A Comparison of Color Formation and Maillard Reaction Products of a Lactose−Lysine and Lactose−N^{α}-Acetyllysine Model System

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The formation of color and Maillard reaction products in two model systems consisting of lactose and lysine or N^{α}-acetyllysine has been investigated. During heating, the blockage of the N^{α} group of lysine determined a faster color and antioxidative ability development compared to the system with free lysine. This is combined to a greater amount of melanoidin formation in the acetylated lysine system, while in the free lysine system a higher amount of pyrraline and hydroxymethyl furfural were detected. The pattern of low molecular weight products suggests that 3-deoxyglucosone and 1-deoxyglucosone degradation pathways are favored for free lysine and N^{α}-acetyllysine, respectively. Whole data allow us to hypothesize that in a lactose−N^{α}-acetyllysine model system the formation of colored high molecular weight polymer proceeds faster because less material is dispersed in reaction pathways, mainly the Strecker degradation, which leads to small and intermediate molecular weight products.

Keywords: *Maillard reaction; lactose; N^{α}-acetyllysine; antioxidative efficiency; melanoidins; color; LC−MS*

INTRODUCTION

The Maillard reaction (MR) between reducing sugars and compounds bearing free amino groups is very important to food processing companies because it strongly influences the quality of the final product. During milk processing, the main effect is the loss of nutritional value due to blockage of the ϵ-amino group of lysine residues (van Boekel, 1998). Severe heating leads to food browning with formation of several colored and flavor-generating compounds that also have antioxidative, mutagenic, and antimutagenic properties (Bressa et al., 1996; Nicoli et al., 1997; Alaiz et al., 1997; Berg et al., 1990; Skog et al., 1998; Yen and Tsai, 1993).

Compounds formed by MR of lactose with different amines were described by Pischetsrieder and Severin (1996). Some disaccharide-specific compounds such as galactosylisomaltol and β-pyranone are formed upon prolonged heating (Kramhöller et al., 1993). Recently, Pischetsrieder and co-workers (1998) report that during a short heating time the aminoreductone 1-alkylamino-1,2-dehydro-4-deoxy-3-hexulose is formed. At the same time, several well-known MR products (MRPs) typical of monosaccharides such as pyrraline and hydroxymethyl furfural (HMF) are formed. The quantification of some of them as markers of thermal treatment has been useful, and it has been attempted by several researchers (for a review, see van Boekel, 1998).

The typical brown color formed by MR is due to chromophores, which have been widely studied in different model systems. In a casein−sugar model system, it is established that color formation is mainly due to the formation of protein oligomers mediated by chromophoric substructures derived from carbohydrates (Hofmann, 1998c,d). In a gluten−glucose model system, it was found that color was mainly due to low molecular weight compounds formed by the ammonia released from glutamine (Izzo and Ho, 1993; Fogliano et al., 1999). In an amino acid sugar model system, the situation is even more complicated. Different hypotheses evoking the polymerization of low molecular weight unit have been put forward (Wedzicha and Kaputo, 1987; Cämmerer and Kroh, 1995; Bailey et al., 1996; Yaylayan and Kaminsky, 1998; Tressl et al., 1998). On the other hand, Hofmann (1998a) reported that when a glucose−alanine solution was heated for 4 h at 95 °C only trace amounts of compounds with molecular weight higher than 3000 Da were formed and that color was almost exclusively due to the low molecular weight fraction. The high molecular weight fraction was also mainly responsible for the antioxidative ability, which a *plethora* of studies ascribed to the Maillard reaction products. This activity is likely due to the metal-chelating ability of this fraction (Wijewickreme et al., 1997).

In the present investigation, we compare lactose−lysine (LL) and lactose−N^{α}-acetyllysine (LLa) model systems, mainly focusing on the contribution to color given by fractions at different molecular range. The antioxidative efficiency of high molecular weight fractions was compared. In addition, the investigation by LC−MS of low molecular weight MRPs formed in the two model systems is shown.

MATERIALS AND METHODS

Materials. D-(+)-Lactose, L-lysine, and N^{α}-acetyllysine were of analytical grade and purchased from Fluka (Fluka Chemie AG, CH). HPLC-grade methanol and water were from Merck (Germany) and filtered through disposable 0.2 μm filters from Acrodisc (Gelman Sciences). Double dialysis was performed with tubes with a cutoff of 3 500 and 10 000 Da (Spectra/Por USA). Absorbance was measured using a UV−vis 2100 Shimadzu (Japan) spectrophotometer.

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10.1021/jf990652a CCC: $19.00
Published on Web 03/07/2000

Preparation of the Model System. N^{α}-Acetyllysine lysine monohydrochloride (5 mmol) and lactose (5 mmol) were dissolved in distilled water (5 mL) in a 50 mL Quickfit round-bottom two-necked flask, equipped with a double-coil water condenser, a lid, and a magnetic stirrer bar. The solution was refluxed in a silicon oil bath for up to 4 h. Samples were collected after each hour and cooled in crushed ice before analysis. The same experiment was also performed in buffered conditions dissolving the reagents in 5 mL of a 0.2 M acetate buffer pH 5.25.

Separation in Different Molecular Weight Fractions. After 4 h reflux, the solution was separated by double dialysis into three fractions: a low molecular weight fraction of less than 3 500 Da (LMW); an intermediate molecular weight fraction (IMW) between 3500−10000 Da; and a fraction containing all high molecular weights (HMW), greater than 10000 Da. A tube of 10000 Da cutoff was filled with lactose−lysine solution and introduced into a larger tube of 3500 Da cutoff added with water. The whole system was introduced into a 5 L flask and dialyzed against water for 3 days at 4 °C under stirring.

Antioxidative Efficiency (AE). The radical scavenging properties of MR mixture were determined according to the method of Pryor et al. (1993) as previously described (Monti et al., 1999) using hexadecyltrimethylammonium bromide (HDTBr) as detergent and 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) as radical initiator. Results were compared with a reference curve obtained measuring AE of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

Determination of Color Dilution (CD_{total}) Factors. To determinate browning intensity of MR mixture, a color dilution factor was defined according to Hofmann (1998b). The freeze-dried material of different molecular weight fractions were dissolved in 5 mL of water and diluted 1:1 by volume until the color difference between the sample and two blanks of tap water could visually be detected in a triangle test. All samples were put in glass vial with 13 mm of diameter.

HPLC Analysis. After heating, the total reaction products were analyzed on a Shimadzu (Japan) Class M10 HPLC system fitted with a Rheodyne injector (20−200 μL loop) and equipped with a Shimadzu SDA-M10A diode array detection (190−600 nm). Data were processed by Shimadzu software M10 HPLC.

The chromatographic separation was achieved on a ODS-(2) Primesphere 5 μm 110 Å, 4.6 mm i.d. × 25 cm column (Phenomenex). The column was equilibrated in 95% phase A (water) and 5% phase B (methanol). Elution was obtained by increasing a linear gradient as follows: time 0 min A 95%, B 5%; time 30 min A 65% B 35%; time 32 min A 0% B 100%; time 37 min A 0% B 100%; time 39 min A 95% B 5%; time 44 min A 95% B 5%. For cleaning the column from material not eluted under our chromatographic conditions, washing with absolute methanol was necessary (Bailey et al., 1996). Raw data were collected from 190 to 600 nm.

HPLC−Mass Spectrometry Analysis. HPLC separation was achieved using the same column and the same solvent gradient described for the analytical separations. Formic acid (2 mM) was added in solvent A to favor sample ionization in the positive-ion mode. A Perkin-Elmer LC series 200 connected to a 785A UV−vis detector and coupled with an API-100 single quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Canada) was used. A flow rate of 40 μL min^{-1} was split from the LC eluent into the ion spray ion source. A probe voltage of 4700 V and a declustering potential of 50 V were used. The instrument mass-to-charge ratio scale was calibrated with ions of ammonium adduct of polypropylene glycol. Full-scan spectra were acquired from 100 to 600 amu using a step size of 0.5 amu and a dwell time of 4.2 ms.

RESULTS AND DISCUSSION

Melanoidin Formation. During heating of the two model systems, a pH drop of about one unit after 4 h was observed. The reaction was accompanied in both cases by brown color formation monitored at 460 nm. As shown in Figure 1 browning formation is faster in the LLa system. The system with free lysine reached after 4 h the absorbance values that LLa system had after two. As the pH intervals during the reaction were slightly different for the two systems, the heating experiments were also performed in buffer at pH 5.25. The color formation was very similar to that obtained using the nonbuffered conditions. It is known that in heated sugar-amino acid systems the browning of the color can be due to low molecular weight colored compounds and to high molecular weight conjugated chromophores, commonly referred as melanoidins (Hodge, 1953; Ledl and Severin, 1978; Nursten and O'Reilly, 1983, 1986). To investigate the influence to color formation of free NH_2^{ϵ} and NH_2^{ϵ} $-NH_2^{\alpha}$ amino groups of lysine (N^{α}-acetyllysine, and lysine) and to evaluate the contribution of low and high molecular weight material to the overall color of the solutions, the reaction mixture of the two systems after 4 h of heating was ranged into three fractions by double dialysis as described under Materials and Methods.

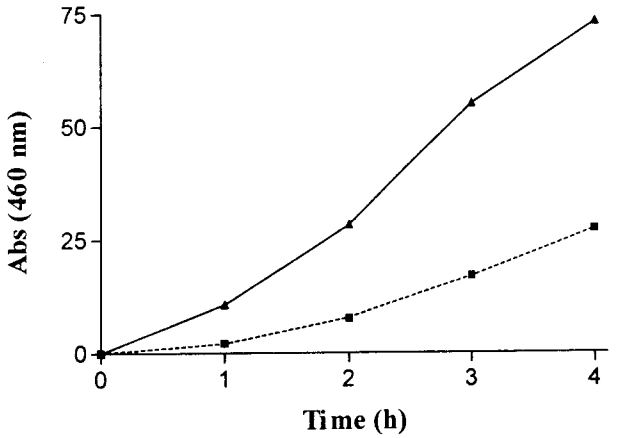

Figure 1. Browning at 460 nm of LL (dotted line) and LLa (straight line) model systems. Corrected absorbance × dilution values; data are means of three replicates.

Table 1. Color Dilution (CD) Factor of Total Mixtures of LL and LLa Model Systems and of Their Different Molecular Weight Fractions (Amount (mg and %) and Antioxidative Efficiency (AE) (μg/mL of Trolox) of HMW Fractions Are Also Reported)[a]

model system (4 h heating)	CD				wt of HMW (mg)	amt of HMW (%)	AE (μg/mL of Trolox)
	total mixture	HMW	IMW	LMW			
LL	5000	200	100	800	22	0.7	0.04
LLa	10 000	1800	200	800	100	3.5	0.13

[a] Values are means of triplicates.

Browning intensity in each fraction was estimated using the color dilution (CD) factor as described by Hofmann (1998b). Results summarized in Table 1 show that in the system containing N^{α}-acetyllysine the main contribution of color is due to the HMW fraction, while in that with free lysine the contribution of high molecular weight fraction is negligible, LMW fraction being the main responsible for color. The amount of melanoidins formed in LLa system is 3.5% of weight of the starting material compared to 0.7% of LL system. As the melanoidins contribution to the total color of the system containing N^{α}-acetylated lysine is relevant, this system behaving more similarly to a protein−carbohydrate system than to an amino acid- carbohydrates

Table 2. Color Dilution (CD) Factor and Amount of HMW Fractions of LL and LLa Model Systems after 1, 2, 3, and 4 h of Heating[a]

	HMW LL			HMW LLa		
time (h)	CD	weight (mg)	amount (%)	CD	weight (mg)	amount (%)
1	2	0.6	0.02	100	45	1.6
2	10	3.6	0.12	200	56	2.0
3	20	4.8	0.17	200	68	2.4
4	200	22	0.70	1800	100	3.5

[a] Values are means of triplicates.

system (Hofmann, 1998a). This result confirms that the formation of HMW colored melanoidins can also occur during MR of sugar and amino acid by polymerization of LMW intermediates. Likely, the formation of HMW melanoidins is not exclusively due to protein-bound chromophores. The formation of HMW compounds was monitored during the 4 h of heating; results are reported in Table 2 where the greater formation of melanoidins of LLa system since the first hour of heating is evident.

To further characterize the two systems, the AE of the brown solutions was investigated by measuring their ability in inhibiting the linoleic acid peroxidation in a micellar system (Pryor et al., 1993). The antioxidative efficiency (AE) is more than 3-fold higher in the system containing N^{α}-acetyllysine. This is in good agreement with previous results which showed that the AE of the brown solution formed by MR was mainly due to melanoidins (Monti et al., 1999).

HPLC−MS Analysis of Low Molecular Weight Fractions. ^{1}H NMR and LC−MS allowed us the identification of the main peaks present in RP-HPLC chromatogram obtained from a LL model system. In Figure 2 the comparison of the chromatograms at 280 nm of the two model systems is reported. The profile of the LLa system is more complex than that of LL where only three major peaks are present and identified by LC−MS as galactosylisomaltol, HMF, and pyrraline (Monti et al., 1999). UV and MS spectra allowed the identification of HMF and acetylated pyrraline in the acetylated system. These two molecules are present in a rather poor way in this system compared to the huge amounts detected in the free-lysine system. The two Amadori products, N^{α}-acetyllactulosyllysine and lactulosyllysine, respectively, were abundant in the two systems and eluted at the void volume of the column.

Mass spectra suggest that in the acetylated system several pyranone, furanones, pyridones, and pyrrolinones are present. In particular, a compound eluted at 16.50 min with a molecular ion $[MH]^+$ at 315.0 uma and Na^+ and K^+ adducts at 337.2 and 353.3 uma, respectively, was evident (Figure 3A). This pattern is compatible with the structure of 2-acetylamino-6-(2,4dihydroxy-2,5-dimethyl-3-oxo-2,3-dihydropyrrol-1-yl)hexanoic acid, a compound that is formed along the 1-DG pathway (Ledl, 1990). A small amount of a compound with a $[MH]^+$ of 477.0 uma was detected that could be likely due to the same compound mentioned above but with the galactose moiety still attached: 2-acetylamino-6-(2,5-dimethyl-2-hydroxy-4-*O*-galactosyl-3-oxo-2,3-dihydropyrrol-1-yl)hexanoic acid (Figure 3B) (Ledl, 1990). Finally, a compound with a $[MH]^+$ of 467.0 uma and its adducts with water and potassium at 485.5 and 506.5 uma, respectively, was detected at 12.06 min retention time. This molecule was referred to as 2-acetylamino-

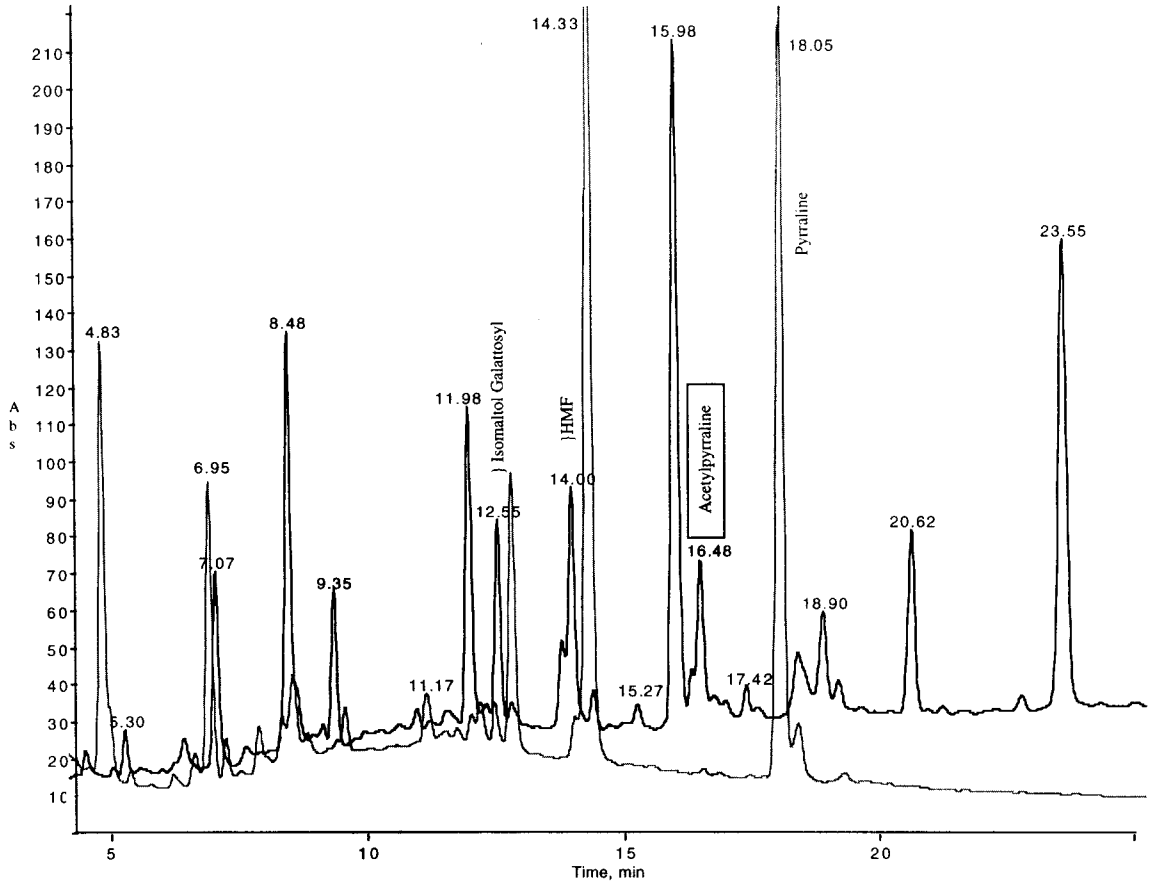

Figure 2. RP-HPLC chromatograms monitored at 280 nm of LL (lighter line) and LLa (heavier line) model systems.

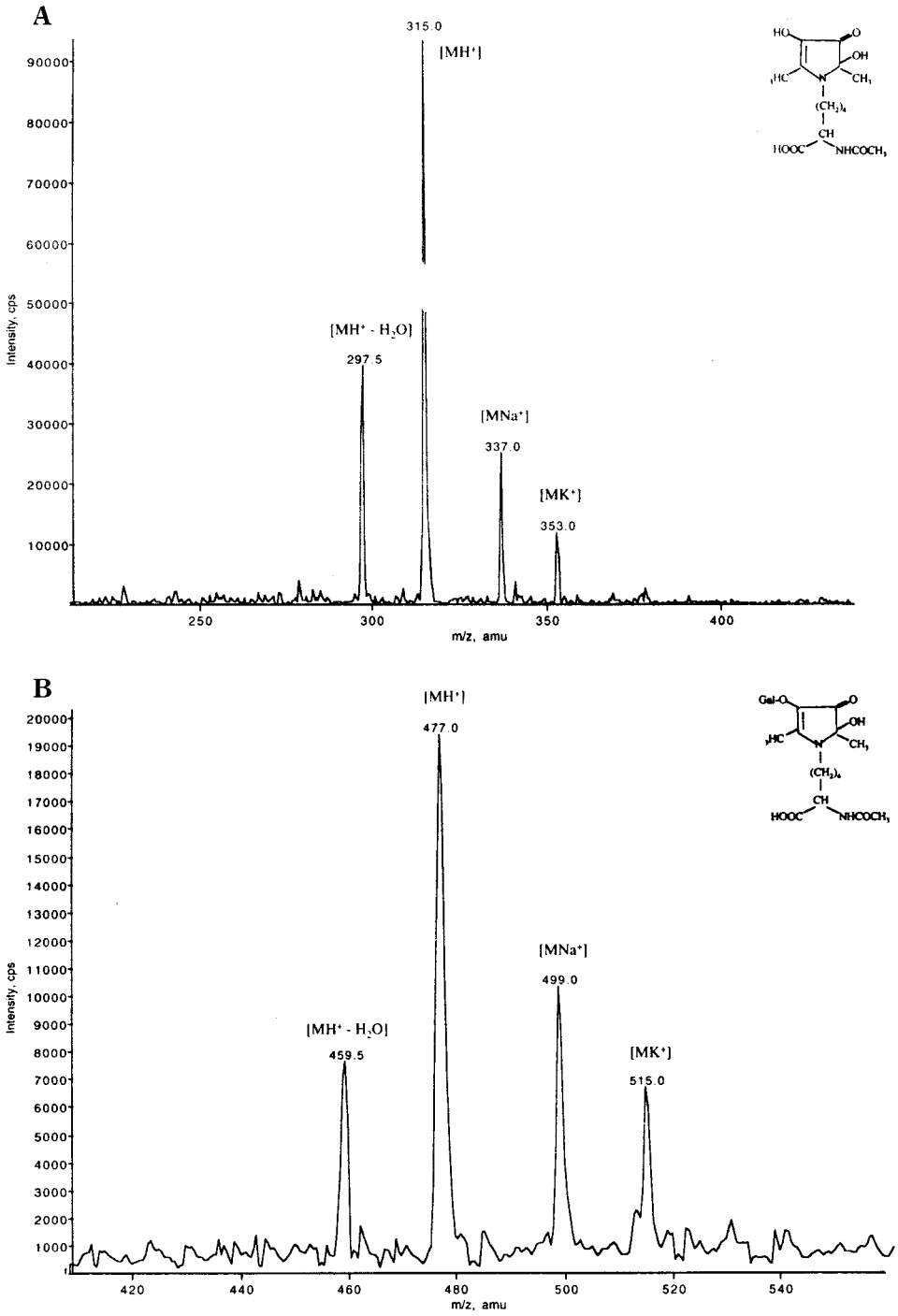

Figure 3. Electrospray mass spectra of tentatively assigned 2-acetylamino-6-(2,4dihydroxy-2,5-dimethyl-3-oxo-2,3- dihydropyrrol-1-yl)hexanoic acid (A) and 2-acetylamino-6-(2,5-dimethyl-2-hydroxy-4-*O*-galactosyl-3-oxo-2,3-dihydropyrrol-1-yl)hexanoic acid (B).

6-[3-hydroxy-2-methyl-4-(2-acetylamino)carboxyhexylimino-4*H*-pyrydin-l-yl]hexanoic acid (Figure 4) according to Pischetsrieder and Severin (1996). Thus, LC−MS analysis of LMW compounds formed in the two systems shows evidence that free lysine model system leads to formation of compounds deriving from the 3-DG pathway, whereas 1-DG pathway is preferentially followed by N^{α}-acetylated lysine system. This is consistent with the pH of the model system, which is slightly higher in the latter case (Pischetsrieder and Severin, 1996).

Hwang et al. (1994), studying the involvement of N^{α}- and N^{ϵ}-amino group of lysine in pyrazine formation, reported that the N^{ϵ} group reacts with minor efficiency with α-dicarbonyl compounds and that the formation of pyrazines through MR on this group is slower compared to the N^{α}-amino group. The rationale for this evidence is that β-decarboxylation in Strecker degradation assists α-amines to generate α-aminoketones, this explaining the greater contribution to pyrazine production of α-amino groups of lysines. On the other hand, α-aminoketones from ϵ-amino groups are formed by an intramolecular rearrangement of Schiff bases and subsequent hydration reaction. These α-aminoketones can further react with each other to form pyrazines. This situation occurs in the model system containing N^{α}-acetylated lysine where the Strecker degradation pathway, which leads to the formation of several LMW compounds and to a slow formation of HMW melanoidins is hampered. It is likely that in the N^{α}-blocked

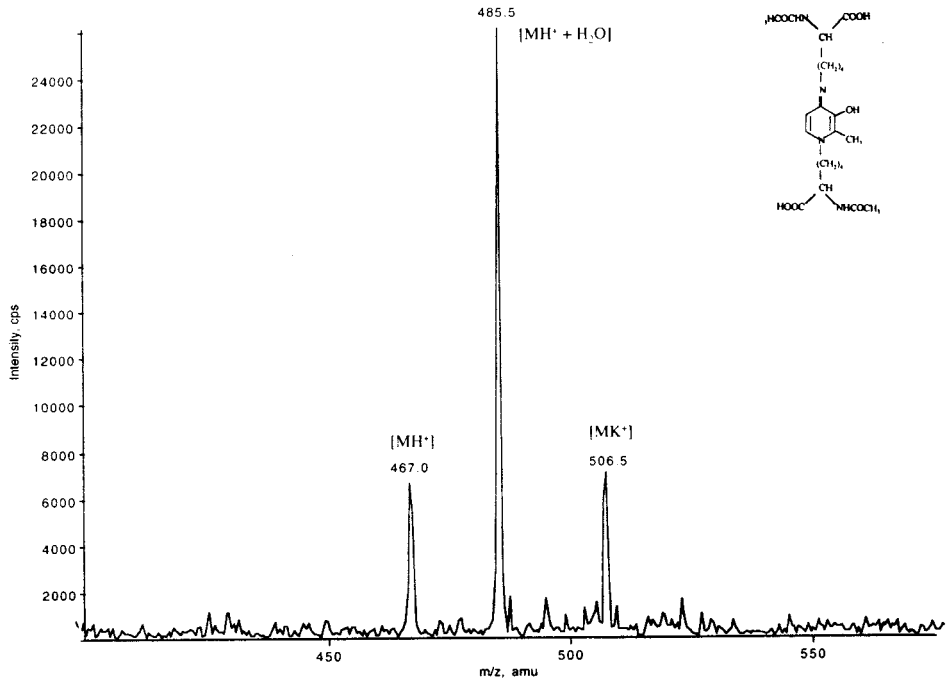

Figure 4. Electrospray mass spectrum of tentatively assigned 2-acetylamino-6-[3-hydroxy-2-methyl-4-(2-acetylamino)carboxyhexylimino-4*H*-pyridin-1-yl]hexanoic acid.

lysine model system a weak competition for the reaction on the ϵ-amino group occurs, leading more material into HMW melanoidins formation.

The results here presented suggest that the impact of the different molecular weight compounds in food color could be strongly dependent on the source of amino group. When free amino acids or free ammonia, as occur in gluten (Fogliano et al., 1999), are present, the contribution of low molecular weight colored compound will tower above the high molecular weight compounds. As in many foods, melanoidins formation mainly involves the ϵ-NH_2 of protein-bound lysine the results obtained with the N^{α}-acetyl lysine model system (more color and more antioxidative ability respect to the free lysine) can better resembling of those occurring in these foods. The deepening of our knowledge of the melanoidin structure is required by their pivotal role in color and antioxidant ability of the whole system. Additionally, they can bind volatile compounds, thus influencing the overall food aroma. Unfortunately, up to now, only hypotheses are available on the molecular structure of the HMW polymers formed from sugar amino acid solution (Tressl et al., 1998; Cämmerer and Kroh, 1995; Yayalan and Kaminsky, 1998), while the structure of some chromophores present in protein-involving melanoidins have been elucidated (Hofmann, 1998a,d). Additional work is necessary to link melanoidins structure to their properties.

ABBREVIATIONS USED

AE, antioxidative efficiency; LC−MS, liquid chromatography−mass spectrometry; HMW, IMW, LMW, high, intermediate, and low molecular weight; LL, lactose−lysine; LLa, lactose−N^{α}-acetyllysine.

ACKNOWLEDGMENT

We are grateful to Prof. M. Tingoli and Dr. L. Rossi of the University of L'Aquila for the helpful discussions.

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Received for review June 18, 1999. Revised manuscript received December 30, 1999. Accepted January 3, 2000. This work was supported by the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific program, CT96-1080, "Optimization of the Maillard reaction. A way to improve quality and safety of thermally processed food".

JF990652A