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Antiradical Efficiency of Maillard Reaction Mixtures in a Hydrophilic Media

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The Maillard reaction (MR) has a clear impact in food science, nutrition, and medical research. Free radical scavenging capacities of several MR mixtures made from single combinations of glucose or lactose and amino acids (gly, his, lys, trp, met, and cys) were evaluated by using the *N*,*N*-dimethyl-*p*-phenylenediamine radical cation assay. Medium-roasted coffee brew was used as reference of a thermally processed food. A novel approach has been applied in order to get more information about the kinetic behavior of the radical scavenging properties of MR mixtures in a watery environment. Antiradical efficiency (AE) concept has been applied, and it takes into consideration the reaction time, apart from the amount of antioxidant necessary to decrease by 50% the radical initial concentration (EC_{50}). Cysteine and histidine reveal as powerful amino acids to exert a high AE in the MR mixtures. No relationship between AE parameter and browning was observed.

KEYWORDS: Maillard reaction; free radical scavenging; DMPD; antiradical efficiency

INTRODUCTION

In the past decade, research on the overall antioxidant capacities of food components and ingredients has been highlighted, since oxidation affects food safety, color, flavor, and texture. In fact, consumers claim an enhanced presence of natural antioxidant in their diet. However, most of the natural antioxidants present in foods are affected by oxidative stresses promoted by processing and preservation methods (*1*, *2*). However, the depletion of the natural occurring antioxidants of a processed food could be equilibrated by the formation of novel compounds with antioxidant activity (*3*). Then, by optimizing the industrial processing conditions, it could be possible to keep the overall antioxidant activity by enhancing the formation of certain Maillard reaction products (MRP) with antioxidant properties.

Because the preliminary work of Lingnert and Eriksson (*4*), much research has been done on the antioxidative properties of MRP by using different approaches, such as polarographic methods (*5*), rancimat (*6*), measurement of conjugated dienes from linoleic acid (*7*), TBARS index (*8*), or the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (*9*). The effect of the food matrix has not always taken into account whether the antioxidant function in aqueous, in bulk lipid, or in heterophasic systems is critically important. Recently, Frankel and Meyer (*10*) reviewed the different factors that affect the measurement of an antioxidant activity in foods and biological systems, such as colloidal properties, stages of oxidation, and the presence of different phases.

Several methods have been carried out to evaluate the antioxidant capacity, and particularly, rapid tests have been developed that measure the ability of antioxidants to intercept free radicals. *N*,*N*-Dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) assay has been successfully applied for assessing the antiradical activity of the water soluble fraction of enteral feeding products (*11*), wines (*12*), tomatoes (*13*), and pomegranate juice (*14*). The aim of this paper is to assess the antiradical efficiency (AE) of several aqueous MR mixtures as compared with a coffee brew in a hydrophilic media by a new kinetic approach. Because the compounds with antiradical activity formed during food processing will be located in the water soluble part of the food (mainly coffee and beer), it is convenient to evaluate the antiradical activity in an aqueous environment.

MATERIALS AND METHODS

Chemicals. D-Glucose, L-tryptophan, L-lysine monohydrochloride, L-glycine monohydrochloride, DMPD, ferric chloride, tannic acid, ferrulic acid, caffeic acid, and gallic acid were purchased from Sigma (Germany); L-histidine, L-methionine, and L-cysteine were purchased from Merck (Germany); D-lactose monohydrate and L-ascorbic acid were purchased from Panreac (Spain); and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was purchased from Aldrich (Germany).

Model Systems. Sugar (0.1 M) and amino acid (0.1 M) were dissolved in 250 mL of 0.05 M sodium phosphate buffer (pH 7). Twelve model systems were prepared as follows: glucose−glycine (GG), glucose−histidine (GH), glucose−lysine (GL), glucose−tryptophan (GT), glucose−cysteine (GC), glucose−methionine (GM), lactose−glycine (LG), lactose−histidine (LH), lactose−lysine (LL), lactose−tryptophan (LT), lactose−cysteine (LC), and lactose−methionine (LM). Model solutions were heated without pH control in tightly stoppered

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Table 1. Parameters That Characterize the Extent of the Browning in the Heated Model Systems[a]

	pH	A_{420}	fluorescence	sugar	amino acid	L* value	b* value	a* value	formic acid	acetic acid
GG	5.52 ± 0.15	0.379 ± 0.033	136.9 ± 19.2	74.7 ± 2.2	23.0 ± 1.1	39.7 ± 0.1	3.76 ± 0.23	7.89 ± 0.01	9.6 ± 0.2	34.5 ± 1.9
GH	6.18 ± 0.18	0.459	247.6	88.1	37.2	42.9 ± 0.1	8.45 ± 0.18	7.42 ± 0.41	10.3	37.2
GL	4.98 ± 0.11	0.649 ± 0.062	159.0 ± 23.5	86.0 ± 1.1	22.9 ± 0.3	38.2 ± 0.2	0.70 ± 0.05	5.24 ± 0.20	7.6 ± 0.7	22.9 ± 0.3
GT	5.89 ± 0.02	0.557 ± 0.044	507.0 ± 31.2	75.3 ± 6.8	21.8 ± 1.4	42.4 ± 0.1	7.38 ± 0.30	8.08 ± 0.23	6.7 ± 0.9	21.8 ± 1.4
GC	5.91 ± 0.05	0.086 ± 0.024	153.8 ± 2.9	57.8 ± 0.2		53.0 ± 0.0	10.26 ± 0.32	−2.81 ± 0.01	1.3 ± 0.1	
GM	5.66 ± 0.10	0.338 ± 0.019	80.6 ± 1.4	82.2 ± 1.0	22.2 ± 4.5	40.9 ± 0.4	6.73 ± 0.64	8.16 ± 0.31	8.6 ± 2.0	22.2 ± 4.5
LG	5.34 ± 0.07	0.344 ± 0.002	146.6 ± 9.5	78.3 ± 0.6	24.8 ± 0.3	40.3 ± 0.3	4.38 ± 0.75	7.73 ± 0.47	33.5 ± 0.6	24.8 ± 0.3
LH	6.09 ± 0.08	0.422 ± 0.019	238.5 ± 3.7	92.3 ± 4.3	48.1 ± 4.9	41.5 ± 0.2	6.33 ± 0.79	7.69 ± 0.25	64.8 ± 0.2	48.1 ± 4.9
LL	4.67 ± 0.01	0.478 ± 0.012	181.8 ± 16.5	82.3 ± 2.3	25.6 ± 0.9	39.4 ± 0.3	2.66 ± 0.38	7.08 ± 0.29	36.9 ± 2.5	25.6 ± 0.9
LT	5.77 ± 0.02	0.512 ± 0.036	189.2 ± 19.6	84.1 ± 0.7	33.2 ± 0.7	39.4 ± 0.2	2.58 ± 0.51	4.92 ± 0.32	40.0 ± 3.9	33.2 ± 0.7
LC	5.95 ± 0.14	0.050 ± 0.005	54.1 ± 1.4	58.3 ± 3.5		52.4 ± 0.7	12.53 ± 1.35	−2.03 ± 0.60		
LM	5.55 ± 0.04	0.269 ± 0.006	91.5 ± 3.5	82.3 ± 2.3	14.7 ± 1.6	42.4 ± 0.1	8.04 ± 0.26	7.94 ± 0.08	46.6 ± 1.5	14.7 ± 1.6

[a] pH value, browning (absorbance at 420 nm), fluorescence (mg quinine sulfate/L), percentage of total sugar and amino acid reacted, formation of formic acid and acetic acid (mM), and CIE lab parameters.

Pyrex flasks, which were immersed in a poly(ethylene glycol) bath kept at 100 °C. After a heating time of 24 h, the samples were rapidly cooled in ice and analyzed.

Coffee Sample. Medium-roasted coffee powder was purchased from a local store. Ground coffee (100 g) was stirred in 300 mL of distilled water at 75 °C for 5 min. The coffee brew was filtered, and an aliquot of filtrate was defatted with dichloromethane (2 × 200 mL).

Browning Measurement. Browning indices of the sample, after appropriate dilution, were recorded by their absorbances at 360, 420, and 505 nm on a Shimadzu UV-1601 (Duisburg, Germany) spectrophotometer.

Fluorescence Measurement. As described by Morales and Jiménez-Pérez (*9*). The results were expressed as quinine sulfate (mg/L).

Sugar and Organic Acids Measurement. Lactose, glucose, isomerization products (lactulose, fructose), galactose (as degradation product), and formic acid and acetic acid were analyzed by ion-exchange high-performance liquid chromatography (HPLC). A filtered sample (20 μL) was injected onto an ION-300 polymeric resin column (100 mm × 7.8 mm, Interaction-Lab, San Jose, CA) kept at 50 °C. A diluted sulfuric acid solution (4.5 mM) was used as mobile phase. Sugars were recorded with a refractive index detector (Erma Inc., Tokyo, Japan) and organic acids at 210 nm.

Amino Acid Measurement. Amino acids were analyzed by the OPA method according to the procedure described by Church et al. (*14*).

Color Measurement. Color was evaluated by using a tristimulus colorimeter MiniScan according to the CIELab. The system gives the values of three color components: the luminosity L* (−black to +white component) and the chromaticness coordinates, a* (+red to −green component) and b* (+yellow to −blue component). Maillard reaction mixtures (5 mL) were added into a glass Petri dish (Ø 5 cm) according to Morales and van Boekel (*16*). The sample was illuminated with D65 artificial daylight (10° standard angle) according to conditions provided by the manufacturer. Each color value reported was the average of three determinations at 22−24 °C.

Free Radical Scavenging Determination. Antiradical activity was measured by the DMPD method as described by Fogliano et al. (*12*). A stock DMPD solution (100 mM) was distributed in fractions of 1 mL each and frozen. Daily, a light-protected DMPD solution (0.75 mM) was prepared from the DMPD stock solution.

AE Determination. It was according to the procedure described by Sánchez-Moreno et al. (*17*) with some minor modifications. The percentage of remaining $DMPD^{\bullet+}$ against different standard dilutions was plotted to obtain the amount of antioxidant necessary to decrease the initial DMPD concentration by 50% (EC_{50}). By plotting the times necessary to reach the steady state against the corresponding dilution factor of the reaction mixtures or concentration of standards, the TEC_{50} was calculated, which was the theoretical time needed by the EC_{50} to reach the steady state. AE was defined as

$$AE = EC_{50}/TEC_{50}$$

Statistical. Analysis of variance and the means separated by Duncan's multiple range test were performed by applying Statgraphics v.2.3 statistical package (Statistical Graphics Corp., Rockwille, MD). All of the statistical procedures were performed at a significance level of 95%. All of the analyses were performed at least in duplicate.

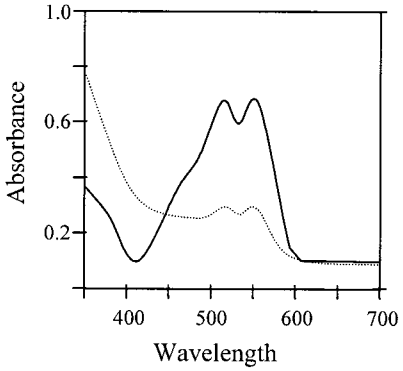

Figure 1. Visible spectrum of the DMPD radical cation ($DMPD^{\bullet+}$) at the reagent solution (solid line) and inhibited by MRP from a GG system (dotted line).

RESULTS AND DISCUSSION

The effect of single combinations of sugars and amino acids on the antiradical capacity of the MR mixtures was studied. Glycine and lysine were selected because they are quite well-described amino acids in most of the studies done on the MR (*18*), histidine, and tryptophan by their hypothetical antioxidant properties (*6*) and methionine and cysteine because they are predominant free amino acids in coffee solutions. **Table 1** summarizes some indices to characterize the extent of the MR, such as pH value, browning, fluorescence of intermediary MRP, percentage of sugar and amino acid reacted, formation of acetic acid and formic acid, and color developed in each heated MR mixture. As expected, model systems containing lysine reached lower pH values and higher browning indices than systems containing histidine or cysteine. It has been reported that acetic acid and formic acid are main degradation products of glucose (via 1,2-enolization) and lactose (via 2,3-enolization), respectively (*19*). At least 70% of the sugar reacted after 24 h of heating at 100 °C, except for systems containing cysteine.

In a previous step, the DMPD method proposed by Fogliano et al. (*12*) was standardized to a concentration of 0.75 mM DMPD in order to get an appropriate inhibition range. For a good extrapolation to calculate both the EC_{50} and the TEC_{50} parameters, it is necessary to cover the range of the dilution factor needed to decrease the initial concentration of $DMPD^{\bullet+}$ by 50%. By using an initial concentration of 0.75 mM $DMPD^{\bullet+}$,

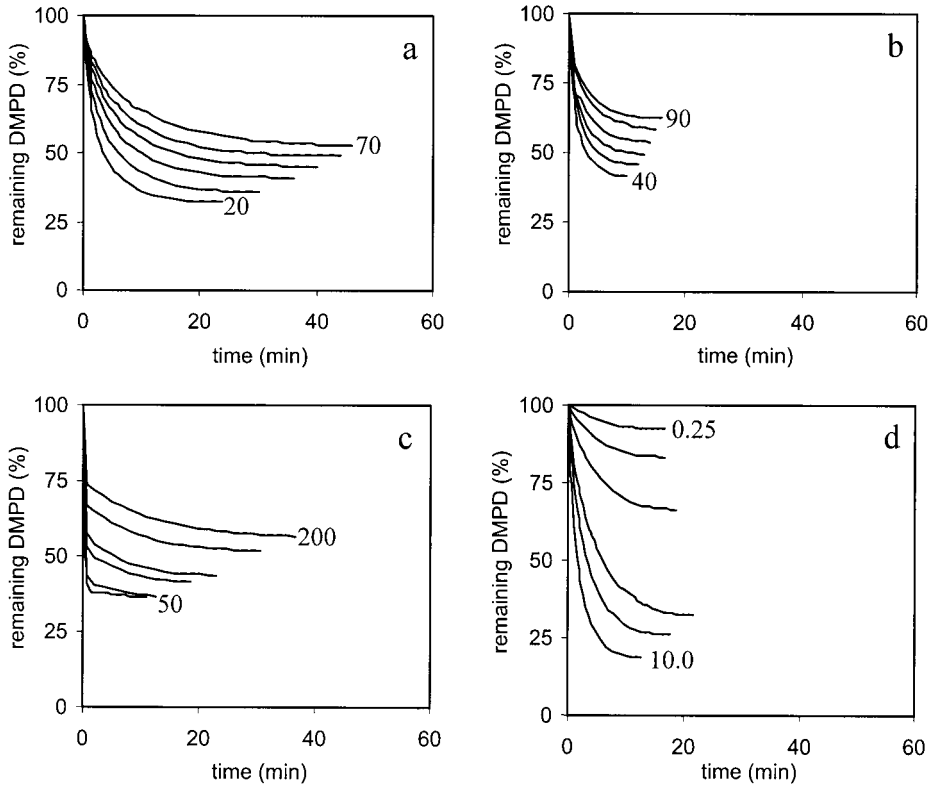

Figure 2. Examples of kinetic behavior. GG system (**a**), GH system (**b**), coffee (**c**), and ferrulic acid (**d**). Numbers describe the higher and lower levels of the dilution factor range applied, except for ferrulic acid expressed as mg/100 mL. Data recorded up to reach the steady state.

it was able to work in a range of dilution lower than 200-fold for the MR mixtures. On the other hand, color of the MR mixtures did not give interference with the assay at the dilution range proposed. Color of the sample is the main limiting factor of the DMPD assay. At these conditions, MRPs formed in each system are able to donate a hydrogen atom and decolorize the radical cation in a measurable range. **Figure 1** shows the visscan of a $DMPD^{\bullet+}$ solution added to water and GG model system after reaching the steady state. It was crucial in order to get highly reproducible values of the use of a fresh $DMPD^{\bullet+}$ solution. A slight loss of color in the daily prepared reagent solution was observed throughout the day, and then, 6−8 h was set as the effective time for the $DMPD^{\bullet+}$ solution; however, this time, new reference solution had to be prepared.

Concerning the relationship between color or fluorescence, as index of the extent of the MR, with the antiradical effect of the MR mixtures, it was unable to find a linear correlation. Literature described contradictory data on this aspect, but this study showed a lack of relationship between browning or fluorescence with the antiradical activity. However, previous studies have shown that browning is not directly related to the free radical scavenging properties of MRP formed at prolonged heating conditions by using DPPH assay in a chloroformic environment (*16*). Then, chromophoric substances should be very unlikely related to the antiradical activity of the formed MRP when different MR mixtures are compared. On the other hand, Antony et al. (*7*) showed the overall antioxidative effect clearly increased with heating time for the same MR mixture, and similar results were obtained by Morales and Jiménez-Pérez (*9*) on the antiradical properties of MR mixtures.

The depletion of the color of the radical cation by different dilutions of the MR mixtures was followed until the reaction reached a plateau. Then, the time for each dilution to reach the steady state was recorded, as well as the residual concentration of $DMPD^{\bullet+}$ at that time. These data for each dilution of the MR mixtures are useful to build the remaining $DMPD^{\bullet+}$ concentration vs time plot and then to calculate the EC_{50} parameter. The EC_{50} parameter is expressed as the dilution factor necessary to decrease the initial concentration of $DMPD^{\bullet+}$ by 50% at the steady state.

Figures 2 and **3** depicted some representative $DMPD^{\bullet+}$ quenching curves with their respective EC_{50} and TEC_{50} plots. In general, a great linearity among the different dilutions carried out was obtained. A change in the antiradical activity according to the ratio antioxidant:radical concentration (e.g., **Figures 2d** and **3d**) when well-described antioxidants (such as ascorbic acid, ferrulic acid, caffeic acid, gallic acid, tannic acid, or trolox) were used was observed . For instance, ferrulic acid showed a dose-dependent kinetic in the range between 0.25 and 10.0 mg/100 mL, as described by the EC_{50} and TEC_{50} parameter. Ferrulic acid was most effective at concentration levels lower than 2 mg/100 mL since higher rates of decolorization of DMPD were reached. This effect has already been shown when DPPH radical was used for assessing the AE of several antioxidants in a methanolic environment (*16*). A dose-dependent effect was also observed in our study in a watery media. It was obtained on average concentrations of 1.19, 1.44, 1.86, 7.81, and 10.81 mg/100 mL for gallic acid, caffeic acid, ferrulic acid, trolox, and tannic acid, respectively, for the EC_{50} parameter. In this case, the lower the value of EC_{50} is, the higher the antiradical power is since lower concentration is necessary to exert the same effect. EC_{50} is a parameter widely used to measure the antiradical power (*20*), but the time at the steady state depends on the antioxidant concentration (*17*). Concerning the TEC_{50} parameter, 55.7, 34.5, 20.1, 38.3, and 13.3 min for gallic acid, caffeic acid, ferrulic acid, trolox, and tannic acid, respectively, were obtained. Ascorbic acid was not analyzed due to the high power of action giving times lower than 1 min to reach the steady state. Taking into account the time to reach the steady state, a different behavior was observed for the most powerful tannic acid. It

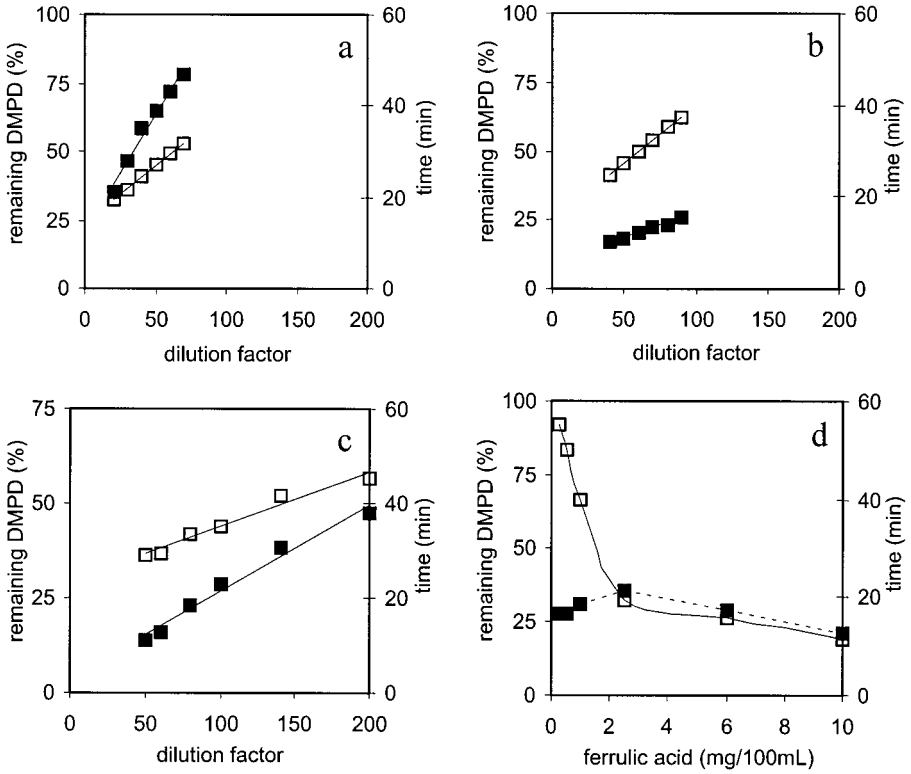

Figure 3. Determination of EC_{50} and TEC_{50} parameter from GG system (**a**), GH system (**b**), coffee (**c**), and ferrulic acid (**d**). Remaining $DMPD^{\bullet+}$ (□), time to reach the steady state (■).

Table 2. Kinetic Parameters for Assessing the AE of the Heated Model Systems as Compared with Coffee in an Aqueous Media[a]

system	EC_{50} (r^2) dilution factor	TEC_{50} (r^2) min	range of dilution factor	data (*n*)	AE
GG	60.4 (0.985)	35.5 (0.963)	(20–80)	7	1.70
GH	62.3 (0.989)	13.1 (0.981)	(40–100)	7	4.75
GL	53.9 (0.994)	33.8 (0.966)	(30–80)	6	1.59
GT	76.9 (0.998)	40.5 (0.758)	(30–100)	8	1.90
GC	159.6 (0.904)	35.7 (0.317)	(20–220)	19	4.47
GM	70.0 (0.988)	37.9 (0.579)	(20–100)	9	1.85
LG	54.1 (0.984)	30.2 (0.993)	(20–70)	6	1.79
LH	86.7 (0.984)	31.7 (0.479)	(20–110)	10	2.73
LL	66.5 (0.985)	36.2 (0.817)	(30–100)	8	1.84
LT	59.7 (0.996)	20.9 (0.993)	(40–100)	7	2.86
LC	62.1 (0.986)	21.5 (0.878)	(40–90)	7	2.80
LM	56.5 (0.964)	19.5 (0.969)	(20–100)	9	2.91
coffee	165.6 (0.958)	42.2 (0.918)	(50–200)	8	3.92

[a] EC_{50} describes the dilution factor needed to decrease by 50% the initial $DMPD^{\bullet+}$ concentration, and TEC_{50} is the time needed to reach the steady state; $P < 0.05$.

can be concluded that the structure of the antioxidant is directly correlated to the concentration (EC_{50}), while no influence of the concentration (EC_{50}) concerning time to reach the steady state (TEC_{50}) was found.

This kinetic approach has been applied to the different MR mixtures and coffee. The activity of natural phenolic compounds (mainly chlorogenic acid) on the overall AE of coffee brew has not been avoided. This assay is based on the rate of reduction and not on the extent of radical cation reduction at a fixed time point as described by Fogliano et al. (*12*). **Table 2** summarized the kinetic parameters obtained. System GC showed a high antiradical response, more than two times the rest of the studied systems. It should be taken into consideration that the EC_{50} parameter is expressed, as dilution factor for the analysis of MR mixtures, since it was not possible to study individual MRP. Anyway, it is not useful to study a single MRP, because each system will form specific compounds more or less efficient to donate a hydrogen atom to the $DMPD^{\bullet+}$. We are interested in analyzing the overall antiradical activity of these solutions as reactant in a complex food matrix. The kinetics parameters (EC_{50} and TEC_{50}) were not able to be determined in most of the solutions treated (100 °C/24 h) or untreated constituted by sugar or amino acid alone, since the undiluted solution of sugar or amino acid could not decrease the initial $DMPD^{\bullet+}$ concentration by 50% for at least 2 h of reaction. An average percentage of remaining $DMPD^{\bullet+}$ of 85.0 ± 5.7% was obtained for lactose and glucose solutions (treated and untreated), which means an AE value lower than 0.01. Similar results were obtained for heated and unheated solutions of glycine, lysine, methionine, and tryptophan. On the other hand, EA values of 0.57 and 0.48 for treated and untreated solutions of histidine, respectively, were found. A significant response was obtained in the cysteine solutions (treated and untreated) where average AE values of 8.9 were reached. Sulfydryl groups of cysteine will play an important role in the antiradical activity of the MRP formed since lower AE values were obtained in the LC and GC systems as compared with cysteine solutions. The GC system reached a similar value for the EC_{50} parameter to coffee.

According to both antiradical power and time of action, the AE of the MR mixtures can be described in three groups (high, intermediate, and low). Systems with low AE (<2) are GT, GM, LL, LG, GG, and GL. Systems with intermediate AE (2−4) are LM, LT, LC, and LH, and systems with high AE (>4) are GH and GC. It can be concluded that glycine and lysine are not appropriate amino acids to promote MRP with antiradical properties in thermally heated foods. However, cysteine and histidine were revealed as the most powerful amino acids to exert MRP with a high radical scavenging capacity, although cysteine solutions (treated and untreated) in absence of sugars exert a significant radical scavenging activity.

CONCLUSIONS

Although several antioxidants are presently used by the food industry, their safety could be questioned by the consumer. The seeking of alternative natural compounds with efficient antioxidant activity has been stressed. The importance of assessing the right processing conditions in order to enhance the formation of new antioxidants from the MR in coffee, beers, or tomato juice has been highlighted as well (*3*). Potent antioxidant could be synthesized from sugars and amino compounds or through optimization of food processing conditions (*2*). Up to now, the main mechanism of antioxidative action of the MRP was not clear; hence, it is necessary to develop model systems in a first step. On the other hand, naturally occurring antioxidant compounds presented in food could quench the antioxidant activity of thermally formed MRP. For instance, antioxidant properties of roasted coffee are mainly related to certain phenolic compounds that are present in green coffee including chlorogenic acid, caffeic acid, and ferrulic acid. There are different effects such as the ability of trapping positively charged electrophilic metabolites, scavenge oxygen radicals, metal chelation, particularly iron and copper, to form inactive complexes, or synergism. Then, it is mandatory to develop a rapid test to characterize the antioxidant properties of different MRP. This paper stated the conditions to use the radical cation DMPD assay as a rapid, low cost, and reliable test for assessing the antiradical activity of colored MR mixtures in aqueous media. On the other hand, the AE concept gives valuable information about the kinetic behavior of the different MR mixtures, and it could be applied to thermally treated foods. The high efficiency of cysteine and histidine to form potent radical scavenging compounds as compared with lysine or glycine has been described. In a next step, the molecular weight fraction of the MRP involved in the antiradical activity should be identified since no chemical nature has, so far, been elucidated.

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