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Peroxyl radical scavenging activity of melanoidins in aqueous systems

Abstract Melanoidins are widely distributed in our diet, due to home or industrial processing of foods. Until recently, melanoidins were considered to be an inert, brown-coloured polymeric component. However, recent research into their nutritional, physiological, and functional properties has suggested that they have antioxidant properties, and we address this issue in this work. A sensitive procedure for assessing the inhibition of lipid peroxidation by melanoidins in watery media has been developed. Main drawbacks and critical steps of the procedure are discussed. Melanoidins can be classified according to the number of peroxyl radicals trapped per molecule. Coffee and sweet wine melanoidins show higher antioxidant activity than melanoidins isolated from beer. For the first time, a linear relationship between the peroxyl radical scavenging activity and the chromophore residues in the melanoidin skeleton responsible for browning has been established.

Keywords Maillard reaction · Melanoidin · Lipid oxidation · AAPH · Colour

Introduction

Melanoidins are polymeric and coloured final products of the Maillard reaction (MR), which are formed during home- and industrial-processing of foods, and are widely distributed in our diet (for instance in coffee, cocoa, bread, malt, and honey). Melanoidins are formed by cyclizations, dehydrations, retroaldolisations, rearrangements, isomerisations, and condensations of Maillard reaction products (MRP), but none of them have been fully characterised yet. As recently reviewed by Martins and Van Boekel [1], three main proposals for the structure of

melanoidins have been put forward: (a) low-molecular-weight (LMW) coloured substances which are able to crosslink free amino groups of lysine or arginine in proteins, to produce high-molecular-weight (HMW) coloured melanoidin [2]; (b) units of furans and/or pyrroles that, through polycondensation reactions, form melanoidin from repeating units [3]; (c) melanoidin skeleton is mainly built up from sugar degradation products formed in the early stages of the MR, polymerised and linked by amino compounds [4].

In recent years, several studies have mainly been focused on the effect of melanoidins on the human diet, and their feasible nutritional, biological, and health implications [5, 6, 7]. But results in the literature are difficult to compare, since models do not resemble food matrices or processing conditions at industry, so the research could not show whether melanoidin or low/intermediate molecular weight compounds are responsible for the studied effects.

Manzocco et al [8] reviewed the relationship between colour changes during the processing of foods due to MR, and the formation of compounds with antioxidant activity. They concluded that a simple positive correlation between colour and antioxidant properties can be found where formation of antioxidant MRPs is the prevalent event during processing. However, the specific role of melanoidin in the overall antioxidant activity of the MRPs has not been addressed. They may act by decreasing oxygen concentration, intercepting singlet oxygen, preventing first-chain initiation by scavenging initial radicals such as hydroxyl radicals, binding metal ions, decomposing primary products to non-radical compounds, chain-breaking to prevent continued hydrogen abstraction from substrates, and synergism [9]. Knowledge of the role of melanoidins in the prevention of lipid oxidation is limited, but they may act as other antioxidants at different levels in the oxidative sequence, in a similar way to polyphenols [10].

The aim of this study is to assess the antiperoxyl radical scavenging properties of melanoidins, by applying an AAPH-induced oxidation of linoleic acid in an aqueous dispersion. As well as the relationship between browning

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and the chromophore residues in the melanoidin skeleton, the ability of melanoidins to prevent lipid peroxidation will be addressed. These findings will help us to understand the properties that melanoidins contribute to the overall antioxidant activity of melanoidin-containing foods.

Materials and methods

Chemicals and reagents

Glucose, tryptophan, lysine, histidine, methionine, cysteine, phenylalanine, tween 20 (polyoxyethylenesorbitan monolaurate) and linoleic acid (99%) were from Sigma; glycine, alanine, tyrosine, aspartic acid, and arginine were from Merck; lactose was from Panreac; AAPH (2,2′-azobis(2-amidinopropane)-dihydrochloride) and trolox were from Aldrich.

Preparation of water-soluble melanoidins from aqueous Maillard reaction model systems

Twenty-two model systems were prepared from a single combination of sugar (glucose or lactose) and amino acid (Ala, Cys, His, Lys, Gly, Met, Phe, Asp, Arg, Typ, Tyr). Water-soluble melanoidins were obtained from different Maillard model systems as described previously [10]. Maillard solutions were subjected to ultrafiltration, using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA, USA), equipped with a 10,000 Da nominal molecular mass cut-off membrane. The retentate was filled up to 200 mL with water and washed again. This washing procedure was repeated at least three times. The high molecular weight fraction corresponding to melanoidins was freeze-dried and stored in a dessicator until analysis. Isolated melanoidins were analysed for the absence of low molecular weight compounds. Melanoidins (M) isolated from model systems were identified by two letters, first related to the sugar (G for glucose, and L for lactose), and the second letter was related to the amino acid. The model named MGK referred to melanoidin isolated from the glucose and lysine model system.

Preparation of water-soluble melanoidins from commercial samples

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. Coffee brew (sample MC) was filtered and 200 mL was de-fatted with dichloromethane (2×200 mL). Beer and sweet wine samples were prepared in a similar way, where 100 mL of beer or sweet wine were mixed with 100 ml of water. These solutions were filtered and treated with dichloromethane. Three widely distributed commercial brands of beer in Europe with different elaboration procedures were selected. A Pilsener-style beer from a Spanish brewery (sample ML), an Abbeys-style beer from a Belgian brewery (sample MT), and a dry-stout beer from a Irish brewery (sample MN) were used. A widely consumed Spanish sweet wine, "Pedro Ximenez", was also used (Sample MW). This Spanish sweet wine is elaborated from dry-grapes by a process called "Soleo".

Browning

Browning indices of the different melanoidins were recorded, after appropriate dilution in water, by their absorbance at 420 nm on a Shimadzu UV-1601 (Duisburg, Germany) spectrophotometer using a 1 cm-path length cell [11]. The absorbance coefficient (a_{420}, mL mg^{-1} cm^{-1}) was deduced from the slope of the curve representing the absorbance at 420 nm versus melanoidin concentration (mg mL^{-1}).

Substrate

An aqueous solution of linoleic acid (LH, 16 mM) was prepared as follows. Under continuous stirring, linoleic acid (0.250 mL) was added dropwise to 10 mL of 50 mM phosphate buffer (pH 7.4) containing 0.250 mL of Tween 20. The dispersion was clarified by adding 30 μL of NaOH (20% w/v). The volume was made up to 50 mL with phosphate buffer. The dispersion was fractionated into 2 mL eppendorf tubes and kept at −20 °C until use. Before use, the dispersion was checked for autooxidation. AAPH decomposes slightly and its products increase the absorbance at 234 nm. A net increase of 0.4020 absorbance units after 200 min was measured. Therefore, this AAPH decomposition absorbance was subtracted from all sample absorbance data from this point on. Note that a scavenging of melanoidins towards AAPH was not detected.

AAPH solution

40 mM AAPH was prepared in 50 mM phosphate buffer (pH 7.4). Portions were distributed in 1 mL test tubes and stored at −20 °C until use.

Antioxidant assay

40 μL of the 16 mM LH dispersion was added to a tube containing 2.80 mL of 0.05 M phosphate buffer (pH 7.4) at 37 °C. 40 μL of the test melanoidin (0.1–1 mg mL^{-1}) was added, followed by 150 μL of the 40 mM AAPH solution. The tube was quickly vortexed for a few seconds, avoiding the formation of foam, and put in a UV cuvette within 30 s. The reading chamber was thermostated at 38 °C. The rate of oxidation of LH was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. A Shimadzu UV-visible 1601 spectrophotometer (Shimadzu) equipped with a thermostated automatic sample positioner was used. Measurements were recorded every 30 s. Traces of metallic ions were masked by ethylenediaminetetraacetic acid (EDTA). The inhibition time (T_{inh}) was calculated with Microsoft Excel software (v97) as the point of intersection between the tangents to the inhibition and propagation phase curves. The antiperoxyl efficiency of melanoidins was expressed as the rate of inhibition (R_{inh}). R_{inh} was calculated from the slope of the curve representing T_{inh} (min) versus melanoidin concentration (mg mL^{-1}).

Statistical analysis

All of the analyses were performed at least in triplicate. The Statgraphics v2.3 statistical package (Statistical Graphics Corp., Rockville, MD) was used, and the statistical procedures were performed at a significance level of 95%.

Results

The water-soluble azo initiator AAPH was applied as a clean and controllable source of thermally-produced alkylperoxyl free radicals (AOO·) in aqueous media. The studies of Pryor et al [12] and Liegeois et al. [13] on the antioxidant efficiency of malt were used as references for setting up a useful procedure for evaluating the peroxyl radical scavenging properties of melanoidins. The method is based on the rate of oxidation of linoleic acid (LH) to its conjugated diene hydroperoxide (LOOH) in aqueous
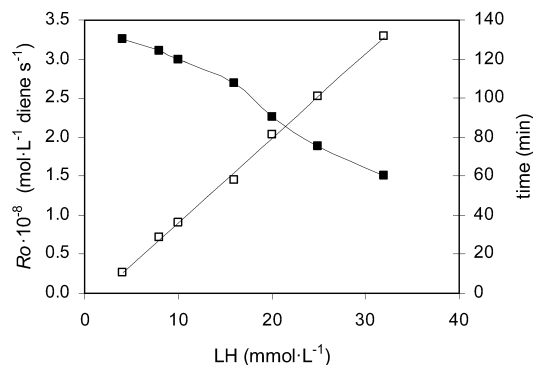
Fig. 1 Initial rate of AAPH-induced oxidation (R_0) of linoleic acid (40 μL) in a phosphate (pH 7.4) dispersion at 38 °C in air, as measured by the kinetics of conjugated diene formation (white squares), and the time taken to reach the end of reaction (black squares). Error bars lay inside the markers ($n=3$).
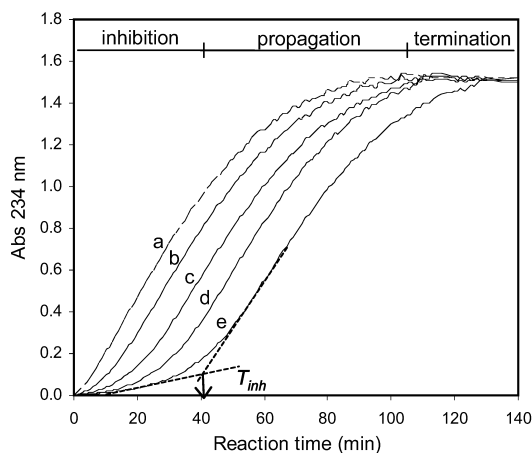

Fig. 2 Classical time course of conjugated diene formation from control (a, in absence of trolox), 0.020 (b), 0.040 (c), 0.060 (d), and 0.080 (e) mg mL^{-1} Trolox. T_{inh} (0.080 mg mL^{-1}, 4.22 μmol L^{-1} in the reaction vessel) is 41.1 min. Residual absorbance from the metabolites of AAPH decomposition is already subtracted

media in the presence or absence of antioxidant activity due to melanoidins (M). Briefly, the process of AAPH-induced oxidation of LH involved a free radical chain reaction mechanism proceeding via initiation, propagation and termination steps. Alkylperoxyl radicals produced in the initiation step by AAPH react with unsaturated bonds of LH by abstracting a hydrogen atom from their molecule in the propagation step [12, 14].

Drawing on our previous experiences with regard to the antiradical efficiency of melanoidins in aqueous media [10], as well as the development of colour in different Maillard reaction media [15],we adjusted the initial proportions of subtract (LH), reactants (AAPH, M), and media (phosphate buffer). The literature indicates an increase in absorbance at 234 nm due to metabolites from AAPH degradation, in addition to the absorbance of AAPH itself. This effect was evaluated and subtracted from all of the raw data. Absorbance due to the degradation of AAPH (150 μL, 40 mmol L^{-1}) at 234 nm was significantly reproducible among trials, and corresponded to a net increment of 0.320 absorbance units after 150 min of reaction. The appropriate molar ratio between LH and AAPH was studied. Addition of AAPH initiates the oxidation of LH, which starts a constant rate of LOOH formation. These experiments were carried out in the absence of M or reference antioxidant (trolox). The initial rates of oxidation (R_0) of LH (from 4–32 mmol L^{-1}) when using 40 mmol L^{-1} AAPH are shown in Fig. 1. LH solutions higher than 35 mM were not considered due to saturation of the detector signal and loss of linearity. Readings did not exceed 1.7 absorbance units at the termination step. Next, we studied the best molar ratio LH:AAPH for obtaining both a measurable conjugated diene formation through all of the time-course, as well as a reasonable time of analysis (Fig. 1). A concentration of 16 mM for LH was selected, which corresponds to an experimental R_0 value of 1.453×10^{-8} mol L^{-1} diene s^{-1}, and a time of termination of 108 min. The effective concentrations in the reaction cuvette were 0.211 mmol L^{-1}, and 1.98 mmol L^{-1} for LH and AAPH, respectively.
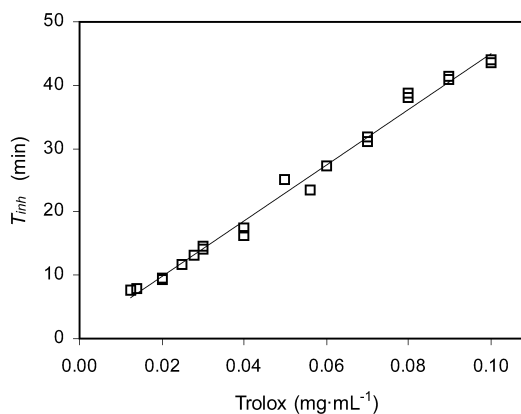

Fig. 3 Effect of Trolox concentration on AAPH-induced linoleic acid oxidation, expressed as inhibition time (T_{inh}) versus Trolox concentration. Every symbol denotes an independent analysis. R_{inh} of 8.62208 min μmol L^{-1} ($n=22$, $r^2=0.9902$) in the reaction vessel

As proposed by Liegeois et al [13], we characterised the analytical procedure by using a water-soluble antioxidant (trolox) as reference. Figure 2 shows a classical representation of conjugated diene formation from AAPH-induced oxidation of LH in the presence of trolox. It also illustrates the significance of the inhibition time (T_{inh}) which corresponds to the point of intersection between the tangents to the inhibition and propagation phase curves. Several concentrations (0.01–0.10 mg mL^{-1}) of trolox were assayed, and the corresponding T_{inh} was calculated (Fig. 3).

A kinetic study of LOOH formation from AAPH-induced LH oxidation was performed for the melanoidin solutions. Melanoidin solutions (0.1–2.0 mg mL^{-1}) with at least six internal data points were prepared, which represent an effective concentration of 1.32–26.40 μg mL^{-1} in the reaction cuvette. In order to ensure the reproducibility between trials, we made sure that the data pas-
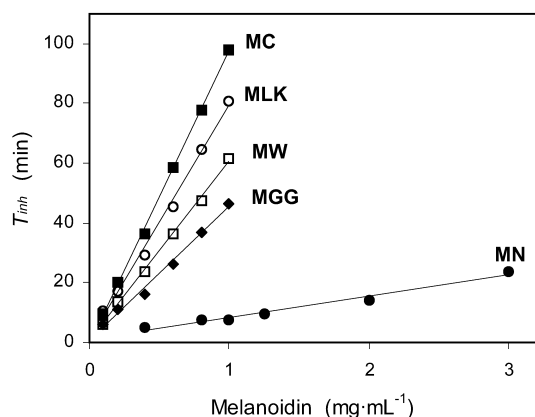
Fig. 4 Representation of some rates of inhibition (R_{inh}) curves of the AAPH-induced oxidation of linoleic acid in the presence of melanoidin

Table 1 Antiperoxyl efficiency (R_{inh}) of melanoidins obtained from model systems and coffee, beer and sweet wine. N represents the stoichiometric number of peroxyl radicals trapped per melanoidin, assuming an average molecular weight of 10 kDa for melanoidins

System	Range (mg mL^{-1})	R_{inh} (min mL mg^{-1})	N
MLA	0.1–1.0	74.47±3.97	13.36
MLH	0.1–2.0	14.17±0.99	2.65
MLK	0.2–1.0	79.73±4.61	14.30
MLG	0.1–1.0	12.80±1.95	2.30
MLM	0.4–2.0	17.23±1.44	3.09
MLP	0.2–1.0	37.09±3.05	6.65
MLQ	0.4–2.0	4.03±0.23	0.72
MLR	0.1–2.0	23.08±2.94	4.14
MLT	0.1–2.0	15.08±1.53	2.71
MLY	0.1–2.0	26.21±1.14	4.70
MGA	0.1–2.0	24.15±2.09	4.33
MGH	0.1–1.0	15.45±1.08	2.77
MGC	0.1–2.0	15.50±1.74	2.78
MGK	0.2–1.0	64.77±3.17	11.62
MGG	0.2–1.0	43.34±1.62	7.77
MGM	0.1–1.0	78.46±7.16	14.07
MGP	0.2–1.0	29.70±2.18	5.33
MGQ	0.4–2.0	3.69±0.25	0.66
MGR	0.4–2.0	17.43±0.91	3.13
MGT	0.4–2.0	6.99±0.44	1.25
MGY	0.4–2.0	19.91±1.14	3.57
MC	0.2–1.0	97.25±5.58	17.44
MW	0.2–1.0	60.73±3.33	10.89
MN	0.2–3.0	7.64±0.33	1.37
MT	0.5–5.0	2.91±0.18	0.52
ML	4.0–10.0	1.58±0.08	0.28

sed several control tests before they were accepted for further calculations; these included: (a) an equal degradation rate of AAPH over reaction time, (b) similar R_0 values, (c) monitoring the analysis until end of the propagation step, and the (d) absence of interaction between melanoidin and AAPH. The increase in the inhibition period was proportional to the melanoidin concentration, which denotes a process of oxidation inhibition. Once the inhibition time is over, the oxidation proceeds at the same rate as in the absence of inhibitor. Although melanoidin itself absorbs at 234 nm, which could be a drawback since could reduce the range of analysis, concentrations up to 2 mg mL^{-1} (40 μL) did not show significant interferences. Levels up to 10 mg mL^{-1} were also considered for melanoidins from beer (ML, MT, MN), since they showed the lowest antiperoxyl activity. No colour interference or sequestering of the AAPH molecule was observed. It has been described that some antioxidants may scavenge the azo initiator and the ratio of LOOH formation may not be constant [12]. For each system, the highest melanoidin concentrations were incubated with AAPH, and the increase of absorbance at 234 from the AAPH metabolites was recorded, in order to assess any quenching effect by melanoidins. No significant deviations were observed from the control (AAPH solution in the absence of melanoidins). The autooxidation of LH, as well as the R_0 value of the control assay were also checked before accepting the results. We obtained a highly reproducible methodology, with an average R_0 value of $1.29\times10^{-8}\pm1.22\times10^{-9}$ mol L^{-1} diene s^{-1} (n=75).

Figure 4 depicts the inhibition curves for some melanoidins, which show the linearity and the different antiperoxyl response of melanoidins. R_{inh} (min mL mg^{-1}) is used as parameter to evaluate the peroxyl scavenging properties of melanoidins. Table 1 lists the R_{inh} values obtained from each melanoidin. Melanoidins may compete with the LH for the peroxyl radical (LOO·) and, as a result, reduce the formation of the lipid radical (L·) that occurs when LH reacts with LOO·. This competition reduces the propagation state of oxidation. Melanoidins (M) may readily transfer a hydrogen atom to the LOO· to produce the more stable LOOH, by acting as a chain-breaking electron donor. Therefore, one or both of the following reactions may be plausible:

a) LOO · +M − OH → LOOh + M − O·

b) LO · +M − O· → LOO − M

LOO· abstracts a hydrogen atom from the melanoidin to yield a melanoidin radical and the respective hydroperoxide (a). In the mechanism (b), a peroxyl and a melanoidin radical (M-O·) react by radical-radical coupling to form a non-radical product.

The stoichiometric coefficient, N, represents the number of peroxyl radicals trapped by each antioxidant molecule. Using trolox as a reference antioxidant (AOH), which removes two radicals per added molecule [16], it was possible to determine the rate of radical generation (R_i) under our reaction conditions from the following equation:

$$R_i = \frac{N[\text{AOH}]}{T_{inh}} \Rightarrow R_i = N\frac{d[\text{AOH}]}{dT_{inh}}$$

An R_i value of 1.9526×10^{-6} [AAPH] s^{-1}, which is comparable with the values obtained by Liegeois et al [13] and Niki et al [16] in similar reaction media, was obtained. Then, applying the above equation, it is possible to deduce the N value for different antioxidants. But this approach is limited to the melanoidin investigation, since the exact molecular weight of melanoidin is unknown.
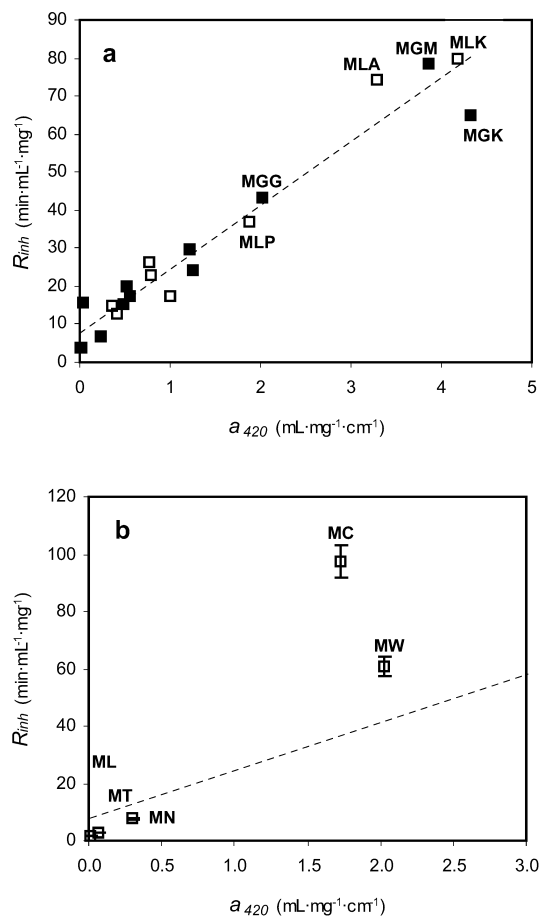
Fig. 5 Relationship between peroxyl radical scavenging activity of melanoidins, expressed as R_{inh}, and browning (expressed as a_{420}), for **a** glucose melanoidins (black squares) and lactose melanoidins (white squares), and **b** melanoidins from coffee, beers and sweet wine. Dotted lines express the linear regression for melanoidins from model systems

We can extrapolate the results, taking into consideration an average molecular weight of 10 kDa. The results are listed in Table 1.

The relationship between browning and the R_{inh} of melanoidins was also studied. Colour is an intrinsic characteristic of melanoidins and their chromophore groups differ according to the reactants and reaction conditions [17]. Melanoidins show significant differences when a_{420} (mL mg^{-1} cm^{-1}) are compared. These results indicate the hetereogenicity of the melanoidins isolated when different chromophoric groups are formed. These results agree with previous experiences, where melanoidins isolated from different sources after equivalent heating treatments could possess similar molecular weights but different charge/mass ratio, suggesting differences in the degree of saturation of the reactive groups in the core structure [15]. Figure 5 shows the results for melanoidins obtained from glucose and lactose model systems (Fig. 5a), and for melanoidins obtained from coffee, beer, and sweet wine (Fig. 5b). In the model systems, results fitted well to a linear model (R_{inh}=16.798·a_{420}+7.59; r^2=0.945, n=21).

Significant differences between the type of reducing sugar (glucose or lactose) and R_{inh} were not observed.

In coffee, wine, and beer, other compounds, mainly polyphenols and carbonyl compounds, can take part in the Maillard reaction itself [18, 19]. The contributions of these alternative reactants to the evolution of the antioxidant properties of processed foods is still unknown. Melanoidins from beers (ML, MT, MN) and sweet wine (MW) clearly followed the same trend, where colour was related to the peroxyl radical quenching properties of the melanoidin. However, MC did not fall in the 95% confidence level, and it showed a higher antiperoxyl radical activity than melanoidins with similar colour. Many complex physical and chemical changes occur during the roasting of green coffee, including the loss of natural antioxidants, but the formation of heat-induced compounds from MR with antioxidant properties is well-known during the advanced stages of roasting [8, 20, 21]. Steinhart et al [21] compared the antioxidant activity of coffee and chlorogenic acid-free coffee as well. Stronger roasting conditions result in a lower amount of chlorogenic acid, but the loss of natural antioxidants was not accompanied by a decrease in the total antioxidative capacity. The roasting process causes higher values of antioxidant activity despite the reduction of chlorogenic acids. In this case, the incorporation of fragments of decomposed chlorogenic acids into the melanoidin structure may be plasible, enhancing its overall antioxidative properties.

Conclusions

A reliable procedure for assessing the peroxyl radical scavenging properties of melanoidins has been developed. Melanoidins are well-known metal quelators [22]. Therefore, traditional metal-catalysed lipid oxidation assays are not appropriate for studying their ability to prevent lipid peroxidation. Using the procedure described here, we are able to categorise the activity of melanoidins according to their N index. On the other hand, a linear correlation between colour and antioxidant activity for MRPs has been described by several authors in model systems [23], during the roasting of soybeans [24], the drying of pasta [25], and when cooking tomato puree [26]. However, the relationship between colour and antioxidant activity due to the melanoidins has not been clearly described. Melanoidins with stronger absorbance at 420 nm were more efficient at inhibiting the AAPH-induced oxidation of an aqueous dispersion of linoleate.

In conclusion, chromophores linked to the melanoidin skeleton are mainly responsible for their peroxyl radical scavenging properties. We are currently performing investigations related to the identification of the functional groups responsible for the peroxyl radical scavenging properties.

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