Chromatographic Determination of Bound Hydroxymethylfurfural as an Index of Milk Protein Glycosylation

Francisco J. Morales,* Carmen Romero, and Salvio Jiménez-Pérez

Departamento de Productos Lácteos, Instituto del Frío (CSIC), Ciudad Universitaria s/n, 28040 Madrid, Spain

5-(Hydroxymethyl)-2-furfuraldehyde (HMF) is formed upon heat treatment of milk and milk-resembling systems by the Maillard reaction, via its Amadori product, and also by isomerization and subsequent degradation of sugars. Traditionally, the HMF content has been used as an indicator of both degradation routes. A new analytical approach has been developed for determining the HMF formed only by the acidic degradation of Amadori products, called bound HMF, and that could be related to the extent of the Maillard reaction due to heat treatment or long-term storage of foods. Optimal conditions for the acidic digestion procedure were determined. Reversed-phase HPLC is applied for accurate measurement of bound HMF.

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

INTRODUCTION

The relatively high concentrations of lactose and lysine-rich proteins in milk make it especially sensitive to thermally induced nonenzymatic browning reactions. In the initial stage of the Maillard reaction, the ϵ-amino group of the essential protein-bound lysine reacts with the aldehyde group of lactose to form the Amadori product, lactulosyllysine (galactose−fructose−lysine, biologically not available) (O'Brien and Morrisey, 1989). Under acidic conditions, the amino group of lactulosyllysine is protonated, and 1,2-enolization through 3-deoxyosulose leads to 5-(hydroxymethyl)-2-furfuraldehyde (HMF). Besides, the acid-catalyzed degradation of lactose to HMF also proceeds via 3-deoxyosulose (Feather, 1970). These are the two main pathways of HMF formation when sugars are heated in solution: via the Amadori products of the Maillard reaction through enolization (in the presence of amino groups) and via lactose isomerization and degradation, known as the Lobry de Bruyn−Alberda van Ekenstein transformation (Ames, 1992).

The Maillard reaction is a complex network of reactions that occur during processing and storage of several foodstuffs. Several methods have been used for determining the extent to which it has progressed, such as the 2-thiobarbituric acid (TBA) method, periodate oxidation, borohydride reduction, and furosine and carboxymethyllysine determination (Furth, 1988). The TBA method has been widely applied in dairies. Keeney and Bassette (1959) developed a spectrophotometric method for determining HMF in dairy products using a TBA reaction product (after hydrolysis with oxalic acid). Since then, many works have appeared on the application of HMF as an index of heat treatments in milk products and foodstuffs. The main drawbacks of the method are the lack of specificity of TBA for HMF, since other aldehydes may take part in the reaction, and the limited stability of the colored complex (Morales et al., 1996). Recently, reversed-phase HPLC methods have been applied, since HMF has a high molar extinction coefficient at 280 nm (van Boekel and Zia-Ur-Rehman, 1987; Morales et al., 1992). Morales et al. (1996) compared the chromatographic and colorimetric methods for HMF determination and found that 71.6% of the amount of HMF measured by the colorimetric method is due to interferences. Hence, the TBA method is not a reliable measurement of HMF content, although it is still used as a quick, cheap measurement of the heat load of milk products.

This research has been supported by the European Community in a Fair program. The overall objective is to understand the factors and inter-relationships between factors that affect the flavor, color, nutritional quality, and toxicological safety of all foods that undergo the Maillard reaction during thermal processing and to obtain a better control via process optimization. On this basis, the development of an alternative analytical approach for protein glycosylation analysis is an important task.

Bound HMF determination has been applied for the determination of nonenzymatically glycosylated (glycated) proteins in such different fields as nutrition, medicine, and food technology. One goal is to develop a quick procedure for determination of bound HMF produced by degradation of lactulosyllysine (the Amadori product of the Maillard reaction) as an indirect measure of the extent of the Maillard reaction in milk proteins, without the interference of lactose degradation that occurs in the traditional method.

This is a preliminary study to establish the best operating conditions for bound HMF determination.

MATERIALS AND METHODS

Sample and Heat Treatments. Sodium caseinate (spray-dried, 92% protein content) was obtained from System Bio-Industries (Barcelona, Spain). Simulated milk solutions were made with 3% sodium caseinate and 5% lactose monohydrate (or glucose) dissolved in phosphate buffer (10 mM, pH 6.65). Sterilized (direct-UHT) skimmed milk (2.98% total protein, 4.8% lactose content, pH 6.62) was heated in stoppered test tubes in a boiling water bath for 3 h (sample SM-I) and 5 h (sample SM-II). Samples were cooled in an ice−water bath and analyzed. Samples were also heated in an oil bath in tightly stoppered stainless steel test tubes (120 × 7 mm length and diameter, respectively) at 110 and 120 °C for times up to 60 min.

* Author to whom correspondence should be addressed (telephone +34.1.549.23.00; fax +34.1.549.36.27; e-mail fjmorales@fresno.csic.es).

S0021-8561(96)00930-2 CCC: $14.00

Preparation of Sugar-Free Sample. The standard procedure for NAP-10 (Sephadex G-25) disposable column (1.3 × 2.6 cm) as described by Pharmacia (Uppsala, Sweden) was used. The cartridge was conditioned with 15 mL of distilled water. After that, 300 μL (~10 mg of protein) of well-mixed milk was loaded on the top of the cartridge with 700 μL of distilled water, and the eluate was discarded. Then 1.5 mL of distilled water was passed and the eluate recovered at ambient temperature into a test tube for subsequent analysis (Dr. T. Henle, Technical University of Munich, Germany, personal communication). Protein recovery was 90%.

Sugar Determination. Lactose, glucose, and galactose were analyzed by ion-exchange HPLC. Samples (1.5 mL) were deproteinized with 3.5 mL of 0.5 M perchloric acid solution. After 5 min at room temperature, the sample was centrifuged at 10 000 rpm for 5 min and the supernatant was filtered through a 0.45 μm acetate filter (13 mm, MSI Inc., Westboro, MA). Twenty microliters of sample was injected into an ION-300 polymeric resin column (300 × 7.8 mm, Interaction-Lab, San Jose, CA) at 50 °C. A sulfuric acid solution (0.22 mL of concentrated H_2SO_4/L) was used as eluent at 0.4 mL/min. Sugars were recorded with a refractive index detector (Erma Inc., Tokyo, Japan).

HMF Determination. Using the RP-HPLC method of Morales et al. (1992) with some minor modifications, 1 mL of milk (for total HMF, T-HMF) or delactosed milk (for bound HMF, B-HMF) was digested with 0.5 mL of 0.3 N oxalic acid solution (or other concentrations, 0.25−1.0 N) for 1 h at 100 °C. After a rapid cooling in ice, the mixtures were slowly deproteinized with 0.5 mL of trichloroacetic acid (TCA) solution (40%, w/v) and centrifuged at 10 000 rpm for 12 min. After filtration through a 0.45 μm filter, the sample was ready for HPLC analysis. For measuring the free HMF (F-HMF) content of the sample, the acid digestion step was skipped. A degassed mobile phase was prepared with sodium acetate buffer (0.08 M), and the pH was adjusted to 3.6 with acetic acid. A Spherisorb ODS-2 S5 analytical column (25 × 0.40 cm, Analytical Tracer, Barcelona, Spain) was used at room temperature. The injection volume was 20 μL for total HMF analysis and 40 μL for bound and free HMF analysis. Detection at 280 nm (0.1 AUFS sensitivity and 0.5 s response time) was selected. A Kontron Instruments (Milan, Italy) chromatographic system was used.

Total Protein Determination. Protein concentration was determined according to the protein dye-binding method of Bradford (1976), using a commercial preparation (Bio-Rad Laboratories, Hercules, CA). Calibration curves (2−25 μg of protein/mL) were made with skimmed milk and sodium caseinate solutions, both checked by Kjeldahl analysis (IDF, 1986).

Statistical Analysis. All samples were analyzed at least in duplicate. Data analysis was carried out with Student's test applying Statgraphic plus v. 1.0 software (Statistical Graphics Corp., Rockwille, MD).

RESULTS AND DISCUSSION

The use of HMF as a specific index of the extent of the Maillard reaction has been limited due to the presence of lactose in the reaction medium. The acidic digestion of the sample in boiling acid promotes the formation of HMF from its precursors via (a) degradation of Amadori compounds (mainly lactulosyllysine) through 1,2-enolization and (b) acid-catalyzed degradation of lactose (Feather and Harris, 1973; Anet, 1964). The HMF itself is acid-labile, and though over 80% of protein-bound radiolabeled glucose can be released as HMF under optimum conditions, in practice yields are often <10% (Yaylayan and Huyghes-Despointes, 1994). Futhermore, Berg and van Boekel (1984) found that in milk and at temperatures lower than 130 °C isomerization and subsequent degradation of lactose are quantitatively more important than the Maillard reaction.

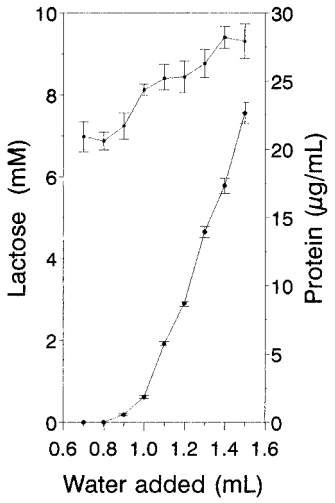

Figure 1. Amounts (with error bars) of lactose (mM) and total protein (μg/mL) recovered from 300 μL of fresh skimmed milk loaded onto a gel filtration NAP-10 column using different elution volumes.

In milk products the 1,2-enolization pathway is not as important as in mildly acid food (e.g. fruit juices), due to the higher pH of the former. Therefore, T-HMF (free + bound + precursors) detected in variable amounts (1.30 ± 0.23 μmol/L, $n = 54$) in raw bulk milk is an artifact generated by the acidic digestion, since unheated milk should not have HMF (Morales et al., 1996). The obtention of a sugar-free solution after gel filtration of the sample could be used to determine the so-called bound HMF, produced exclusively by decomposition of the Amadori product. B-HMF is an indirect measurement of lactulosyllysine in the glycated protein (nonenzymatic glycosylation) and therefore an index of protein quality after a heat treatment.

Obtention of a Sugar-Free Sample. One of the main objectives was to obtain a delactosed sample. In a preliminary step, the most appropriate conditions for using the NAP column with milk or milk-resembling systems (sodium caseinate plus lactose) were established. Following the standard protocol, 300 μL of sample (milk or simulated milk solution, containing about 15 mg of lactose) loaded into the NAP-10 cartridge was not lactose-free when the sample was recovered with 1.5 mL of water. A similar result was obtained with 200 μL of sample (containing about 10 mg of lactose). We observed that the residual lactose appeared in the drops eluted last, after most of the protein had been already came out (data not shown). Hence, the elution volumes were varied between 1.5 and 0.7 mL.

The results with skimmed milk are shown in Figure 1. The residual amount of lactose in the solution after the gel permeation stage decreases linearly with decreasing elution volume. The presence of lactose was not significant (chromatographic peak area was less than twice the basal noise) with an elution volume of 0.8 mL, but 0.7 mL was chosen for safety (Figure 2). Obviously, the recovery of total protein (mainly casein) decreased linearly with decreasing elution volume. Figure 1 shows the concentration of total protein (dilution factors were taken) for different elution volumes. All of the samples were set up to a final volume of 1.5 mL to simplify comparison of the results. The average recovery of protein in the sample eluted with 0.7 mL was 87.6%, as obtained by Kjeldahl analysis, and this value was used for the following analyses. The delac-

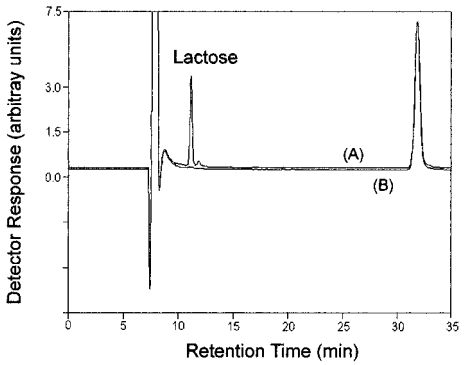

Figure 2. HPLC profile from sugar elution of sample eluted with 1.5 mL (7.5 mM lactose, A) and 0.7 mL (B) of distilled water.

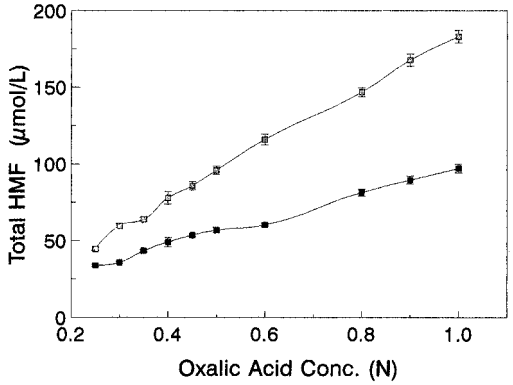

Figure 3. Effect of oxalic acid concentration on total HMF (μmol/L) formation during acidic digestion (1 h in a boiling water bath) of two preheated skimmed milk samples: SM-I (■); SM-II (□).

tosed samples were stable for analysis during at least 4 days under refrigeration, no precipitation being observed.

Optimum Conditions for Acidic Digestion of Milk. The Amadori compound is relatively stable under mild conditions, and the Maillard reaction in milk practically stops at this stage as long as heating is not drastic or the reaction time not very long (Finot and Mauron, 1972). The release of HMF from protein-bound lactulosyllysine is promoted by acidic conditions, the rate depending on the concentration and type of acid. Traditionally, oxalic acid has been used to promote the Maillard reaction through 1,2-enolization followed by elimination of the hydroxyl group at C-3 and deamination at C-1 of the Amadori products yielding 3-deoxyosones, HMF, and furfural; acetic acid (2 M) is also optimal for the release of HMF from N-substituted fructoseamines (Gottschalk, 1952; van Boekel and Zia-Ur-Rehman, 1987). However, HMF is just an intermediary of the reaction, which can also be degraded to other end products (e.g. formic acid, levulinic acid) or condensed into brown polymers (Ames, 1992).

To measure bound HMF in heated milk samples, 0.7 mL of sugar-free sample was made up to 1 mL with distilled water and digested with 0.5 mL of variable concentrations (0.25−1.0 N) of oxalic acid. The strength of the acid solution was tested on two independent samples of milk heated under controlled conditions. Figure 3 shows the formation of total HMF by different concentrations of oxalic acid for two different initial heat pretreatments (SM-I and SM-II). As expected, the amount of total HMF increases gradually, since the reaction medium contains enough lactose and the degradation of formed HMF was not important. Figure

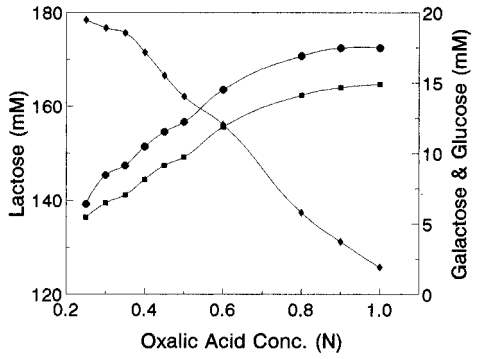

Figure 4. Variation in sugar content (lactose, galactose, and glucose; mM) during acid-catalyzed release of total HMF in sample SM-II: lactose (◆), galactose (●), glucose (■).

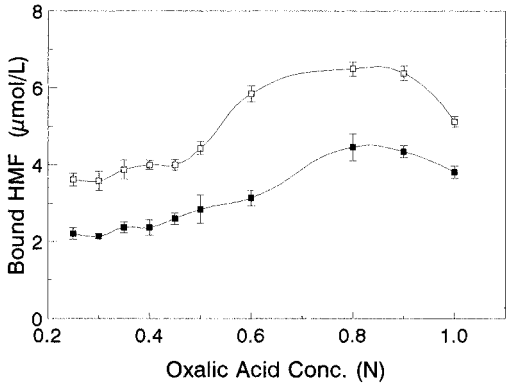

Figure 5. Effect of oxalic acid concentration on the formation of bound HMF released from lactulosyllysine in samples SM-I (■) and SM-II (□).

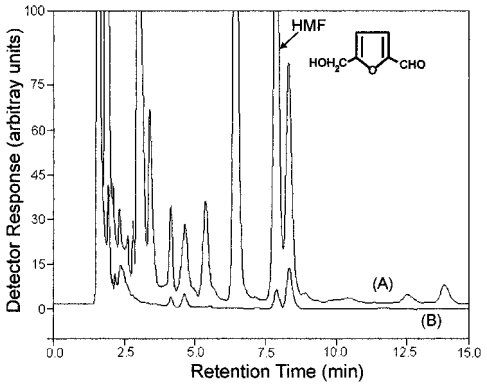

Figure 6. Reversed-phase HPLC chromatogram obtained from total (A) and bound HMF (B) determination.

4 shows that the acidic degradation of lactose and the subsequent formation of glucose and galactose increase linearly with the oxalic acid concentration. An equimolar formation of glucose and galactose from lactose degradation is not observed. Possibly, glucose can contribute to the formation of HMF, as does lactose. When a sugar-free sample is heated under acidic conditions to promote the lactulosyllysine degradation through HMF formation, the results are different (Figure 5). The formation of bound HMF increases linearly until it reaches a maximum at around 0.8 N oxalic acid, after which it decreases in comparison with Figure 4. The levels of B-HMF are always lower than those of T-HMF. These findings show that the acidic degradation of sugar is the main route for T-HMF formation, the Maillard reaction being a minor route. Figure 6 shows a typical chromatogram of T- and B-HMF.

Determination of Bound HMF (B-HMF). Two

Table 1. Formation of Total HMF (T-HMF), Free HMF (F-HMF), and Bound HMF (B-HMF) in Skimmed Milk (M) and Glucose (5%, w/v) plus Sodium Caseinate (3%, w/v) Model System (GC) Heated in an Oil Bath at 110 °C for 30 min (1) or for 60 min (2) and at 120 °C for 30 min (3) or for 60 min (4)[a]

	T-HMF	F-HMF	B-HMF
M-1	42.36 ± 2.44	1.15 ± 0.11	1.34 ± 0.07
M-2	64.25 ± 0.30	2.86 ± 0.00	1.77 ± 0.06
M-3	72.76 ± 2.10	4.72 ± 0.49	1.93 ± 0.14
M-4	124.28 ± 1.68	5.82 ± 0.40	2.40 ± 0.20
GC-1	218.47 ± 1.64	2.41 ± 0.01	11.79 ± 0.23
GC-2	296.09 ± 3.57	3.79 ± 0.11	12.31 ± 0.19
GC-3	293.88 ± 0.84	4.23 ± 0.07	11.63 ± 0.12
GC-4	388.04 ± 2.70	13.00 ± 0.53	8.53 ± 0.65

[a]Concentrations are expressed as μmol/L with the standard deviation for three independent trials.

samples, skimmed milk and a glucose plus sodium caseinate system, were heated in an oil bath at different controlled conditions of temperature and time. Table 1 shows the amounts of total, free, and bound HMF found in each system. To determine bound and free HMF, 40 μL of filtered sample was injected into the HPLC instead of the 20 μL used for T-HMF analysis. The HMF formation (total, free, and bound) in both samples increased with the extent of heating. Glucose promotes more efficiently the Maillard reaction and the acidic degradation of sugars, since higher values of HMF were found in all samples. Most of the T-HMF came from sugar degradation, which again illustrates the Maillard reaction as a minor route for its formation. The formation of B-HMF is of the same order as that of F-HMF in unheated milk and lower in heated milk.

It has been established that lactulosyllysine formation is related to the heating conditions [e.g. Berg and van Boekel (1994)], since it degrades under severe heating conditions. This effect can be observed in the GC system heated at 120 °C for 60 min, for which the amount of B-HMF is lower than after heating at 120 °C for 30 min.

Conclusion. HMF is a well-known intermediary of the 1,2-enolization route of decomposition of Amadori rearrangement products. The acidic degradation (by boiling with oxalic acid) of lactose during analysis promotes the formation of HMF. A new approach has been developed for the direct determination of so-called bound HMF from Amadori product degradation which gives reliable information on the extent of the Maillard reaction. Since the traditional HMF value is not a precise measurement of the extent of the Maillard reaction in milk and milk products, B-HMF might be a useful, sensitive indicator of the Maillard reaction in milk and milk-resembling systems or generally in foods subjected to heat treatment or long-term storage. The proposed method offers several benefits since it does not require dialysis of samples, only small amounts of sample are needed, and the time necessary for analysis is short. A kinetic study of B-HMF formation will be the object of future research.

ACKNOWLEDGMENT

We are indebted to Miss Dolores Gómez for technical assistance.

LITERATURE CITED

Ames, J. M. The Maillard reaction. In *Biochemistry of Food Proteins*; Hudson, B. J. F., Ed.; Elsevier: London, 1992; pp 99−153.

Anet, E. F. L. J. 3-Deoxy-glycosuloses (3-deoxyglycosones) and the degradation of carbohydrates. *Adv. Carbohydr. Chem.* **1964**, *19*, 181−218.

Berg, H. E.; Boekel, M. A. J. S., van. Degradation of lactose during heating of milk. I. Reaction pathways. *Neth. Milk Dairy J.* **1994**, *48*, 157−175.

Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Feather, M. S. The conversion of D-xylose and D-glucoronic acid to 2-furaldehyde. *Tetrahedron Lett.* **1970**, *48*, 4143−4145.

Feather, M. S.; Harris, J. F. Dehydration reactions of carbohydrates. *Adv. Carbohydr. Chem.* **1973**, *28*, 161-224.

Finot, P. A.; Mauron, J. Le blocage de la lysine pour la reaction de Maillard: II- Proprites chimiques des derives *N*-(desoxy-1-D-fructosyl-1) et *N*-(deoxy-1-lactulosyl-1) de la lysine. *Helv. Chim. Acta* **1972**, *55*, 1153−1164.

Furth, A. J. Methods of assaying nonenzymatic glycosylation. *Anal. Biochem.* **1988**, *175*, 347−360.

Gottschalk, A. Some biochemically revelant properties of N-subtituted fructosamines derived from N-glucosylamino-acids and N-arylglucosylamines. *Biochem. J.* **1952**, *52*, 455-460.

IDF 20A/86. Standard method for determination of nitrogen content in milk. International Dairy Federation: Brussels, 1986.

Keeney, M.; Bassette, R. Detection of intermediate compounds in the early stages of browning reaction in milk products. *J. Dairy Sci.* **1959**, *42*, 945−960.

Morales, F. J.; Romero, C.; Jiménez-Pérez, S. An enhanced liquid chromatographic method for 5-hydroxymethylfurfural determination in UHT milk. *Chromatographia* **1992**, *33*, 45−48.

Morales, F. J.; Romero, C.; Jiménez-Pérez, S. Study on 5-hydroxymethylfurfural formation in milk during UHT treatment measured by two analytical procedures. In *Heat Treatments and Alternative Methods*; International Dairy Federation: Brussels, 1996; pp 354−357.

O'Brien, J. M.; Morrisey P. A. The Maillard reaction in milk products. *Bull. Int. Dairy Fed.* **1989**, *238*, 53−61.

van Boekel, M. A. J. S.; Zia-Ur-Rehman. Determination of HMF in heated milk by HPLC. *Neth. Milk Dairy J.* **1987**, *41*, 297−306.

Yaylayan, V. A.; Huyghues-Despointes, A. Chemistry of Amadori rearrangement products: Analysis, synthesis, kinetic reactions and spectroscopic properties. *CRC Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 321−369.

Received for review December 11, 1996. Revised manuscript received February 17, 1997. Accepted February 21, 1997.[⊗] Research supported by the European Community, Project FAIR-CT-96-1080.

JF960930V

[⊗] Abstract published in *Advance ACS Abstracts*, April 1, 1997.