JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Properties and Metal Chelating Activity of Glucose-Lysine Heated Mixtures: Relationships with Mineral Absorption Across Caco-2 Cell Monolayers

BEATRIZ RUIZ-ROCA, MARÍA PILAR NAVARRO, AND ISABEL SEIQUER*

Unidad de Nutrición Animal, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Camino del Jueves, 18100 Armilla, Granada, Spain

Model Maillard reaction products were generated by heating glucose-lysine mixtures (GL) at 150 °C for different times (15, 30, 60, and 90 min). Samples were characterized by free lysine, browning, and UV−visible spectra and assessed for antioxidant properties, metal chelating ability, and effects on mineral absorption across Caco-2 monolayers. It was found that the capacity to retard lipid peroxidation in a model linoleic acid emulsion system increased with heating time up to 60 min and then leveled off, whereas the scavenging activity toward 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals increased in early periods of the reaction (15 and 30 min of heating) and decreased thereafter. The iron binding affinity of the different samples was not correlated with antioxidant properties, and iron transport in Caco-2 cells was unchanged between samples. On the contrary, copper chelating activity showed significant correlation with free radical scavenging activity and with copper absorption across intestinal cells. It can be concluded that severe heat treatment of GL mixtures maintained the ability to reduce lipid peroxidation but decreased the free radical scavenging activity. Moreover, antiradical activity, copper chelation ability, and positive effects on copper absorption were correlated and associated to compounds formed at early stages of the Maillard reaction.

KEYWORDS: Maillard reaction products; antioxidant activity; mineral absorption; Caco-2 cells

INTRODUCTION

The Maillard reaction (MR), or nonenzymatic browning, starts when carbonyl groups from reducing sugars condense with amino groups from amino acids and progress into subsequent complex reaction products, collectively known as Maillard reaction products (MRPs). These products are formed during food processing and storage and are widely present in the human diet (*1*). Moreover, heat processing is commonly applied by the animal feedstuff industry via extrusion and pelleting methods, and MRPs are consequently formed (*2*).

Apart from their contribution to sensorial features of thermally treated foods such as aroma, color, and taste, certain biological effects have been attributed to MRPs, such as decreases in protein digestibility (*3*), modifications in mineral bioavailability (*4*), antioxidant activities (*5*), etc. Many in vitro studies (*6*) and assays in cell cultures (*7, 8*) evidence the antioxidant properties of MRPs. Most of these studies were carried out with model systems (*5, 7, 9*) or compounds isolated from foods (*10*). However, the mechanisms by which MRPs exert antioxidant activity are still unclear; some authors have related this effect with their free radical scavenging effect (*5, 11, 12*) or their effectiveness as metal chelating agents, as it is known that MRPs may behave like anionic polymers that are able to complex minerals (*13, 14*). Several authors have shown that MRPs can form complexes with copper (*15, 16*) and iron (*17*), and it has been observed that the presence of glucose−lysine (GL) heated mixtures strongly affects mineral solubility (*18*). Moreover, studies performed in rats reveal the influence of MRPs intake on copper and iron bioavailability (*19, 20*). Therefore, the metal chelating ability of MRPs may have two different aspects: on the one hand, a positive antioxidant effect in retarding metal-catalyzed reactions and, on the other hand, a possible negative effect on trace metal ions bioavailability. Thus, there is a need to ascertain whether there is a correlation between the antioxidant activity of model system MRP and the metal availability.

Reaction conditions, such as temperature, time of heating, pH, water activity, and source of reactant, have been shown to influence the characteristics and behavior of the MRPs formed (*14*). It has been shown that melanoidins from model systems have different iron and copper chelating abilities and different antioxidant properties depending on the reaction conditions used for synthesis (*17, 21*). Development of browning is usually associated with an increase in the antioxidant properties in systems where the MR is prevalent (*22, 23*), although some authors do not find a correlation between browning and

* To whom correspondence should be addressed. Tel: 34-58-572757. Fax: 34-58-572753. E-mail: iseiquer@eez.csic.es.

10.1021/jf801718h CCC: $40.75
Published on Web 09/10/2008

antioxidant capacity in assays performed in foods such as coffee (*11, 24*).

The objectives of the present work were to study the relationships between the antioxidant activity and the chelating ability of MRPs from GL model systems heated at different times and to evaluate whether these properties affect mineral availability. The antioxidant activity was evaluated by the capacity to reduce lipid oxidation in a lipid emulsion (measuring the thiobarbituric acid reactive substances, TBARS) and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging properties of the samples. Effects of the GL mixtures on mineral absorption were studied using an intestinal cell culture model, the Caco-2 cell line.

MATERIALS AND METHODS

All of the chemical products and solvents, for all of the analyses, were of the highest grade available and were acquired from Sigma (Sigma-Aldrich, St. Louis, MO) and Merck (Darmstadt, Germany).

Preparation and Characterization of Samples. Glucose (G) and lysine (L) were used to prepare the samples. Equimolar mixtures of GL monohydrate, 40% moisture in unbuffered systems, were heated in an oven (Selecta 2000210, Barcelona, Spain) at 150 °C for 15, 30, 60, and 90 min, using open recipients. The chosen temperature and time of heating corresponded to those commonly used for cooking food. After the samples were heated, the reaction was stopped by cooling in an ice bath, and the mixtures were removed, frozen, lyophilized (FTS System, Inc., TDS-3, NY), and stored in polyethylene bags at −80 °C until they were used.

Samples were characterized by means of weight loss, pH, absorbance measurements, and residual free L. Samples (50 g) were weighed (Mettler Toledo, AB420, Madrid, Spain) before and after heating to determine the percentage of weight loss. All of the mixtures, raw (GL0) and heated (GL15, GL30, GL60, and GL90), were suspended in demineralized water (Milli-Q Ultrapure Water System, Millipore Corp., Bedford, MA) (1 mg mL^{-1}), vigorously shaken for 10 min in a vortex, and centrifuged at 3080*g* for 45 min. In these conditions, all of the GL samples were totally soluble, except GL90, in which a little dark pellet was observed. pH (pHM250 ion analyzer MeterLab, Radiometer, Copenhagen, Denmark) and absorbance values at 280 and 420 nm (Shimadzu UV-1700, model TCC-240A, Duisburg, Germany) were measured in sample solutions or the corresponding supernatants.

The free L content in the GL mixtures was determined by high-performance liquid chromatography (HPLC) according to the Waters Pico Tag method (*25*) with precolumn derivatization with phenyl-isothiocianate using a Waters 2695 separation module (Waters Cromatografía, S. A., Madrid, Spain), without the hydrolysis step. A Millenium 32 chromatography manager system (Waters Cromatografía) was used for gradient control and data processing.

In the assays described below, the contribution of G and L by themselves to the different processes was also assessed. Both were prepared in the same proportions (quantity and moisture) as those present in the raw mixture.

TBARS Assay. The inhibitory effect on lipid peroxidation of the samples (GL mixtures, G and L) was measured in a linoleic acid emulsion system, following the procedure described by Wijewickreme and Kitts (*15*), with some modifications. First, the linoleic acid emulsion was prepared by adding linoleic acid (1.5 g) to a mixture of 0.1 M potassium phosphate buffer (PBS), pH 6.8 (200 mL), and Tween 80 (0.4 g). Then, the samples (4 mg) were incubated with 5 mL of linoleic acid emulsion and 5 mL of PBS at 45 °C for 24 h under dark conditions. Following incubation, the solution was diluted (1:1) with 25 mM Tris buffer (pH 7.4) containing 0.02% sodium azide, vigorously shaken, and used to measure the TBARS. One milliliter of the mixture containing the sample was added to 0.8% TBA (1 mL) in test tubes with marble caps and incubated in a water bath (100 °C) for 15 min. To lower the metal-catalyzed autoxidation of lipids, BHT (0.02%) was added to the TBA reagent. After the mixture was cooled, 1.5 mL of *n*-butanol was added and the mixture was shaken vigorously. The samples were centrifuged (15 min, 1700*g*, 4 °C), and the absorbance of the upper layer was measured at 532 nm using a Shimadzu 1700 UV−visible spectrophotometer. A standard curve prepared from 1,1,3,3,-tetraethoxypropane in 1% sulfuric acid was used. Controls without samples were prepared in the same manner. Results were expressed as the percentage of lipid peroxidation inhibition, calculated as $(TBARS_{control} - TBARS_{sample})/TBARS_{control} \times 100$.

DPPH Assay. The DPPH radical scavenging activity of the different samples was estimated according to the method reported by Jing and Kitts (*5*), slightly modified. One milliliter of the samples dissolved in PBS (1 mg/mL) was mixed with 1 mL of 0.1 mM DPPH in ethanol. The mixture was shaken vigorously and kept at room temperature for 30 min under dark conditions. The absorbance of the resulting solution was measured at 517 nm using a UV−visible spectrophotometer (Shimazdu, UV-1700, Columbia, United States). The control was prepared in the same manner, using PBS instead of the samples. The capability to scavenge the DPPH free radical of the sample (% of scavenging activity) was expressed as the percentage of disappearance of the initial purple color and was calculated according to the following equation: scavenging activity (%) = $(absorbance_{control} - absorbance_{sample})/absorbance_{control} \times 100$.

Copper Chelation Activity. The metal chelating activity of MRPs, G, and L was determined according to the tetramethyl murexide (TMM) method (*16*). Copper sulfate (0.1 mM $CuSO_4$), MRPs (200 μg mL^{-1}), and TMM (1 mM) were each dissolved in 10 mM hexamine·HCL (pH 5) buffer containing 10 mM potassium chloride (KCl). One milliliter of $CuSO_4$, 1 mL of MRPs, and 0.1 mL of TMM solutions were shaken in a vortex and allowed to settle for 10 min. Free copper in the samples was obtained by measuring the absorbance ratio between 460 and 530 nm and reading the amount of free copper from a standard curve ($CuSO_4$, 0.01−0.2 mM) (Shimazdu UV-1700). The amount of copper bound to the samples was obtained by calculating the difference between total copper and free copper in the system. The percentage of copper chelation activity was expressed according to the following expression: % copper chelation activity = (total copper − free copper) × 100/total copper.

Iron Chelation Activity. The method described by Yoshimura et al. (*13*) with some modifications was applied to measure the iron chelating power of the GL mixtures, G and L. Samples (0.05 g) and 1.4 mg of $(NH4)_2 \cdot Fe(SO_4)_2 \cdot 6H_2O$ (final concentration, 357 μM) were dissolved in 10 mL of Hepes buffer (30 mM, pH 7.4). The solutions were mixed with 10 mL of toluene containing 10 mM dibenzoylmethane (DBM) and 1 mM tri-*n*-octylphosphine oxide (TOPO), vigorously shaken, and centrifuged (1500*g*, 20 °C, 30 min). Free iron in the samples was obtained by measuring the absorbance at 480 nm and using a standard curve prepared from $(NH4)_2 \cdot Fe(SO_4)_2 \cdot 6H_2O$ (Shimadzu UV-1700). The percentage of iron chelation activity was expressed according to the following expression: % iron chelation activity = (total iron − free iron) × 100/total iron.

Cell Culture. Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) through the Cell Bank of Granada University at passage 20 and used in experiments at passages 30−35. All cell culture media and cell culture-grade chemicals were obtained from Sigma Chemical Co. Culture flasks and Petri dishes were purchased from Corning Costar (Cambridge, MA). Cells were grown in 75 cm^2 plastic flasks containing high glucose Dulbecco's modified minimal essential medium (DMEM), supplemented with heat-inactivated fetal bovine serum (10%), sodium bicarbonate (3.7 g L^{-1}), nonessential amino acids (1%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (15 mM), bovine insulin (0.1 UI mL^{-1}), and 1% antibiotic−antymycotic solution (Sigma A-5955). The cells were maintained at 37 °C in an incubator in a 95:5 atm of air−CO_2 at 90% humidity and given fresh medium every 2 or 3 days. Trypsinization and seeding of cells into permeable polycarbonate filter supports (Transwell, 24 mm diameter, 4.7 cm^2 area, 3 μm pore size, Costar) were performed as described elsewhere (*26*). The medium was changed every second day, and cells were used for transport and uptake experiments after 21 days of culture. The cell monolayer integrity during differentiation of Caco-2 cells was monitored by determining transepithelial electrical resistance (TEER) of filter-grown cell monolayers on different days after seeding, using a Minicell electrical resistance system

(Millipore, Bedford, MA). The monolayers used in this study exhibited adequate TEER values ranging from 500 to 650 Ω/cm^2. In addition to TEER measurements, the poorly absorbed phenol red marker, at a concentration of 42 μM in the culture medium, was used to confirm the integrity of the Caco-2 cell monolayers. To evaluate the rates of phenol red absorption, 1.5 mL of culture medium was added to the apical side, and 2.5 mL of transport buffer (130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES, pH 7) was added to the basolateral side. After incubation for 1 h at 37 °C, spectrophotometric measurements ($\lambda = 559$ nm) of phenol red in both apical and basolateral samples, following adjustment at pH 10 with 5 N NaOH where only the basic form of phenol red exists, were carried out. Cell monolayers were used when the leakage rate of phenol red was lower than 2.5% per hour.

Copper and Iron Uptake and Transport Experiments. Cell viability in the presence of solutions of GL mixtures, G, L, Cu ($CuSO_4$), and Fe ($FeSO_4$) in transport buffer (see above) was assayed prior to the experiments to select concentrations of metals and samples that do not affect the integrity of the cell monolayer in the bicameral cell culture system. Concentrations of iron and copper were chosen among those that could be physiologically reached at gastrointestinal levels. Metal solutions were freshly prepared before each assay. The viability of the Caco-2 cells was assessed by a neutral red (NR) cytotoxicity assay procedure, based on the ability of viable uninjured cells to actively incorporate NR, a supravital dye, into lysosomes. Cells were seeded in 96 well microtiter plates at a density of 75000 cells/well in 100 μL of media and maintained overnight to allow adherence to the wells. Growth medium was removed, and 100 μL of metal and sample solutions was added to the cells. The control wells received growth medium. Caco-2 cells were harvested after 3 h of exposure, during which time the growth medium was not changed. Cell viability was assessed by staining with NR (2 h at 37 °C), followed by cell fixation (0.5% formaldehyde and 0.1% $CaCl_2$ for 30 s at room temperature). Microtiter plates were washed by three brief immersions in phosphate-buffered saline, and the cells were lysed (50% ethanol and 1% acetic acid overnight at 4 °C). The optical densities of the resulting solutions were measured at 550 nm using a BioRad model 550 microplate reader (BioRad, CA). Cell viability data were expressed as a percentage as compared to control data from at least two independent experiments ($n \geq 5$ per experiment).

Uptake and absorption experiments were carried out 20 days after initial seeding into bicameral chambers. Spent culture medium was aspirated from the apical and basolateral chambers, and both cell surfaces of the monolayer were washed three times with HBSS at 37 °C. A 2.5 mL amount of transport buffer (see above) was then added to the basolateral chambers. Cell monolayers were covered with 1.5 mL of solutions of iron (0.1 mM) or copper (60 μM), alone (control solutions) or containing GL mixtures, G, or L (1 mg mL^{-1}) (three replicates/solution). Cell cultures were then incubated at 37 °C in a humidified air/CO_2 atmosphere for 3 h. After incubation, medium from the apical compartment was aspirated, the filter insert was removed, and the cell surface was washed twice with ice-cold buffer containing 150 mM NaCl, 1 mM EDTA, and 10 mM HEPES, pH 7, to remove nonspecifically bound metal and residual medium. The membrane with the cell monolayer was cut out, transferred to melting pots, and reserved to determine the amounts of Cu and Fe internalized in cells. To calculate the Cu and Fe transported across the cell monolayer, buffer from the basolateral chamber was removed; to ensure complete collection, the wells were washed twice with demineralized water.

Absorbance measurement at 280 and 420 nm was carried out in solutions before the experiments and in the transport buffer removed from the basal chamber after the incubation time. The absorbance recovery (%) was calculated taking into account the corresponding dilution factors.

Mineral Analysis. All materials used for analysis were previously soaked overnight in dilute nitric acid (10 N) and carefully rinsed with demineralized water. Iron and copper analyses in cell monolayers and basal chamber solutions were performed by flame atomic absorption spectrophotometry using a Perkin-Elmer Analyst 700 Spectrophotometer (Norwalk, CT). Previously, the samples were completely digested by the addition of concentrated HNO_3 and $HClO_4$ and by heating at high temperatures (180−220 °C) in a sand beaker (Block Digestor Selecta S-509; J. P. Selecta, Barcelona, Spain). Standard solutions were prepared from stock Tritisol solutions ($FeCl_3$ in 15% HCl, 1000 mg of Fe, $CuCl_2$ in H_2O, and 1000 mg of Cu, Merck). External standards from the Community Bureau of Reference (Brussels) were used to test iron and copper recovery (Pig kidney, CRM no. 186 and bovine liver CRM no. 185R, respectively) (Fe: certified value, 299 ± 10.00 $\mu g\ g^{-1}$; measured value, 295.31 ± 4.77 $\mu g\ g^{-1}$; Cu: certified value, 277 ± 5.00 $\mu g\ g^{-1}$; measured value, 287.27 ± 5.34 $\mu g\ g^{-1}$).

Table 1. Samples Characterization[a]

sample[b]	pH	absorbance 280 nm	absorbance 420 nm	free lysine (%)	weight loss (%)
GL0	9.71 ± 0.00 a	0.33 ± 0.00 a	0.05 ± 0.00 a	44.6 ± 0.84 a	
GL15	8.80 ± 0.00 b	1.14 ± 0.00 b	0.10 ± 0.00 b	25.8 ± 0.09 b	6.52 ± 0.02 a
GL30	7.73 ± 0.00 c	3.61 ± 0.01 c	1.02 ± 0.00 c	13.5 ± 0.28 c	14.06 ± 0.05 b
GL60	6.30 ± 0.01 d	3.62 ± 0.01 c	1.95 ± 0.01 d	12.2 ± 0.11 c	35.20 ± 0.05 c
GL90	5.43 ± 0.00 e	3.61 ± 0.01 c	2.29 ± 0.01 e	8.9 ± 0.05 d	49.18 ± 0.30 d

[a] Values are means ± SE ($n = 3$). Different letters in each column indicate significant differences (one-way ANOVA followed by a Duncan test, $P > 0.05$). [b] GL0, raw GL mixture; GL15, GL30, GL60, and GL90, GL mixtures heated at 150 °C for 15, 30, 60, and 90 min, respectively.

Statistical Analysis. All analyses were run in triplicate. One-way analysis of variance (ANOVA) was performed between the GL mixture data, and means comparisons were carried out using Duncan's multiple-range test ($P < 0.05$). In addition, in the Caco-2 cells experiments, the results of GL mixtures were compared with the controls by means of one-way ANOVA, followed by Duncan's multiple-range test to compare significant variations between means ($P < 0.05$). Analyses were performed using Statgraphics Plus, version 5.1, 2001. Results of G and L alone were not taken into account to the statistical analysis.

RESULTS AND DISCUSSION

Characterization of Samples. Characteristics of the GL mixtures are shown in **Table 1**. In our experimental conditions, MR was allowed to proceed without external pH control during heating. The pH of the heated mixtures decreased gradually and significantly as the heating time increased up to 90 min. This result was in accordance with those of other authors, who have reported, using model systems from different sugar−amino acids (*18, 27*) or sugar−proteins (*23*), a drop in pH values as heating time is increased. The decrease in pH during thermal treatment has been attributed to the formation of organic acids (mainly acetic acid and formic acid) (*28*). The disappearance of basic amino groups of lysine could also be implicated, as a relationship between pH values and free lysine ($r = 0.8924$; $P = 0.0002$) was observed among the samples of the present assay.

The measurement of the absorbance is the parameter most frequently used to monitor the progress of the MR (*18, 27*). The 280−290 nm absorbance range is related with early stage and low molecular weight MRP (*5*), whereas absorbance at 420 nm is an index for the formation of later stage and colored compounds of the MR (*27, 29*). Our results show that heat treatment of the GL mixtures caused an increase in absorbance at 280 nm, reaching a plateau at 30 min of heating (**Table 1**). Conversely, other authors have found a continuous increase in absorbance at 294 nm of protein−sugar MRPs heated at 100 °C up to 5 h (*23*). In the present assay, heating GL mixtures at 150 °C led to progressively higher formation of uncolored compounds up to 30 min of heating, but with longer heating times, no further formation of early and intermediate products was detected, probably because these compounds, which are the precursors of the browned ones, are rapidly transformed into advanced and colored MRPs in more drastic conditions. The development of browning, as measured by absorbance at 420 nm, increased continuously as a function of heating time,

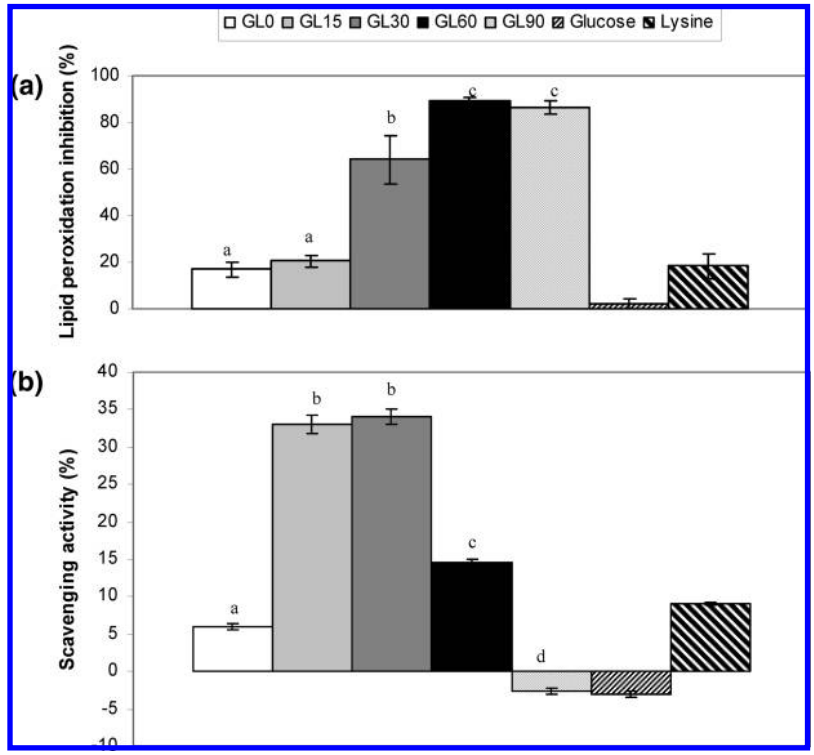

Figure 1. Percentages of lipid peroxidation inhibition (**a**) and DPPH scavenging activity (**b**) of glucose, lysine, and GL mixtures. The inhibitory effect on lipid peroxidation was measured in a linoleic acid emulsion system by the TBA method. Results are expressed as the percentage of lipid peroxidation inhibition calculated as (TBARS control − TBARS sample)/TBARS control × 100. The scavenging activity was measured by means of the DPPH method and is expressed as the percentage of disappearance of the initial purple color, calculated by the following equation: scavenging activity (%) = (absorbance control − absorbance sample)/absorbance control × 100; $n = 4$. Different letters indicate significant differences (one-way ANOVA, followed by Duncan's test, $P < 0.05$). GL0, raw GL mixture; GL15, GL30, GL60, and GL90, GL mixtures heated at 150 °C for 15, 30, 60, and 90 min, respectively.

according with previous assays carried out by other investigators with model MRPs (*27, 30*). However, in certain conditions, a decrease in 420 nm absorbance values after an initial increase has been observed in model MRPs at prolonged heating times (*18, 31*). Apart from the MR, caramelization of sugar could contribute to the browning of the mixture (*29*), although it has been shown that in sugar−amino acid mixtures that browning is almost completely due to the MR (*27, 30*).

On the other hand, values of browning and absorbance at 280 nm were inversely correlated with the free L content of MRPs ($r = -0.9282$, $P = 0.0000$ and $r = -0.9694$, $P = 0.0000$, respectively). Our results are in agreement with those of other authors who have reported free L losses in model systems during extended heating (*23, 32*), confirming that heat treatment catalyzes the interaction between ϵ-NH_2 group of lysine and sugar via the glycation process.

Antioxidant Activity. The ability of the GL mixtures, G and L, to reduce the lipid peroxidation in a model linoleic acid emulsion system is presented in **Figure 1a**. As has been shown for GL and fructose-lysine MRP obtained in different conditions (*15*), all of the GL mixtures of the present assay revealed antioxidant activity as assessed by the TBARS method, confirming that the presence of MRPs contributes to slowing the development of lipid oxidation (*22*). The antioxidant activity was strongly correlated with the degree of browning of the samples ($r = 0.9075$, $P = 0.0000$); thus, the stronger the color, the greater the antioxidant activity is. Similar results have been obtained when the inhibition of an induced-lipid oxidation has been investigated in the presence of model melanoidins and melanoidins isolated from beer and sweet wine (*6, 22*). In a similar way, the ability of coffee brews to suppress oxidation in a linoleic acid model system increases with the degree of roasting (*33*). Therefore, compounds formed in the later stages of the MR seem to play an essential role in the prevention of lipid oxidation, and the use of MRPs has been proposed in food technology as antioxidants to prevent the deterioration of food quality (*34*), although our knowledge of the mechanism of action remains limited. Although it has been suggested that the effectiveness of MRPs in reducing the propagation of lipid autoxidation reactions could signify an ability to scavenge free radicals (*21*), no statistical correlation between the reduction of lipid peroxidation and the antiradical activity was found in the present assay. The capacity of GL mixtures to reduce the stable radical DPPH increased drastically at the beginning of heating (15 and 30 min) but dropped significantly at more prolonged heating (**Figure 1b**). Moreover, a negative relationship was found between free radical scavenging properties and browning ($r = -0.8539$, $P = 0.0008$). Therefore, browning may not be a good index to evaluate the free radical scavenging activity of the MRPs formed, according to some authors (*27*) but in disagreement with others (*10, 23*), who found that the DPPH radical scavenging activity of model MRPs increases linearly as browning and heating time increase. In this line, Jing and Kitts (*5*) observe that the high molecular weight fraction isolated from sugar−L MRPs has a greater DPPH scavenging activity than the low molecular weight fraction. Free radical scavenging activity in relation to roasting and degree of browning has also been studied in foods, where antioxidants other than MRP are present, such as polyphenols and phenolic acids. Studies carried out with malts show that antioxidant activity increases with the intensity of heating, in parallel with color formation (*35*), whereas reports have been made of a loss

Table 2. Iron and Copper Chelating Activity of Samples[a]

	iron		copper	
sample[b]	free (μg)	chelating activity (%)	free (μg)	chelating activity (%)
GL0	5.85 ± 0.33 a	97.07 ± 0.16 a	3.90 ± 0.05 a	38.43 ± 0.86 a
GL15	20.20 ± 1.30 b	89.89 ± 0.65 b	2.12 ± 0.01 b	66.59 ± 0.25 b
GL30	36.81 ± 1.97 c	81.60 ± 0.99 c	2.39 ± 0.03 c	62.21 ± 0.56 c
GL60	26.35 ± 2.01 b	86.83 ± 1.00 b	3.56 ± 0.02 d	43.83 ± 0.38 d
GL90	34.34 ± 2.08 c	82.83 ± 1.04 c	3.48 ± 0.07 d	45.17 ± 1.11 d
lysine	0.85 ± 0.07	99.86 ± 0.03	2.53 ± 0.11	60.17 ± 1.78
glucose	85.54 ± 7.21	57.23 ± 3.60	6.31 ± 0.01	0.63 ± 0.17

[a] Values are means ± SE ($n = 3$). The chelating activity was estimated from the initial amount of free iron and copper, respectively. Different letters in each column indicate significant differences between GL mixtures (one-way ANOVA followed by a Duncan test, $P > 0.05$). [b] GL0, raw GL mixture; GL15, GL30, GL60, and GL90, GL mixtures heated at 150 °C for 15, 30, 60, and 90 min, respectively.

of antioxidant capacity during the advanced phases of roasting in coffee brews (*24, 33*). Our results agree with those of Anese et al. (*36*), who suggest that the highest scavenging properties of glucose−glycine heated mixtures are located at the first stages of the MR and with Yilmaz and Toledo (*31*) who stated that heating glucose−histidine mixtures at high temperatures over long periods degrades the antioxidant MRPs formed in the early stages of the reaction. Samaras et al. (*30*) also found increased scavenging properties of glucose−proline mixtures when these were heated at 220 °C for 10 min, followed by a loss of antioxidant activity at longer heating times. Therefore, it has been proposed that compounds that are thermally induced by severe temperature and/or time conditions may contribute to color development but not to antioxidant activity (*30*). On the other hand, the amino acid plays an important role in the final antiradical activity of the compounds (*37*), as shown in the present assay (**Figure 1**). Therefore, the disappearance of free lysine with increased heating time (**Table 1**) could have contributed to the reduced antiradical properties of the samples. The DPPH assay showed that the glucose has no antioxidant activity.

Chelating Activity. The iron and copper chelating activity of the samples is summarized in **Table 2**. MRPs are known metal chelators (*20*), and their metal ion binding affinity has been proposed as a possible mechanism for their antioxidant activity (*5*), as transition metals, especially iron and copper, are implicated in the generation of free radicals by Fenton reactions. All of the GL mixtures showed a high iron chelating ability, ranging from 82 to 97% (**Table 2**). Moreover, L by itself was able to complex nearly all of the iron present, thus confirming its recognized capacity to chelate iron (*38*). Therefore, the significant decrease in iron chelating activity shown by the heated mixtures as compared to the raw sample and L could be partially due to the losses of free L; however, the progressive decrease in free L was not accompanied by a similar decrease in chelating ability, which means that new compounds with iron binding properties were formed during thermal treatment. Previously, other authors have shown the ability of different sugar−amino acid model MRPs to chelate iron (*13, 17*), and even equations for the effect of melanoidins on the chelation of iron have been proposed (*17*). Although iron complexation has been related to the antioxidant properties of MRPs (*13, 5*), as chelating iron could afford protection against oxidative damage, no statistical correlations between iron chelation and lipid oxidation inhibition or efficiency for scavenging free radicals were found in the present assay. In line with Morales et al. (*17*), no relationship was found between browning and iron binding ability of the samples. The sugar, although to a lesser extent than the amino acid, was also found to contribute to the iron chelating power, as has been pointed out by others (*13, 17*).

When the GL mixture was heated for 15 min, the copper-chelating ability of the sample enhanced significantly with respect to the raw sample, but at more prolonged heating times, a progressive decrease was observed (**Table 2**), although always maintaining higher values than in the nonheated mixture. A negative relationship was found between 420 nm absorbance values and copper chelation ability ($r = -0.9513$, $P = 0.0000$). The special affinity of MRP to chelate copper has been described by several authors (*39*). Seifert et al. (*40*) studied the complex formation of some MRPs (Nϵ-fructoselysine and Nϵ-carboxymethyllysine) with copper and zinc and reported the formation of moderately stable copper complexes, whereas no complex formation with zinc was observed, thus concluding that new copper-binding centers are formed on the glycation of proteins. Moreover, increased copper insolubilization associated with the thermal treatment of GL mixtures has been reported in intestinal conditions (*18*). The chelation of copper has been related with the antioxidant activity in model MRPs (*15*) and in other compounds such as hydrolyzed potato protein (*41*). A different behavior of iron and copper binding ability associated with the thermal treatment would indicate a different MRPs−metal chelating mechanism. In this line, different protein binding properties have been described for copper and iron in metal-catalyzed oxidation of proteins (*42*). Copper chelating activity was significantly related to the scavenging ability of the samples in the present assay ($r = 0.6826$, $P = 0.0025$), but no correlation was found with lipid peroxidation reduction. As shown for the scavenging properties, the compounds showing better copper chelating activity were those formed in the initial stages of the MR. Decreases of copper affinity associated with the heat treatment of the samples could be somewhat attributed to the loss of free lysine, as it has been shown that lysine is capable of forming chelates with copper (*43*), which is in accordance with our results (**Table 2**). In fact, Cu−Lys complexes have been used in animal nutrition to improve copper bioavailability (*44*). No copper chelating activity could be attributed to the sugar.

Mineral Uptake and Transport in Cell Cultures. Several reports have validated the usefulness of the Caco-2 cell culture system to study iron metabolism (*45*). Iron uptake by Caco-2 cells was drastically reduced in the presence of GL mixtures as compared to the control ($FeSO_4$) (**Table 3**), which could be attributed to the formation of iron chelates, previously demonstrated (**Table 2**). According to some authors, the efficiency of iron uptake is determined by the affinity of the Fe complex for the iron uptake proteins and the tendency of the chelator to release the iron to the proteins (*46*). Thus, the MRPs from GL mixtures seem to chelate iron with a high affinity so that it is poorly available for uptake by brush border iron transporter proteins, impairing its uptake by the enterocyte in a similar way to that described for other dietary components (*47*). Among the GL mixtures, it may be observed that compounds formed at prolonged heating times favored the internalization of iron into the cells with respect to the shorter heating times, in agreement with previous results (*20*). The amount of mineral internalized by intestinal cells cannot be considered totally available, because many cells in the intestinal mucosa are desquamated and renewed daily; therefore, variations in the mineral uptake by the enterocyte may have a relative impact on availability.

Table 3. Iron Uptake and Transport in Caco-2 Cell Monolayers After 3 h of Incubation with 0.1 mM Fe Solutions Containing the Different Samples[a]

	uptake		transport	
sample[b]	μg/well	%	μg/well	%
control	1.88 ± 0.37	22.48 ± 4.49	1.91 ± 0.37	22.79 ± 4.40
GL0	0.19 ± 0.02 a*	2.29 ± 0.35 a*	2.22 ± 0.05 a	26.62 ± 0.68 a
GL15	0.15 ± 0.07 ab*	3.22 ± 0.08 ab*	1.48 ± 0.27 a	17.74 ± 3.32 a
GL30	0.19 ± 0.07 a*	2.32 ± 0.79 a*	2.15 ± 0.36 a	25.70 ± 4.36 a
GL60	0.51 ± 0.08 c*	6.13 ± 0.91 c*	1.29 ± 0.17 a	15.45 ± 2.09 a
GL90	0.47 ± 0.08 bc*	5.60 ± 0.98 bc*	1.92 ± 0.46 a	22.98 ± 5.56 a
lysine	0.09 ± 0.03	1.01 ± 0.31	2.12 ± 0.37	25.43 ± 4.53
glucose	0.20 ± 0.05	2.45 ± 0.60	3.68 ± 0.57	43.99 ± 6.92

[a] Values are means ± SE ($n = 3$). Different letters in each column indicate significant differences between GL mixtures; the asterisk indicates significant differences between the GL mixtures and the control ($FeSO_4$) (one-way ANOVA followed by a Duncan test, $P > 0.05$). [b] Control, transport buffer with $FeSO_4$; GL0, raw GL mixture; GL15, GL30, GL60, and GL90, GL mixtures heated at 150 °C for 15, 30, 60, and 90 min, respectively.

Table 4. Absorbance Recovery Across Caco-2 Cell Monolayers After 3 h of Incubation with 0.1 mM Fe Solutions[a]

	apical chamber	basal chamber	
sample[b]	initial Abs	final Abs	recovery (%)
		280 nm	
GL15	1.626 ± 0.00 a	0.150 ± 0.03 a	9.27 ± 1.95 a
GL30	4.822 ± 0.00 b	0.958 ± 0.07 b	19.87 ± 1.43 b
GL60	4.822 ± 0.00 b	1.205 ± 0.29 bc	24.99 ± 3.47 b
GL90	4.823 ± 0.00 b	1.394 ± 0.26 c	28.92 ± 4.55 b
		420 nm	
GL15	0.15 ± 0.00 a	0.000 ± 0.00 a	0.000 ± 0.00 a
GL30	1.48 ± 0.00 b	0.012 ± 0.01 b	0.810 ± 0.12 b
GL60	2.73 ± 0.00 c	0.052 ± 0.01 c	1.922 ± 0.51 b
GL90	3.27 ± 0.00 d	0.052 ± 0.01 c	1.581 ± 0.52 b

[a] Values are means ± SE ($n = 3$). Different letters indicate significant differences between samples (one-way ANOVA followed by a Duncan test, $P > 0.05$). [b] GL15, GL30, GL60, and GL90, GL mixtures heated at 150 °C for 15, 30, 60, and 90 min, respectively.

However, considerable quantities of iron crossed the cell monolayer and were transported to the basolateral chambers, both in the presence of GL mixtures and with the control solution, with no differences between them. It seems likely that most of the iron transported to the basal chamber in the presence of GL mixtures was in ionic form, nonchelated iron, and has crossed the cell monolayer moving transcellularly. Although it is known that there is little paracellular iron transport under normal circumstances, we cannot discount the possibility of the absorption of certain iron−MRPs as intact chelates through a paracellular route, in a similar fashion manner to that proposed for iron−EDTA (*48*). The absorbance recovery in the basal chambers (**Table 4**) confirmed that early and intermediate Maillard compounds were absorbed to some extent, as 280 nm absorbance values progressively increased with thermal treatment of samples reaching nearly 30% of recovery. However, the lack of correlation between "absorbance recovery" (280 and 420 nm) and "iron transport" suggest that the transport of iron complexes was not the main route of iron absorption. The 420 nm absorbance values were little or not at all detected in solutions collected from the basal chambers, which would mean a negligible flux of advanced MRPs, in agreement with other authors who have found extremely low transport levels across Caco-2 cell monolayers of Maillard products such as carboxymethyllisine (*49*). On the other hand, these findings agree with the MRPs metabolic transit studies performed mainly on rats, which have shown that at least 30% of low molecular weight compounds are absorbed, whereas high molecular weight compounds are absorbed to a much lesser extent (*50*).

Table 5. Copper Uptake and Transport in Caco-2 Cell Monolayers After 3 h of Incubation with 60 μM Cu Solutions[a]

	uptake		transport	
sample[b]	μg/well	%	μg/well	%
control	0.73 ± 0.09	12.26 ± 2.05	0.43 ± 0.09	7.54 ± 2.33
GL0	0.22 ± 0.04 a*	3.78 ± 0.69 a*	0.31 ± 0.05 a	5.44 ± 0.88 a
GL15	0.11 ± 0.01 b*	1.92 ± 0.29 b*	0.62 ± 0.02 bc	10.94 ± 0.42 bc
GL30	0.13 ± 0.03 b*	2.21 ± 0.53 b*	0.76 ± 0.15 c*	13.34 ± 2.74 c*
GL60	0.08 ± 0.01 b*	1.48 ± 0.28 b*	0.45 ± 0.07 ab	7.85 ± 1.28 ab
GL90	0.05 ± 0.00 b*	0.96 ± 0.12 b*	0.22 ± 0.01 a	3.98 ± 0.28 a
lysine	0.15 ± 0.01	2.71 ± 0.17 b	0.18 ± 0.00	3.14 ± 0.15 bc
glucose	0.25 ± 0.01	4.39 ± 0.24 b	0.11 ± 0.00	1.93 ± 0.02 c

[a] Values are means ± SE ($n = 3$). Different letters indicate significant differences between GL mixtures; the asterisk indicates significant differences between the GL mixtures and the control ($CuSO_4$) (one-way ANOVA followed by a Duncan test, $P > 0.05$). [b] Control, transport buffer with $CuSO_4$; GL0, raw GL mixture; GL15, GL30, GL60, and GL90, GL mixtures heated at 150 °C for 15, 30, 60, and 90 min, respectively.

Table 6. Absorbance Recovery Across Caco-2 Cell Monolayers After 3 h of Incubation at 37 °C with Cu-MRP Solutions[a]

	apical chamber	basal chamber	
sample[b]	initial Abs	final Abs	recovery (%)
		280 nm	
GL15	1.419 ± 0.00 a	−0.003 ± 0.01 a	−0.248 ± 0.46 a
GL30	4.702 ± 0.00 b	1.769 ± 0.25 b	37.63 ± 5.47 b
GL60	4.602 ± 0.00 c	2.19 ± 0.29 b	47.69 ± 6.44 b
GL90	4.602 ± 0.00 c	1.87 ± 0.26 b	40.67 ± 5.74 b
		420 nm	
GL15	0.402 ± 0.00 a	0.001 ± 0.00 a	0.000 ± 0.00 a
GL30	2.04 ± 0.00 b	0.381 ± 0.05 b	18.68 ± 2.69 b
GL60	3.91 ± 0.00 c	0.497 ± 0.06 b	12.67 ± 1.58 c
GL90	4.30 ± 0.00 d	0.445 ± 0.06 b	10.34 ± 1.51 c

[a] Values are means ± SE ($n = 3$). Different letters indicate significant differences between samples (one-way ANOVA followed by a Duncan test, $P > 0.05$). [b] GL15, GL30, GL60, and GL90, GL mixtures heated at 150 °C for 15, 30, 60, and 90 min, respectively.

A positive effect of glucose on iron absorption was observed (**Table 3**). Accordingly, studies performed in rats have found increased values of iron absorption when diets contained glucose and $FeSO_4$ as the iron source (*51*).

Although the Caco-2 cell line is a useful model to study copper metabolism (*52*) and Cu transporter proteins have been shown to be expressed in the villi of Caco-2 cells in culture (*52*), little is known regarding the effects of MRPs in copper absorption across Caco-2 cells. Our results show that copper uptake by Caco-2 cells was significantly lower after incubation in the presence of GL mixtures, or L and G separately, than in the presence of copper sulfate (**Table 5**). Conversely, copper transferred to the basal chamber was enhanced during incubation with GL heated mixtures, reaching significant differences with respect to the control in the presence of GL30, although dropping progressively with longer heating times. The absorbance recovery data revealed again that early MRPs crossed the cell monolayer in considerable proportions, except in the presence of GL15, as measured by the absorbance values at 280 nm of basal chamber solutions (**Table 6**). Browning compounds seem to have been absorbed to a lesser extent, as indicated by absorbance measured at 420 nm, although in the presence of GL30 values were significantly increased, in coincidence with higher copper absorption after incubation with this sample. Thus, it may be suggested that the formation of certain Cu−MRP complexes enhances copper absorption,

although the mechanism remains as yet unknown. Moreover, a relationship was observed between copper chelating ability and copper absorption ($r = 0.5389$, $P = 0.0210$). In accordance with our results, other experiments carried out on rats fed a rich MRPs diet have also found increased values of copper absorption, with copper accumulating in some organs (*19*). The physiological consequences of such increased absorption of copper bound to MRPs remains to be studied, as apart from the likely beneficial role of copper in oxidative stress, excess can also be toxic (*53*). On the other hand, including pharmacological levels of copper in animal feed has been shown to increase growth performance, especially of weanling pigs (*54*). Copper sources other than copper sulfate should be studied, to reduce feeding levels and, consequently, decrease copper excretion in manure and the potential environmental threat (*55*).

We may conclude that severe heat treatment of GL mixtures maintained the ability to reduce lipid peroxidation but decreased the free radical scavenging activity. The iron binding affinity of the different samples was not correlated with antioxidant properties and did not modify iron absorption across Caco-2 cells. Conversely, copper chelating ability, free radical scavenging activity, and positive effects on copper absorption were correlated and associated to compounds formed at early stages of the MR.

LITERATURE CITED

(1) Delgado-Andrade, C.; Seiquer, I.; Navarro, M. P.; Morales, F. J. Maillard reaction indicators in diets usually consumed by adolescent population. *Mol. Nutr. Food Res.* **2007**, *51*, 341–351.

(2) Stein, H. H.; Bohlke, R. A. The effects of thermal treatment of field peas (*Pisum sativum* L.) on nutrient and energy digestibility by growing pigs. *J. Anim. Sci.* **2007**, *85*, 1424–1431.

(3) Seiquer, I.; Díaz-Alguacil, J.; Delgado-Andrade, C.; López-Frías, M.; Muñoz Hoyos, A.; Galdó, G.; Navarro, M. P. Diets rich in Maillard reaction products affect protein digestibility in adolescent males aged 11−14 y. *Am. J. Clin. Nutr.* **2006**, *830*, 1082–1088.

(4) Delgado-Andrade, C.; Seiquer, I.; Navarro, P. Comparative effects of glucose-lysine versus glucose-methionine Maillard reaction products consumption: In vitro and in vivo calcium availability. *Mol. Nutr. Food Res.* **2005**, *49*, 679–84.

(5) Jing, H.; Kitts, D. D. Antioxidant activity of sugar-lysine Maillard reaction products in cell free and cell culture systems. *Arch. Biochem. Biophys.* **2004**, *429*, 154–163.

(6) Morales, F. J.; Jiménez-Pérez, S. Peroxyl radical scavenging activity of melanoidins in aqueos systems. *Eur. Food Res. Technol.* **2004**, *218*, 515–520.

(7) Jing, H.; Kitts, D. D. Redox-related cytotoxic response to different casein glycation products in Caco-2 and Int-407 cells. *J. Agric. Food. Chem.* **2004**, *52*, 3577–3582.

(8) Valls-Bellés, V.; Torres, M. C.; Muñiz, P.; Boix, L.; González-Sanjosé, M. L.; Codoñer-Franch, P. The protective effects of melanoidins in adriamycin-induced oxidative stress in rat isolated hepatocytes. *J. Sci. Food Agric.* **2004**, *84*, 1701–1707.

(9) Jing, H.; Kitts, D. D. Comparison of the antioxidative and cytotoxic properties of glucose-lysine and fructose-lysine Maillard reaction products. *Food Res. Int.* **2000**, *33*, 509–516.

(10) Nicoli, M. C.; Toniolo, R.; Anese, M. Relationship between redox potential and chain-breaking activity of model systems and foods. *Food Chem.* **2004**, *88*, 79–83.

(11) Morales, F. J. Assesing the non-specific hydroxyl radical scavenging properties of melanoidins in a Fenton-type reaction system. *Anal. Chim. Acta* **2005**, *534*, 171–176.

(12) Papetti, A.; Daglia, M.; Aceti, C.; Quaglia, M.; Gregotti, C.; Gazzani, G. Isolation of an in vitro and ex vivo antiradical melanoidin from roasted barley. *J. Agric. Food Chem.* **2006**, *54*, 1209–1216.

(13) Yoshimura, Y.; Iijima, T.; Watanabe, T.; Nakazawa, H. Antioxidative effect of Maillard reaction products using glucose-glycine model system. *J. Agric. Food Chem.* **1997**, *45*, 4106–4109.

(14) Wijewickreme, A. N.; Kitts, D. D.; Durance, T. D. Reaction conditions influence the elementary composition and metal chelating affinity of nondialyzable model Maillard reaction products. *J. Agric. Food Chem.* **1997**, *45*, 4577–4583.

(15) Wijewickreme, A. N.; Kitts, D. D. Metal chelating and antioxidant activity of model Maillard reaction products. *Adv. Exp. Med. Biol.* **1998**, *36*, 543–553.

(16) Terasawa, N.; Murata, M.; Homma, S. Separation of model melanoidin into components with copper chelating Sepharose 6B column chromatography and comparison of chelating activity. *Agric. Biol. Chem.* **1991**, *55*, 1507–1514.

(17) Morales, F. J.; Fernández-Fraguas, C.; Jiménez-Pérez, S. Iron-binding ability of melanoidins from food and model systems. *Food Chem.* **2005**, *90*, 821–827.

(18) Delgado-Andrade, C.; Seiquer, I.; Nieto, R.; Navarro, M. P. Effects of heated glucose-lysine and glucose-methionine model-systems on mineral solubility. *Food Chem.* **2004**, *87*, 329–337.

(19) Delgado-Andrade, C.; Seiquer, I.; Navarro, M. P. Copper metabolism in rats fed diets containing Maillard reaction products. *J. Food Sci.* **2002**, *67*, 855–860.

(20) Delgado-Andrade, C.; Seiquer, I.; Navarro, M. P. Bioavailability of iron from a heat treated glucose-lysine model food system: Assays in rats and in Caco-2 cells. *J. Sci. Food Agric.* **2004**, *84*, 1507–1513.

(21) Wijewickreme, A. N.; Kitts, D. D. Influence of reactions conditions on the oxidative behaviour of model Maillard reaction products. *J. Agric. Food Chem.* **1997**, *45*, 4571–4576.

(22) Mastrocola, D.; Munari, M. Progress of the Maillard reaction and antioxidant action of Maillard reaction products in preheated model systems during storage. *J. Agric. Food Chem.* **2000**, *48*, 3555–3559.

(23) Benjakul, S.; Lertittikul, W.; Bauer, F. Antioxidant activity of Maillard reaction products from a porcine plasma protein-sugar model system. *Food Chem.* **2005**, *93*, 189–196.

(24) Del Castillo, M. D.; Ames, J. M.; Gordon, M. H. Effect of roasting on the antioxidant activity of coffee brews. *J. Agric. Food Chem.* **2002**, *50*, 3698–3703.

(25) Cohen, S. A.; Meys, M.; Tarvin, T. L. *The Pico-Tag Method. A Manual of Advanced Techniques for Amino Acid Analysis*; Millipore Corporation: Bedford, MA, 1989.

(26) Navarro, M. P.; Aspe, T.; Seiquer, I. Zinc transport in Caco-2 cells and zinc balance in rats: Influence of the heat treatment of a casein-glucose-fructose mixture. *J. Agric. Food Chem.* **2000**, *48*, 3589–3596.

(27) Morales, F. J.; Jiménez-Pérez, S. Free radical scavenging capacity of Maillard reaction products as related to colour and fluorescence. *Food Chem.* **2001**, *72*, 119–125.

(28) Martins, S. I. F. S.; Van Boekel, M. A. J. S. A kinetic model for the glucose/glycine Maillard reaction pathways. *Food Chem.* **2005**, *90*, 257–269.

(29) Ajandouz, E. H.; Tchiakpe, L. S.; Dalle-Ore, F.; Benajiba, A.; Puigserver, A. Effects of pH on caramelization and Maillard reaction kinetics in fructose-lysine model system. *J. Food Sci.* **2001**, *66*, 926–931.

(30) Samaras, T. S.; Camburn, P. A.; Chandra, S. X.; Gordon, M. H.; Ames, J. M. Antioxidant properties of kilned and roasted malts. *J. Agric. Food Chem.* **2005**, *53*, 8068–8074.

(31) Yilmaz, Y.; Toledo, R. Antioxidant activity of water-soluble Maillard reaction products. *Food Chem.* **2005**, *93*, 273–278.

(32) Naranjo, G. B.; Malec, L. S.; Vigo, M. S. Reducing sugars effect on available lysine loss of casein by moderate heat treatment. *Food Chem.* **1998**, *62*, 309–313.

(33) Daglia, M.; Papetti, A.; Gregotti, C.; Berte, F.; Gazzani, G. In vitro antioxidant and ex vivo protective activities of green and roasted coffee. *J. Agric. Food Chem.* **2000**, *48*, 1449–1454.

(34) Bressa, F.; Tesson, N.; Dalla Rosa, M.; Sensidoni, A.; Tubaro, F. Antioxidant effect of Maillard reaction products: Application to

a butter cookie of a competition kinetics analysis. *J. Agric. Food Chem.* **1996**, *50*, 6997–7006.

(35) Samaras, T. S.; Gordon, M. H.; Ames, J. M. Antioxidant properties of malt model systems. *J. Agric. Food Chem.* **2005**, *53*, 4938–4945.

(36) Anese, M.; Pittia, P.; Nicoli, M. C. Oxygen consuming properties of heated glucose/glycine aqueous solutions. *Ital. J. Food Sci.* **1993**, *1*, 75–79.

(37) Morales, F. J.; Babbel, M. B. Melanoidins exert a weak antiradical activity in watery fluids. *J. Agric. Food Chem.* **2002**, *50*, 4657–4661.

(38) Van Campen, D. Enhancement of iron absorption from ligated segments of rat intestine by histidine, cysteine and lysine: Effects of removing ionizing groups and of stereoisomerism. *J. Nutr.* **1973**, *103*, 139–142.

(39) O'Brien, J.; Morrisey, P. A. Metal ion complexation byproducts of the Maillard reaction. *Food Chem.* **1997**, *58*, 17–27.

(40) Seifert, S. T.; Krause, R. M.; Gloe, K.; Henle, T. Metal complexation by the peptide-bound maillard reaction products N(ϵ)-fructoselysine and N(ϵ)-carboxymethyllysine. *J. Agric. Food Chem.* **2004**, *52*, 2347–2350.

(41) Wang, L. L.; Xiong, Y. L. Inhibition of lipid oxidation in cooked beef patties by hydrolyzed potato protein is related to its reducing and radical scavenging ability. *J. Agric. Food Chem.* **2005**, *53*, 9186–9192.

(42) Stadtman, E. R.; Levine, R. L. Protein oxidation. *Ann. N. Y. Acad. Sci.* **2000**, *899*, 191–208.

(43) Berthon, G.; Hacht, B.; Blais, M. J.; May, P. M. Copper-histidine ternary complex equilibrium with glutamine asparagines and serine. The implications for computer-simulated distributions of copper(II) in blood plasma. *Inorg. Chim. Acta* **1986**, *125*, 219–227.

(44) Guo, R.; Henry, P. R.; Holwerda, R. A.; Cao, J.; Littell, R. C.; Miles, R. D.; Ammerman, C. B. Chemical characteristics and relative bioavailability of supplemental organic copper sources for poultry. *J. Anim. Sci.* **2001**, *79*, 1132–1141.

(45) García-Casal, M. N.; Leets, I.; Layrisse, M. Beta-carotene and inhibitors of iron absorption modify iron uptake by Caco-2 cells. *J. Nutr.* **2000**, *130*, 5–9.

(46) Salovaara, S.; Sandberg, A. S.; Andlid, T. Organic acids influence iron uptake in the human epithelial cell line Caco-2. *J. Agric. Food Chem.* **2002**, *50*, 6233–6238.

(47) Glahn, R. P.; Wien, E. M.; Van Campen, D. R.; Miller, D. D. Caco-2 cell iron uptake from meat and casein digests parallels *in vivo* studies: use of a novel in vitro method for rapid estimation of iron bioavailability. *J. Nutr.* **1996**, *126*, 332–339.

(48) South, P. K.; Miller, D. D. Iron binding by tannic acid: Effects of selected ligands. *Food Chem.* **1998**, *63*, 167–172.

(49) Grundwald, S.; Krause, R.; Bruch, M.; Henle, T.; Brandsch, M. Transepithelial flux of early and advanced glycation compounds across Caco-2 cell monolayers and their interaction with intestinal amino acid and peptide transport systems. *Br. J. Nutr.* **2006**, *95*, 1221–1228.

(50) Finot, P. A.; Magnenat, E. Metabolic transit of early and advanced Maillard products. *Prog. Food Nutr. Sci.* **1981**, *5*, 193–207.

(51) Brouwer, I. A.; Lemmens, A. G.; Beyne, A. C. Dietary fructose v. glucose lowers ferrous-iron absorption in rats. *Br. J. Nutr.* **1993**, *70*, 171–178.

(52) Klomp, A. E.; Tops, B. B.; Van Denberg, I. E.; Berger, R.; Klomp, L. W. Biochemical characterization and subcellular localization of human copper transporter 1 (hCTR1). *Biochem. J.* **2002**, *364*, 497–505.

(53) Linder, M. C.; Hazegh-Azam, M. Copper biochemistry and molecular biology. *Am. J. Clin. Nutr.* **1996**, *63*, S797–S811.

(54) Crownwell, G. L.; Lindermman, M. D.; Monegue, H. J.; Hall, D. D.; Orr, D. E., Jr. Tribasic copper chloride and copper aulfate as copper source for wealing pigs. *J. Anim. Sci.* **1998**, *76*, 118–123.

(55) Kornegay, E. T.; Verstegen, M. W. A. Swine nutrition and environmental pollution and odor control. In *Swine Nutrition*; Lewis, A. J., Southern, L. L., Eds.; CRC Press: Boca Raton, FL, 2001; Vol. 1.

Received for review June 4, 2008. Revised manuscript received July 31, 2008. Accepted August 14, 2008.

JF801718H