any other peptide group, the reaction might be used for measuring the available lysine content (García et al., 1989). This procedure offers a reliable measurement of the blocking reactions of lysine that are taking place, such as Maillard reaction with lactose and reaction with MAD.

As expected, the available lysine of model systems contents is clearly affected by the extent of the heat treatment of the sample (Figure 1). Lysine, one of the eight amino acids essential for humans and other animals, is easily damaged by processing or storage, causing reduction of the nutritional quality of the food, as shown by Morales et al. (1995) for milk systems. Multifactor ANOVA was performed on the variables MAD content, temperature, and heating time for studying the effect of the addition of MAD up to 1 mM. Previously, raw data (expressed as millimolar) were transformed taking exponential (EXP) since homocedasticity and, consequently, the power of the derived models was fairly improved. Normality of the derived models was accepted from the analysis of residuals, and the contribution of each factor (MAD, temperature, and heating time) was measured after the effects of all other factors had been removed.

Figure 2 shows the plot of means obtained for each model system with MAD concentration the classification factor. *F* test in the ANOVA table gave an *F* ratio of 5.95 (3 df) with a *p* value of 0.0008, which means a significant effect of the presence of MAD on the availability of lysine in the model systems. MRS0 and MRS1 systems were significantly different from the MRS3 system. Goodness of the ANOVA analysis was performed by analyzing the normality plot of the residuals, giving a significance value of 0.661 for the Chi-square test (3 df), indicating the normal distribution of the residuals.

Although lysine is relatively abundant in milk protein, heat treatment may promote its irreversible reaction with carbonyl compounds present in foods such as reducing sugars (i.e., lactose) and sugar breakdown products (methylglyoxal) or lipid oxidation products (i.e., MAD). Moreover, during the Maillard reaction, the formation of other advanced Maillard compounds, such as pyrroles or furans, which could condense with the protein, had been described. Hence, not all of the blocked lysine will drive to furosine because the LALY intermediary is not formed. Furosine was derived from the nonoxidative acidic degradation of the LALY formed

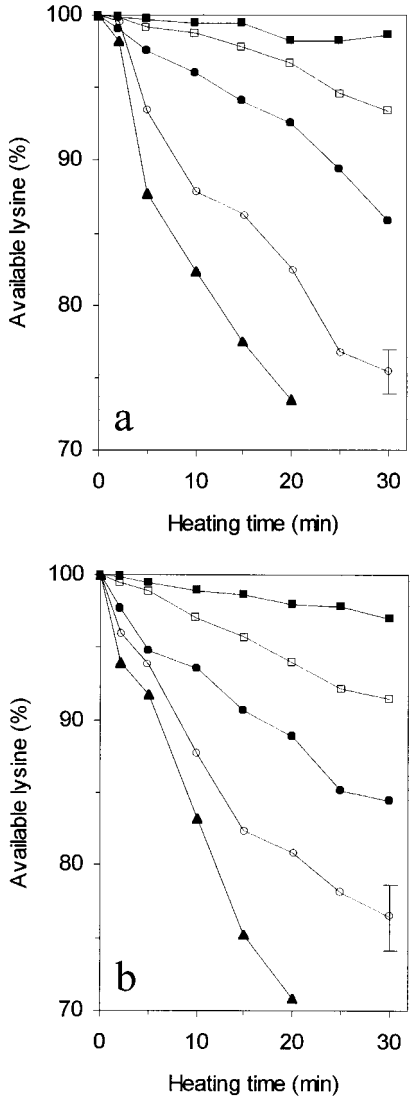

Figure 1. Percentage of available lysine in the MRS0 (a) and MRS3 (b) model systems heated at 110 °C (■), 120 °C (□), 130 °C (●), 140 °C (○), and 150 °C (▲) for up to 30 min. Error bars, included on the 140 °C/30 min curve, represent the maximum standard deviation observed between duplicate analyses for all results.

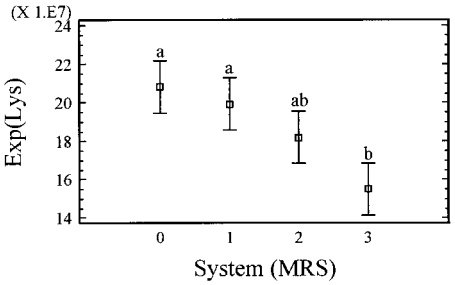

Figure 2. Mean plot from the multifactor ANOVA analysis according to the malondialdehyde content (0, 0.01, 0.1, and 1.0 mM) for available lysine in system MRS. Confidence intervals are shown as error bars, and letters indicate significant differences between groups at $p < 0.05$.

from the reaction of lactose and the lysyl residues of casein in the initial stage of the Maillard reaction. Lysine can be used biologically when the ϵ-amino group is free. In milk, radiolabeled lactose analysis has been used to determine that the ϵ-amino residue of lysine of casein (mainly, κ-casein) the main amino acid involved in the early stage of the Maillard reaction (Turner et

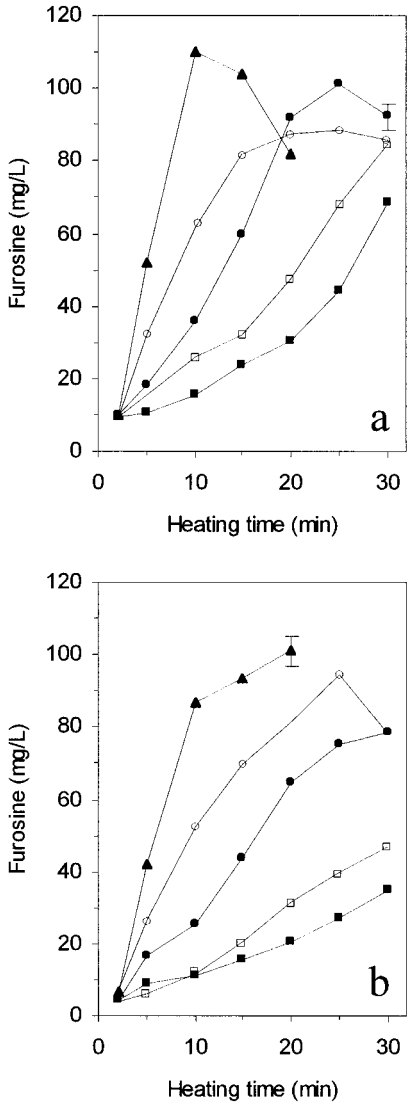

Figure 3. Furosine concentration (milligrams per liter) in the MRS0 (a) and MRS3 (b) model systems heated at 110 °C (■), 120 °C (□), 130 °C (●), 140 °C (○), and 150 °C (▲) for up to 30 min. Error bars represent the maximum standard deviation observed between duplicate analyses for all results.

al., 1978) and, subsequently, LALY is formed as a stable Amadori compound [e.g., Finot et al. (1981)].

Figure 3 describes the results of the furosine content in the model systems MRS0 and MRS3. Furosine values are not linearly independent of the thermal conditions applied. At severe heat conditions, the quick degradation of the Amadori product through the Maillard reaction gives lower values of furosine. By applying multifactor ANOVA analysis, the power of the analysis was improved by transforming data by taking the logarithm of the raw data of furosine (expressed as milligrams per liter). Figure 4 shows the plot of means for furosine obtained for each model system with MAD concentration the classification factor. *F* test in the ANOVA table gave an *F* ratio of 8.56 (3 df) with a *p* value < 0.0000, which indicates a significant effect of the presence of MAD on the formation of furosine in the model systems. The MRS0 system was significantly different from the MRS1, MRS2, and MRS3 systems. Goodness of the ANOVA was performed by analyzing the normality plot of the residuals giving a significance value of 0.5652 for the Chi-square test (3 df), indicating the normal distribution of the residuals. Clearer differ-

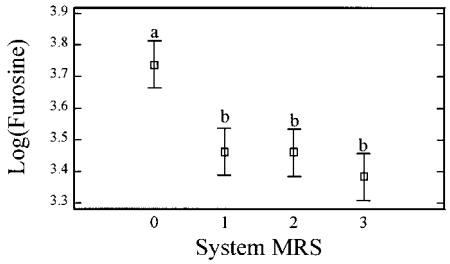

Figure 4. Mean plot from the multifactor ANOVA analysis according to the malondialdehyde content (0, 0.01, 0.1, and 1.0 mM) for furosine in system MRS. Confidence intervals are shown as error bars, and letters indicate significant differences between groups at $p < 0.05$.

ences between the above-described groups were obtained when the temperature at 150 °C was omitted from the statistical analysis, giving an *F* ratio of 11.47 (3 df). At severe heating conditions LALY is not stable and degrades through different pathways such as 3-deoxyosone or 1-deoxyosone routes to form a wide family of advanced Maillard products. At this point, since LALY is indirectly analyzed as furosine we observed a decrease in the values.

Underestimated LALY, from furosine analysis, in the presence of MAD has been calculated at low−medium heat treatment conditions to avoid the effect of LALY degradation toward intermediary and/or advanced Maillard products as well as protein polymerization reactions. An average value of 25.25 ± 15.10% of LALY was obtained by using the furosine methods in MRS1, MRS2, and MRS3. Higher differences were calculated for model system MRS3: 32.82 ± 14.26% of LALY was underestimated by using the furosine method in the presence of 1 mM MAD.

In the light of these results, it is shown that determination of furosine is affected by the presence of low amounts of MAD. Multifactor ANOVA analysis cannot distinguish clear differences in the values of furosine related with an increase in the MAD amounts up to 1 mM. These results are different from those obtained for available lysine, which showed a proportional effect with the level of MAD in the reaction mixture. Low levels of MAD drastically decreased the formation of LALY bound to casein. A competitive effect of the MAD with lactose could exist to condense the lysyl groups, MAD being a more effective binding agent than lactose, even at low levels such as 0.01 mM. These results could also affect the application of the levels of LALY (furosine) coupled with that of undenatured β-lactoglobulin as a means of detecting the addition of reconstituted milk powder in pasteurized milks added with fats different from those of milk fat.

CONCLUSION

Furosine has been largely used as a thermal index of the deterioration of milk products (Erbersdobler et al., 1987; Resmini et al., 1990a). However, PUFAs from vegetable or fish oils, iron, and vitamin C are common ingredients in newly designed products. Recently, new milk-based products recombined with fish oil have been marketed to cover a group of so-called functional foods enriched in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. A dilute aqueous solution consisting of casein, lactose, and MAD has been applied to the study of the effect of bifunctional aldehydes on the usefulness of furosine as a food quality marker. The exposure of protein to peroxidizing lipids or their secondary breakdown products, such as MAD, alkanals, 2-alkenals, 2,4-aldienals, and 4-hydroxyalkenals, can produce changes in proteins, such as loss of enzyme activity, polymerization, insolubilization, fragmentation, browning, damage to certain amino residues, and formation of lipid−protein complexes (Hidalgo and Kinsella, 1989).

Results reported in this work show that indicators of protein quality based on the measurement of modified lysine through the formation of Amadori products in the Maillard reaction could be underestimated due to the presence of MAD. Temperature had the most important effect on lysine loss, but the MAD concentration of the reaction medium also influenced the rate. Levels of 0.01 mM MAD can significantly affect the determination of furosine in a milk-based system. Bifunctional aldehydes are very reactive at low concentration. These results are important for milk, which is enriched with iron, vitamin C, lactose or maltodextrin, and PUFAs. A future task will be to identify the lysine−MAD adducts or their degradation products by chemical or immunocytochemical techniques to advance in this area. On the other hand, similar results could be expected for methylglyoxal, which often appears in foods, and it has the same empirical formula and molecular weight as MAD.

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