Antioxidants from a Heated Histidine–Glucose Model System. Investigation of the Copper(II) Binding Ability

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ABSTRACT: The metal binding ability of Maillard reaction products (MRP) was investigated by spectrophotometric monitoring for complex/chelate formation. In addition, the antioxidant activity of MRP, in the presence of Cu(II), was evaluated on a lipid model system consisting of sunflower seed oil/water (1:2). Results from the Rancimat test indicated a significant decrease in pro-oxidant ability of Cu(II) in the presence of MRP in contrast to histidine alone, whether heated or not. Results from the diphenylpicrylhydrazyl method showed that, in contrast to histidine (heated or not), the antiradical ability of MRP is reduced in the presence of Cu(II), but depends on the reaction time between MRP and Cu(II).

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KEY WORDS: Copper(II), 2,2-diphenyl-1-picrylhydrazyl radical, glucose, histidine, lipid oxidation, Maillard reaction products.

Nonenzymic browning or Maillard reaction occurs between amino compounds (e.g., proteins or amino acids) and carbonyl compounds (e.g., reducing sugars) in heated food systems. Both undesirable and beneficial effects may result from Maillard reaction. The formation of antioxidative Maillard reaction products (MRP) has been extensively observed. Mechanisms of antioxidative ability include radical chain-breaking activity through hydrogen atom donation (1,2) and scavenging reactive oxygen species (3–5). Furthermore, there is strong evidence that MRP have the ability to bind transition metals (5–9), which are possibly involved in the initiation and propagation steps of lipid oxidation. Transition metal binding activity may be significant as an antioxidant mechanism (1,10).

The antioxidative properties of MRP obtained from a histidine–glucose model system were observed in a previous study (11). Additionally, the MRP were able to reduce the stable diphenylpicrylhydrazine radical (DPPH$^{\cdot}$), possibly through a hydrogen donation mechanism or by radical combination. This paper extends that investigation to the effect of copper(II) ions on the antioxidant ability of the MRP.

MATERIALS AND METHODS

Materials. Antifoaming silicone emulsion, anhydrous D-glucose, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$), L-histidine monohydrochloride monohydrate, *n*-propyl gallate, butylated hydroxytoluene (BHT), and sunflower seed oil (antioxidant free) were purchased from Sigma Chemicals Co. (Poole, Dorset, United Kingdom). Potassium dihydrogen orthophosphate, cupric sulfate pentahydrate, potassium hydroxide, and solvents were purchased from Fisher Scientific UK (Loughborough, Leicestershire, United Kingdom).

Preparation of MRP. MRP were obtained by refluxing potassium dihydrogen orthophosphate (0.1 M) buffer containing L-histidine monohydrochloride monohydrate (33.3 mM) and anhydrous D-glucose (100.0 mM) for 10 h at 105 ± 2°C. The pH of the system was adjusted to 7.00 with a 4 M potassium hydroxide solution and dropped to 6.53 during the reaction. The crude MRP solution was a clear solution, and no insoluble material was observed. Similarly, a 33.3 mM L-histidine monohydrochloride monohydrate solution (HIS) was heated for 10 h (heated HIS) under identical conditions to the preparation of MRP. The pH of the system remained consistent with an initial and final pH of 7.00 and 7.01, respectively. Samples of the reaction mixtures were flushed with nitrogen (oxygen free) and stored at −20°C until used in antioxidant tests.

Spectrophotometric monitoring of complex/chelate formation. The electronic absorption spectra (200 to 800 nm) of 2.00% (vol/vol) MRP, 2.00% (vol/vol) heated HIS, or 2.00% (vol/vol) HIS solutions containing 0, 4, 8, 12, 16, or 20 mg/L of Cu(II) were determined with a Shimadzu UV-1601A Model spectrophotometer (Shimadzu Corp., Kyoto, Japan) after 60 min at ambient temperature. The spectra were recorded against the corresponding concentrations of Cu(II), with no pH control.

Preparation of the lipid model system. Peroxides originally present in the sunflower seed oil were partially removed with silicic acid (12). The oil was treated twice, and the final peroxide value (13) was 2.21 meq/kg (±0.15 meq/kg) (n = 3). Samples of the sunflower seed oil were flushed with nitrogen (oxygen free) and stored at −50°C until used in the Rancimat test. The peroxide value of the oil increased by 0.64 meq/kg (±0.11 meq/kg) over 6 mon. Sunflower seed oil-in-water emulsions (SSOE) were prepared by homogenizing, for 1 min, sunflower seed oil (12.50 g), polyoxyethylene-sorbitan monopalmitate (Tween 40) (1.16 g), and distilled water (25.00 g) with a X10/20 Ystral homogenizer (Ystral GmbH, Dottingen, Germany) equipped with a T1500 speed regulator (speed 6) and a 23/T stainless steel shaft with a generator for low-viscosity media. Similarly, sunflower seed oil-in-water emulsions containing 10 mg/kg Cu(II) [SSOECu(II)] were prepared by dissolving cupric sulfate pentahydrate in the aqueous phase of the SSOE prior to homogenization.

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Measurement of antioxidative activity. The oxidative stability of SSOE treated with either 1.00% (vol/wt) MRP, 1.00% (vol/wt) HIS, 1.00% (vol/wt) heated HIS, or 100 mg/kg *n*-propyl gallate was compared to that of an identical set made from SSOECu(II). A level of 1% (vol/wt) MRP in SSOE corresponded to *ca.* 430 mg solids/kg SSOE, whereas 1% (vol/wt) heated HIS corresponded to *ca.* 240 mg solids/kg SSOE which is equal to the initial amount of histidine (33.3 mM) in the histidine–glucose reaction mixture before heating. The oxidative stability was evaluated by means of Rancimat apparatus where 10 mL of each SSOE or SSOECu(II) was separately placed in the reaction vessel of a Metrohm Rancimat apparatus (model 617) (Metrohm AG, Herisau, Switzerland) set at a heating temperature of 50°C and an air flow of 20 L/h. A drop of antifoaming silicone emulsion was also added to each reaction vessel. Induction times (in hours) were determined, and the antioxidant activity was expressed as protection factor (PF), defined in Equation 1 as

$$PF = \frac{\text{(induction time of treated emulsion)}}{\text{(induction time of control emulsion)}} \quad [1]$$

A PF > 1 indicates an inhibition of lipid oxidation; the greater the PF, the better the oxidative stability.

Determination of antiradical ability. The antiradical ability of a methanol solution of *n*-propyl gallate (100 mg/L) and methanol solutions of MRP, HIS, and heated HIS at a level equivalent to 100 mg solids/mL was evaluated in the presence of 10 mg/L Cu(II) using the stable radical DPPH$^{\cdot}$ (14,15). The antiradical activity of a sample was expressed as % disappearance of DPPH$^{\cdot}$ calculated as described below; the greater the % disappearance of the initial purple color, the greater the antiradical activity (Eq. 2):

$$\%\ \text{disappearance} = \frac{DPPH^{\cdot}_{Blank} - \left(DPPH^{\cdot}_{Sample} + Control_{Sample}\right)}{DPPH^{\cdot}_{Blank}} \times 100 \quad [2]$$

where $DPPH^{\cdot}_{Blank}$ = absorbance at 517 nm of 4 mL distilled water + 1 mL of 0.1 mM methanolic DPPH$^{\cdot}$ solution. $DPPH^{\cdot}_{Sample}$ = absorbance at 517 nm of 4 mL aqueous sample + 10 mg/L Cu(II) + 1 mL of 0.1 mM methanolic DPPH$^{\cdot}$ solution. $Control_{Sample}$ = absorbance at 517 nm of 4 mL aqueous sample + 1 mL of methanol.

The respective mixtures were shaken vigorously and left to stand for 30 min in a water bath at 25.0 ± 0.2°C). $DPPH^{\cdot}_{Blank}$, $DPPH^{\cdot}_{Sample}$, and $Control_{Sample}$ were obtained by determining the absorbance at 517 nm against a control (4 mL distilled water + 1 mL methanol), using a Shimadzu UV-160A Model spectrophotometer.

Statistical analysis. Regression analysis and Student's *t*-test for significance were performed with a Microsoft Excel version 8.0 package (Microsoft Corporation, Redmond, WA). Significance level is $P < 0.05$.

RESULTS AND DISCUSSION

Spectrophotometric monitoring of complex/chelate formation. A crude MRP solution prepared from a histidine–glucose mixture was examined for its Cu(II) binding ability. The electronic rearrangement resulting from such metal binding may be observed spectrophotometrically by monitoring shifts in absorption bands. A summary of the data obtained from the absorption spectra (200–360 nm) of 2% (vol/vol) MRP, 2% (vol/vol) heated HIS, or 2% (vol/vol) HIS containing a range of concentrations of Cu(II) is displayed in Table 1.

TABLE 1
Absorption Band Shifts (in nm) in the Absorption Spectra of 2% (vol/vol) MRP, 2% (vol/vol) HIS, or 2% (vol/vol) Heated HIS Solutions Containing 0, 4, 8, 12, 16, or 20 mg/L Cu(II)

2% (vol/vol) solution	Hyperchromic shift	Hypochromic shift	Increasing shoulder	Decreasing shoulder
MRP	270–252	212–222	600–602	342[a]
Heated HIS	210–218		246–240	
	630–650[b]			
HIS	210–218		240	
	640–646[b]			

[a]Shoulder disappears for Cu(II) concentration ≥4 mg/L.
[b]Low intensity (maximum absorbance <0.025 absorbance units). MRP, Maillard reaction products solution; HIS, L-histidine monohydrochloride monohydrate solution; Cu(II), cupric ions; heated HIS, HIS solution heated for 10 h at 105 ± 2°C.

The addition of increasing concentrations of Cu(II) to the MRP, HIS, and heated HIS solutions resulted in systematic spectral changes. These involved intensity shifts (hyperchromic and hypochromic) and also wavelength shifts (bathochromic and hypsochromic shifts) for specific wavelengths. These shifts denote a copper binding activity from MRP, heated HIS, and HIS. The low-intensity hyperchromic shifts observed at λ_{max}= 640–646 and 630–650 nm for HIS and heated HIS, respectively, may be attributed to the typical complex formation between copper(II) and the amino group/carboxylic group of histidine (16).

Further indication of metal-binding ability from MRP is provided by the relationship between the concentration of Cu(II) and the increase of absorbance at λ_{max} 270–252 (R = 0.999) and 600–602 nm (R = 0.993) (Fig. 1). Similarly, correlations obtained for HIS [histidine in molar excess compared to Cu(II)] were R = 0.9998 at 240 and R = 0.987 at 640–646 nm. The correlations for heated HIS were R = 0.999 and R =

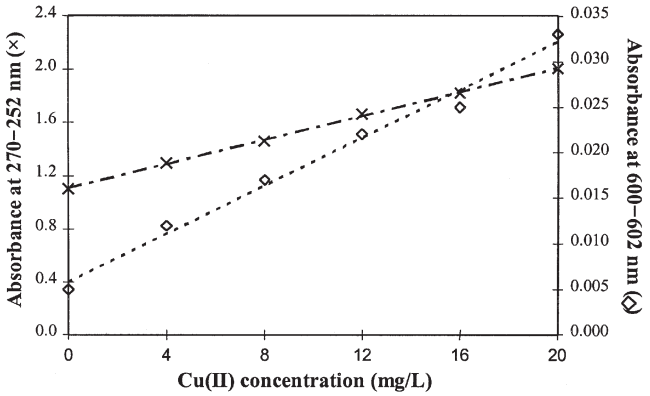

FIG. 1. Relationship between the increase of Cu(II) concentrations and absorbance of a 2% (vol/vol) Maillard reaction products solution at 270–252 nm and 600–602 nm.

0.982 at 246–240 and 630–650 nm, respectively. These data indicate a strong correlation ($P < 0.05$) between the increase of copper(II) concentration and absorbance at λ_{max} where hyperchromic shifts were observed, therefore confirming a Cu(II) complexation/chelation ability of MRP, HIS, and heated HIS. However, metal binding ability does not imply a protection against lipid oxidation. Amino acids, for example, may exhibit pro-oxidative effects in the presence of transition metals (17–20). Therefore, the effect of Cu(II) on the rate of lipid oxidation in the presence of MRP or histidine, heated or not, was evaluated by a Rancimat measurement of the induction period.

Rancimat test. The effect of 10 mg/kg Cu(II) on the rate of oxidation of SSOE containing either 1% (vol/wt) MRP, 1% (vol/wt) HIS, 1% (vol/wt) heated HIS, 100 mg/kg *n*-propyl gallate, or 100 mg/kg BHT was studied (Table 2). All the treated SSOE without added Cu(II) showed significant ($P < 0.05$) protection when compared to the control SSOE, with HIS and heated HIS being the most effective antioxidants. The addition of 10 mg/kg Cu(II) to the SSOE resulted in a decrease of protection toward lipid oxidation in the presence of HIS or heated HIS. The SSOECu(II) with *n*-propyl gallate showed protection, as *n*-propyl gallate has the ability to chelate transition metals. No significant protection was observed for SSOECu(II) containing BHT, this result was anticipated, as BHT does not complex transition metals. The MRP-treated SSOECu(II) had a significantly ($P < 0.05$) higher protection factor (+30.8%) than that of the MRP-treated SSOE without added Cu(II). Additionally, the mean induction time observed for the MRP-treated SSOECu(II) (87.7 ± 13.7 h, $n = 3$) was not significantly different from that of the control SSOE (91.5 ± 8.4 h, $n = 3$). This shows that the binding of Cu(II) by MRP reduces the pro-oxidant role of Cu(II) in contrast to HIS and heated HIS. Therefore, the MRP prepared from histidine and glucose serve as Cu(II) chelators.

DPPH· test. The effect of cupric ions on the radical donating ability of MRP, HIS, heated HIS, *n*-propyl gallate, or BHT was investigated using the stable free radical, DPPH·. The addition of Cu(II) to MRP resulted in a decrease in antiradical activity (13.4% disappearance) compared to the system without Cu(II) (Table 3). Additionally, this decrease in antiradical activity was found to be more distinct when the interaction time between MRP and Cu(II) was extended before performing the DPPH• test. An increase in interaction time between Cu(II) and MRP prior to the DPPH• test resulted in a progressive decrease of antiradical activity, the % disappearance decreasing from 53.4% after 0 min to 36.0% after 60 min interaction time (Fig. 2). The binding of Cu(II) by MRP appears to limit the interaction with the DPPH·. A possible mechanism is that the reactive sites of MRP may be involved in chelating Cu(II) and blocking the reaction site to DPPH•.

In contrast to MRP, the addition of 10 mg/L Cu(II) to the 100 mg/L *n*-propyl gallate solution produced a substantial increase in antiradical activity as the % disappearance increased (+29.9%). The increased antiradical ability of *n*-propyl gallate in the presence of Cu(II) may be due to the formation of a copper-propyl gallate chelate, which may favor the abstraction of hydrogen atoms from the hydroxy groups involved in the copper-propyl gallate chelate. Thus, *n*-propyl gallate may be effective in protecting lipid systems as a result of a

TABLE 2
Rancimat Protection Factors of MRP, HIS, Heated HIS, *n*-Propyl Gallate, or BHT, in the presence of 10 mg/L Cu(II) or not in SSOE

	Protection factor[a] [±%RSD]	
	SSOE	SSOECu(II)
Control[b]	1.00[c] [± 15.4]	1.00[c] [± 32.5]
1% (vol/wt) MRP	2.76[d] [± 2.6]	3.61[e] [± 4.0]
1% (vol/wt) heated HIS	3.76[e] [± 2.4]	2.16[g] [± 2.8]
1% (vol/wt) HIS	3.61[e] [± 8.8]	2.06[h] [± 0.5]
100 mg/kg *n*-propyl gallate	2.58[f] [± 5.8]	2.29[d,f,g,h,i] [± 34.8]
100 mg/kg BHT	2.73[d,f] [± 11.7]	1.30[c,i] [± 13.4]

[a]Mean values of three determinations [±%RSD].
[b]Mean values of 15 determinations [±%RSD]. Values followed by different roman superscripts (c–i) are significantly different ($P < 0.05$). The average ($n = 15$) induction time was 82.0 h (±12.7 h) for the control SSOE and 24.3 h (±7.9 h) for the control SSOECu(II); SSOE, sunflower seed oil-in-water emulsion; SSOECu(II), sunflower seed oil-in-water emulsions + 10 mg/kg cupric ions; BHT, butylated hydroxy toluene; see Table 1 for other abbreviations.

TABLE 3
Effect of 10 mg/L Cu(II) on the Antiradical Activity of MRP, HIS, Heated HIS, *n*-Propyl Gallate, or BHT as Measured by the DPPH· Test

	Disappearance (%)[a] [±SD]			
	Without Cu(II)		With Cu(II)	
MRP	54.4	[± 1.2]	47.1	[± 1.8]
Heated HIS	3.2	[± 0.3]	10.7	[± 0.4]
HIS	5.6	[± 0.0]	11.2	[± 0.1]
n-Propyl gallate	62.9	[± 0.8]	81.7	[± 0.8]
BHT	95.8	[± 0.6]	96.8	[± 0.1]
Control	0.0	[± 0.0]	2.3	[± 0.7]

[a]Mean values of three determinations [± standard deviation]; DPPH·, 2,2-diphenyl-1-picrylhydrazyl; see Tables 1 and 2 for other abbreviations.

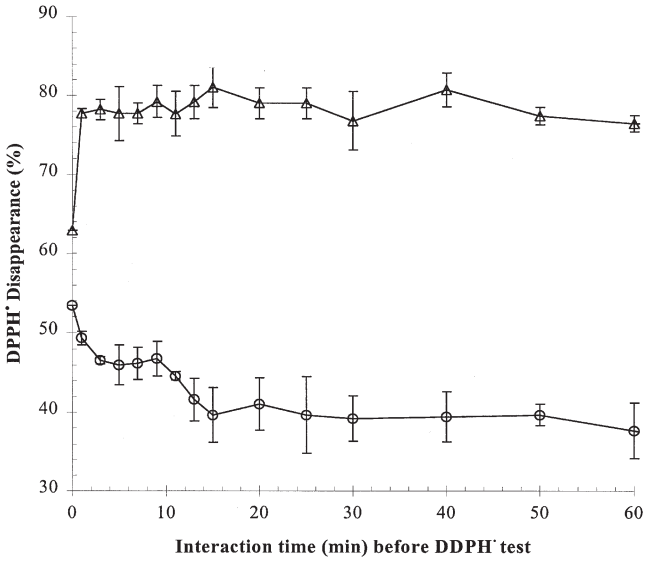

FIG. 2. Effect of reaction time [for constant 2,2-diphenyl-1-picrylhydrazyl (DPPH·) test time of 30 min] between 10 mg/L Cu(II) and 100 mg/L Maillard reaction products (○) (MRP) or 100 mg/L *n*-propyl gallate (△) on the antiradical ability.

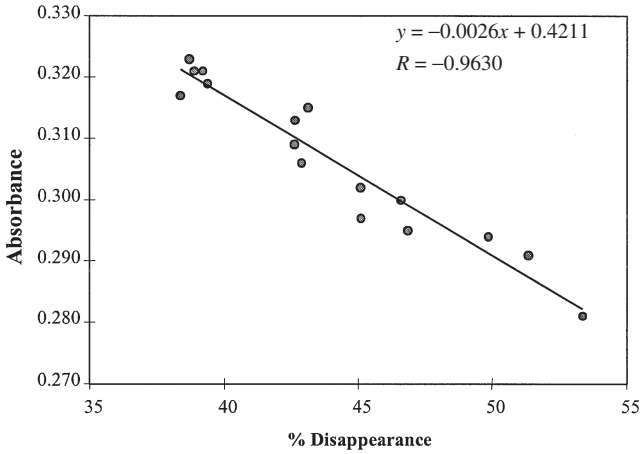

FIG. 3. Correlation between % DPPH· disappearance for a mixture of 100 mg/L MRP and 10 mg/L Cu(II) solution, and the absorbance at 260 nm over a period of 110 min. [Absorbance at 260 nm indicates interaction between MRP and Cu(II).] For abbreviations see Figure 2.

combined Cu(II) chelation and, possibly, an increased hydrogen-donating ability resulting from the Cu(II) chelation. However, this could not be confirmed with the Rancimat test as great variations in protection factors were observed, but only when *n*-propyl gallate was added to the SSOECu(II).

Increases in the antiradical activity of histidine (100% increase) and heated histidine (234% increase) were observed when Cu(II) was added, but the antiradical activity never exceeded 11%. A mechanism similar to that suggested for *n*-propyl gallate may take place whereby histidine–copper complexes could favor the donation of radical species to DPPH·. Finally, no significant variation in % disappearance of the DPPH· radical was observed for BHT-treated samples.

To further investigate the relationship between the decrease of antiradical activity over reaction time between MRP and Cu(II) solutions and metal binding ability of MRP, the changes in absorbance of a mixture of 100 mg/L MRP and 10 mg/L Cu(II) were monitored at λ_{max} 260 nm over 110 min. These results were correlated with the loss of antiradical activity of the same solution (Fig. 3). A significant correlation ($R = -0.963$) between antiradical activity and metal binding ability of MRP was found. The negative slope confirms that the MRP involved in the copper complexing have a lower ability to reduce DPPH·, thereby suggesting that the mechanism involved is different from that of copper(II) chelation by *n*-propyl gallate. However, the relevance of the DPPH· radical in relation to allylic radicals involved in lipid oxidation and where large complex macromolecules such as MRP are concerned is not clear. Care must be taken in relating results from the disappearance of DPPH· with actual antioxidant activity in actual lipid systems.

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