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# Free radical scavenging capacity of Maillard reaction products as related to colour and fluorescence

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**Abstract**

The free radical scavenging activity of Maillard reaction products (MRPs) produced by heating glucose or lactose with lysine, alanine or glycine was estimated directly by means of a 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) method. Model systems of sugar (lactose and glucose) and amino acid (alanine, glycine, and lysine) were heated without pH control at 100°C up to 24 h. Fluorescence (347 and 415 nm), browning (absorbance at 420 nm), colour parameters in the CIELab scale, and free radical scavenging activity were determined. Browning is not directly related to the free radical scavenging properties of MRPs formed at prolonged heating conditions. This study shows that fluorescence measurement of heated sugar/amino acid systems is more effective than browning to follow the formation of MRPs with free radical scavenging activities. This approach could be applied to optimise the formation of natural antioxidants during food processing where browning is not desirable for consumers. 

*Keywords:* Colour; Fluorescence; Radical scavenging activity; Maillard reaction

## 1. Introduction

It is well known that the Maillard reaction products (MRPs) may influence the oxidative stability and shelf life of several foods, such as cereals (Lerici & Nicoli, 1996; Lingnert, 1980), milk (Hansen & Hemphill, 1984), and meat (Bedinghaus & Ockerman, 1995). The antioxidant capacity of MRPs was observed for the first time by Franzke and Iwainsky (1954) and some fractions were reported to have strong antioxidant properties comparable to those of commonly used food antioxidants (Lingnert & Hall, 1986). Potent antioxidants could be synthesised from sugars and amino compounds or through optimisation of food processing conditions (Anese, Manzocco, Nicoli & Lerici, 1999; Lingnert & Hall). It has been shown that the antioxidant efficiency of MRPs is influenced by several factors, such as the ratio and type of amino compounds and sugars involved, temperature, pH and water activity (i.e. Lingert & Hall). Furthermore, they are effective synergists in combination with phenolic antioxidants used in the food industry, acting both as scavengers of heavy metals and as promoters of the decomposition of hydroperoxides (Pokorný, 1991). It is supposed that the main mechanism of action is the ability of trapping positively charged electrophilic metabolites, scavenge oxygen radicals or metal chelation, particularly iron, to form inactive complexes. However, the compounds accounting for this effect have not been identified and the mechanism of antioxidant effect is still under study.

The Maillard reaction take place in three major stages (early, advanced and final stage) and it is dependent upon factors such as pH, time, temperature, concentration of reactants and reactants type (Ames, 1992). While the development of colour is an important and obvious feature of the reaction, few studies have been carried out on the development of fluorescence (Morales, Romero & Jiménez-Pérez, 1996). Coloured and fluorescent compounds need not be identical and fluorogens may be precursors of brown pigments showing a shorter induction period (Baisier & Labuza, 1992; Morales & van Boekel, 1997). An increment in the antioxidant activity of MRP is mostly related to browning that is not acceptable for consumers in certain foods. Hence, a balance between

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positive and negative effects should be taken into account before stimulating their formation during processing.

Increasing interest has been directed towards the utilisation of normal food constituents with antioxidative properties where MRPs are widely present in foods. On the other hand, it is well documented that naturally occurring antioxidant could be significantly lost as a consequence of processing and storage (Anese et al., 1999; Liao & Seib, 1988), but antioxidant MRPs are formed (Yen & Chung, 1999). Hence, functional properties of MRPs have been reviewed with reference to their use as potential new food ingredients. The purpose of this paper was to study the relationship between colour and fluorescence with the free radical scavenging properties of MRPs in order to gain more insight on the development of these beneficial properties in processed foods for human health.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals used were the purest available. D-(+)-Glucose, L-lysine monohydrochloride, L-alanine, L-glycine monohydrochloride, and 2,2-diphenyl-1-pycrylhydrazyl (DPPH·) were purchased from Sigma (St. Louis, MO, USA). D-(+)-lactose monohydrate and chloroform from Panreac (Barcelona, Spain).

### 2.2. Preparation of model MRP systems

Sugar (0.1 mol) and amino acid (0.1 mol) were dissolved in 1 l of a 100 mM phosphate buffer (pH 7) solution. Eight model systems were prepared, being glucose (G), glucose-alanine (GA), glucose-glycine (GG), glucose-lysine (GL), lactose (L), lactose-alanine (LA), glucose-glycine (LG), and lactose-lysine (LL). Model solutions were heated without pH control in tightly stoppered Pirex flasks which were immersed in a glycerol bath kept at 100°C. After a predetermined heating time (3, 6, 12, and 24 h), a sample is obtained and immersed in ice for a rapid cooling.

### 2.3. Chemical analyses

#### 2.3.1. Measurement of browning

Browning indices of the samples were recorded by their absorbance at 420 nm on a Shimadzu UV-1601 (Duisburg, Germany) spectrophotometer using a 1 cm path length cell after appropriate dilution with distilled water.

#### 2.3.2. Measurement of colour

The evaluation of colour of the heated sugar/amino acid mixtures were carried out using a tristimulus colorimeter MiniScan MS/S-4000S (Associates Laboratory Inc., Reston, VA, USA) according to the CIE Lab scale (CIE Colorimetry Committee, 1974; McLaren & Riggs, 1976). The system provides the values of three colour components; $L^*$ (black–white component, luminosity), and the chromaticness coordinates, $a^*$ (+red to −green component) and $b^*$ (+yellow to −blue component) (Hunter, 1942). Samples were pipetted into a 5 cm diam. glass Petri dish, as described by Morales and van Boekel (1998). The sample was illuminated with D65-artificial daylight (10° standard angle) according to conditions provided by the manufacturer. The $E$ index is calculated from the equation: $E=(L^{*2}+a^{*2}+b^{*2})^{1/2}$ and Chroma value according to the next equation: $C=(a^{*2}+b^{*2})^{1/2}$. Solid colours are named according to Kelly and Judd (1976). Each colour value reported was the mean of four determinations at 22–24°C.

#### 2.3.3. Measurement of fluorescence

Procedure was as described by Morales et al. (1996) with some minor modifications. Model system (100 μl) was taken and diluted with 10 ml of milli-Q water, to prevent quenching effects. The solution was measured at an excitation wavelength of 347 nm and emission wavelength of 415 nm. The linearity of fluorescence response was checked with a quinine sulphate solution dissolved in 0.1 mol/l $H_2SO_4$. The results were expressed as quinine sulphate (mg/l) amounts. A fluorescence spectrophotometer (SMF-25, Kontron Instruments, Milan, Italy) was used for determination of fluorescence. Quarzglass cuvettes (QS-1.000 Suprasil, Hellma GmbH & Co, DE) with light path of 1 cm were used. An average of three readings was recorded.

#### 2.3.4. Free radical scavenging method

The antiradical capacity of the reaction mixtures, previously heated at different temperatures, was estimated according to the procedure reported by Cämmerer and Kroh (1996) that was slightly modified. An aliquot of sample (200 μl) was added to 1 ml of DPPH· 74 mg/l in chloroform. A daily-prepared solution of DPPH· gave a final absorption at 520 nm of ± 1.8 AU. Mixture was shaken vigorously for 1 h, then absorption of the organic layer was measured at 520 nm. Temperature in the measurement chamber was set at 30°C. The DPPH· concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression; $[DPPH\cdot]_t = 0.2563\ (A_{520\ nm}) + 0.0203$ ($r^2 = 0.9995$), where $[DPPH\cdot]_t$ was expressed as mg/100 ml. The antiradical activity of sample was expressed as percentage disappearance of DPPH·; the greater the percentage disappearance of the initial purple colour, the greater antiradical activity. The percentage of remaining DPPH· ($\%DPPH\cdot_{rem}$) was calculated as follows; $\%DPPH\cdot_{rem} = ([DPPH\cdot]_t/[DPPH\cdot]_o)\times 100$, where $[DPPH\cdot]_o$ is the concentration at time zero. The percentage of

remaining DPPH· against the time was plotted to obtain the reaction time necessary to decrease the initial DPPH· concentration by 50% ($TC_{50}$).

### 2.4. Statistical analysis

Analysis of variance and the means separated by Duncan's multiple range test was performed by applying Statgraphic v.7.0 statistical package (Statistical Graphics Corp., Rockwille, MD, US). All the statistical procedures were performed at a significance level of 95%. All the analyses were performed at least in duplicate.

## 3. Results and discussion

The Maillard reaction is known to be greatly influenced by the pH of medium (Ashoor & Zent, 1984). Hence the rate and profile of MRP formed, with chemical and physical effects on the matrix, depends on the pH of the media. Model systems were adjusted at pH 7 because most of the previous studies on model systems used neutral values. Fig. 1 depicts the time course of pH according to the heating time for each sugar/amino acid model system. MR was allowed to proceed without external pH control during heating. In this sense, pH effect is analogous to food processing conditions. Model systems constituted by lysine showed a significant ($P > 0.05$) drop in the pH value as compared with others.

The extent of browning as a function of heating time is shown in Fig. 2. Browning showed a slight induction period, depending on the type of amino acid and sugar, being more significant for model systems constituted by alanine apart from heated sugar solutions alone. Concerning browning, glucose was more reactive than lactose independently of the type of amino acid used, but lysine was more reactive than glycine and alanine. Browning was clearly related to the descent in the pH value, where LL and GL reached significantly ($P > 0.05$) the lowest pH values after heating for 24 h, 4.11 and 4.33, respectively. By heating lactose or glucose alone, only a little browning was observed, confirming that in the sugar/amino acid mixtures, browning is almost completely due to the MR, without any significant caramelisation of the sugar.

The single wavelength measurements (optical density) of browned solutions at $\lambda_{max}$ between 470 and 360 nm are frequently used to measure the rate and extent of formation of the coloured stage of the Maillard reaction. However, it is not a reliable way to describe visual colour changes in terms of visual properties of the coloured compounds (MacDougall & Granov, 1998). Several authors have postulated equations to relate visual colour with browning by applying the colour parameters provided by a tristimulus colorimeter (e.g. Giangiacomo & Messina, 1988). CIELAB system established a system of numerical coordinates to locate individual colours in a uniform visual colour spacing (CIE Colorimetry Committee, 1974). The colour index ($E$) is mainly influenced by the colour lightness ($L^*$); a decrease in $E$ index is related to a loss of lightness in the sample. The $E$ index could describe how far apart two colours are in the colour space. The values of $E$ index against time for each system are depicted in Fig. 3a. The $E$ index decreased significantly during heating time for all samples, which indicates increased darkness at the advanced stage of the MR. Systems GL, GG and LL darken faster than LG, GA and LA although they reached similar values at 24 h of heating. Colour formation is likely due both

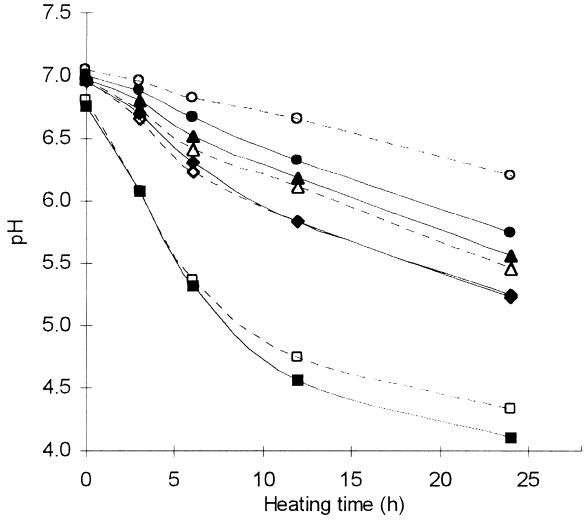


Fig. 1. Development of pH in sugar and sugar/amino acid mixtures as function of time (hour). Glucose systems (dotted line, empty symbols) and lactose systems (solid line, full symbols), sugar (●), alanine (▲), glycine (◆), and lysine (■).

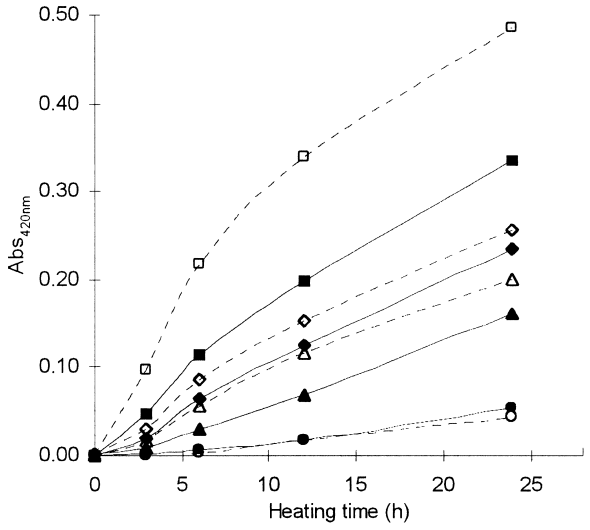


Fig. 2. Development of browning (as measured by absorbance at 420 nm) in sugar and sugar/amino acid mixtures as function of time (h). Symbols as in Fig. 1.

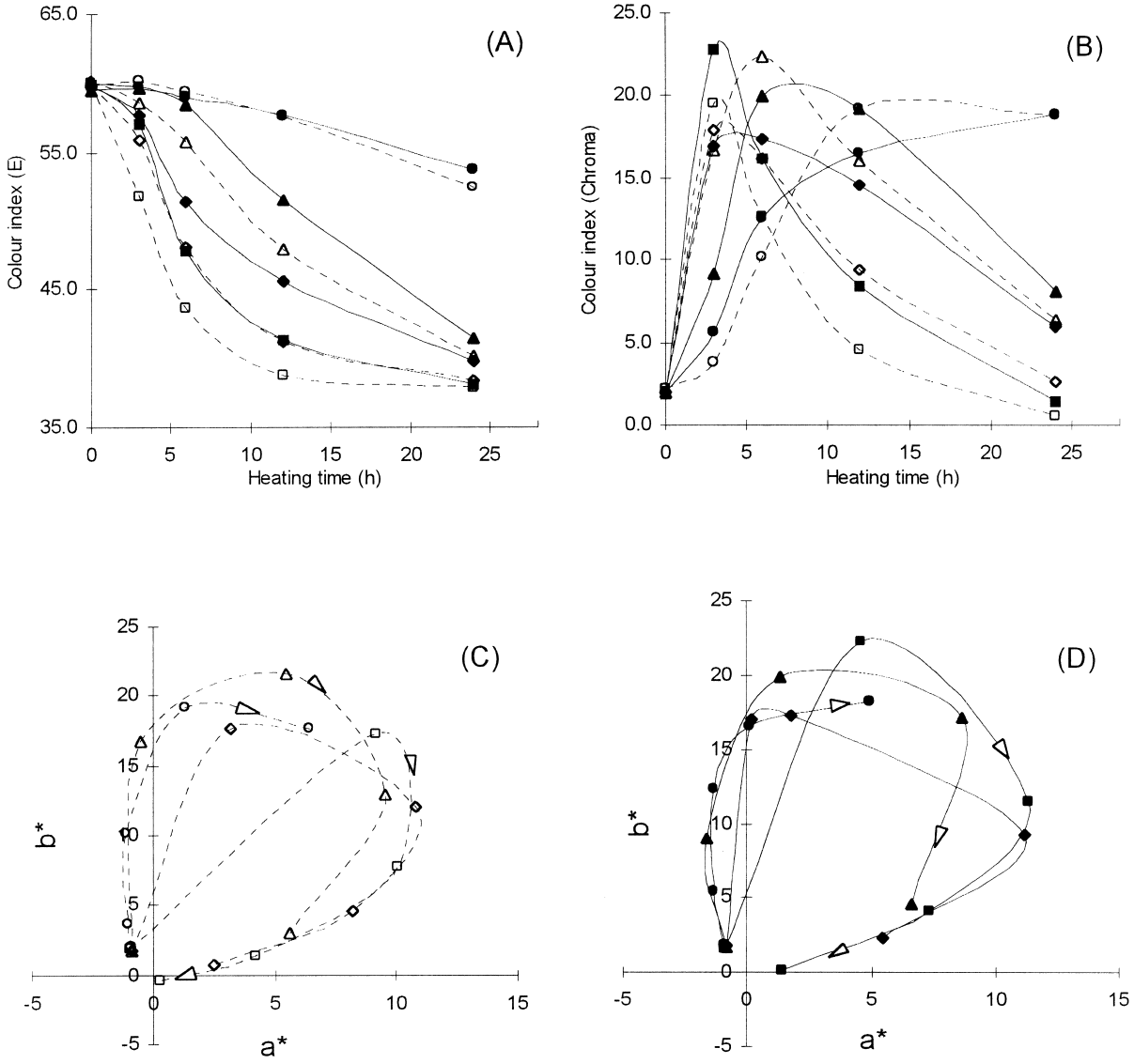


Fig. 3. CIELAB colour index ($E$), chroma index ($C$), and chroma ($a^*b^*$) plots of colour development of sugar and sugar/amino acid mixtures heated at 100°C up to 24 h. Arrow indicates increasing heating time up to 24 h (clockwise). Symbols as in Fig. 1.

to the formation of low molecular weight compounds and to the presence of melanoidins with high molecular weight (Ames, 1992).

$C^*$ indicates the degree of saturation, purity or intensity of visual colour and is defined as degree of departure from grey ($a^*$ and $b^* = 0$) toward pure chromatic colour. The increase in the $C^*$ value denotes that the heated model systems have more red and yellow characteristics till they reach a maximum where the visual colour of the system becomes much more complicated (Fig. 3b). Systems LL, GL and GG showed a maximum of $a^*$ and $b^*$ coordinates at 3 h of heating where GA, LG and LA systems showed the maximum after 6 h of heating. Then, chromaticity coordinates decreased progressively towards reddish and blue colours. The chroma ($a^*b^*$) plot gives more information on the colour development in the model systems (Fig. 3c and d). Curves proceed with the clockwise as well as the heating time. Mostly, a net increase in yellow–brown colour is observed during the first hours of heating reaching a maximum. Then the curve forms a loop with changing from yellow–brown to orange–brown, even purplish red for the sample GL and LL at higher heating times. Again, GL and LL systems showed the darkest colour.

In a previous paper, Morales et al. (1996) stated that non-protein bound fluorescence in milk had two different reaction routes depending on the presence of amino groups and that fluorescence related to the MR is quantitatively more important than sugar caramelisation. The same statement may be made for the model systems with sugars alone (G, L), where fluorescent compounds are also formed from the Lobry de Bruyn–Alberda van

Ekenstein transformation and subsequent degradation, as depicted in Fig. 4, but fluorescence shown in the heated sugar mixtures was minimal as compared with model systems containing sugar and amino acid. In this case, all the systems shown the same behaviour, reaching a maximum about 6 h and a plateau at prolonged heating. Notably, measurement of fluorescence remains constant, thus suggesting that the fluorescent molecules are rather stable at prolonged heating. The levels of fluorescence were similar when observed with browning or colour appearance where systems containing lysine reached the highest values and model systems constituted by alanine the lowest response. It can be considered that a predominant fluorescent chemical structure does not exist and different MRPs with fluorescent chemical structures should be formed related to increasing heating conditions, as stated by Morales and van Boekel (1997). At prolonged heating, some of this MRP could be involved in the formation of coloured MRPs or melanoidins. Fluorescence is related to browning in the sense that model systems containing lysine were the most reactive and alanine shown the lowest reactivity, but the fluorescent compounds did not follow the same trend as coloured compounds in the model systems.

Obviously, it is expected that different MRPs with different browning properties, colour appearance and fluorescent properties be formed in each system. This fact could be applied to understand the free radical scavenging properties of the MRPs. Lingnert and Hall (1986) have stated that is important to study the antioxidant capacity of MRPs in order to be applied as food ingredients but several points have to be considered, such as high cost of raw material, low solubility in lipid fraction, dark colour and characteristic flavour. To evaluate the free radical scavenging properties of the MRPs formed, model systems are allowed to react with a stable radical, DPPH· in a chloroform solution. The reduction of DPPH·, indicating a positive antiradical activity of the MRPs, is followed by monitoring the decrease in its absorbance at 520 nm during the reaction. When DPPH· is reduced by an active MRP the absorption disappears.

$$DPPH_{(coloured)} + MRP \rightarrow DPPH\text{-}H_{(uncoloured)} + MRP\cdot$$

Several experiments were performed in a preliminary step in order to set the most appropriate initial concentration of DPPH· and the ratio between sugar/amino acid and DPPH·. The kinetics of radical scavenging activity of samples to determine the time needed to reach the steady state were established (data not shown). The sample LL heated for 24 h reduced to 50%

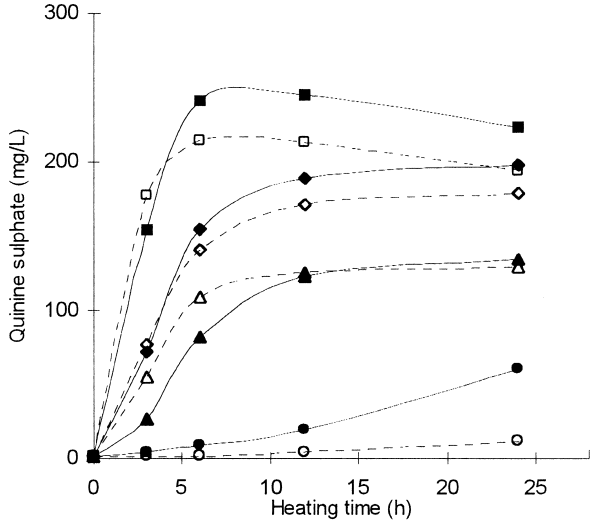


Fig. 4. Development of fluorescence (expressed as sulphate quinine amounts) in sugar and sugar/amino acid mixtures as function of time (h). Symbols as Fig. 1.

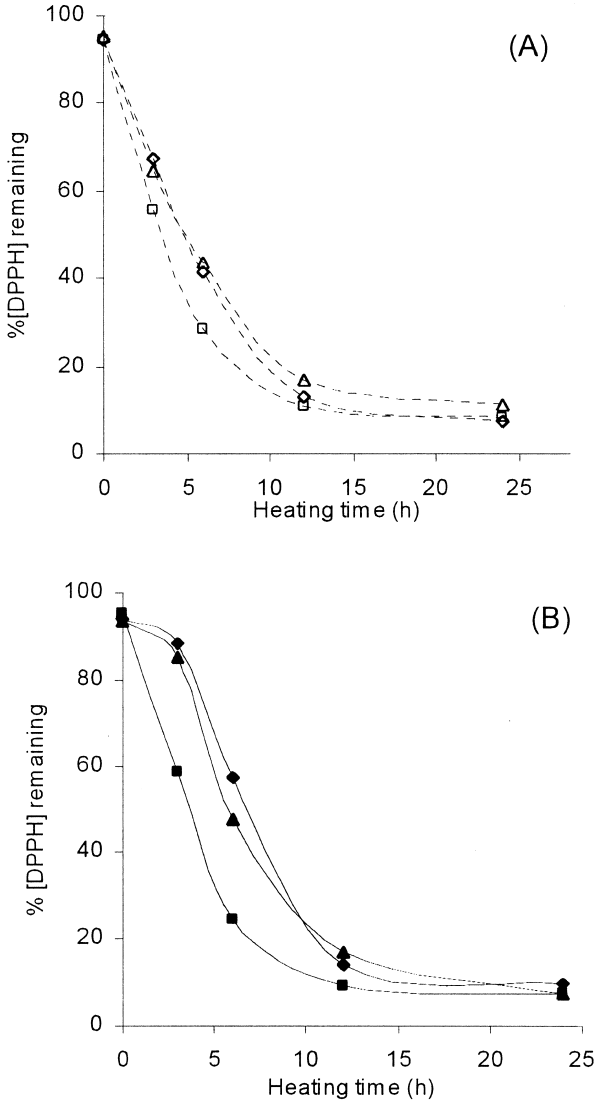


Fig. 5. Free radical scavenging activity in sugar/amino acid mixtures heated at 100°C up to 24 h. Symbols as in Fig. 1.

the initial DPPH· concentration at about 8.5 min, reaching a plateau at 20 min, and the rest of the samples reached the steady-state before 60 min. Hence, 60 min of reaction with DPPH· solution was acceptable to reach the steady step. On the other hand, it was found in tests that coloured MRPs did not show interference with the analytical procedure at the experimental conditions described. Different MRPs were incubated with chloroform for 60 min and colouring in the organic layer was not observed. Finally, the reproducibility of the assay was tested on samples heated for 6 h. The higher coefficient of variation obtained was $3.2 \pm 0.05\%$ ($n = 6$) in the LG (6 h) sample. Fig. 5a and b shows the free radical scavenging activity for the model systems. The free radical scavenging activity of the MRPs formed in the different model systems increased drastically from the first minutes of heating until 12 h, reaching a plateau after that. The free radical scavenging activities of each model system was compared according to theirs $TC_{50}$ value. Fig. 6 depicted the $TC_{50}$ values for each model system. It can be described four different groups according to their antiradical activity, GL and LL (high activity), GA and GG (medium activity), LA and LG (medium–low activity), glucose and lactose (low activity).

Under the same conditions, the browning of the systems was increasing almost linearly with the heating time, as opposed to fluorescence and the antiradical activity which follow a similar trend. Browning was not related to the free-radical scavenging activity of the MRPs at prolonged heating conditions. Browning is not a quality feature accepted for some foods such as dairy products. In this sense, browning could not be a good indirect index to monitor the formation of MRPs with free radical scavenging activity. It could be applied the measurement of fluorescence to set the optimum degree of antioxidant compounds in the heated solutions since maximum fluorescence in the system is related to an acceptable free radical scavenging activity. However, it cannot be concluded that free radical scavenging properties shown in the model systems are directly due to formation of certain fluorescent compounds. However, values of $TC_{50}$ are connected to model systems at maximum fluorescence. In conclusion, this study shows that fluorescence measurement of heated sugar/amino acid systems is more effective than browning to follow the formation of MRPs with free radical scavenging activities in systems where browning is not a characteristic of quality.

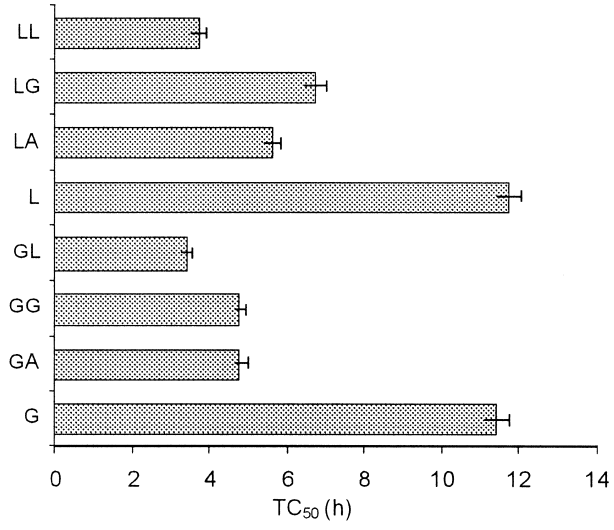


Fig. 6. Effective time ($TC_{50}$, hours) for sugar and sugar/amino acid mixtures heated at 100°C up to 24 h.

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