Reaction Conditions Influence the Elementary Composition and Metal Chelating Affinity of Nondialyzable Model Maillard Reaction Products

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Nondialyzable glucose−lysine (Glu−Lys) and fructose−lysine (Fru−Lys) Maillard reaction products (MRPs) were synthesized by reacting 0.8 M L-lysine with 0.8M D-glucose or 0.8 M D-fructose using 14 different combinations of reaction time, temperature, initial pH, and initial water activity. The extent of browning in each reaction was monitored by UV−vis absorbance spectra, absorbance at 420 nm, and the yield of nondialyzable MRPs. Crude MRPs were fractionated further by immobilized metal affinity chromatography. Although the relative concentration and the number of unidentified intermediate compounds present in Glu−Lys and Fru−Lys MRP mixtures varied considerably, two principal components for both Glu−Lys and Fru−Lys MRP mixtures with molecular masses of approximately 5700 and 12 400 Da were identified by MALDI-MS. Elementary composition and copper chelating affinity of crude and fractionated MRPs were greatly influenced by type of reactant sugar and reaction conditions used for synthesis. Glucose, in general, produced MRPs with relatively higher copper chelating affinity, possessing higher percentages of carbon and lesser percentages of nitrogen compared to counterpart Fru−Lys MRPs.

Keywords: *Maillard reaction; nondialyzable; chelation; chromatography; elementary compositio*

INTRODUCTION

The Maillard reaction (MR) occurs nonenzymatically in foods between reducing sugars and available amino groups during thermal processing and home cooking operations. The reaction can occur under severe or mild heating conditions and can contain many complex chemical intermediates, which ultimately lead to the production of brown polymeric compounds known as melanoidins (Pomeranz *et al*., 1962). The formation of Maillard reaction products (MRPs) is greatly influenced by both the reaction conditions and the source of reactant sugars. Due to the complexity of this reaction, partly because of the large number of chemical compounds derived, even the most advanced separation and analytical techniques do not enable complete characterization of all melanoidins or associated intermediate products. As such, a more general formula (sugar + amino acid ≈2−3 H_2O) and an empirical formula (e.g. $C_8H_{11}NO_6$) have been proposed for melanoidins by Kato and Tsuchida (1981) and Motai and Inoue (1974), respectively. A fundamental structure for melanoidin has also been proposed for melanoidins by Kato and Tsuchida (1981) and Cammerer and Kroh (1995). Since MR affect color, flavor, functional properties, and nutritional value of foods, as well as hold a multitude of potential bioactive properties, this reaction has been the subject of considerable research for many years (Rendleman, 1987; Kitts *et al*., 1993a,b; Bedinghaus and Ockerman, 1995).

Published observations strongly indicate that melanoidins behave as chelating agents for polyvalent metal cations and thereby may influence the bioavailability of these ions (Rendleman, 1987). Rendleman (1987) and Rendleman and Inglett (1990) proposed that two H^+ ions are released for each Cu^{2+} or Ca^{2+} bound to the melanoidin. In addition, the amount of metal bound to melanoidin on the basis of nitrogen content of melanoidin was found to be Ca/4N and Cu/4N, which signifies the number of Ca^{2+} or Cu^{2+} ions bound per four nitrogen atoms. These findings, therefore, indicate that immobilized metal affinity chromatography (IMAC) may be a useful method for further chemical characterization of MRPs. Since very little effort has been given to determine the significance of reaction conditions on metal chelating affinity of derived MRPs, the objective of this study was to investigate the influence of chemical reaction conditions on the elementary composition and copper chelating affinity of two model MRPs, prepared similarly at relatively short reaction time and temperature combinations. These reaction conditions were chosen to simulate typical thermal processes conducted in many cooking situations.

MATERIALS AND METHODS

Materials. L-Lysine, D-glucose, and D-fructose were purchased from Sigma Chemical Co. (St. Louis, MO). Glycerol, cupric sulfate ($CuSO_4 \cdot 5H_2O$), ethylenediaminetetraacetic acid (EDTA), tetramethyl murexide (TMM), copper reference solutions for atomic absorption spectroscopy, and hexamine were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Analytical grade hydrochloric acid, sodium hydroxide, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, sodium acetate, sodium chloride, and potassium chloride were purchased from BDH Chemical Co. (Toronto, ON). Dialysis tubing [molecular weight cutoff (MWCO) = 6000−8000 and 3500] was obtained from Spectrum Scientific Co. (Houston, TX). Chelating Sepharose fast flow resin was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Distilled water used to prepare model MRPs was further purified by a Barnstead E-pure system and used throughout the study. Relative humidity was measured using a Ro-tronic Hygroscope DT relative humidity detector (Rotronic Instrument Corp., Huntington, NY), and the readings were converted to water activity (a_w) values by dividing by 100. A Bio-Rad

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Table 1. Initial and Final Experimental Conditions [Reaction Time, Oven Temperature, Initial Water Activity (a_w), and Initial pH] Used and Obtained in the Preparation of Glu−Lys and Fru−Lys Nondialyzable Model MRP Mixtures and the Yield Gained at the End of the Reaction

	initial study conditions				final study conditions							
					Gly−Lys		Fru−Lys		equivalent reaction time[a] (min)		yield[b] (g)	
expt no.	time (min)	oven temp[c] (°C)	pH	a_w	pH	a_w	pH	a_w	Gly−Lys	Fru−Lys	Glu−Lys	Fru−Lys
1	65	110	6.37	0.58	3.20	0.56	3.20	0.56	1.10	1.05	2.41 ± 4.2	0.75 ± 3.2
2	90	146	6.18	0.81	3.15	0.80	3.16	0.78	14.95	14.82	0.50 ± 6.0	0.05 ± 2.8
3	119	127	6.14	0.74	3.14	0.71	3.12	0.68	15.38	13.41	7.03 ± 2.1	0.55 ± 1.1
4	86	112	6.97	0.68	3.51	0.65	3.51	0.65	4.95	2.79	0.18 ± 1.1	1.48 ± 2.1
5	107	157	8.51	0.57	3.62	0.53	3.75	0.51	60.1	63.1	2.00 ± 3.2	5.11 ± 1.7
6	85	86	7.98	0.68	3.53	0.66	3.54	0.67	0.95	0.87	0.13 ± 2.0	0.01 ± 1.1
7	45	91	6.67	0.94	3.32	0.93	3.30	0.94	1.50	1.41	nd[d]	nd
8	58	143	6.98	0.95	3.51	0.92	3.53	0.91	6.31	5.86	0.70 ± 1.5	0.51 ± 2.0
9	116	99	7.81	0.80	3.60	0.76	3.61	0.77	0.10	0.08	0.10 ± 1.3	0.45 ± 3.1
10	43	112	6.44	0.88	3.24	0.87	3.22	0.88	0.09	0.06	1.30 ± 4.1	nd
11	43	159	8.57	0.62	3.54	0.59	3.51	0.61	22.6	22.8	4.10 ± 3.0	6.65 ± 2.8
12	30	144	7.14	0.81	3.53	0.80	3.53	0.79	0.58	0.61	0.23 ± 1.8	0.60 ± 1.6
13	71	129	8.41	0.78	3.86	0.74	3.84	0.73	7.81	6.54	7.50 ± 2.3	0.40 ± 3.2
14	108	80	7.66	0.88	3.58	0.84	3.56	0.85	0.30	0.19	1.21 ± 2.1	2.09 ± 3.7

[a] Equivalent reaction time calculated for $T_{ref} = 393$ K; $z = 19.1$ °C. [b] Yield in grams of nondialyzable MRP per 100 mL of final reaction solution. Values represent mean ± SD ($n = 3$). [c] Ambient temperature in the oven. [d] Not detected.

Econo pump controller and a Bio-Rad Econo UV detector (Bio-Rad Laboratories, Richmond, CA) were used in the immobilized copper affinity chromatography to adjust the pH gradient of the mobile phase and to measure the MRP contents in the eluent, respectively.

Production of Model MRP Mixtures. MRP mixtures were prepared by heating a solution of 0.8 M D-glucose or 0.8 M D-fructose with 0.8 M L-lysine (Kitts *et al.*, 1993a) in a Perma View hot air oven. Since it is difficult to isolate the effect of a single factor influencing the production of MRP mixtures (Lingert, 1990), a randomly selected experimental design was generated using random-centroid optimization program (Dou *et al.*, 1993). The four variable experimental factors used were time, temperature, a_w, and pH (Table 1). The initial a_w values of reactions were adjusted according to the method of Eichner and Karel (1972), and the initial pH values were adjusted with 5 M NaOH. All experiments were conducted in 500 mL round-bottom flasks containing 100 mL of reactants. The internal heating pattern of the reaction solution and the ambient temperature of the oven were recorded with sheathed Type-T copper/constantan thermocouples.

At the end of the reaction time, the resulting brown solution was rapidly cooled on ice, dialyzed (MWCO = 6000−8000) against 20−30 changes of double distilled deionized water at 4 °C for 7 days, and the nondialyzable retentate was lyophilized. The produced Glu−Lys and Fru−Lys MRP mixtures were weighed and stored in a desiccator at 0 °C until further use.

Correction for Thermal Lags. Since the ambient temperature of the oven does not represent the reaction solution temperature, the temperature histories of the reaction solutions were corrected for the heating lag, and an equivalent reaction time (U) for a reaction temperature of 120 °C (393 K) was calculated according to the method described by Hayakawa *et al.* (1977) and Ramaswamy *et al.* (1989). The equivalent heat process times (U) calculated for each individual experiment using the reference temperature of 393 K, in addition to the ambient oven temperature values used in this study, are given in Table 1.

In addition to the heat penetration data acquired using thermocouples, two other parameters, namely, activation energy (E_a) and z value (temperature change required to change the decimal reduction time by a factor of 10), were used to calculate U values. An E_a value of 147 kJ/(mol K) (Labuza and Baisier, 1992), which represented an activation energy parameter for protein quality loss in a soy−glucose model (a_w = 0.3−0.8; reaction temperature = 80−130 °C) Maillard reaction, was chosen for the computation of U in this study since this particular E_a value represented a MR with a_w and reaction temperatures similar to the present study. The appropriate z value was calculated using eq 1 (Lund, 1975)

$$z = (2.303RT_1T_2/E_a) \quad (1)$$

where R is the universal gas constant [8.314 J/(mol K)], T_1 is the selected reference temperature (393 K), and T_2 is 373.9 K (a temperature z degrees less than T_1).

$$U = \Delta\sum_{t=t_b}^{t=0} 10^{(T-T_1)/z}\Delta t \quad (2)$$

In eq 2, T_1 is the reference temperature (393 K), T is the reaction temperature, t_b is the unmodified heating time (minutes), and $z = 19.1$ °C. U was thus the equivalent time of the total reaction at 393 K for a reaction with $E_a = 147$ kJ/(mol K).

Spectral Characteristics of Model MRP Mixtures. Absorption spectra of crude MRP mixtures (0.01 mg/mL) and absorbance readings of MR solutions at 420 nm were measured using a Shimadzu UV-160 spectrophotometer (Tekscience, ON).

Metal Chelating Affinity of Model MRP Mixtures. Solutions consisting of $CuSO_4$ (0.05−0.4 mM), MRP mixtures (100 μg/mL), and TMM (1 mM) were prepared in 10 mM hexamine−HCl buffer (pH 5) containing 10 mM KCl. The MRP samples (1 mL) were individually mixed with 1 mL of $CuSO_4$ (0.05−0.4 mM) for 10 min at room temperature. The total copper present in each solution was measured by atomic absorption spectrophotometry. The amount of free copper in the solutions was obtained from a standard curve, where the absorbance ratio, A_{460}/A_{530}, in a solution of 1 mL of $CuSO_4$ (0.05−0.4 mM), 1 mL of hexamine−HCl buffer, and 0.1 mL of TMM was plotted against the amount of total copper added. The amount of copper bound to MRP mixtures at different copper concentrations was calculated as the difference between the amount of total and free copper present in the solution.

The binding activity of copper to MRP mixtures was calculated from a Scatchard curve, where the ratio of bound copper (micromoles of Cu^{2+} per milligram of MRP) to free copper (micromoles) was plotted against the concentration of bound copper (micromoles of Cu^{2+} per milligram of MRP). The number of association sites (n) and the dissociation constant (K_d) of MRPs were calculated from the x-intercept and the slope (slope = $1/K_d$) of the Scatchard curve, respectively.

Fractionation of Model MRP Mixtures by Immobilized Copper Affinity Chromatography. Fractionation of crude MRP mixtures into copper chelated components was conducted by immobilized copper affinity chromatography. A chelating Sepharose 6B column (3.5 × 7 cm) was activated with 0.1 M $CuSO_4$ solution and washed with several volumes

of deionized distilled water at a flow rate of 3.2 mL/min until there was no free copper present in the eluate when analyzed by atomic absorption spectrophotometry. The resulting copper loaded column was subsequently equilibrated with 0.05 M (pH 7.65) phosphate buffer (buffer A) containing 0.5 M NaCl. MRP mixtures (250 mg) dissolved in 2 mL of buffer A were applied to the column and washed with 250 mL of the same buffer. Elution of the bound MRP mixtures from the column was conducted at the same flow rate, with a linear pH gradient ranging from pH 7.65 (buffer A) to pH 5.5 (buffer B; 0.05 M acetate buffer with 0.5 M NaCl). The column eluates were collected into 7 mL fractions. The fractions having an absorbance at 280 nm were pooled together, dialyzed (MWCO = 3500) against deionized distilled water for 7 days at 4 °C, and then lyophilized. The first peak eluted during the wash phase with pH 7.65 buffer was designated component 0, and the subsequent peaks eluted during the gradient pH fall were designated components 1−5, respectively. Following each MRP application, the column was flushed with EDTA, followed by 4−6 volumes of deionized distilled water, and then resaturated with $CuSO_4$ before the next sample was applied.

Elementary Composition and Molecular Weight Determination of Model MRP Mixtures and MRP Mixture Components Fractionated by Chromatography. Elementary composition of dialyzed crude MRP mixtures and their metal chelated components was obtained by pyrolyzing the compounds at 1100 °C in helium using a microanalytical (EA Model 1108, Italy) elemental analyzer (Department of Chemistry, UBC). Gases that escaped from the compounds during pyrolysis were quantitated by gas chromatography. The molecular weights were determined by a Kratos Kompact matrix assisted laser desorption ionization mass spectrophotometer (MALDI-MS) (Department of Chemistry, UBC) using sinnapinic acid as the matrix (3,5-dimethoxy-4-hydroxycinnamic acid). Mass accuracy was ±0.1−0.01%.

Statistical Analysis. One-way analysis of variance (ANOVA) followed by Tukey multiple-range test (Systat Inc., Evanston, IL) was used in data analysis. The level of confidence required for significance was selected as $p \leq 0.05$. Each experiment was replicated three times, with internal controls.

RESULTS

Yield of Glucose−Lysine (Glu−Lys) and Fructose−Lysine (Fru−Lys) MRP Mixtures. The initial and final pH and a_w values, reaction times, and ambient temperatures of the oven used in the production of MRP mixtures, along with equivalent reaction times and nondialyzable melanoidin yields, are given in Table 1. An acidic final pH was obtained in all experimental mixtures regardless of the initial pH. The a_w values remained relatively unchanged from initial values. The calculated *U* values were similar for both Glu−Lys and Fru−Lys MRP synthesis experiments. Nondialyzable MRP mixture yields of different synthesis experiments varied depending on the combination of reaction conditions and source of the reducing sugar. Nondialyzable melanoidin was not present in sufficient quantities for recovery from Glu−Lys synthesis experiment 7 and experiments 7 and 10 of the Fru−Lys reaction. The greatest MRP yields were obtained from synthesis experiments 3 and 13 for the Glu−Lys reaction. Although similar initial oven temperature and water activity values were used for all of the above experiments, both the initial pH and reaction times used were substantially different. The highest MRP yields for Fru−Lys reactions were obtained from synthesis experiments 5 and 11. Both of these Fru−Lys experiments were characterized by similar heating temperatures and initial pH values but large differences in reaction times.

The use of short reaction times and low oven temperatures (e.g. experiments 6 and 7) produced the smallest quantities of nondialyzable MRP mixtures. In addition, experiments having either a short reaction time or a low oven temperature (i.e. experiments 9, 12, and 14), as well as those experiments possessing an a_w value >0.8 (experiments 2, 7−10, 12, and 14), also produced low quantities of nondialyzable MRP mixtures. The *U* values computed for Glu−Lys MRP mixtures were similar to *U* values calculated for Fru−Lys MRP mixtures.

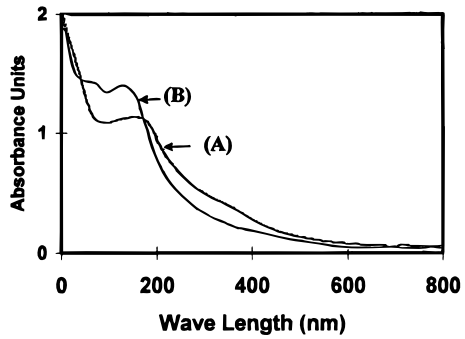

Figure 1. Spectral patterns of crude Glu−Lys and Fru−Lys MRP mixtures: (A) spectral pattern of all Fru−Lys MRP mixtures and Glu−Lys MRP mixtures except Glu−Lys MRP mixtures 4 and 6; (B) spectral pattern of Glu−Lys MRP mixtures 4 and 6.

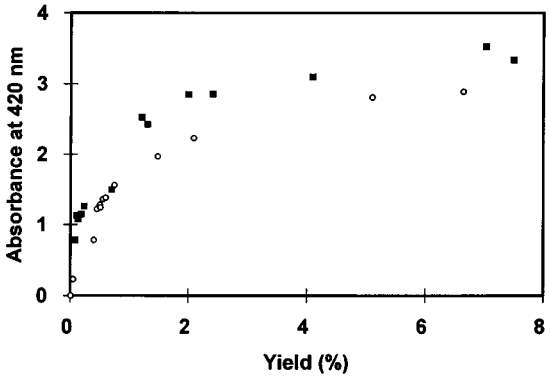

Figure 2. Absorbance readings of crude Glu−Lys and Fru−Lys MRP mixtures at 420 nm versus their nondialyzable MRP mixture yields: (○) Fru−Lys MRP mixtures; (■) Glu−Lys MRP mixtures.

Spectral Characteristics of MRP Mixtures. Spectral patterns for both derived Glu−Lys and Fru−Lys MRP mixtures are given in Figure 1. The spectral pattern shown in curve A had one absorption shoulder at 320 nm, while the spectral pattern shown in curve B had two absorption shoulders (e.g. at 280 and 320 nm). Glu−Lys experiments 4 and 6 had spectral patterns similar to curve B, whereas all other Glu−Lys experiments and all Fru−Lys experiments produced spectral patterns similar to curve A.

The 420 nm absorbance readings obtained for different Glu−Lys and Fru−Lys MRP mixtures are given in Figure 2. As the results demonstrate, high absorbance readings at 420 nm were detected for experiments that have resulted in high nondialyzable MRP yields. In addition, large differences in the 420 nm absorbance readings between the two Glu−Lys and Fru−Lys model systems were observed as the nondialyzable yield of MRP increased above 1%.

Metal Chelating Affinity of Model MRP Mixtures. Binding Activity of Copper Ions to MRP Mixtures. Many of the MRP mixtures derived from different synthesis experiments possessed detectable copper binding activity (Table 2). In addition, MRP mixtures synthesized from Glu−Lys experiments 3 and

Table 2. Bound Copper, Dissociation Constants (K_d), and Number of Copper Binding Sites (n) in Crude Glu−Lys and Fru−Lys MRP Mixtures[a]

expt no.	bound copper (μmol of Cu/mg of MRP)		K_d (mol)		n	
	Glu−Lys	Fru−Lys	Glu−Lys	Fru−Lys	Glu−Lys	Fru−Lys
1	0.47 ± 0.01	0.31 ± 0.12	5.3×10^{-6}	8.9×10^{-7}	0.54	0.31
2[a,b]	0.03 ± 0.23					
3	1.10 ± 0.17	0.16 ± 0.05	2.4×10^{-4}	5.9×10^{-7}	1.37	0.82
4	0.19 ± 0.51	2.27 ± 0.24	3.7×10^{-6}	1.1×10^{-6}	0.51	0.23
5	0.31 ± 0.34	0.66 ± 1.10	8.0×10^{-6}	2.9×10^{-6}	0.54	1.44
6[a,b]	0.06 ± 1.40	0.00				
7[a,b]	0.00	0.00				
8[b]	0.16 ± 0.62	0.05 ± 0.61	3.9×10^{-6}		0.17	
9[a]	0.00	0.19 ± 0.44		8.0×10^{-6}		0.64
10[a,b]	0.03 ± 0.04	0.00				
11	0.47 ± 1.03	1.42 ± 1.16	4.8×10^{-6}	1.8×10^{-6}	0.51	1.25
12	0.63 ± 2.02	0.16 ± 0.04	8.1×10^{-5}	2.8×10^{-6}	0.58	0.14
13[b]	1.57 ± 0.93	0.02 ± 0.65	2.1×10^{-4}		1.24	
14[b]	0.16 ± 0.71	0.08 ± 0.38	2.7×10^{-6}		0.36	

[a] Glu−Lys experiments with bound copper values <0.08 μmol/mg of MRP. [b] Fru−Lys experiments with bound copper values <0.08 μmol/mg of MRP.

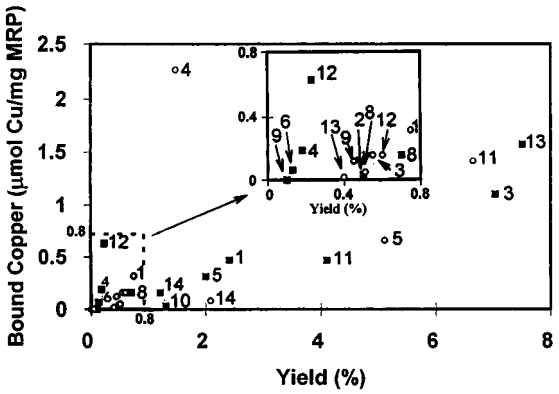

Figure 3. Amount of copper bound to crude MRP mixtures vs nondialyzable yield: (○) Fru−Lys MRP mixtures; (■) Glu−Lys MRP mixtures. The numbers beside symbols denote the experiment number for each set of conditions used in MRP synthesis. The inset shows an expanded scale.

13 and from Fru−Lys experiments *5* and 11 had the greatest MRP yields and high metal chelating affinity (Figure 3).

In general, many of the synthesis experiments used to produce Glu−Lys MRP mixtures were characterized by a K_d value that was ∼10-fold greater than that for the corresponding Fru−Lys MRP mixture synthesis experiments (Table 2). The number of copper binding sites also varied with specific MRP synthesis conditions. Within individual Glu−Lys and Fru−Lys experiments, the greatest number of copper binding sites were obtained for Glu−Lys reaction experiments 3 and 13 and for Fru−Lys reaction experiments 3, 5, and 11.

Fractionation of Model MRP Mixtures by Immobilized Copper Affinity Chromatography. Only the crude MRP mixtures that possessed a chelating value of >0.08 μmol/mg were further fractionated by immobilized copper affinity chromatography. The percentage of melanoidin recovered from the immobilized copper affinity column increased as the gradient pH lowered from 7.65 to 5.5.

The K_d values and the number of copper binding sites (n) for components 1 and 5 collected from the column eluate are given in Table 3. Crude dialyzed MRP mixtures had comparatively lower K_d and n values than associated fractionated components. Thus, immobilized copper affinity chromatography was effective in fractionating crude dialyzed MRP mixtures into components

Table 3. Dissociation Constants (K_d) and Number of Copper Binding Sites (n) in Crude and Fractionated Glu−Lys and Fru−Lys MRP Mixtures

expt no.	component	bound Cu (μmol of Cu/mg of MRP)	K_d (mol)	n
Glu−Lys MRP mixture 3	crude[a]	0.10	2.4×10^{-4}	1.37
	1[b]	0.02	6.2×10^{-4}	4.31
	5[c]	0.03	5.1×10^{-3}	9.47
Glu−Lys MRP mixture 13	crude	1.57	2.1×10^{-4}	1.24
	1	0.02	2.2×10^{-4}	6.42
	5	0.05	1.6×10^{-4}	9.60
Fru−Lys MRP mixture 5	crude	6.61	2.9×10^{-6}	1.44
	1	0.02	5.1×10^{-5}	3.10
	5	0.05	6.7×10^{-4}	6.22
Fru−Lys MRP mixture 11	crude	1.42	1.8×10^{-6}	1.25
	1	0.01	1.3×10^{-3}	2.45
	5	0.02	1.6×10^{-3}	8.48

[a] Crude dialyzed MRP mixture. [b] Component 1 (i.e., first peak eluted from the column during gradient pH change; fractions 1−20 were pooled together). [c] Component 5 (i.e., last peak eluted from the column during gradient pH change; fractions 107−117 were pooled together).

with specific copper binding activity. In addition, the components eluted from the column at a lower pH value possessed a greater chelating activity. As a result, higher K_d and n values were obtained for eluate component 5, compared to component 1 for both Glu−Lys and Fru−Lys MRP mixtures.

Since components 1 and 5 fractionated by immobilized copper affinity chromatography from both crude Glu−Lys and Fru−Lys MRP mixtures yielded the greatest recovery for Glu−Lys experiment 3 and Fru−Lys experiment 5, further characterization by MALDI-MS was possible only for these fractions in this study. Components 1 and 5 in both Glu−Lys and Fru−Lys MRP mixtures contained two common principal peaks with apparent molecular weights of ∼5700 (major) and ∼12 400 (minor), respectively (Figure 4). Accompanying these two peaks were many additional smaller peaks indicating the presence of other trace compounds produced during different stages of the Maillard polymerization reaction.

Elemental Analysis of Crude MRP Mixtures and MRP Mixture Components. Results of the elemental analysis together with the estimated average empirical formulas for both crude MRP mixtures and fractionated MRP mixture components obtained from different Glu−Lys and Fru−Lys MRP synthesis experiments are presented in Tables 4 and 5, respectively. Fru−Lys MRP mixtures consistently exhibited a smaller number of carbon atoms compared to similarly synthesized Glu−Lys MRP mixtures. Despite this difference, the C to O ratio was approximately 2:1 for all eight experiments. In MRP fractionated components, a C to O ratio of approximately 2:>1, with a lesser number of carbon atoms in the average empirical formulas, was observed. The C to N ratio also varied depending on the sugar and experimental condition used to generate different MRP mixtures. Fru−Lys MRP mixtures exhibited higher C to N ratios compared to their Glu−Lys counterpart, although this difference was not noticed in copper chelated MRP components.

DISCUSSION

The effect of various reaction conditions on the composition and yield of MRP mixtures was assessed by examining the nondialyzable melanoidins derived from both Glu−Lys and Fru−Lys browning mixtures. Our findings confirmed former investigations, that the

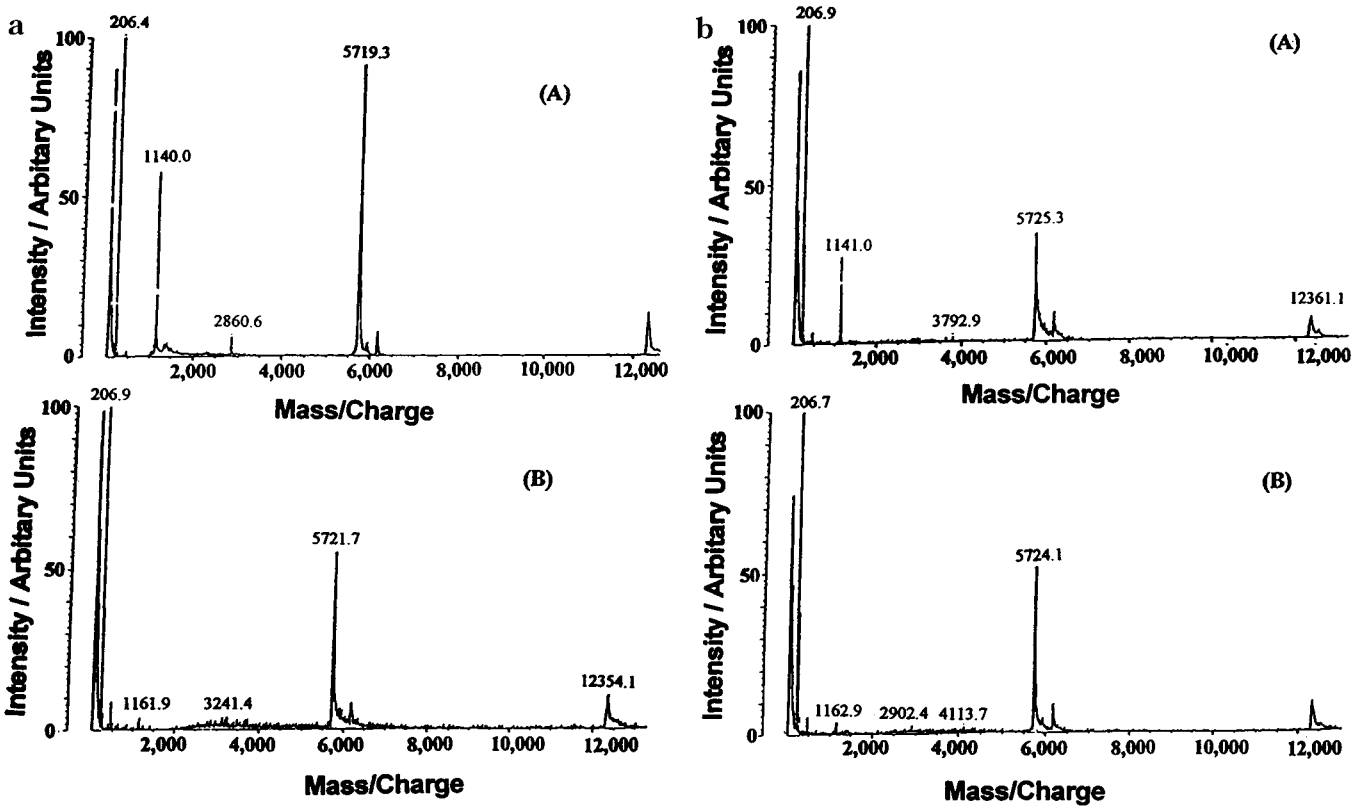

Figure 4. MALDI mass spectra of (a) component 1 and (b) component 5 of (A) Glu−Lys MRP mixture 3 and (B) Fru−Lys MRP mixture 5 fractionated by chelation chromatography.

Table 4. Results of Elemental Analysis and Empirical Formulas of Crude Glu−Lys and Fru−Lys MRP Mixtures

type of MRP mixture	% C	% H	% O[a]	% N	av empirical formula[b]	C:O[c]	C:N[d]
Glu−Lys MRP mixture 3	51.87	6.74	35.03	6.36	$C_{9.50}H_{14.83}NO_{4.82}$	2:1.00	1: 0.12
Fru−Lys MRP mixture 3	51.62	6.72	34.04	7.62	$C_{7.95}H_{12.83}NO_{3.91}$	2:0.98	1: 0.15
Glu−Lys MRP mixture 5	53.48	6.17	33.92	6.43	$C_{9.70}H_{13.43}NO_{4.60}$	2:0.95	1: 0.12
Fru−Lys MRP mixture 5	53.21	6.42	33.69	7.76	$C_{8.02}H_{13.29}NO_{4.36}$	2:1.09	1: 0.15
Glu−Lys MRP mixture 11	48.58	6.75	38.01	6.66	$C_{8.51}H_{14.12}NO_{5.00}$	2:1.18	1: 0.14
Fru−Lys MRP mixture 11	47.63	7.39	37.22	7.76	$C_{7.16}H_{13.33}NO_{4.20}$	2:1.17	1: 0.16
Glu−Lys MRP mixture 13	50.06	7.27	35.84	6.83	$C_{8.56}H_{14.90}NO_{4.59}$	2:1.07	1: 0.14
Fru−Lys MRP mixture 13	50.39	7.60	33.66	8.35	$C_{7.04}H_{12.75}NO_{3.53}$	2:1.00	1: 0.17

[a] Calculated from C, H, and N%. [b] Calculated as N = 1. [c] Carbon/oxygen ratio. [d] Carbon/nitrogen ratio.

Table 5. Elementary Composition and Empirical Formulas of Crude MRP Mixtures and MRP Mixtures Fractionated by Immobilized Copper Affinity Chromatography

synthesis expt no.	component	% C	% H	% O[a]	% N	empirical formula[b]	C:O[f]	C:N[g]
Glu−Lys MRP mixture 3	crude[c]	51.87	6.74	35.03	6.36	$C_{9.50}H_{14.83}NO_{4.82}$	2:1.00	1:0.12
	1[d]	44.97	6.17	42.16	6.70	$C_{7.83}H_{12.89}NO_{5.51}$	2:1.41	1:0.15
	5[e]	44.94	6.82	42.18	6.05	$C_{8.66}H_{15.78}NO_{6.10}$	2:1.41	1:0.13
Glu−Lys MRP mixture 13	crude	50.06	7.27	35.84	6.83	$C_{8.6}H_{14.90}NO_{4.59}$	2:1.07	1:0.14
	1	46.36	6.81	40.80	6.01	$C_{9.0}H_{15.87}NO_{5.94}$	2:1.32	1:0.13
	5	46.30	6.26	41.43	6.00	$C_{9.0}H_{14.60}NO_{6.04}$	2:1.34	1:0.13
Fru−Lys MRP mixture 5	crude	53.21	6.42	33.69	7.76	$C_{8.02}H_{13.29}NO_{4.36}$	2:1.09	1:0.15
	1	46.35	6.17	40.57	6.89	$C_{7.84}H_{11.53}NO_{5.15}$	2:1.31	1:0.15
	5	45.94	6.82	40.74	6.49	$C_{7.76}H_{12.31}NO_{4.75}$	2:1.33	1:0.14
Fru−Lys MRP mixture 11	crude	47.63	7.39	37.22	7.76	$C_{7.16}H_{13.33}NO_{4.20}$	2:1.17	1:0.16
	1	47.60	6.30	38.89	7.16	$C_{7.76}H_{12.31}NO_{4.75}$	2:1.22	1:0.15
	5	45.84	6.81	40.28	7.05	$C_{7.58}H_{13.52}NO_{4.99}$	2:1.32	1:0.15

[a] Calculated from C, H, and N%. [b] Calculated as N = 1. [c] Crude, crude MRP mixture. [d] MRP component 1 eluted from the copper chelating column. [e] MRP component 6 eluted from the copper chelating column. [f] Carbon/oxygen ratio. [g] Carbon/nitrogen ratio.

nondialyzable MRP mixture yields are influenced by the initial reaction conditions used to generate brown pigments (Eichner and Karel, 1972; Resnik *et al.*, 1979). Moreover, the greater MRP yields and higher absorbance readings at 420 nm for Glu−Lys MRP mixtures compared to Fru−Lys MRP mixtures generated under identical reaction conditions in this study demonstrate the relative importance of the source of reducing sugar in synthesis of Maillard intermediate products. Similar observations have been made by Cerrutti *et al.* (1985) and Baxter (1995). The MRPs derived from different glucose and fructose sugars also corresponded to different average empirical formulas, with Fru−Lys having one carbon atom less but a higher C to N ratio than

Glu−Lys counterparts. These observed differences in yield and empirical formulas of MRP mixtures derived from glucose and fructose sugars when reacted with the same amino acid may be a result of the greater reactivity and different thermal fragmentation activities of aldose reducing sugars when compared to ketose reducing sugars as described by Cammerer and Kroh (1995). Alternatively, these results could be due to the steric hindrance of the carbonyl group present in keto sugars as demonstrated by O'Brien and Morrissey (1989). Keto sugars proceed through imine intermediates, which favor the formation of Heyns products, while aldose sugars proceed through Amadori products (Hodge, 1953). According to Pilkova *et al.* (1990), different rates of browning between aldo and keto sugars could also be attributed to the characteristically slower browning rate of Heyns products compared to Amadori products. As such, lower C to N ratios observed for Glu−Lys MRP mixtures compared to similarly synthesized Fru−Lys MRP mixtures herein are likely the result of rapid polymerization of the intermediary compounds formed in the Glu−Lys reaction giving rise to MRP polymers with more carbon atoms. In addition, the observation made by Rewicki *et al.* (1994) that the MRPs derived from fructose are relatively rich in pyrazine and are much different in composition to MRPs generated from glucose under similar experimental conditions further supports the findings made in our study.

MRP mixtures are a composite of several discrete chromophores that exhibit reduced absorbance capacity with increased wavelength (O'Brien and Morissey, 1989). The first stage of MRP formation is characterized by an absorption maximum at 280 nm, which has been partly attributed to the presence of heterocylic derivatives (Cuzzoni *et al.*, 1988). As the reaction continues, absorbance values at 280 nm progressively decline along with the appearance of more complex soluble pre-melanoidins possessing a maximum absorption at 320 nm. The precursors of more complex melanoidins likely accumulated to a measurable extent before a detectable quantity of high molecular weight melanoidin appears (Rendleman and Inglett, 1990). Therefore, detection of a visible absorption shoulder at 320 nm for both Glu−Lys and Fru−Lys MRP mixtures in the present study reflects a similar generation of more complex soluble MRP compounds regardless of the different reaction conditions used. The measurement of MRPs at 420 nm in this study was used as an endpoint measurement for quantifying the yield of melanoidins. The relatively higher absorbance readings at 420 nm consistently obtained in all experiments with model Glu−Lys MRP mixtures, compared to Fru−Lys MRP mixtures, indicated a greater melanoidin concentration in Glu−Lys MRPs. Unsaturated carbonyl and furfural compounds (Reynolds, 1965) giving rise to heterocylic amines (Mauron, 1981) are examples of higher molecular weight compounds derived from polymerization of reactive Maillard intermediate compounds. Moreover, the absence of a single definitive absorption maximum in all MRP mixtures tested herein may signify the simultaneous formation and condensation of chromophores on a single polymeric molecule, previously referred to as the mixed chromophore hypothesis (Clark and Tanenbaum, 1974).

IMAC was found to be a useful technique for separating crude MRP mixtures into smaller components. Crude dialyzed MRP mixtures tested herein were fractionated into five or six distinct components, similar to a study done previously using glucose−glycine-derived melanoidins (Terasawa *et al.*, 1991). Going by the elementary analysis data, fractionation of MRPs by chelation chromatography may have improved the accuracy in determining the composition of complex MRP polymers. Characteristically different empirical formulas obtained for both crude and fractionated MRP mixtures support the recent findings that the composition of derived MRP polymers is more dependent on the reaction conditions than the molar ratio of the reactants (Cammerer and Kroh, 1995). It is therefore feasible to conclude that the characteristic chelation behavior observed for Glu−Lys and Fru−Lys MRPs derived from the different experimental conditions in our study was attributed to the compositional variations in differently synthesized Glu−Lys and Fru−Lys model MRPs. In addition, fractionation of crude dialyzed MRPs by immobilized metal affinity chromatography also resulted in an enrichment of MRP components possessing high numbers of copper binding sites and thus greater copper binding activity. Therefore, assessing the metal chelation activity of crude MRPs may underestimate the true potential of more purified MRP components to bind trace minerals.

The use of MALDI-MS to determine the molecular weight size of a principal component present in a MRP component is, to our knowledge, the first attempt made using this technology. A high-intensity MALDI-MS signal around 5000 molecular weight range, observed herein for the fractionated MRP components, corresponded to a high copper binding activity in that molecular weight range. Yamaguchi *et al.* (1981) reported the presence of an antioxidative MRP component with a molecular weight of 4500 in a glucose−glycine MRP model system. Moreover, other studies have also shown that maltol and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone compounds present in MRP mixtures are associated with Fe^{3+} binding (Hashiba, 1985). Therefore, further studies are required to investigate the mechanism of copper binding by various model MRPs and its relevance to antioxidant activity.

CONCLUSION

This study demonstrated that the metal chelating affinity and elementary composition of MRPs are largely determined by the experimental conditions and reactant sugars used in the synthesis reaction. Although different structural compounds may exist within various complex MRPs, the principal components were found to possess similar molecular weights but characteristically different copper chelation activities.

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