Study of Hydroxymethylfurfural Formation from Acid Degradation of the Amadori Product in Milk-Resembling Systems

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5-(Hydroxymethyl)-2-furfuraldehyde (HMF) is formed upon heat treatment of milk and milk-resembling systems by the Maillard reaction, via its Amadori product lactulosyllysine, as well as by isomerization and subsequent degradation of sugars. An improved method for the evaluation of the lysine modification caused by the Maillard reaction is presented. It is based on the indirect determination of the Amadori product as "bound" HMF by reverse-phase HPLC. The best digestion conditions have been settled at 2 mg of freeze-dried protein/mL of 0.3 N oxalic acid. In this work the "bound" HMF formation from acidic degradation of the Amadori product at temperatures between 110 and 150 °C and for times up to 30 min in a milk-resembling system has been studied.

Keywords: *Hydroxymethylfurfural; milk; Maillard reaction; HPLC analysis*

INTRODUCTION

Sterilization treatments, usual for foodstuffs for human consumption and necessary for long-life products, produce a number of organoleptic and physicochemical changes, most of which are undesirable for the consumer. The Maillard reaction is a very complex set of reactions that take place during processing and storage of most foods and cause deterioration of their nutritive value (Carpenter and Booth, 1973). In dairy products, even in the absence of browning or off-flavors, their nutritive value may be considerably reduced due to the formation of the protein-bound Amadori product (lactulosyllysine = galactose-fructose-lysine, designated as LALY) formed by reaction of lactose and the ϵ-amino group of lysine (Finot and Mauron, 1969) in the first stage of the Maillard reaction, almost selectively blocking the lysine residues during industrial treatments of milk.

Four possible routes of degradation of the Amadori products in foodstuffs have been identified. The first three involve the formation of deoxyosones, namely 1-deoxyosones, 1-amino-1,4-dideoxyosones, and 3-deoxyosones, while the fourth pathway involves fusion and the Strecker degradation (Ames, 1992). 3-Deoxyosones are formed by 1,2-enolization of LALY with β-elimination of the C-3 hydroxyl group at low pH and are reactive α-dicarbonyl intermediates which decompose to hydroxymethylfurfural (HMF) with loss of three water molecules. The HMF formed by acidic degradation of LALY in the absence of sugars is designated "bound" HMF (b-HMF) to differentiate it from total and free HMF. It should be remembered that HMF has two formation routes: by degradation of lactose and by the Maillard reaction. Therefore, the usefulness of total HMF as a specific index of the extent of the Maillard reaction in milk and milk-like products is limited, since HMF is also formed by the acid-catalyzed degradation of lactose (Anet, 1964).

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Several methods for the measurement of LALY have been described. There is a direct measurement by ion-exchange chromatography of enzymatically released LALY after complete hydrolysis of the protein (Henle et al., 1991). LALY is degraded during acid hydrolysis of protein with concentrated HCl and can be indirectly estimated by the measurement of furosine (ϵ-N-2-furoylmethyl-L-lysine) (Finot et al., 1968), formed with a yield of about 40% from the fructoselysine moiety when 8 M HCl is used (Erbersdobler, 1986). Several techniques have been described for furosine analysis: (a) GLC after derivatization to heptafluorobutyryl-isobutyl esters (Büser and Erbersdobler, 1985), (b) amino acid analysis (Dehn-Müller et al., 1991), (c) HPLC (Chiang, 1983; Resmini et al., 1990), and (d) capillary zone electrophoresis (Tirelli and Pellegrino, 1995). Carboxymethyllysine, formed by oxidative cleavage of the fructose-lysine residue and determinable by GLC or HPLC, also gives an indirect measurement of Amadori product formation (Büser and Erbersdobler, 1986).

Under mildly acidic conditions LALY is degraded to HMF (Gottschalk, 1952). In a dialyzed lactose−caseinate solution containing 2.3 mM lactulosyllysine, preheated for 72 h at 60 °C, 5.6 μM HMF is formed at 130 °C/2 min, and this value decreases to 2.9 μM HMF at 130 °C/15 min. (Morales and van Boekel, unpublished results). In a previous paper we have considered the possibility of using the levels of HMF formed by acidic degradation of LALY, and designed as b-HMF, as an index of protein glycosylation (Morales et al., 1997). The sugar-free milk of a milk-like solution was obtained by gel filtration.

In this work we have modified the procedure of obtention of the sugar-free sample and have carried out a complete study of the HMF formation by acidic degradation of LALY at 110−150 °C and for times up to 30 min. Factors affecting analysis of b-HMF were tested for achieving the best analytical conditions, and the method was applied to the analysis of heat treatments in milk and milk products. All the work reported here concerns the protein-bound Amadori product LALY.

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MATERIALS AND METHODS

Samples and Heat Treatments. Sodium caseinate (spray-dried, 94% of total protein (N × 6.38 in the dry matter) was obtained from DMV-International (Veghel, The Netherlands). Simulated milk solutions were made with 3% sodium caseinate and 5% lactose monohydrate dissolved in a simulated milk ultrafiltrate solution (Jenness and Koops, 1962), and the pH was adjusted to 6.65. The solution was reconstituted with washed milk fat from raw milk until 1.5% total fat content. Then, the solution was homogenized at 35 °C at 30 MPa bar for the first stage and 8 MPa bar for the second stage in a high-pressure laboratory homogenizer from APV (8.30H-Rannie, Albertslund, Denmark). Simulated milk solutions were heated in an oil bath in tightly stoppered stainless steel test tubes (120 × 7 mm^2) at controlled temperatures between 110 and 150 °C for times up to 30 min. Pasteurized skimmed milk (2.98% total protein, 4.8% lactose content, 0.2% fat content, pH 6.67, sample S1) was heated in stoppered test tubes in a boiling water bath for 30 min (sample S2) or 60 min (sample S3).

Preparation of a Dried Delactosed Sample. Milk or milk-resembling sample (20 mL) was deproteinized with 10 mL of trichloroacetic acid (TCA) (40%, w/v) and centrifuged at 15 000 rpm for 15 min at 4 °C in a RT6000B Sorvall centrifuge (Du Pont Co., Wilmington, DE). The supernatant was discarded, and the precipitated casein was washed with 15 mL of distilled water and centrifuged in the same conditions, this step being repeated twice. The casein fraction was dissolved in a dichloromethane:water (1:4) solution for 15 min. After centrifugation, the protein fraction was washed twice with 15 mL of water. Finally, the protein fraction was freeze-dried and analyzed by ion-exchange HPLC for absence of residual amounts of sugars.

Sugar Determination. Lactose was analyzed by ion-exchange HPLC as described by Morales et al. (1997).

Organic Acids Determination. Acetic and formic acids were analyzed by ion-exchange HPLC using an ION-300 polymeric resin column (300 × 7.8 mm^2, Interaction-Lab, San Jose, CA) at 50 °C. Organic acids were analyzed by monitoring their UV absorbance at 210 nm.

HMF Determination. Free and total HMF were analyzed by reversed-phase (RP-HPLC) as described by Morales et al. (1992). An Extrasyl ODS-2 S5 analytical column (25 × 0.40 cm^2, 5 μm, Barcelona, Spain) was used for HMF separation at room temperature.

Furosine Determination. The 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. (1990).

Available Lysyl Residues Determination. Available lysyl residues in casein were determined fluorimetrically after derivatization with *o*-phthaldialdehyde as described by Morales et al. (1995).

HPLC Equipment. The chromatographic system consisted of a MD-420 pump, a MD-465 autosampler, a refractive index detector (Erma Inc., Tokyo, Japan), a M-4322 UV−vis spectrophotometer, and a MT-2 computing integrator connected to a personal computer, all from Kontron Instruments (Milan, Italy).

RESULTS AND DISCUSSION

In a first step, the optimal ratio between the concentrations of lyophilized protein and oxalic acid (0.3 N) during the digestion of sample was determined. Increasing amounts of delactosed protein in the range 5−600 mg were digested in the presence of 2.5 mL of 0.3 N oxalic acid for 30, 60, and 120 min in a boiling water bath. Afterward, the solution was deproteinized with 0.2 mL of TCA solution (40% w/v) and the supernatant analyzed for its b-HMF content. Results obtained for 2 h of hydrolysis time are shown in Figure 1 for samples S1 and S3. Up to 0.2 g of protein/2.5 mL of oxalic acid there is a proportional relationship between HMF formation from the acidic degradation of LALY formed during the early stage of the Maillard reaction against the concentration of protein. Furthermore, the formation of b-HMF from LALY through 3-deoxyosulose as intermediary increases with preheating since sample S3, preheated for 60 min in a boiling water bath, reached higher levels of HMF than sample S1, which was not preheated. The plateau in Figure 1 is due to inhibition by excess of substrate.

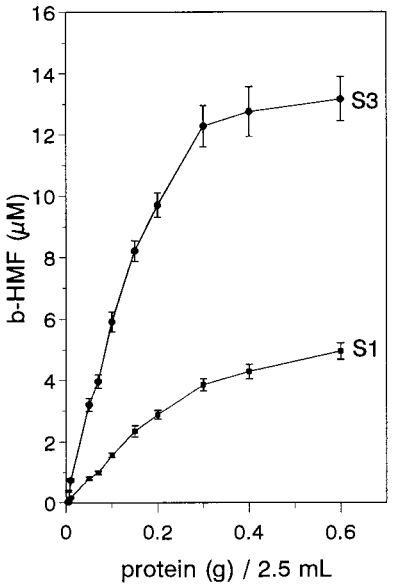

Figure 1. Effect of the protein concentration on the formation of b-HMF (μmol/L) originating from degradation of the Amadori product in 0.3 N oxalic acid for 2 h in the samples S1 and S3. Data shows the average and error bar of two independent analyses.

Obviously, if the formation of b-HMF is referred to grams of protein, decreasing curves are obtained (graph not shown), especially in sample S3. Cross-linking during heating should have a protective effect, since both inter- and intraprotein cross-linking may reduce the effectiveness of the acidic digestion due to a smaller accessibility of the lactosylated residues in the protein. A proportion of 2 mg of lyophilized protein/mL of oxalic acid is acceptable, although the limiting factor is the limit of detection of the chromatographic technique, which is close to 0.15 μmol/L for an injection volume of 40 μL.

The release of b-HMF from LALY is promoted by acids, the rate depending on the concentration and type of acid (Gottschalk, 1952). The effect of the oxalic acid concentration on the degradation of LALY is shown in Figure 2. Protein (5 mg) from samples S1 and S3 was digested with 2.5 mL of oxalic acid solution for 1 h. In sample S1, degradation of LALY to b-HMF reaches a maximum for a concentration of oxalic acid close to 0.4 N, but in sample S3 b-HMF is still increasing at higher oxalic acid concentrations. Nevertheless, an oxalic acid concentration of 0.3 N was chosen because it produces an acceptable degradation rate of LALY with good reproducibility. Amounts of 51.9 ± 3.4 pmol/g of protein (n = 5) and 147.3 6.8 pmol/g of protein (n = 5) were obtained for S1 and S3, respectively. It is important to notice that not all the LALY formed during heat

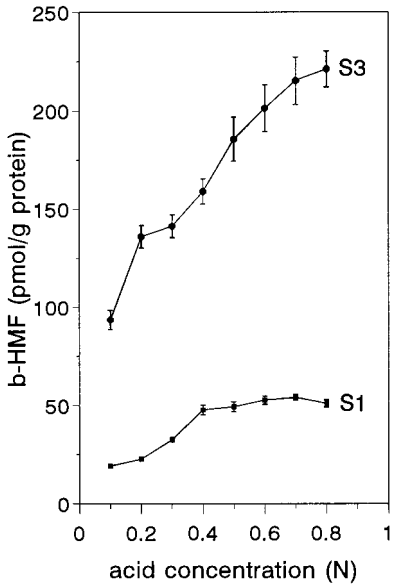

Figure 2. Effect of the concentration of oxalic acid on the formation of b-HMF (pmol/g of protein) from LALY degradation for 1 h in a boiling water bath in samples S1 and S3. Data shows the average and error bar of two independent analyses.

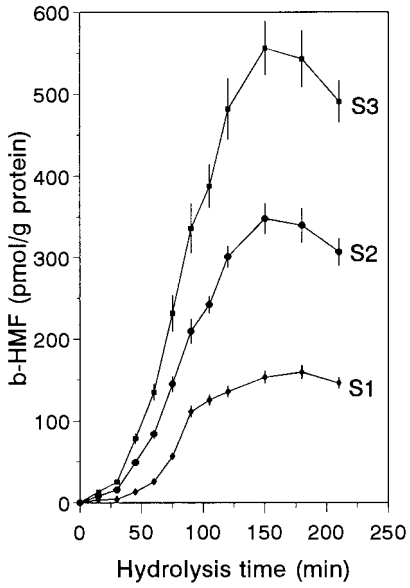

Figure 3. Effect of the heating time on the formation of b-HMF (pmol/g of protein) during digestion of LALY in 0.3 N oxalic acid solution in the samples S1, S2, and S3. Data shows the average and min−max bar of two independent analyses.

treatment and bound to protein will be degraded to b-HMF under these experimental conditions since the degradation products of 3-deoxyosones include other compounds such as pyrroaldehydes, pyridiniumbetaine, pyranones, lactones, and lactams (i.e., Ledl, 1990). Furthermore, it has been described that 3-deoxyosones can also react with Amadori products to give further pyrroaldehydes and other structures formed by competing reactions (Farmar et al., 1988).

Figure 3 shows the effect of the digestion time in a 0.3 N oxalic acid solution in a boiling water bath on the formation of b-HMF from LALY degradation through 3-deoxyglucosone via 1,2-enolization. Temperature and time are the main variables, since at 60 °C (1 h, 1 N trifluoroacetic acid) less than 10% of 3-deoxyglucosone is converted into HMF, and at 100 °C complete decomposition was noted (Weenen and Tjan, 1992; Anet, 1960). The sample preheated for 60 min in boiling water (S3) shows a maximum of b-HMF formation at around 150 min, after which b-HMF formation decreases. Under these conditions HMF is stable, since a solution of HMF (25 and 100 μmol/L) in a 0.3 N oxalic acid solution does not degrade for up to 3 h of digestion (data not shown). We have no clear explanation for this lack of linearity, although it is possible that HMF may interact with reactive groups of compounds also formed during the acidic digestion, forming other products bound or not bound to protein, as described above (Ledl, 1990; Ames, 1992). The coefficient of variation at 120 min of heating ranged from 3.81% for sample S1 to 7.70% for sample S3. A digestion time of 120 min was selected as a good compromise between duration of analysis and rate of degradation of LALY to b-HMF.

Table 1. Levels of Total, Free, and "Bound" HMF, Furosine, and Lactulose in the Milk Samples S1, S2, and S3

sample	total HMF (pmol/g)	free HMF (pmol/g)	"bound" HMF (pmol/g)	furosine (mmol/g)	lactulose (mg/L)
S1	39454.1	8063.2	52.8	0.6	72.0
S2	124118.1	17134.4	114.2	22.7	166.2
S3	248467.9	25438.5	182.9	68.2	282.6

b-HMF was compared with other heat-induced indices frequently used for milk products such as free and total HMF, furosine, and lactulose. Furosine is a classical parameter of the early stage of the Maillard reaction, since it is only formed by complete hydrolysis of LALY with 8 M HCl. As for lactulose, it is a well-described marker of the extent of isomerization in milk products. The denomination of b-HMF could be misleading, since bound HMF originates from degradation of LALY bound to protein but is released in the reaction medium. In Table 1 the levels of "bound", free, and total HMF, furosine, and lactulose found in samples S1, S2, and S3 are given; it can be concluded that all these parameters are good markers of the heat treatment applied to the milk samples. Taking into account that furosine is formed with a yield of 40% during the acidic hydrolysis of LALY (Erbersdobler, 1986), the yield of formation of b-HMF from LALY is 3.5%, 0.2%, and 0.1% in samples S1, S2, and S3, respectively.

Application of "Bound" HMF as Heating Index. The conditions for the analysis of b-HMF formed by acidic degradation of LALY being established, the relationship between b-HMF and the heat treatment applied was studied over a wide range of temperature and time.

Figure 4 shows the chromatograms obtained from a sample of lactose−caseinate heated at 130 °C for 10, 15, and 30 min where other compounds besides b-HMF can be separated. We have focused on two chromatographic peaks, designated peak B (eluted at 4.4 min) and peak Y (eluted at 8.5 min), that show a positive relationship with the heat treatment of the sample. Peak B has no characteristic UV−vis spectrum. Peak Y shows an absorbance maximum at 273 nm without shoulders in a spectrum obtained by coupling a diode array to the chromatograph (0.08 M Na-acetate, pH 3.6). A 5-methyl-3(2H)furanone-like, a furan-like, or a pyranone-like compound could be good candidates for peak Y according to the relative retention time from HMF. Isolation and identification of this unknown compound is being carried out.

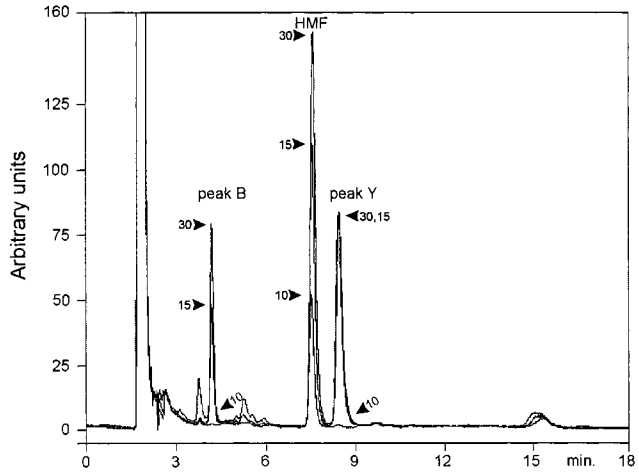

Figure 4. Typical chromatogram obtained from b-HMF analysis in the lactose−casein model system heated at 130 °C for 10, 15, and 30 min.

Figure 5a shows the dependence of heating time and temperature on the formation of b-HMF in the lactose−caseinate system. Results obtained (average of two analyses) show at low temperatures a linear increase with time, which at higher temperatures is followed by an apparent degradation of b-HMF. These results could have three possible explanations: (a) degradation of LALY by severe heating; (b) interaction of b-HMF with other highly reactive compounds (bound or not bound to protein) formed in the reaction medium, as pointed out above (hydrolysis of sample S3 in Figure 3), or (c) a combination of both effects.

Furosine is a widely accepted indirect index related to the amount of LALY. LALY can be estimated by analyzing the furosine formed by its hydrolysis with concentrated HCl as described by Erbersdobler (1986); results are shown in Figure 6a. Under more severe heating, the furosine level decreases because levels of LALY decrease. Nevertheless, a decrease in the LALY formation in heated samples does not show that the Maillard reaction has stopped but shows that other intermediary or advanced compounds can also react with the available ϵ-amino group of lysine in the casein, resulting in a decrease of the available lysine (Figure 6b).

During the heat treatment usually applied to milk, the Maillard reaction mostly stops at the early stage with the formation of LALY, neither browning nor off-flavors being observed at this step. With more heating the milk shows browning due to the advance of the Maillard reaction beyond the Amadori product stage, and consequently the levels of LALY (indirectly measured by the furosine content) decrease. The progress of the Maillard reaction explains the apparent discrepancy between furosine formation (Figure 6a) and loss of available lysine (Figure 6b). Due to the diversity of reactive intermediates in this Maillard reaction mixture, numerous protein-bound cross-linked structures, such as lysilpyrraline or pentosidine, other than dehydroalanine-derived amino acids such as lysinoalanine, can be expected to form (Henle and Klostermeyer, 1993; Henle et al., 1997). Morales and van Boekel (1996) described the formation of lysylpyrraline in milk-like model systems similar to those studied in this work. 3-Deoxoglucosone and lysine are precursors of lysylpyrraline formation. Lysyl residues are required in the first steps of protein polymerization, which are followed by interactions involving arginyl residues (i.e., Mohammad et al., 1949; Eble et al., 1983).

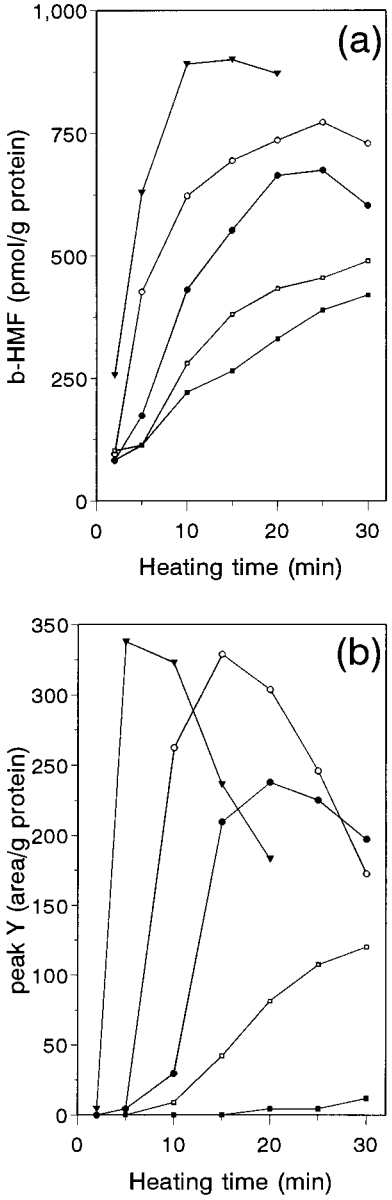

Figure 5. Formation of b-HMF (a) and unknown compound peak Y (b) in the lactose−caseinate model system heated at 110−150 °C for up to 30 min: 110 (■), 120 (□), 130 (●), 140 (○), and 150 °C (▼).

An additional experiment was performed to study the breakdown of LALY during heat treatment by monitoring the formation of organic acids in samples S1, S2, and S3 during acidic digestion of the delactosed protein. Acetic acid was detected in samples S2 and S3 (0.10 and 0.31 mmol/g of protein, respectively), but no formic acid was detected.

Regarding the second explanation stated above (b), it may also be possible that HMF ("bound" or free or total) is involved in other chemical pathways. Berg (1993) reported that during heating (140 °C for up to 23 min) of model systems consisting of HMF (357 μmol/L) and sodium caseinate (2.6%) some HMF was slowly degraded, but in the absence of casein the HMF concentration did not change. It has also been described that HMF may react with reducing sugars at high temperatures by both the aldehyde and hydroxymethyl groups (Urashima et al., 1988). Recently, the identifica-

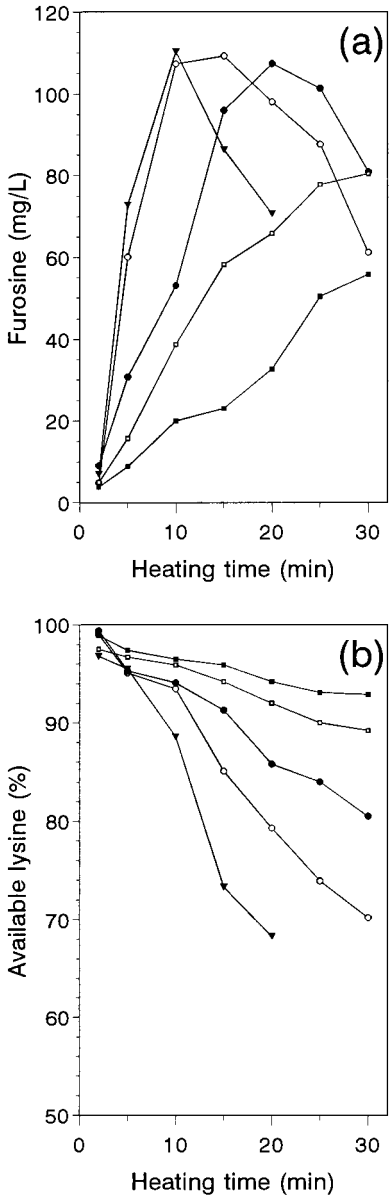

Figure 6. Formation of furosine (a) and loss of available lysine (b) in the lactose−caseinate model system heated at 110−150 °C for up to 30 min: 110 (■), 120 (□), 130 (●), 140 (○), and 150 °C (▼).

tion of a colored substructure protein-bound from a thermally treated model system of casein and furans has been confirmed by NMR, UV, and IR techniques (Hofmann, 1998). Therefore, decreases of b-HMF levels in samples heated at high temperatures are mainly due to degradation of LALY formed by the Maillard reaction, and secondly to reaction of bound HMF with other advanced Maillard products. In conclusion, it could be assumed that the loss of linearity with time of formation of b-HMF at high temperatures (Figure 5a) is explained by both statements (a) and (b).

A residual formation in the unheated sample of 41.8 pmol of bound HMF/g of protein (39.5−44.1, $n = 3$) was observed, which agrees with the finding of small levels of furosine, about 4 mg/100 mg of protein, in raw bulk milk and in lactose-free sodium caseinate solutions (Erbersdobler et al., 1984; Resmini et al., 1992). Finot et al. (1977) reported that the amount of lysyl residues bound to lactose in raw milk is about 2.5% of the total lysine present.

Our interest has also been focused on the formation of two unknown compounds during the analysis of b-HMF, designated as peak Y and peak B. In Figure 5b the evolution of peak Y as a function of temperature and time of heating is shown. Formation of peak Y necessitates more drastic conditions than formation of b-HMF, since at moderate temperatures peak Y was not detected.

Conclusion. The possibilities of the application of "bound" HMF, a minor compound from acidic degradation of LALY, as a reliable index of the extent of the Maillard reaction mainly in milk and milk-like systems has been enhanced. Gel filtration techniques as introduced by Morales et al. (1997) for obtaining a delactosed milk fraction are suitable for routine analysis of milk systems where whey proteins are not severely degraded (such as during thermization, pasteurization, or UHT sterilization at dairies). The procedure described is suitable for analysis of b-HMF in milk systems under more severe heating conditions. In conclusion, bound HMF determination could provide a sensitive and accurate means of measuring the amount of lysyl residues in milk protein that has condensed with lactose during thermal processing. Furosine and b-HMF are equivalent in the sense that both are indexes of the early stages of the Maillard reaction.

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