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Iron-binding ability of melanoidins from food and model systems ☆

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Abstract

Soluble high molecular weight fractions isolated from the development of the Maillard reaction in 22 different model systems and food matrices were studied in order to assess their iron-chelating ability in vitro. Melanoidins and iron were incubated in a Na-acetate buffer (0.1 M, pH 5) at different weight ratios and free iron was measured by FAAS and the bathophenantroline procedure at the steady state. Melanoidins were classified at three levels according to their number of co-ordination sites for iron. Melanoidins from coffee (medium roasted), different types of beer (Pilsener, Abbeys, and dry-stout styles), and sweet wine (Pedro Ximenez) exerted a low iron-binding effect compared with melanoidins from model systems. The type of sugar was shown to be a significant parameter for obtaining melanoidins with high iron ability, and glucose was more efficient than lactose. No relationship was observed between browning and iron binding ability of melanoidins from model systems. The chromophoric residues were not the main co-ordination sites for iron complexation in the melanoidin structure.

Keywords: Maillard reaction; Melanoidin; Iron ability; Kinetics; Color

1. Introduction

Melanoidins are polymeric brown compounds formed in the last stage of the Maillard reaction (MR) that have a direct effect on food acceptability by the consumer. Apart from being involved in the colour properties of thermally treated foods, melanoidins show remarkable binding properties toward low molecular weight substances in the food matrix, which are interesting from technological (Petracco et al., 1999), nutritional (Jägerstad, Skog, & Solyakov, 2002) and physiological (Faist & Erbersdobler, 2001) points of view. This property has recently been used for the stabilisation of aroma compounds, such as furfural or pyrrole derivatives and aromatic thiols, which have an impact on the overall organoleptic properties of coffee, beer and sweet wine (Hofmann, 1998; Rivero-Perez, Pérez-Magariño, & González-San José, 2002).

Gomyo and Horikoshi (1976) reported that the melanoidins behave as anionic hydrophilic polymers, which can form stable complexes with metal cations. At pH values, close to those found in most foods, melanoidins have a negative net charge and are able to bind metallic ions (Migo, del Rosario, & Matsumura, 1997). Many approaches have been applied to study the metal-binding ability of melanoidins, e.g. titration, dialysis equilibrium, and spectrometry (Gomyo & Horikoshi, 1976; Homma, Aida, & Fujimaki, 1986). Recently, immobilised metal affinity chromatography (IMAC) has been proposed as an useful tool for the fractionation of melanoidins in homogeneous fractions according to their metal binding ability (Borelli, Fogliano, Monti, &

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Ames, 2002; Homma et al., 1986; Terasawa, Murata, & Homma, 1991; Wijewickreme, Kitts, & Durance, 1997). Hashiba (1986) reported that ketone or hydroxyl groups of pyranone or pyridone residues act as donors groups in melanoidins and participate in the chelation with metals. However, in these experiments, iron was also present in the reaction media as reactant and participated in the MR, not only as a catalyst, but also as a chromophore.

The results that appear in the literature are difficult to compare since different variables, such as starting material (where sometimes metal was also present in the reaction media) and reaction conditions (such as pH or temperature) were used, and mostly were not realistic for foodstuffs. The aim of this study is to define a screening procedure to assess the iron-binding properties of melanoidins in vitro. To this end, we study the iron-chelating ability of melanoidins isolated from different Maillard model systems as well as commercial coffee, beer and sweet wine.

2. Materials and methods

2.1. Chemicals and reagents

Glucose, L-tryptophan, L-lysine, L-histidine, L-methionine, L-cysteine, L-phenylalanine, bathophenanthroline (BpH, 4,7-diphenyl-1,10-phenanthroline disulfonic acid salt), hydroxylamine hydrochloride, and ferrous chloride were from Sigma (St. Louis, MO, USA); L-glycine, L-alanine, L-tyrosine, L-aspartic acid, and L-arginine were from Merck (Darmstadt, Germany); lactose was from Panreac (Barcelona, Spain).

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

Twenty-two water-soluble melanoidins were obtained from different Maillard model systems as described previously (Morales & Babbel, 2002). 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). Melanoidin fractions were analysed for absence of low molecular weight compounds (Morales & Babbel, 2002). Lyophilised samples (initial Maillard reaction mixture and correspondent melanoidin) were stored at −20 °C in a desiccator prior to analysis.

2.3. 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 was filtered and an aliquot of filtrate was de-fatted with dichloromethane (2×200 ml). Beer and sweet wine samples were prepared in a similar way, where 100 ml of beer sample (L/T/N) or sweet wine sample (W) were previously mixed with 100 ml of water. Three widely distributed commercial brands of beer in Europe with different preparation procedures were selected. A Pilsener style beer from a Spanish brewery (sample L), Abbeys style beer from a Belgian brewery (sample T), and a dry-stout beer from an Irish brewery (sample N). A widely consumed Spanish sweet wine, so-called *Pedro Ximenez*, was used (Sample W). This Spanish sweet wine is prepared from dry-grapes by a process called "*Soleo*".

2.4. Determination of total iron and ionic species

Determination of ionic state of iron was performed according to the bathophenanthroline method (Lee & Clydesdale, 1979). Determination was performed with or without hydroxylamine addition, depending on whether data for total ionic Fe (Fe^*, sum of Fe^{2+} and Fe^{3+}) was desired. The Fe(II)-tris bathophenanthroline complex has a maximum absorptivity at 535 nm in the buffer 15 (0.1 M sodium acetate, pH 5.0). Limits of detection were established at 0.04 and 0.07 mg/l for Fe^{2+} and Fe^* determination, respectively. A Shimadzu UV-601 (Germany) spectrophotometer was used. Linearity of the response was obtained up to 15 mg/l in 0.1 M Na-acetate (pH 5.0). Additionally, total soluble iron was determined by atomic absorption spectrometry using a Model 5100PC atomic absorption spectrometer (Perkin–Elmer, Norwalk, CT, USA) in an air-acetylene flame ($\lambda = 248.3$ nm). An iron standard solution (1.000 ± 0.002 g/l, Panreac) for spectrometry was used for external calibration.

2.5. Measurement of Fe^{2+} binding to melanoidins

Melanoidin solutions (up to 10 mg/ml in 0.1 M sodium acetate buffer at pH 5.0) were incubated with variable amounts of $FeCl_2$ (up to 100 mg/l of Fe^{2+}) at room temperature (unless otherwise indicated). After vortexing for 2 h, samples were allow to stand for 22 h until the steady state was reached. Sample was ultrafiltered by using Amicon Ultrafiltration cones (YM-10 from Amicon-Millipore, Germany) with a 10,000 molecular weight cut-off at 14,000*g* for 30 min (Hezzich, Germany). Ultrafiltrate were diluted in 0.1 M Na-acetate buffer (pH 5.0) by 20 times, then total soluble iron content and Fe^{2+} were analysed by FAAS and the bathophenanthroline (BpH) procedures, respectively. Iron bound (Fe_B) to melanoidin was calculated as the difference between iron contents before and after the ultrafiltration step. Finally, the sets of data obtained were plotted according to the Scatchard method to examine the kinetic characteristics of binding (Scatchard, 1949).

2.6. Statistical analysis

Analysis of variance and the means separated by Duncan's multiple range test was performed by applying the 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.

3. Results and discussion

Melanoidin structure has not yet been totally elucidated. Melanoidins obtained from model systems, as compared with coffee, beer and sweet wine, have a different origin and should have a different structure, when phenolic subunits are expected to be present in a significant proportion and subsequently exert specific effects. Intermediary compounds from the MR and from chlorogenic acid decomposition are expected to be linked to the carbohydrate skeleton of coffee melanoidins (Nunes & Coimbra, 2001). Thus it cannot be absolutely refuted that a small proportion of non-reacted material may still be bound non-covalently to melanoidins. On the other hand, exhaustive ultrafiltration is necessary to reduce the presence of salts that could decrease the binding ability of metals. Melanoidin fractions from commercial samples were analysed by HPGPC to check the absence of low molecular weight material (Fig. 1). It has been reported that of low molecular weight fraction is mainly responsible fraction for mineral chelating in coffee (e.g., Nakamura-Takada et al., 1994).

Colour is the most obvious characteristic of melanoidins. This attribute was used to characterise the melanoidins obtained from coffee, beer, and sweet wine, in which a complex structure could be expected as compared to that from sugar–amino acid Maillard systems. Fig. 2 shows significant differences where apparent absorptivity indices (a_{420}, l/g/cm) at 420 nm are compared. Apparent absorptivity index can be deduced from the slope in Fig. 2. Melanoidins from coffee (MC) and sweet wine (MW) show higher browning than melanoidins from beer (ML, MT, MN) at similar melanoidin concentrations. In the melanoidins from model systems, a_{420} index ranged from 0.007 ± 0.002 (MLD system) to 4.212 ± 0.331 (MGK system). Model systems containing lysine, alanine, glycine, and phenylalanine show a_{420} *values* above 1000 l/g/cm. These results indicate the heterogenicity of the melanoidins isolated when different chromophoric groups were formed under the same reaction conditions (temperature, time, molar ratio). This results agree with previous experiences in which melanoidins isolated from different sources after an equivalent heating treatment could possess the same apparent molecular weight but different charge/mass ratio, suggesting differences in their degree of saturation of reactive groups at the core structure (Morales, 2002).

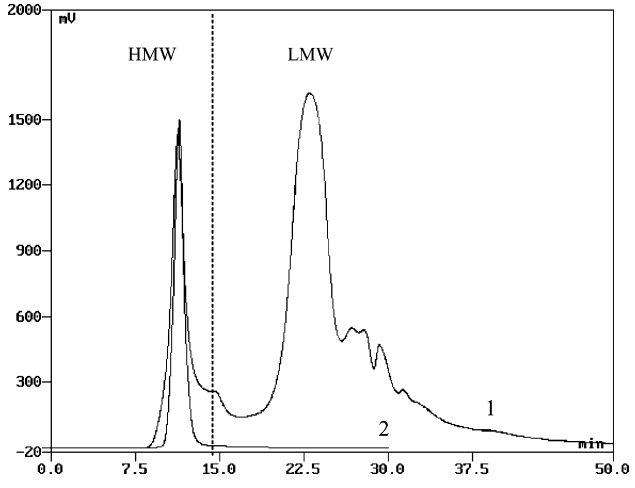

Fig. 1. Classical HPGPC chromatogram of coffee brew (1) and coffee melanoidin (2, MC). Dotted line denotes the nominal cut-off at 10 kDa. HMW (high molecular weight fraction), LMW (low molecular weight fraction).

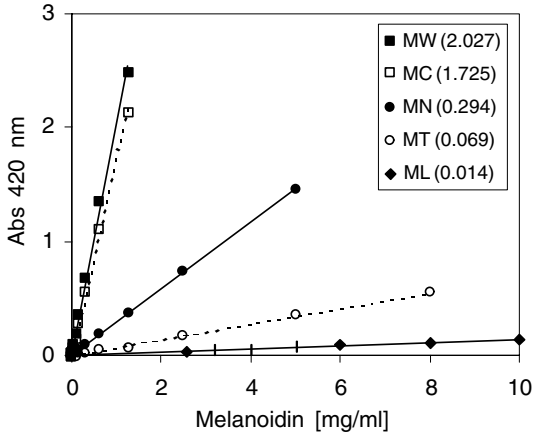

Fig. 2. Browning as function of melanoidin concentration for commercial samples. Apparent absorptivity index (l/g/cm) in brackets.

The BpH procedure for ionic iron determination was evaluated for any interference from melanoidins. The main limitation factor was the interference of colour of the melanoidin solution itself at the maximum of absorbance of the Fe(II)–tris–BpH complex. Different BpH:melanoidin:iron ratios were studied to obtain the higher response. Melanoidins were removed from solution by an ultrafiltration process (10,000 Da cut-off) where a slightly coloured dialyzate was obtained. A BpH concentration of 0.8 mg/ml was selected to chelate the total amount of iron non-bound iron to melanoidin in the dialyzate (Fig. 3). Recovery of the process was established as 95.8% (95.3–97.2, $n = 5$) for samples containing up to 100 mg/l of iron and diluted 20-fold before FAAS or BpH analysis.

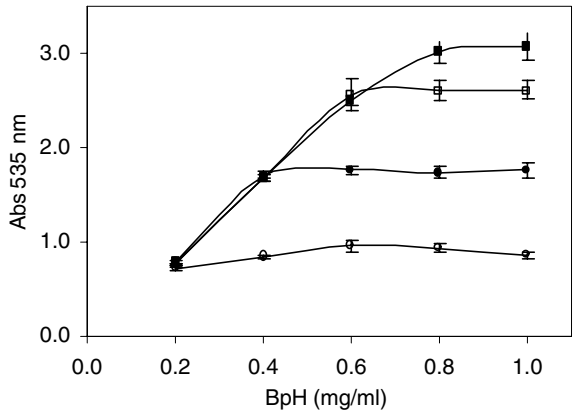

Fig. 3. Effect of BpH concentration on binding of an iron in solution. Iron concentration of 10 (■), 15 (□), 10 (●), and 5 (○) mg/l.

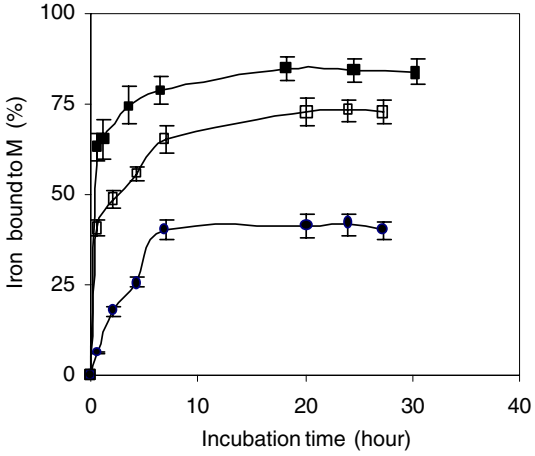

Fig. 4. Time to reach the steady state of iron solution (100 mg/l) incubated with different amounts of MC. 10 mg/ml (■), 5 mg/ml (□), and 1 mg/ml (●).

Food-related melanoidins, (coffee, beer and sweet-wine) comprise groups of ligands, such as tannins, polysaccharides, peptides and combinations of them. Food-related melanoidins are expected to be constituted of more complex skeletons than model melanoidins. The effect of the melanoidin, apart from the type of reactive groups forming the structure, on the chelation of iron can be described in the next equation:

$$[Fe_I] + [M] \longleftrightarrow [M - Fe] + [Fe_F] \quad (1)$$

$$K_d = [M - Fe]/[Fe_F][M] = [Fe_B]/[Fe_F][M] \quad (2)$$

$$[Fe_B] = [Fe_I] - [Fe_F] \quad (3)$$

where, Fe_I is the initial concentration of Fe^{2+}; Fe_F is the concentration of Fe^{2+} at the steady state; Fe_B the proportion of iron bound to the melanoidin structure at the steady state; K_d is named as apparent dissociation constant. K_d is valid only for the experimental conditions used (pH, T, ionic strength, and ligand and metal concentration range), and it is an arithmetic mean of the apparent equilibrium constants of the different specific sites in the melanoidin. Melanoidins exert a net negative electric charge at pH 5.0, and melanoidins become more negative at higher pH values (Migo et al., 1997). The equilibrium was reached when the rate of formation of M–Fe was equal to the rate of dissociation. The steady state condition was determined by measuring the amount of iron bound to melanoidin no longer increasing with time (Fig. 4). The time-binding curve shows that the complexation reaction between melanoidin reactive residues and iron required a prolonged incubation time to reach the steady state. It was found that 24 h of incubation, after the addition of iron to the melanoidin solution, was time enough to reach the steady state condition.

In a first step, the binding capacity of melanoidins toward Fe^{2+} was investigated by applying saturation experiments. Saturation binding experiments measure non-specific Fe^{2+} binding, at equilibrium, at various ratios of iron:melanoidin. By analysing these data, the affinity of each melanoidin toward Fe^{2+} can be determined. In most systems, ratios of iron/melanoidin from 0.002 to 2 (9 steps) were tested (up to 0.1 mg/ml of Fe^{2+} and 50 mg/ml of melanoidin). In samples MC, MN, MT, ML, 50 mg/ml was the highest concentration of melanoidin assayed due to their low iron-binding ability. In order to avoid interference in the ultrafiltration process, concentrations above 50 mg/ml were not used. Furthermore, there would be metal-induced precipitation at high melanoidin concentrations. This effect was clearly observed in MC samples (80 mg/ml) after the incubation period. In these experiments, three different parameters were determined:

- M_{50}: concentration of melanoidin (mg/ml) necessary to bind 50% of the Fe_I.
- Max-B: maximum percentage of iron bound to melanoidin.
- M-max: concentration of melanoidin (mg/ml) necessary to reach Max-B.

Some characteristic profiles of percentage of iron bound to melanoidin against concentration of melanoidin solution are shown in Fig. 5. Fig. 5 also graphically represents the parameters M_{50}, Max-B, and M-max. Melanoidins from model systems have stronger binding ability toward iron than coffee, beer or sweet wine, and Max-B is mainly reached about 1 mg/ml of melanoidin. It is important to note that it was not possible to reach the saturation for samples MN, MT, and ML. At melanoidin concentrations above 50 mg/ml the saturation was not reached although it seems that it was not far from this value. It was also noticeable that, once Max-B was reached, some decrease in the percentage of Fe_B was detected (MC, MW in Fig. 5). This could be due

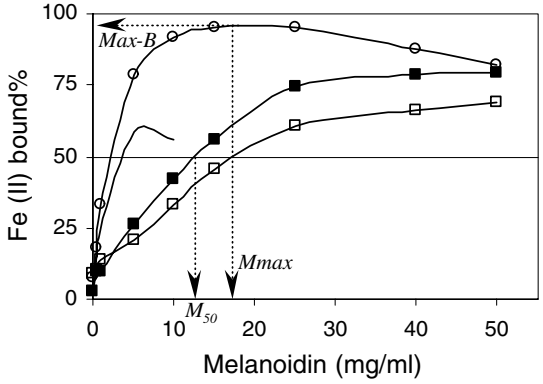

Fig. 5. Saturation curves toward iron of melanoidins from coffee (○, MC), Abbey style beer (■, MT), lager style beer (□, ML), and sweet wine (no marker, MW).

to residual metal-induced precipitation of the melanoidin structure under prolonged saturation conditions.

Analysis of iron was performed by both the BpH colorimetric procedure and FAAS, and agreement was significant between ionic iron and total soluble iron determinations ($r^2 = 0.9977$, $n = 28$) under the experimental conditions applied. Table 1 summarises the results obtained. Most systems were able to bind more than 85% of the starting iron (100 mg Fe^{2+}/l) before reaching the saturation level, except for the systems that contain cysteine (MGC, MLC) and MW. Results show that there were no significant differences between the different melanoidins from the Maillard model systems, but widely different behaviour was observed in the ability to bind iron among melanoidins isolated from commercial coffee, sweet wine and beer. Lower M_{50} values indicated higher efficiency in binding iron for a shorter time, but M_{50} and Max-B indices are not necessarily related.

Table 1
Iron-chelating capacity of various melanoidins according to the parameters M_{50}, Max-B, and n

System	M_{50} (mg/ml)	Max-B (%)	$n \times 1000$
MGA	0.40 ± 0.02	93.7	131 ± 5.7
MGC	0.42 ± 0.03	76.1	108 ± 6.6
MGD	2.11 ± 0.07	92.5	23.5 ± 0.7
MGF	0.42 ± 0.02	93.9	121 ± 5.9
MGG	0.52 ± 0.02	93.1	94.3 ± 7.0
MGH	0.47 ± 0.04	86.7	113 ± 8.1
MGK	0.72 ± 0.03	94.0	71.2 ± 2.7
MGM	0.55 ± 0.01	94.7	88.8 ± 1.9
MGR	0.78 ± 0.01	93.1	65.4 ± 1.2
MGW	0.47 ± 0.04	91.3	102 ± 5.8
MGY	0.50 ± 0.03	95.7	86.0 ± 4.3
MLA	0.68 ± 0.04	88.9	77.8 ± 3.2
MLC	0.54	72.1	81.3
MLD	2.59 ± 0.13	90.0	19.7 ± 0.6
MLF	0.68 ± 0.04	98.7	19.7 ± 1.3
MLG	0.85 ± 0.01	92.1	59.3 ± 1.1
MLH	0.68 ± 0.03	85.9	84.9 ± 3.5
MLK	1.55 ± 0.01	94.8	48.7 ± 0.6
MLM	0.75 ± 0.03	93.0	72.8 ± 2.5
MLR	0.75 ± 0.04	91.9	62.3 ± 2.9
MLW	0.61 ± 0.01	91.9	97.6 ± 1.0
MLY	0.74 ± 0.04	94.4	69.8 ± 3.2
MC	2.47 ± 0.09	95.1	18.6 ± 1.8
MW	4.00 ± 0.21	58.4	32.9 ± 2.2
MN[a]	15.8 ± 1.13	83.1	7.4 ± 0.6
MT[a]	13.7 ± 0.46	79.6	5.7 ± 0.3
ML[a]	19.3 ± 1.12	68.9	4.7 ± 0.2

[a] Denotes systems in which Max-B was not reached at 50 mg/ml.

To gain more insight in the results obtained, data were linearised by applying the well-known Scatchard plot procedure. It should be kept in mind that this is an approximation since non-linear regression analysis is more accurate, but the Scatchard plot is useful for assessing rates of iron binding abilities among systems. Next, Eq. (4) was applied:

$$Fe_B/Fe_F = [n/K_d] - [Fe_B/K_d] \quad (4)$$

n being the apparent number of co-ordination sites per unit of weight of melanoidin. When Fe_B/Fe_F is plotted against Fe_B, extrapolation to intersection on the x-axis, it yields n/K_d. Calculation of the slopes gives the reciprocal of the dissociation constant of iron–melanoidin interaction. By applying this mathematical procedure, a set of conditions was assumed; (a) the equilibrium was reached after 24 h of incubation, (b) there was no cooperativity among co-ordination sites and (c) binding was reversible according to the law of mass action.

Fig. 6 shows some examples of different types of curves obtained. It is noteworthy that bilinear behaviour was clearly observed in the coffee melanoidin, which means that at least two different types of binding sites (with high and low co-ordination strength toward iron) are present in the polymer structure. The total value of co-ordination sites is the sum of partial co-ordination sites, but for further calculations the second type of co-ordination site (low strength) was not taken into account. Table 1 summarises the apparent number of co-ordination sites (n) obtained for each melanoidin. The n values are expressed as milligrammes of iron bound

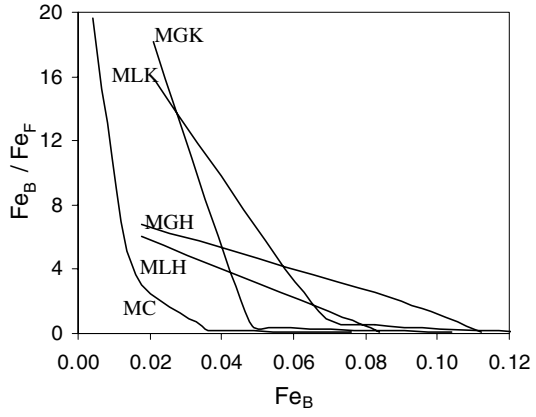

Fig. 6. Scatchard representation for MC, MGK, MLK, MGH, and MLH systems.

per gramme of melanoidin instead of molar ratios since the molecular weight of each melanoidin is unknown. It was possible to group the melanoidins in three levels according to number of apparent co-ordination sites; these being low ($n\times10^3<50$), medium ($50<n\times10^3<100$) and high iron chelating activity ($n\times10^3>100$) melanoidins at pH 5.0. Melanoidins from sweet wine, coffee and beer showed lower activity than most of melanoidins isolated from the model system. Results are of the same order of magnitude as obtained by studying Cu^{2+} chelating activity of a glycose:glycine melanoidin (Terasawa et al., 1991), although the two systems are difficult to compare. Authors have obtained a co-ordination number of 7, which correspond to 44.5 mg/g of melanoidin, if we consider 10,000 Da as the average molecular weight of MGG (Terasawa et al., 1991). On the other hand, results cannot be compared with those obtained by IMAC techniques since the initial crude extract of melanoidin was used in all the experiments.

ANOVA analysis was applied to determine the effect of starting material on the iron-binding properties of melanoidins isolated from model systems. Melanoidins from model systems with glucose had significantly stronger iron-binding properties than melanoidins from lactose, regardless of the type of amino acid used. These results are in line with Wijewickreme et al. (1997) who showed that the Cu^{2+}-chelating ability of melanoidins was influenced by the type of sugar and the reaction conditions used for synthesis. This observation also agrees with the melanoidin structure proposed by Cämmerer, Jalyschko, and Kroh (2002). Lactose is less reactive than glucose and will mostly be incorporated into the melanoidin skeleton as the whole molecule. However, glucose shows an important extent of retroaldol reaction, leading to the formation of mostly C3 dicarbonyl compounds with a higher reactivity than an glucose itself (Cämmerer, Wedzicha, & Kroh, 1999). Therefore, glucose melanoidins have glucose side chains but also very reactive residues in the backbone. These residues, probably rich in hydroxyl groups, could be responsible for higher iron-binding properties than the lactose molecule.

On the other hand, no relationship was observed a relationship between colour, expressed as browning at 420 nm, and the iron-binding capacity of melanoidins, expressed as apparent number of co-ordination sites. For instance, MGK and MLG show similar a_{420} values, 4.212 and 4.109, respectively, but different $n\times10^3$ values, 71.2 and 59.3, respectively. Similarly, for MGG and MLG, a_{420} values were of 2.009 and 2.351, and $n\times10^3$ values were 94.3 and 59.3, respectively. The effect of the heating conditions on the metal-binding properties of melanoidins will be investigated in the future.

In conclusion, the full characterisation of melanoidin structures is still unknown issue. Therefore it is necessary to apply indirect strategies to assess structural differences in the melanoidin backbone, such as their ability to chelate metal ions. Colour (Hofmann, Czerny, Calligaris, & Schierberle, 2001) and, more recently, antioxidant properties (e.g., Morales & Babbel, 2002), as well as the charge/mass ratio (e.g., Morales, 2002) have been used for melanoidin classification. Results shows that melanoidins could be classified according to their iron-binding ability by using an in vitro procedure. It is noteworthy that melanoidins from model systems have stronger iron-ability than those from coffee, beer or sweet wine. On the other hand, there is a homogeneity of the type of functional groups involved in the chelating properties since a mono-linear response was observed for all model systems under the experimental conditions (pH 5), except for coffee, in which at least two types of co-ordination sites were observed. This observation was expected since complex Maillard reactions are taking place in during the roasting of coffee. It is possible that chromophoric residues are not the main co-ordination sites for iron complexation in the melanoidin structure at pH 5. The lack of relationship between the browning and iron-binding ability of melanoidins described in this work needs to be investigated in detail the future.

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