LC/MS Analysis and Antioxidative Efficiency of Maillard Reaction Products from a Lactose−Lysine Model System

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Aqueous solutions of lactose and lysine were refluxed for up to 4 h without pH control. Samples were collected every hour, and the reaction was monitored by measuring the pH, the optical density at 420 nm, and the relative antioxidative efficiency (RAE). The greatest change in optical density and antioxidative efficiency occurred for the mixture heated for 4 h. The 4 h solution was separated into three fractions according to the molecular weights of the components and tested for RAE. The high molecular weight fraction was more colored, and it had the highest antioxidative activity. The low molecular weight fraction was separated by high-performance liquid chromatography (HPLC). RAE values were measured for each purified compound. HPLC coupled with diode array and electrospray mass spectrometry allowed a rapid screening of the solutions and a tentative identification of several peaks. Nuclear magnetic resonance analysis allowed the identification of galactosylisomaltol and pyrraline. The resonance assignments for these compounds were revised.

Keywords: *Maillard reaction; lactose; lysine; antioxidative efficiency; pyrraline; galactosylisomaltol; HPLC/MS*

INTRODUCTION

Because of its importance in determining color and flavor properties of foods, the Maillard reaction (MR) plays a central role in food chemistry (Feather, 1989; Ames, 1990; Bailey et al., 1995; Arnoldi et al., 1997). Previously, this reaction was investigated by studying the reaction of several sugars with amino acids and proteins (O'Brien and Morissey, 1989; Namiki, 1988; Friedman, 1996). To investigate the chemistry of the different stages of MR and the effects of heat treatments in real foods, a valuable tool is to monitor the formation of MR products (MRPs) in model systems. When MR is undesired, as is the case with several milk-based products (Erbersdobler and Anderson, 1983; Resmini and Pellegrino, 1991), this approach allows the identification of markers of heat treatments.

Lactose, a reducing disaccharide abundant in milk, reacts extensively with the ϵ-amino group of lysines through MR. As a result, a marked off-flavor develops accompanied by changes in color and/or stability. MRPs obtained from disaccharides have been studied by the group of Ledl and Severin (Ledl, 1984; Ledl et al., 1989; Klein et al., 1992; Kramhöller et al., 1993; Pischetsrieder and Severin, 1994, 1996; Pischetsrieder et al., 1998). The initial product of the MR between lactose and amino acids is the so-called Amadori compound. This product after prolonged heating or storage undergoes degradation to different dicarbonyl compounds. Yield, composition, and rates of this step of MR depend mainly on pH. Under pH control the main products are 1-deoxyglucosone (neutral pH), 3-deoxyglucosone (slightly acidic pH), and 4-deoxyglucosone, which is typical of β-1−4 glycosidic linked disaccharides (slightly basic pH).

Further degradation of deoxyglucosones leads to the formation of nitrogen-containing compounds such as pyrraline, furanoneamine, and pyridone or to the formation of nitrogen-free compounds such as hydroxymethylfurfural (HMF), galactosylisomaltol, and β-pyranone. Efforts have been made to quantify MRPs or its derivatives in different foods (Resmini and Pellegrino, 1991; Erbersdobler et al., 1996). However, there is still a need for a method to simultaneously detect different MR markers in complex matrices.

The objective of this study was to develop a high-performance liquid chromatography/mass spectrometry (HPLC/MS) protocol of analysis suitable for a rapid identification and quantification of the main products formed in the lactose−lysine (LL) system. The correlation between color formation and antioxidative activity was also investigated. We have also investigated the antioxidative ability of isolated low molecular weight (LMW) MRPs.

MATERIALS AND METHODS

Materials. D-(+)-Lactose and L-lysine monohydrochloride were of analytical grade and purchased from Fluka Chemie AG (Buchs, Switzerland). Linoleic acid, sodium dodecyl sulfate, and HDTBr were purchased from Sigma (St. Louis, MO). ABAP was from Wako Chemicals (USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was from Aldrich (Germany). HPLC-grade methanol and water were from Merck (Darmstadt, Germany) and filtered through disposable

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0.2 μm filters from Acrodisc (Gelman Sciences, Ann Arbor, MI). Double dialysis was performed with tubes with a cutoff of 3500 and 10000 Da (Spectra-Por, Los Angeles, CA). Absorbance was measured using a UV−vis 2100 Shimadzu (Tokyo, Japan) spectrophotometer.

Preparation of the Model System. An LL model system was prepared and analyzed according to the method of Monti et al. (1998) with slight modifications. Lysine monohydrochloride (7.3 g, 0.04 mol) and lactose (14.4 g, 0.04 mol) were dissolved in distilled water (40 mL) in a 50 mL Quickfit round-bottom two-neck flask, equipped with a double-coil water condenser, a lid, and a magnetic stirrer bar. The solution was heated for up to 4 h at 100 °C under reflux. Samples were collected after each hour and cooled in crushed ice before analysis. The reaction was monitored by measuring the final pH, the optical density at 420 nm, and the antioxidative activity.

Separation in Different Molecular Weight Fractions. After 4 h of reflux, the solution was separated by double dialysis into three fractions: a low molecular weight fraction of <3500 Da (LMW); an intermediate molecular weight fraction (IMW) between 3500 and 10000 Da; and a fraction containing all high molecular weights (HMW), that is, ≥10 kDa. A tube of 10000 Da cutoff was filled with LL solution and introduced into a larger tube of 3500 Da cutoff with water. The whole system was introduced into a flask and dialyzed against water for 3 days at 4 °C with stirring.

Relative Antioxidative Efficiency (RAE). The radical scavenging properties of raw fractions and pure single MRPs were analyzed according to the method of Pryor et al. (1993) with slight modifications. Kinetic parameters were recorded on a Shimadzu 2100 spectrophotometer equipped with a thermostated cuvette and continuous stirring. The system was controlled by Shimadzu kinetics software UV 2101 PC.

A phosphate buffer (2.6 mL, 50 mM, pH 7.4) micellar suspension of linoleic acid (2.6 mM) was pipetted into a cuvette and heated at 50 °C under stirring using 0.1 M HDTBr as detergent. The surfactant−phosphate solution was used as a blank. After 5 min of equilibration, 10 μL of ABAP (86 mM) was added, and peroxidation was monitored at 234 nm. After 15 min, 5−10 μL of antioxidant dissolved in methanol at 1−10 mg/mL concentration was added, and the kinetic parameters were monitored for 15 min. The ratio between the slope of the linear plot of absorbance versus time (d*A*/d*t*) after and before the addition of the antioxidant solution gives the antioxidant efficiency (AE)

$$AE = 1 - (S_{inh}/S_{ABAP})] \times 100 \quad (1)$$

where S_{inh} and S_{ABAP} are the slope after and before the addition of the antioxidant solution, respectively. A reference curve was obtained for trolox. For raw solutions, fractions, and purified compounds, RAE was obtained as the ratio between the slope obtained by plotting the concentration of the antioxidative sample (slope sample) versus its AE and that from trolox (slope trol):

$$RAE_{trol} = (S_{sample}/S_{trol}) \times 100 \quad (2)$$

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

The chromatographic separation was achieved on an ODS-(2) Primesphere 5 μm, 110 Å column (Phenomenex, Torrence, CA) (4.6 mm i.d. × 25 cm). The column was equilibrated in 95% phase (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 70%/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 of material not eluted under our chromatographic conditions (Bailey et al., 1996), washing with absolute methanol was necessary. Chromatograms were monitored at 254, 280, 360, and 420 nm, and raw data were collected from 190 to 600 nm.

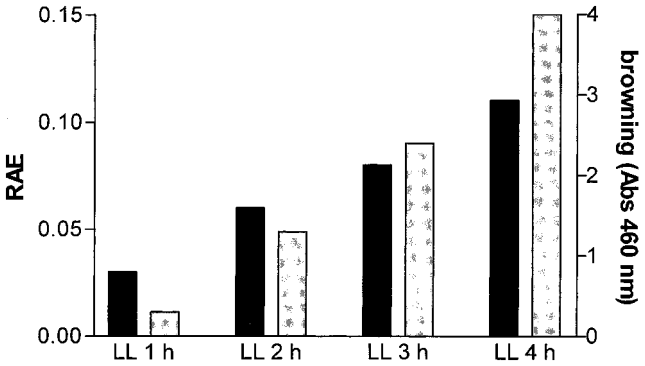

Figure 1. RAE value (black bars) and absorbance at 460 nm (gray bars) of LL raw samples collected at different hours of reflux. Values are calculated with respect to the AE of trolox.

Preparative HPLC. Preparative separation was achieved using a Supelco LC18 5 μm, 100 Å reverse-phase column (10 mm i.d. × 25 cm length) and the same solvent gradient used for the analytical separations. Peaks were manually collected, freeze-dried, dissolved in water, and successively repurified on the analytical column. Purified compounds were freeze-dried and then analyzed by NMR and AE.

HPLC/Mass Spectrometry (HPLC/MS) 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 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 the ions of the 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.

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded in D_2O on a Brucker AMX-600 instrument operating at 600.13 MHz. Pulse sequences from the literature were used for the two-dimensional experiments (Bax and Davis, 1985; Ernst et al., 1987). Two-dimensional spectra were obtained using the following acquisition parameters. $^1H-^1H$ correlated spectroscopy (COSY): time domain, 512 in F1 and 1024 in F2; 512 × 512 data matrix size; number of scansn 32; dummy scansn 4. $^1H-^1H$ nuclear Overhauser effect spectroscopy (NOESY): time domain, 512 in F1 and 1024 in F2; 512 × 512 data matrix size; number of scans, 176; dummy scans, 8; mixing time, 300 ms. Heteronuclear multiple quantum correlation (HMQC): time domain, 512 in F1 and 1024 in F2; 512 × 512 data matrix size; number of scans, 76; dummy scans, 4; delay for evolution of coupling constant, 3.3 ms. Heteronuclear multiple bond correlation (HMBC) optimized on long-range coupling: time domain, 512 in F1 and 1024 in F2; 512 × 512 data matrix size; number of scans, 96; dummy scans, 8; low pass filter, 3.3 ms; delay for evolution of long-range couplings constant, 80 ms. Data were acquired and processed in the phase-sensitive mode (TPPI) (Bodenhousen et al., 1980).

RESULTS AND DISCUSSION

Color Formation Parallels Antioxidant Activity. During the heating of the LL model system, a pH decrease from 4.9 to 3.9 and a progressive browning of the reaction were observed (Figure 1). These observations are consistent with similar ones previously reported for other carbohydrate−amino acid model sys-

tems (Ames et al., 1997). Color formation is likely due both to the formation of LMW colored compounds and to the presence of melanoidins, which are conjugated chromophores at a high molecular weight (Nursten and Reilly, 1983, 1986). Note that the absorbance at 460 nm has already been used as the end-point measurement for quantifying the yield of the HMW melanoidins (Wijewickreme et al., 1997).

Model compounds starting either from pure lactose (1 M) or pure lysine (1 M) were also prepared. By heating lactose alone, only a little browning was observed, confirming that in the sugar−amino acid system, browning is almost completely due to the Maillard reaction, without any significant caramelization of the sugar (Bailey et al., 1996). No browning was observed by heating the amino acid alone.

It is known that colored compounds formed through MR also have other important properties such as mutagenic/dismutagenic effects (Kasai et al., 1980; Yen and Tsai, 1993; Yen et al., 1993) and antioxidative ability (Eichner, 1981; Hayase et al., 1989; Anese et al., 1993; Yen and Hsieh, 1995). Using the experimental system described above (Pryor et al., 1993), we can obtain a measure of AE, which depends on the ability of the antioxidant compounds to transfer a hydrogen atom to the linoleic peroxyl radicals (LOO•) species. In particular, water soluble antioxidants react either with the small fraction of radicals produced in the water phase from ABAP or at the surface of micelles with either primary radicals or lipid peroxyl radicals (Pryor et al., 1993). It is worth noting the effect of the micellar charges on the RAEs. MRPs from the LL model system display a significant AE using a cationic detergent (HDTBr); to obtain the same AE in anionic detergent (SDS) micelles, concentrations of MRPs 10−50 times higher have to be tested. Only compounds that interact with the micelles where the LOO• are formed can exert their effect. The MRPs are likely attracted to the positively charged HDTBr micelles where autoxidation is occurring.

By comparing solutions collected at different times, noticeable differences were observed. The solution collected after 4 h has the greater ability to inhibit linoleic autoxidation ("chain breaking activity"). As shown in Figure 1, this trend is the same occurring during color formation. This result suggests that the HMW compounds formed through MR are also responsible for the color formation and the AE of MRP solutions.

HMW Fraction Shows the Highest AE. The model system solution showing the greatest values for RAE and browning (4 h of heating) was ranked into three fractions (HMW, IMW, LMW) by double dialyses. After freeze-drying, all samples were dissolved at a concentration of 1 mg mL^{-1} and their RAEs measured. Figure 2 shows that the HMW fraction, presumably containing melanoidins, has the highest RAE. In this case, although the 420 nm absorbance of the LMW fraction was half that of the IMW fraction, the observed RAE was the same. This finding suggests that although the HMW compounds are mainly responsible for the AE of MR solutions, the IMW compound and the noncolored LMW compounds also contribute to it.

Purified MRPs Present in the LMW Fraction Exert Antioxidant Effects. The MRPs present in the raw mixture after 4 h of refluxing were separated by reverse-phase HPLC with a diode array detection. The

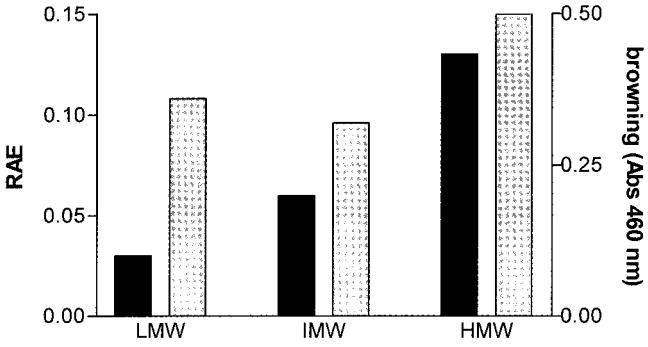

Figure 2. RAE value (black bars) and absorbance at 460 nm (gray bars) of 1 mg/mL solution of HMW, IMW, and LMW fractions obtained from LL 4 h.

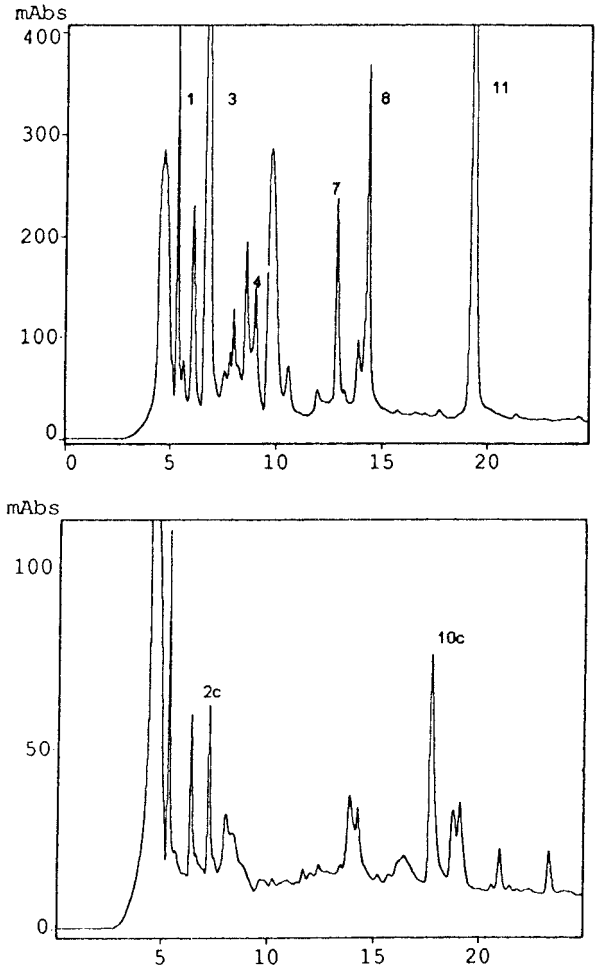

Figure 3. Chromatograms of the LL model system after 4 h of reflux: (top) detection at 280 nm; (bottom) detection at 360 nm. Only the most stable peaks were numbered.

chromatograms obtained at 280 and 360 nm are shown in Figure 3. The profile obtained by loading the LMW fraction was substantially identical to that of the solution heated for 4 h except for broadening due to polymeric material formed during reflux (Bailey et al., 1996). The most representative, reproducible, and stable peaks are indicated by numbers 1, 3, 4, 7, 8, and 11 on the UV trace and numbers 2c and 10c (c means colored) on the vis trace. The area of each peak was measured after different times of heating. All reached a maximum after 3 h except for peaks 7 and 8, the areas of which increased up to 4 h.

Table 1. RAE of Purified Peaks from Fraction LL < 3500 Da

sample	RAE trolox	sample	RAE trolox
trolox	100	peak 7	5.5
peak 1	2.0	peak 8	2.0
peak 2c	0.4	peak 10c	0.25
peak 3	bl[a]	peak 11	3.6
peak 4	2.0		

[a] bl, below limit of detection.

To evaluate the contribution of single compounds to the AE of the MR mixtures, a preparative HPLC purification was carried out and the RAE of each HPLC purified peak was measured. Results are shown in Table 1. Peak 7 showed the highest RAE value immediately followed by peak 11. The structural features of the compounds as well as the partition into the heterogeneous system used (water/micelles) may account for the different RAEs expressed by the purified compounds (Foti et al., 1996). The different activities of the eight purified compounds are mainly due to their intrinsic abilities to transfer a hydrogen atom to $LOO^{\bullet}$.

HPLC/MS Analysis of the LMW Fraction Allows a Tentative Identification of Some Peaks. To obtain a preliminary indication of the nature of the compounds present in the sample, UV spectra of each peak were compared with a spectral library obtained with reference compounds (Bailey et al., 1996): peak 7 showed a furanone-like spectrum, peak 8 a furan-like spectrum, and peak 11 a pyrrole-like spectrum. Further information was achieved by coupling to the HPLC an electrospray mass spectrometer equipped with an ion spray source. This technique is particularly useful for the complex mixture of highly hydrophilic compounds present in the model systems usually adopted to study the MR (Benson et al., 1998). A tentative identification of the compounds, based on the mass of the molecular ion, is shown in Table 2. The MS spectra of some peaks present in the chromatogram are shown in Figures 4–6.

Table 2. Molecular Ion, λ_{max}, and Tentative Identification of the Main Peaks Present in the LMW Fraction of the Lactose–Lysine Model System

sample	t_R (min)	molecular mass	λ_{max} (nm)	identification
unretained material		470		lactulosyl-lysine
peak 1	5.44	272	298, 337sh[a]	
peak 2c	7.50	nd[b]	298, 346	
peak 3	6.94	236	296	
peak 4	9.10	515.5	259	
peak 7	12.77	288	279	hydroxymethyl-furfural
peak 8	14.55	126	227, 284	galactosyliso maltol
peak 10c	18.30	nd	281, 376	
peak 11	19.73	254	298, 258sh	pyrraline

[a] sh, shoulder. [b] nd, not detected.

The unretained material eluted on the front of the LC column gave by MS a major peak at 471 uma and the corresponding adducts with water (Figure 4). The MS spectrum was identical to that of the $N_{\alpha}H_2$-N_{ϵ}-(1-deoxy-D-lactulosyl-1)-L-lysine standard synthesized in our laboratories (manuscript in preparation). Therefore, the compound eluted in the front of the column was identified as the Amadori product between lactose and lysine, which has a molecular ion of 471 uma. Unfortunately, its elution was very tailed and a residual signal at 471 uma was detectable up to 10 min of

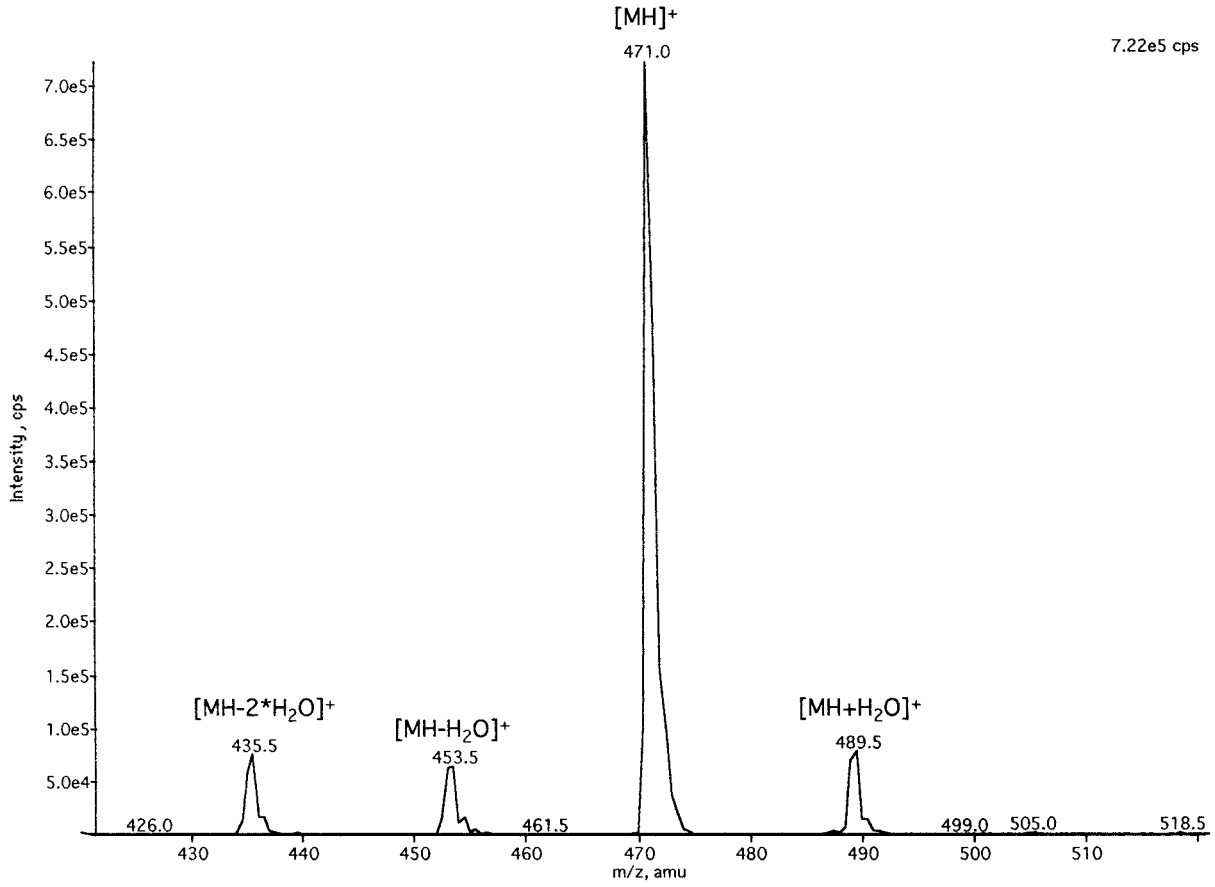

Figure 4. Mass spectrum of lactulosyl-lysine. Peak assignment is highlighted.

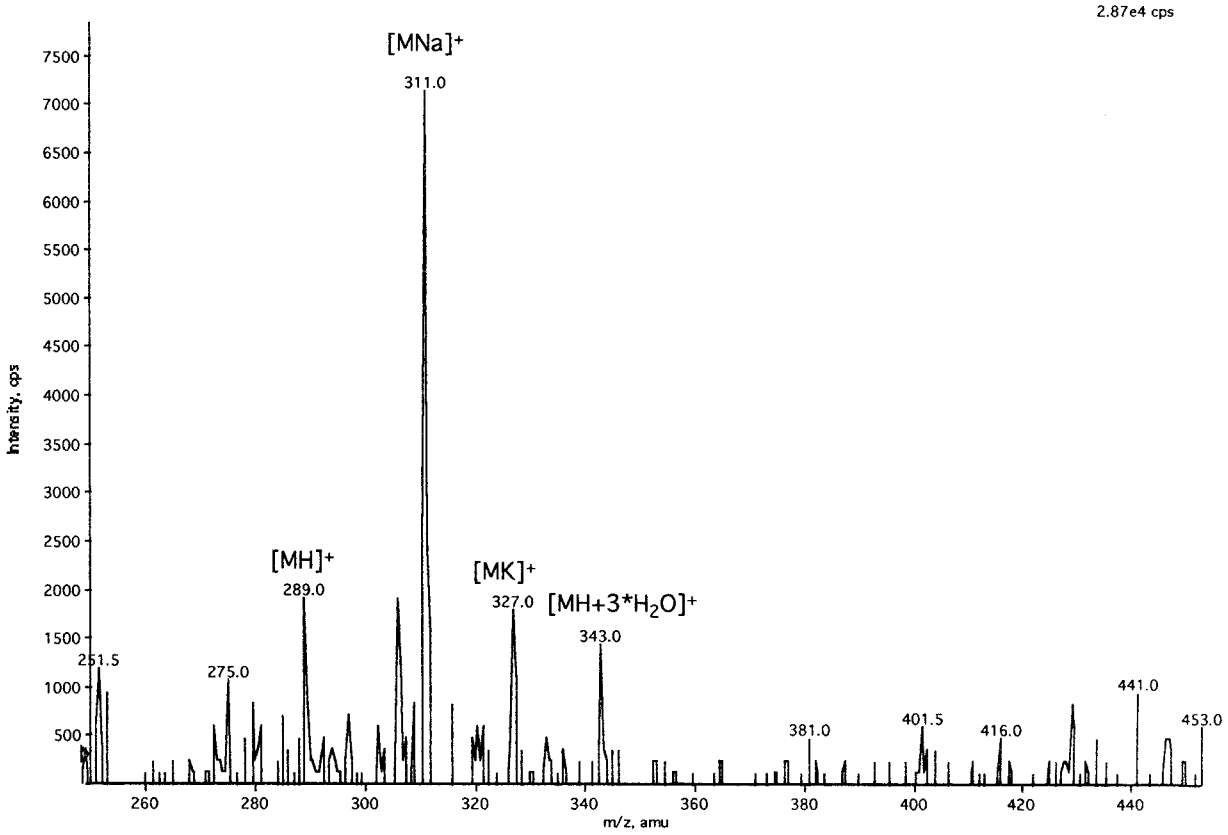

Figure 5. Mass spectrum of galactosylisomaltol. Peak assignment is highlighted.

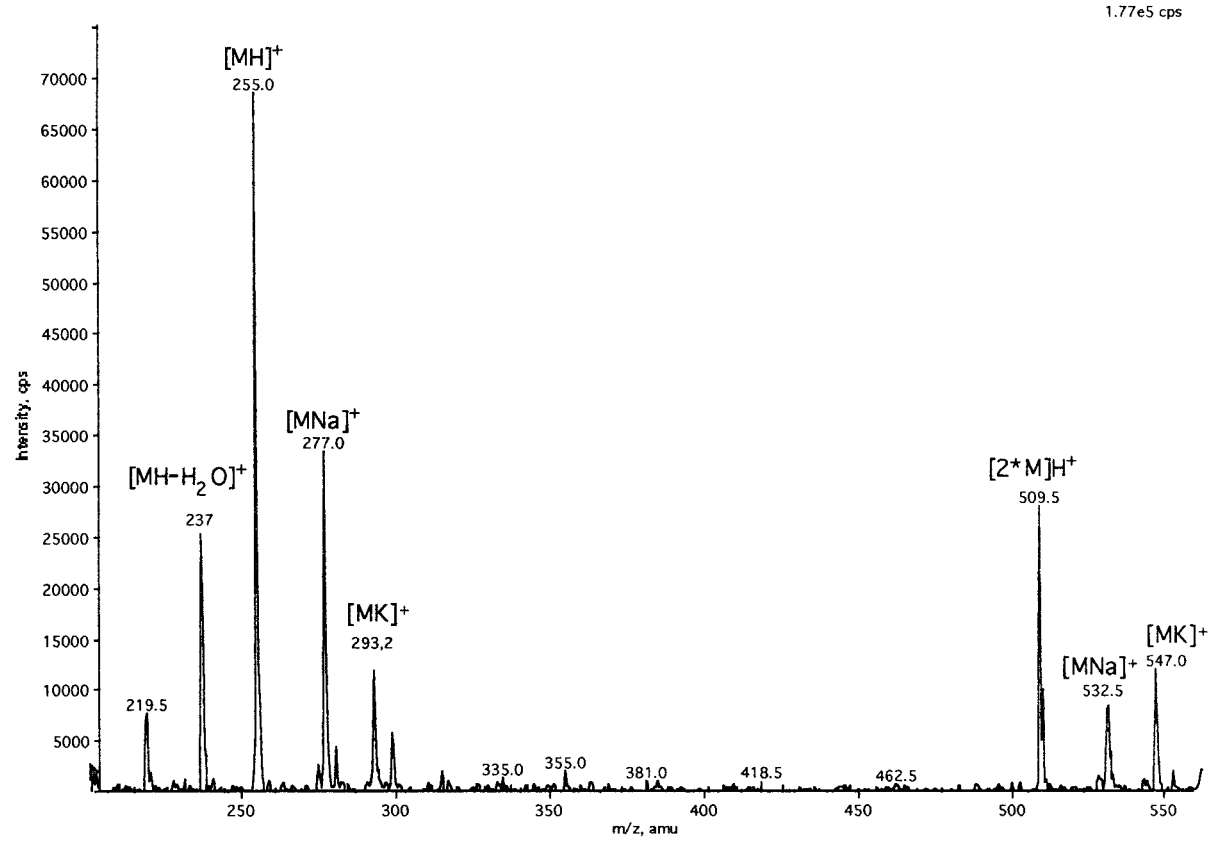

Figure 6. Mass spectrum of pyrraline. Peak assignment is highlighted.

elution. This phenomenon, which is not detectable by on-line UV detection, results in a contamination of other compounds and is particularly undesired during structural elucidation by NMR. The molecular ion of lactose and lysine and the relative adducts were also detected in the unretained material.

Peak 7 (Figure 5), which has a furan-like UV spectrum, gives a molecular ion MH^+ at 289 uma and peaks

Table 3. ^{1}H, ^{13}C, and 2D Data of Pyrraline (Peak 11)

	type	^{13}C	^{1}H	m	*J* (Hz)	COSY	HMBC optimized on long-range coupling
a	CH_2	59.95	3.379	tr	6.4	b	c; b
b	CH_2	36.82	1.603	m		a, c	c; d; a
c	CH_2	26.80	1.272	m		d, b	b; d; a; e
d	CH_2	35.22	1.644	m		e,c	b; e
e	CH_2	49.73	4.201	m		d	m; l; c; b
f	CH_2	59.71	4.485	s			m; g
g	CH	115.55	6.263	d	4.2	h	f
h	CH	130.98	7.034	d	4.2	g	
i	COH	185.82	9.250	s			
l	C	136.45					f; e
m	C	148.06					e

Table 4. ^{1}H, ^{13}C, and 2D Data of Galactosylisomaltol (Peak 7)

	type	^{13}C	^{1}H	m	*J* (Hz)	COSY	NOESY	HMBC optimized on long-range coupling
1	CH_3	27.03	2.552	s				2
2	CO	190.14						1
3	C	154.82						1′; 6
4	C	137.83						5
5	CH	105.12	6.738	d	2.0	6	6; 1′; 3′; 5′	4; 6
6	CH	149.68	7.740	d	2.0	5	5	5; 3
1′	CH	102.76	5.180	d	7.8	2′	5; 2′; 4′; 5′; 6′	6
2′	CH	71.13	3.930	dd	7.8; 10.0	1′; 3′	1′	
3′	CH	73.46	3.824	dd	10.0; 3.5	2′; 4′	5; 4′	4′; 5′
4′	CH	69.20	4.061	dd	3.5; 1.0	3′; 5′	1′; 3′; 5′	3′; 5′
5′	CH	76.80	3.951	dt	1.0; 5.7	4′; 6′	5; 1′; 4′; 6′	4′; 6′
6′	CH_2	61.40	3.833	m		5′	1′; 5′	5′

at 311 uma and 327 uma due to the adducts with Na^+ and K^+, respectively. This suggests that peak 7 is galactosylisomaltol, which is formed through MR of lactose upon prolonged heating (Pischetsrieder and Severin, 1996).

Peak 8 was identified by HPLC co-injection with pure standard as 5-hydroxymethylfurfural (HMF). The mass spectrum (not shown) has a peak at 127 uma ($[MH]^+$) and at 109 uma ($[MH - H_2O]^+$).

Peak 11 (Figure 6) gave a very informative mass spectrum. The $[MH]^+$ is detectable at 255 uma, the $[MNa]^+$ at 277 uma, and the $[MK]^+$ at 293 uma. The peak at 509 uma due to the protonated dimeric form of the compound and the adducts with Na^+ and K^+ were evident. As the UV spectrum is characteristic of pyrrolic compounds, this peak was tentatively identified as pyrraline (Henle and Bachmann, 1996).

Some of the peaks visible on the UV trace, mainly eluting between 5 and 10 min of our chromatographic conditions, correspond to nonionizable peaks. This is due to the ion source used, which does not give a good ionization yield for uncharged compounds. This fact can explain why lactulosyl lysine and pyrraline gave very good peaks, whereas galactosylisomaltol or HMF, although present in great amount, showed less clear MS spectra. The use of different sources such as the turbo ion spray or the heated nebulizer, which use a different ionization mechanism, could enhance the ionization of uncharged compounds.

Identification of Pyrraline and Galactosylisomaltol Was Confirmed by NMR Analysis. Due to the low degree of purity of the compounds, **t**he direct NMR analysis of MRPs collected after preparative separation was unsatisfactory. A further purification of each peak on a 4.6 mm column was used to obtain a sufficient amount of peaks 7 and 11. These were analyzed by NMR to confirm their identities.

Structure elucidation of peak 11, obtained using 2D experiments, confirmed that the compound was pyrraline. Data are reported in Table 3. ^{1}H data are in accordance with those reported by Henle and Bachmann (1996) except for the assignments of βCH_2 and γCH_2 of the lysine chain, which were inverted. In fact, COSY experiments clearly show correlation between the signal due to αCH at 3.37 ppm and the multiplet at 1.63 ppm, which can be assigned to βCH_2. Moreover, the HMBC optimized on long-range coupling showed cross-peaks between the C at 36.8 ppm and the H at 3.37, 1.60, and 1.64 and between the C at 26.8 and all of the protons of the lysine chain. This figure suggests that the C at 26.8, bearing protons at 1.27, is in the middle of the chain.

NMR structure elucidation of peak 7 confirmed that this compound was galactosylisomaltol. The ^{13}C data were in accordance with those reported in the literature (Ledl et al., 1986). However, for this compound NMR data were not complete, because they were obtained at low field (60 MHz) in other experimental conditions. Therefore, the complete reassignment of the structure was carried out, and data are summarized in Table 4. The NOESY experiment was very informative because it showed cross-peaks between the proton in position 5 of the furanic ring and the protons at 1′, 3′, and 5′ of galactose.

Concluding Remarks. HMF, galactosylisomaltol and pyrraline are major compounds produced in the Maillard reaction. They are found in a variety of dairy foods (Pischetsrieder and Severin, 1996). The antioxidative activity of each nonvolatile compound was weaker compared to that of trolox; however, their combined effects with each other and with other nonvolatile compounds in the Maillard mixture may be comparable to those of known antioxidants. In fact, synergetic effects can occur when the AE of a complex mixture is measured. Results of this study confirm that foods and beverages containing antioxidative MRPs may be more resistant to oxidative degradation (Nicoli et al., 1997). They also suggest that LC/MS has the potential for a

large-scale use as a tool to identify and quantify directly LMW chemical markers of MR in complex matrices.

ABBREVIATIONS USED

APAB, 2,2′-azobis(2-amidinopropane) dihydrochloride; HDTBr, hexadecyltrimethylammonium bromide; AE, antioxidative efficiency; RAE, relative antioxidative efficiency; RP-HPLC, reverse-phase high-performance liquid chromatography; LC/MS: liquid chromatography/mass spectrometry; MRPs, Maillard reaction products; HMW, IMW, LMW, high, intermediate, and low molecular weight.

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