Antioxidants from a Heated Histidine–Glucose Model System. I: Investigation of the Antioxidant Role of Histidine and Isolation of Antioxidants by High-Performance Liquid Chromatography

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ABSTRACT: The radical-combining activity of Maillard reaction products [$MRP_{(aq)}$], produced by heating D-glucose and L-histidine (3:1) in a 0.1 M phosphate buffer for 10 h at 105°C (final pH 6.53), was estimated directly by means of a diphenylpicrylhydrazyl radical ($DPPH^{\bullet}$) method. Additionally, the indirect methods of peroxide values changes (oven test), hexanal formation, and protection factors (Rancimat method) were determined on a lipid model system that consisted of sunflower seed oil/water (1:2), emulsified with 3% (w/w) Tween 40. Results from the $DPPH^{\bullet}$ method showed a potential antioxidant activity of $MRP_{(aq)}$, which was confirmed by the indirect methods. Surprisingly, histidine in solution alone (heated or not) exhibited an antioxidant activity greater than or similar to the $MRP_{(aq)}$ activity in the indirect methods with the lipid model system, in contrast to the results from the $DPPH^{\bullet}$ method. The suitability of various solvents for extraction of potential antioxidant compounds from freeze-dried $MRP_{(aq)}$ was examined, and ethanol extracts showed the greatest activity by the $DPPH^{\bullet}$ method. Consequently, the ethanol extract of freeze-dried $MRP_{(aq)}$ was separated by means of preparative reverse-phase high-performance liquid chromatography (HPLC) with a 0.05 M phosphate buffer (pH 4.4)/water/acetonitrile gradient system. The antioxidant activity of the eluate was measured through the $DPPH^{\bullet}$ method, and a fraction (Fraction A) with antiradical activity was further purified by preparative HPLC. Fraction B was collected, and its freeze-dried residue exhibited potent antiradical activity, significantly greater than that of the same level of *n*-propyl gallate.

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KEY WORDS: Antioxidative activity, 2,2-diphenyl-1-picrylhydrazyl radical, glucose, histidine, HPLC separation, lipid oxidation, Maillard reaction, model system.

Products of the nonenzymic browning reaction between carbonyl compounds and amino compounds, also known as the Maillard reaction, have been reported to inhibit lipid oxidation in foodstuffs (1–7) and therefore may prevent associated off-odors, losses of nutritional value, and the development of toxicity.

Various researchers have investigated the antioxidative effects of Maillard reaction products (MRP) over the last 30 yr, and the main factors that affect the yield of antioxidant compounds are known to be pH (8–10), nature of initial reactants (9,11,12), molar ratio of initial reactants (3,9), water activity (13,14), temperature, and heating time (8,10,15–17).

The antioxidant activity of MRP is believed to be a result of multiple mechanisms, such as (i) free-radical termination (2,10,18), (ii) metal-chelating ability (2,10,19–21), (iii) active oxygen species scavenging (22–24), (iv) hydroperoxide–destroying ability (10,18), and (v) synergism (2,3,10, 25,26).

However, little is known about the structural characteristics of these antioxidative MRP. Reductones and amino reductones, produced during the advanced stage of the Maillard reaction, are possibly involved (13,27), with their antioxidant activity being related to their hydrogen-donating ability, peroxide-reducing activity, and metal-chelating activity (10,13,18); hydroxypyridones and hydroxypyranones have been shown to chelate transition metals, such as iron (19).

The aims of this work were to investigate the antioxidative properties of Maillard reaction products, obtained from a histidine–glucose model system, and to isolate components of potential antioxidative ability.

MATERIALS AND METHODS

Materials. Antifoaming silicone emulsion, anhydrous D-glucose, 2,2-diphenyl-1-picrylhydrazyl, L-histidine monohydrochloride monohydrate, *n*-propyl gallate, butylated hydroxytoluene, and sunflower seed oil were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Potassium dihydrogen orthophosphate and solvents were purchased from Fisher Scientific UK (Loughborough, Leicestershire, United Kingdom).

Preparation of MRP. $MRP_{(aq)}$ were obtained by refluxing 0.1 M potassium dihydrogen orthophosphate buffer that contained 33.3 mM of L-histidine monohydrochloride monohy-

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drate and 100.0 mM of anhydrous D-glucose (initial pH 7.00) for 10 h at 105°C (±2°C) (final pH 6.53). $MRP_{(aq)}$ was a transparent solution in which no insoluble polymers were observed. Similarly, a 33.3 mM L-histidine monohydrochloride monohydrate solution (pH 7.00) [$HIS_{(aq)}$] was heated for 10 h [heated $HIS_{(aq)}$] (final pH 7.01) under identical conditions as the preparation of $MRP_{(aq)}$. Samples of the reaction mixtures were withdrawn after 10 h of heating and were stored at –20°C, after nitrogen (oxygen-free) flushing, until further utilization.

Assessment of antioxidant activity. Preparation of the lipid model system. A sunflower seed oil-in-water emulsion (SSOE) was prepared by homogenizing for 1 min 12.50 g sunflower seed oil, 1.16 g polyoxyethylene-sorbitan monopalmitate (Tween 40), and 25.00 g distilled water with a X10/20 Ystral homogenizer (Ystral homogenizer, Dottingen, Germany), equipped with a T1500 speed regulator (speed 6) and a 23/T stainless steel shaft with a generator for low-viscosity media.

Measurement of antioxidative activity. The oxidative stability of SSOE without antioxidants (Control) was compared to that of SSOE treated with either 1% (vol/wt) $MRP_{(aq)}$, 1% (vol/wt) $HIS_{(aq)}$, 1% (vol/wt) heated $HIS_{(aq)}$, or 100 $mg \cdot kg^{-1}$ *n*-propyl gallate. A level of 1% (vol/wt) $MRP_{(aq)}$ in SSOE corresponded to *ca.* 430 mg $solids \cdot kg^{-1}$ SSOE, while 1% (vol/wt) heated $HIS_{(aq)}$ corresponded to *ca.* 240 mg $solids \cdot kg^{-1}$ SSOE, which is equal to the initial amount of histidine (33.3 mM) in the histidine–glucose reaction mixture before heating. The antioxidative activity was evaluated by means of Rancimat apparatus, by oven test, and by monitoring the hexanal formation.

In the Rancimat test, 10 mL of each SSOE was 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 $L \cdot h^{-1}$. 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), which is defined as (Equation 1)

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

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

In the oven test, 10-mL portions of each SSOE were placed in 50-mL stoppered flasks and incubated in the dark at 50°C (±2°C) for up to 4 d. Peroxide values were determined by an iodometric method (28) after chloroform extraction at 0, 1, 2, 3, and 4 d of incubation. The antioxidant activity of the various additives was evaluated graphically (interpolation) to give the time required for the various SSOE to reach a peroxide value of 10 $meq \cdot kg^{-1}$. The interpolation was performed by determining the equation of the best-fitting line of each set of results, after checking the linearity (correlation square, r^2) of the curves.

Hexanal formation was determined by static headspace gas chromatography–mass spectrometry (GC–MS). Emulsion samples of 1 mL of each SSOE were sealed with a crimper in 22-mL headspace vials with silicone rubber Teflon caps and incubated at 50°C (±0.2°C) in a shaking water bath (120 $shakes \cdot min^{-1}$) for up to 10 d, vials being withdrawn every 24 h. Subsequently, the vials were heated at 80°C for 10 min in the headspace magazine of a Varian Genesis Headspace Autosampler (Varian, Walnut Creek, CA) and pressurized for 30 s before the volatiles were automatically transferred at 230°C to a Varian Model 3400CX gas chromatograph, equipped with a fused-silica capillary DB-5MS column (30 m × 0.25 mm i.d. with 0.25-µm thickness; J&W Scientific, Folsom, CA) and coupled to a Saturn MS detector (Varian). The column was heated from 35° to 75°C at $10°C \cdot min^{-1}$, followed by a temperature rate of $20°C \cdot min^{-1}$ up to 230°C. The GC conditions were as follows: helium head column pressure, 30 psi; splitless injector temperature, 230°C. The mass spectrometer was operated in the electron impact mode at 70 eV and scanned from 37 to 270 amu with 1-s scan time cycles during the GC run. The total ion signal was integrated electronically by means of Varian Saturn data system software. Peaks for hexanal were eluted after 2.75 min, and peak heights were standardized with known amounts of hexanal, purified through a Waters Sep-Pak silica cartridge (Waters Associates, Milford, MA) (29) and added at various concentrations to SSOE, which contained 200 mg butylated hydroxytoluene per kg oil to prevent oxidation/hexanal formation during the static headspace procedure. Results were calculated as hexanal in millimoles per kilogram of oil.

DPPH• method. The antiradical ability of 100 $mg \cdot l^{-1}$ *n*-propyl gallate and the solutions of $MRP_{(aq)}$, $HIS_{(aq)}$, and heated $HIS_{(aq)}$ at a level equivalent to 0.1 mg solids per mL were evaluated by using the stable radical 2,2-diphenyl-1-picrylhydrazyl in a methanol solution (23,25,30,31). The antiradical activity of a sample was expressed as percentage disappearance of DPPH•, calculated as described below; the greater the percentage disappearance of the initial purple color, the greater the antiradical activity (Equation 2).

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

where $DPPH^{\bullet}_{Blank}$ = absorbance at 517 nm of 4 mL distilled water + 1 mL of 0.1 mm methanolic DPPH• solution, $DPPH^{\bullet}_{Sample}$ = absorbance at 517 nm of 4 mL aqueous sample + 1 mL of 0.1 mM methanolic DPPH• solution, and $Control_{Sample}$ = 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°C (±0.2°C). $DPPH^{\bullet}_{Blank}$, $DPPH^{\bullet}_{Sample}$, and $Control_{Sample}$ were obtained by determining the absorbance at 517 nm against a Control (4 mL distilled water + 1 mL methanol) with a Shimadzu UV-160A Model spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Isolation of potential antioxidative MRP. A scheme for the isolation of antioxidative MRP from a heated histidine–glucose model system is provided in Figure 1.

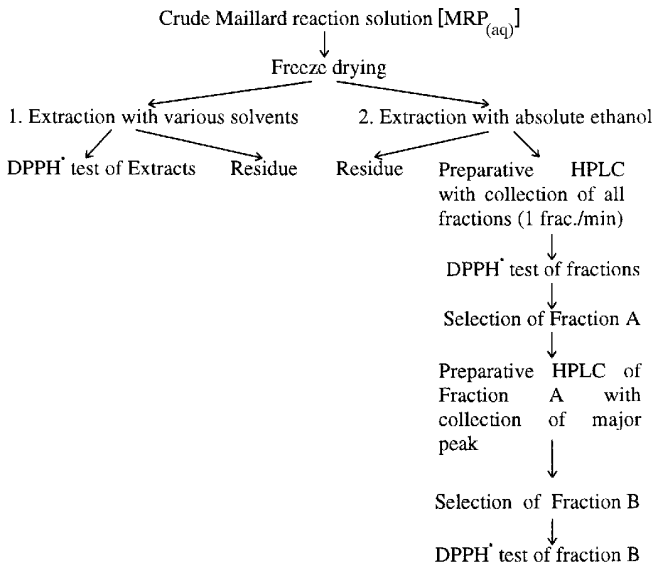

FIG. 1. Isolation of antioxidative Maillard reaction product (MRP) from a heated histidine–glucose model system. DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical; HPLC, high-performance liquid chromatography.

Solvent extraction of MRP. Approximately 100 mL of the crude mixture of $MRP_{(aq)}$ was freeze-dried at *ca.* −40°C and *ca.* 5 mbar for 72 h in a Modulyo freeze drier (Edwards, Crawley, United Kingdom). The freeze-dried residue formed a crust, which was crushed into a thin powder before extraction.

Solid–liquid extraction of freeze-dried $MRP_{(aq)}$ was achieved through a batch process. For this, 20 mL of either methanol (HPLC-grade), absolute ethanol (AR-grade), propan-2-ol (HPLC-grade), or ethyl acetate (HPLC-grade) were added to *ca.* 0.5 g of freeze-dried $MRP_{(aq)}$ and shaken for 5 min (Gallenkhamp flask shaker, speed 8) at ambient temperature, filtered through 0.45-µm cellulose acetate filters (Sartorius, Goettingen, Germany) or through 0.45-µm FP Vericel™ membrane filters (Gelman Sciences, Ann Arbor, MI), depending on the nature of the solvent. The residue was returned to the respective fresh solvent for a further two extractions. The combined solvent extracts were then evaporated at 50°C under reduced pressure in a rotary film evaporator. The dried extracted compounds thus obtained were tested for their antiradical activity by means of the DPPH• test at a concentration of 100 $mg \cdot L^{-1}$.

Ethanol extraction of MRP ($MRP_{(EtOH)}$). Approximately 50 g of freeze-dried $MRP_{(aq)}$ was extracted by batch process with absolute ethanol (20 × 50 mL), and the solvent was removed under reduced pressure in a rotary film evaporator at 50°C. Residues were dissolved in water at a 10% (wt/vol) level [$MRP_{(EtOH)}$], flushed with oxygen-free nitrogen, and stored at −20°C.

Preparative HPLC. Preparative HPLC was performed through a 250 × 22.5 mm i.d. Econosphere C18 column with a particle size of 10 µm (Alltech, Deerfield, IL). The HPLC system comprised a low-pressure Merck–Hitachi Model L-6200A Intelligent Pump (Merck, Darmstadt, Germany–Hitachi, Tokyo, Japan), set at a flow rate of 10 $mL \cdot min^{-1}$. Detection of the separated compounds was achieved with a Merck–Hitachi diode array detector, model L-4500 (Merck, Hitachi) with a flow cell of 1.0 × 10 mm (8 µL). The diode array detector was coupled to a Merck–Hitachi Model D-6500 chromatography data station (Merck, Hitachi), with scanning over the range 190–450 nm and fixed recording at 209.9 nm. A Rheodyne syringe-loading injector model 7125 (Rheodyne Incorporated, Cotati, CA) was used for sample injection, connected to a 1-mL Rheodyne loop. Samples were injected with a 1-mL Hamilton Gastight® valve flushing glass syringe (Hamilton Company, Reno, NV).

Purification of $MRP_{(EtOH)}$. Fractionation of the aqueous 10% (wt/vol) $MRP_{(EtOH)}$ solution was initially achieved by injecting 19 × 1 mL into the HPLC system, described above by using the gradient system displayed in Table 1. Fractions were collected manually every minute (10 mL per fraction) over a period of 60 min and tested for their antiradical activity with the appropriate mobile phase as controls.

A fraction (Fraction A) that eluted at a retention time R_T = 56 min with high antiradical activity (70.3% disappearance) was selected, freeze-dried (*ca.* −40°C and *ca.* 5 mbar for 72 h) and redissolved in HPLC-grade water, at a concentration of 0.5% (wt/vol) [Fraction $A_{(aq)}$].

Subsequently, Fraction $A_{(aq)}$ was further purified by means of preparative reverse-phase HPLC with the gradient system described in Table 2 (flow rate = 10 $mL \cdot min^{-1}$).

The major peak of Fraction $A_{(aq)}$ [R_T = 32.1 min (± 0.2 min)] was collected manually, the collection being made from the start of the peak [R_T = 31.7 min (± 0.0 min)] to its end [R_T = 33.3 min (± 0.2 min)]. This fraction recovered was named Fraction B and was placed into solid carbon dioxide immediately after collection and freeze-dried at −40°C (*ca.* 5 mbar) for 48 h in the dark. The freeze-dried residue of Fraction B was subsequently tested for its antiradical activity at a concentration of 100 $mg \cdot L^{-1}$ by the DPPH• test.

Statistical analysis. Regression analysis and Student's *t*-test for significance were performed with a Microsoft Excel-version 5.0*a* package (Microsoft Corporation, Redmond, WA). Significance level is $P < 0.05$.

TABLE 1
Ternary Gradient System Used to Separate an Ethanolic Extract [$MRP_{(EtOH)}$] of Maillard Reaction Products, by Means of Preparative Reverse-Phase HPLC[a]

Time	Solvents % (vol/vol)		
(min)	0.05 M Phosphate buffer (pH 4.4)	Water	Acetonitrile (far UV)
0	100	0	0
23	100	0	0
28	0	100	0
33	0	100	0
43	0	90	10
48	0	80	20
53	0	60	40
55	0	0	100
65	0	0	100

[a]$MRP_{(EtOH)}$, Maillard reaction products (absolute ethanol extract); HPLC, high-performance liquid chromatography; UV, ultraviolet.

TABLE 2
Binary Gradient System Used to Purify Fraction A by Means of Preparative Reverse-Phase HPLC[a]

Time (min)	Solvents % (vol/vol)	
	Water	Acetonitrile (far UV)
0	100	0
5	100	0
45	60	40

[a]See Table 1 for abbreviations.

RESULTS AND DISCUSSION

Evaluation of antioxidant activity. The first step of this study was to establish whether a crude solution of MRP from histidine–glucose [$MRP_{(aq)}$] exhibited any antioxidant ability. This was compared to a commercial antioxidant, *n*-propyl gallate, and to the initial amount of histidine, heated [heated $HIS_{(aq)}$] or unheated [$HIS_{(aq)}$]. Antioxidant activity was evaluated through the formation of peroxides (oven test), hexanal (GC–MS), and secondary oxidation products (Rancimat test) and through a more direct method with a stable free radical ($DPPH^{\bullet}$ test).

The effects of either $MRP_{(aq)}$, $HIS_{(aq)}$, heated $HIS_{(aq)}$, or *n*-propyl gallate on the peroxide value (PV) of SSOE were investigated at 50°C (Fig. 2). The times to attain a PV of 10 meq·kg^{-1} oil are displayed in Table 3, along with average square correlation values r^2 obtained from the best fitting lines. All the additives protected the SSOE significantly based on the time required to reach a PV of 10 meq·kg^{-1} oil, the order of antioxidant activity being ($P < 0.05$): $HIS_{(aq)}$ = heated $HIS_{(aq)}$ > $MRP_{(aq)}$ > *n*-propyl gallate.

TABLE 3
Time to Reach a Peroxide Value of 10 meq·kg^{-1} for Sunflower Seed Oil-in-Water Emulsions (SSOE) with Various Additives, After Incubation (at 50°C up to 4 d)

SSOE	r^2	Time (h)[a]
Control	0.991	18.5[a] [±1.0]
1% (vol/wt) $MRP_{(aq)}$	0.998	45.2[b] [±1.0]
1% (vol/wt) $HIS_{(aq)}$	0.982	66.8[c] [±0.3]
1% (vol/wt) heated $HIS_{(aq)}$	0.997	59.4[c] [±2.4]
100 mg·kg^{-1} *n*-propyl gallate	0.974	32.0[d] [±0.2]

[a]Mean values of two determinations [±standard deviation]. Values followed by different roman superscripts are significantly different ($P < 0.05$). $MRP_{(aq)}$, Maillard reaction products (aqueous solution); $HIS_{(aq)}$, L-histidine monohydrochloride monohydrate solution.

$MRP_{(aq)}$ had a greater protective effect on SSOE than *n*-propyl gallate, an antioxidant commonly used in foodstuffs. However, $HIS_{(aq)}$, heated or unheated, exhibited the greatest antioxidant activity. Although the antioxidative ability of histidine has been reported previously (32–36), the fact that $HIS_{(aq)}$ on its own (heated or unheated) had a greater inhibiting effect than $MRP_{(aq)}$ is unexpected. This suggests that unreacted histidine or histidine decomposition products, present in the $MRP_{(aq)}$ solution, may be responsible, partially or totally, for the protective effect observed for $MRP_{(aq)}$.

The protection factors, obtained from the Rancimat test with SSOE containing either $MRP_{(aq)}$, $HIS_{(aq)}$, heated $HIS_{(aq)}$ or *n*-propyl gallate, are compared in Table 4. Of all additives, the addition of 1% (vol/wt) heated $HIS_{(aq)}$ and 1% (vol/wt) $HIS_{(aq)}$ conferred the greatest protection, significantly greater than that of SSOE containing either 1% (vol/wt) $MRP_{(aq)}$ or the reference antioxidant *n*-propyl gallate. These results agree with those of the oven test in that $HIS_{(aq)}$, heated or unheated, had a significantly greater antioxidant effectiveness than $MRP_{(aq)}$.

On the basis of hexanal formation, no significant difference ($P < 0.05$) in antioxidant activity was observed between SSOE that contained $MRP_{(aq)}$, $HIS_{(aq)}$, or heated $HIS_{(aq)}$ (Fig. 3), although their effectiveness was significantly greater ($P > 0.05$) than that of *n*-propyl gallate after 4 d oxidation time.

The antiradical activities of $MRP_{(aq)}$, $HIS_{(aq)}$, and heated

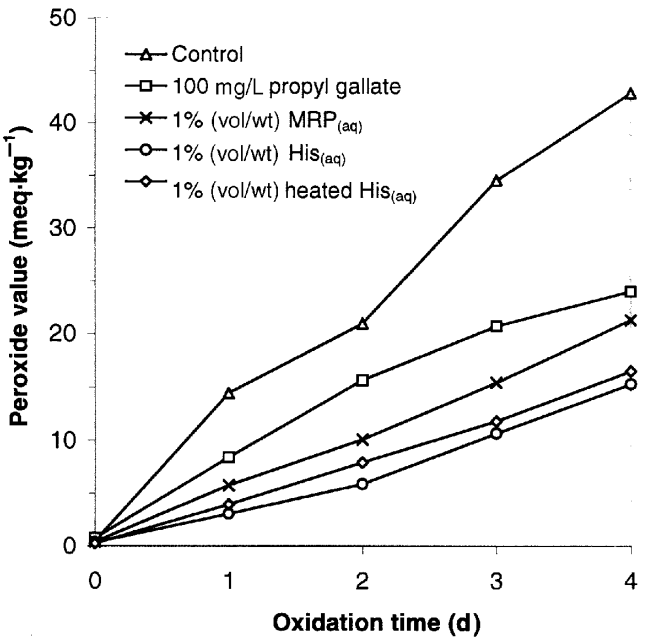

FIG. 2. Effect of 1% (vol/wt) crude solution of Maillard reaction products [$MRP_{(aq)}$], 1% (vol/wt) unheated histidine [$HIS_{(aq)}$], 1% (vol/wt) heated histidine [heated $HIS_{(aq)}$], and 100 mg·l^{-1} *n*-propyl gallate on the peroxide values (mean values of two determinations) of sunflower seed oil-in-water emulsions (SSOE).

TABLE 4
Protection Factor Obtained for SSOE, with Either a Crude Solution of $MRP_{(aq)}$, $HIS_{(aq)}$, Heated $HIS_{(aq)}$, or *n*-Propyl Gallate, by Means of Rancimat Test at 50°C

SSOE	Protection factor[a]
Control	1.00[a] [±0.09]
1% (vol/wt) $MRP_{(aq)}$	2.76[b] [±0.07]
1% (vol/wt) $HIS_{(aq)}$	3.76[c] [±0.32]
1% (vol/wt) heated $HIS_{(aq)}$	3.61[c] [±0.09]
100 mg·kg^{-1} *n*-propyl gallate	2.58[d] [±0.15]

[a]Mean values of three determinations [±standard deviation]. Values followed by different roman superscripts are significantly different ($P < 0.05$). The average ($n = 12$) induction time of the control SSOE was 83.6 h (±7.7 h). See Table 3 for abbreviations.

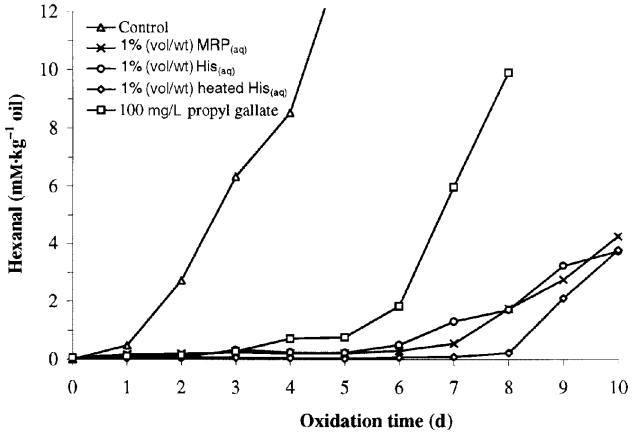

FIG. 3. Effect of 1% (vol/wt) crude solution of [$MRP_{(aq)}$], 1% (vol/wt) $HIS_{(aq)}$, 1% (vol/wt) heated $HIS_{(aq)}$, and 100 mg·L^{-1} *n*-propyl gallate on the hexanal formation of SSOE. See Figure 2 for abbreviations.

$HIS_{(aq)}$ were determined by the DPPH$^{\bullet}$ test and compared to *n*-propyl gallate as a reference (Table 5). The greatest antiradical activity was observed with *n*-propyl gallate and $MRP_{(aq)}$, which contrasts with the low activity observed for $HIS_{(aq)}$ and heated $HIS_{(aq)}$. Although the mass of $MRP_{(aq)}$ used was equal to that of the synthetic antioxidants, a realistic comparison of their respective antiradical activities would be achieved by using equimolar solutions of each. However, this is not possible because the molarity of the complex $MRP_{(aq)}$ solution remains unknown.

The antiradical activity observed for $MRP_{(aq)}$ shows an ability to reduce DPPH$^{\bullet}$, possibly either by donating hydrogen atoms or by combination with radicals produced in the course of the Maillard reaction (2,9,37–39) and could potentially represent a mechanism of antioxidant activity.

The weak antiradical activity observed for $HIS_{(aq)}$ (5.6%) and heated $HIS_{(aq)}$ (3.2%) was unexpected and conflicts with the results obtained by means of the oven test, hexanal test, and the Rancimat test (which monitor various products of oxidation). Histidine has been postulated to have the ability to act as a primary antioxidant through the donation of a hydrogen radical from, possibly, the imidazole ring nitrogen or the β-carbon (34). However, steric hindrance (40) might hinder histidine from reacting with DPPH$^{\bullet}$ and therefore fail to show a significant antiradical activity.

Overall, the results from the oven test, determination of hexanal, Rancimat test, and DPPH$^{\bullet}$ test confirm the presence of antioxidative MRP, which cannot be accounted for from the presence of histidine in the $MRP_{(aq)}$ mixture.

Isolation of potentially antioxidative MRP. The second part of the work focused on the development of a method to readily screen potentially antioxidative products from a fractionated histidine–glucose reaction mixture. The method is based upon the reduction of DPPH$^{\bullet}$ by components of freeze-dried $MRP_{(aq)}$, fractionated by solid–liquid extraction and subsequently purified by preparative HPLC.

Various solvents were tested for their suitability in extracting the most DPPH$^{\bullet}$-active components from freeze-dried $MRP_{(aq)}$. The antiradical activity of MRP extracts, obtained using either methanol, absolute ethanol, propan-2-ol or ethyl acetate, is displayed in Table 6. Absolute ethanol had the best selectivity for the extraction from freeze-dried $MRP_{(aq)}$ of compounds that reduce DPPH$^{\bullet}$. The absolute ethanol extract [$MRP_{(EtOH)}$] is the only extract that exhibits a greater antiradical activity than that of the same level of $MRP_{(aq)}$.

Subsequently, $MRP_{(EtOH)}$ was separated by means of preparative HPLC, a typical chromatogram being illustrated in Figure 4. The $MRP_{(EtOH)}$ fractions, collected every minute after HPLC separation, were screened for their antiradical activity by the DPPH$^{\bullet}$ test. Because of its high antiradical activity (70.3% disappearance) and the stability of its components, Fraction A (Fig. 4) was selected for further preparative HPLC separation, and the major peak was recovered and named Fraction B (see the Materials and Methods section).

The antiradical activity, obtained for 100 mg·L^{-1} freeze-dried Fraction B, was 90.5% disappearance, which was significantly greater ($P < 0.05$) than that of 100 mg·L^{-1} *n*-propyl gallate (62.9%) or 100 mg·L^{-1} $MRP_{(EtOH)}$ (86.8%). However, the recovery of Fraction B was too low [0.032% (w/w) of the solids in $MRP_{(aq)}$] to evaluate its antioxidant activity with SSOE.

Fraction B represents only a small part of the intricate mixture that comprises $MRP_{(aq)}$. Several preparative HPLC fractions had great antiradical activity, which suggests the presence of many active components. Nevertheless, the ability to isolate well-resolved components of $MRP_{(EtOH)}$, as demonstrated by this HPLC method, will substantially assist further study on the exact structure and properties of these reaction products. The identification of MRP from histidine and glu-

TABLE 5
Antiradical Activity of Either a Crude Solution of $MRP_{(aq)}$, Unheated $HIS_{(aq)}$, Heated $HIS_{(aq)}$, or *n*-Propyl Gallate as Determined by the 2,2-Diphenyl-1-picrylhydrazyl Radical (DPPH$^{\bullet}$) Method

Sample (100 mg·l^{-1})	% Disappearance[a]
$MRP_{(aq)}$	54.4[a] [±1.2]
$HIS_{(aq)}$	5.6[b] [±0.0]
Heated $HIS_{(aq)}$	3.2[c] [±0.2]
n-Propyl gallate	62.9[d] [±0.8]

[a]Mean values of three determinations [±standard deviation]. Values followed by different roman superscripts are significantly different ($P < 0.05$). See Table 3 for abbreviations.

TABLE 6
Antiradical Activity of Various Solvent Extracts of a Crude Solution of Maillard $MRP_{(aq)}$ as Determined by the DPPH$^{\bullet}$ Method

Extract (100 mg·L^{-1})	Disappeance (%)[a]
$MRP_{(aq)}$	54.4[a] [±1.2]
Methanol	52.7[b] [±0.1]
Absolute ethanol	86.8[c] [±0.3]
Propan-2-ol	21.1[d] [±0.3]
Ethyl acetate	8.3[e] [±0.6]

[a]Mean values of three determinations [±standard deviation]. Values followed by different roman superscripts are significantly different ($P < 0.05$). See Tables 3 and 5 for abbreviations.

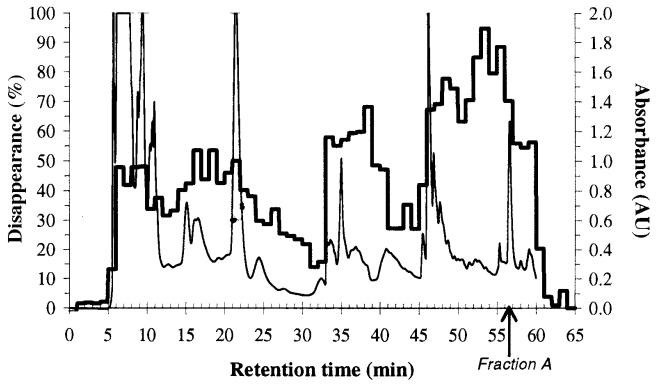

FIG. 4. Antiradical activity (▬ disappearance %) and corresponding chromatogram monitored at 209.9 nm (—) of 5% (wt/vol) ethanolic extract ($MRP_{(EtOH)}$) of $MRP_{(aq)}$, separated by preparative reversed-phase-HPLC. See Figures 1 and 2 for abbreviations.

cose, whether antioxidative or not, could contribute to a better understanding of the later stages of the Maillard reaction and allow a greater control over the progression of the reaction and the products formed.

Work is currently proceeding toward the structural determination and antioxidative effect of these compounds in lipid model systems.

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