Food Sci. Biotechnol. 23(2): 561-568 (2014)
DOI 10.1007/s10068-014-0077-5

RESEARCH ARTICLE

Relationship between the Chemical Composition and the Biological Activities of Food Melanoidins

Davide Tagliazucchi and Elena Verzelloni

Received May 31, 2013; revised July 31, 2013; accepted August 26, 2013; published online April 30, 2014

Abstract The relationship between the chemical composition and the biological activities of food melanoidin-rich fractions was investigated. Melanoidin-rich fractions were extracted using ultrafiltration (a 10 kDa cut-off) from coffee, barley coffee, dark beer, and traditional balsamic vinegar. All the food melanoidin-rich fractions were formed mainly of carbohydrates, phenolic compounds, and proteins. In dark beer, barley coffee, and traditional balsamic vinegar melanoidins, glucose was the most abundant sugar incorporated into melanoidins. Coffee melanoidins contained the largest amount of phenolic groups, followed by traditional balsamic vinegar melanoidins. The radical scavenging, Fe^{2+}-chelating, and heme binding abilities of food melanoidins were investigated under gastric conditions. The melanoidin-rich fraction extracted from coffee was the most active, showing the highest radical scavenging, Fe^{2+}-chelating, and heme binding activities, compared to barley coffee, dark beer, and traditional balsamic vinegar. The radical scavenging and Fe^{2+}-chelating abilities were assigned to the phenolic groups present in food melanoidins.

Keywords: melanoidin, stomach, antioxidant, coffee, phenol

Introduction

Thermally treated foods are an important component of the daily human diet. During thermal treatment, flavored and brown-colored compounds are formed as a consequence of Maillard reactions (1) occurring between an amino acid or protein and a reducing sugar. This is a common food reaction that takes place during storage, cooking, and heat processing (1) that may produce a large number of colorless aroma compounds, ultra-violet absorbing intermediates, and dark-brown polymeric compounds (2). The end-products of Maillard reactions are called melanoidins and are generally defined as brown-colored, nitrogen-containing, and, high molecular weight (usually more than 5 kDa) compounds. They are involved in the color, flavor, and texture of thermally treated foods, such as coffee, dark beer, sweet wine, traditional balsamic vinegar, cocoa, and bread (3-5). Recently, scientific interest in the field of high molecular weight melanoidins has increased because of varied biological activities and functional properties (6). Some of these properties are antioxidant and chelating (7-9), antimicrobial (10), antihypertensive (3), antiglycative (11), and prebiotic (5) activities.

Despite efforts in recent years, the chemical structure of melanoidins is largely unknown and different melanoidin populations may coexist within food. Different structures are present simultaneously in food as a consequence of the presence of many chemical substances that participate in the reactions that form melanoidins (12).

Several studies had been carried out with the aim of detecting specific chemical substituents in food melanoidins. The melanoidins from coffee are by far the most widely investigated (12). Polysaccharides (mainly galactomannans and arabinogalactans), proteins, and chlorogenic acids contribute to formation of coffee melanoidins, although most of the weight of coffee melanoidins (up to 90%) is imparted by unknown materials (12). Some low molecular weight compounds (chlorogenic acid and low molecular weight Maillard reaction products) have been found to be ionically linked to a melanoidin core (13).

Two major factors limit the actual physiological relevance of the biological activities of coffee melanoidins. First,

Davide Tagliazucchi (✉), Elena Verzelloni
Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola 2, 42100 Reggio Emilia, Italy
Tel: +39-0522-522060; Fax: +39-0522-522053
E-mail: davide.tagliazucchi@unimore.it

limited knowledge of the structure of coffee melanoidins makes it difficult to identify the active components responsible for specific biological activities. Second, although melanoidins are consumed regularly as part of the daily human diet, they are generally considered to be poorly absorbable bio-available compounds (14). For these reasons, it is unlikely that coffee melanoidins act as biologically active compounds in the bloodstream or organs. More important, most of the consumed melanoidins remain in the gastrointestinal tract. Therefore, it may be a key site for their antioxidant and biological actions (15).

Recently, dietary melanoidins (isolated by ultrafiltration >10 kDa) were shown to inhibit lipid peroxidation in an *in vitro* model of the gut during digestion of meat (15,16). In particular, coffee melanoidins were able to completely inhibit lipid hydroperoxides and advanced lipoxidation end product formation at a concentration of 3 mg/mL. Coffee melanoidins were more efficient in inhibiting lipid peroxidation during simulated digestion of turkey meat than melanoidins from dark beer, barley coffee, and traditional balsamic vinegar. Reduction in the formation of these pro-atherogenic compounds has been shown to be followed by a decrease in their absorption in humans after simultaneous consumption of coffee and meat (17).

The aim of this work was to determine levels of total sugars, glucose, fructose, total nitrogen, total proteins, and phenolic groups in high molecular weight melanoidins extracted from dark beer, barley coffee, coffee, and traditional balsamic vinegar. The antioxidant activity, iron-chelating ability, and heme-binding capacity were also determined under gastric conditions, and relationships between biological activities and chemical compositions were analyzed.

Materials and Methods

Materials Gallic acid, $HmFe^{III}$ (hemin), TPTZ, and bovine serum albumin (BSA) were supplied by Sigma (Milan, Italy). ABTS was supplied by Calbiochem (Darmstadt, Germany). All other chemical reagents were from Carlo Erba (Milan, Italy). A kit for D-glucose and D-fructose determination was obtained from Roche (Darmstadt, Germany). Amicon Ultra-4 and Microcon YM-10 (regenerated cellulose, 10 kDa) were supplied by Millipore (Milan, Italy). Whatman filter paper No. 4 was supplied by Whatman (Maidstone, Kent, UK). Coffee, barley coffee, and dark beer were purchased in a local supermarket in Reggio Emilia, Italy in December of 2012. Traditional balsamic vinegar samples were supplied by "Consorzio fra produttori di Aceto Balsamico Tradizionale di Reggio Emilia" (Reggio Emilia, Italy). Absorbance values were read using a Jasco V-550 UV/Vis spectrophotometer (Tokyo, Japan).

Sample preparation Coffee and barley coffee were prepared by dissolving 2 g of soluble coffee or barley coffee in 100 mL of boiling water followed by dilution at 1:2 with water, then filtering using Whatman filter paper No. 4. Dark beer was diluted 1:2 using water and also filtered. Traditional balsamic vinegar (TBV) samples were prepared by a 5× dilution in water (1 g of TBV brought to 5 g using water) and subsequent filtration with Whatman filter paper No. 4. Samples were diluted and filtered to avoid clogging of the ultrafiltration units.

Isolation of high molecular weight material from the food samples High molecular weight material (>10 kDa) was extracted from filtered samples as previously described (15). Filtered samples (4 mL) were subjected to ultrafiltration using an Amicon Ultra-4 (Millipore) at 7,500×*g* for 70 min at 4ºC. The retentate was refilled with water and ultrafiltered again. This washing procedure (diafiltration) was repeated three times to reduce the concentration of contaminating low molecular weight compounds. At the end of washing, retentates containing high molecular weight materials were made up to 4 mL using distilled water. These fractions were then used for determination of chemical composition, antioxidant activity analysis, and heme-binding and Fe^{2+}-chelation ability determinations.

Retentates were freeze-dried and solid residues were weighed in some experiments to quantify melanoidins extracted from different food samples.

Determination of total protein, nitrogen, and phenolic groups The protein content of retentates extracted from different food samples was assayed using the Bradford method (18) with bovine serum albumin as a standard. The total nitrogen (TN) content was determined using a Kjeldhal automated apparatus (Instrument Lab Control, Reggio Emilia) (19). The protein nitrogen (PN) content was calculated by dividing the amount of protein determined using a Bradford assay by 6.25 (protein/nitrogen ratio of 6.25) (19). The non-protein nitrogen (NPN) content was calculated by subtracting the protein nitrogen content from the total nitrogen content. The total phenolic group content of retentates extracted from different food samples was determined using Folin-Ciocalteu reagent (19). Gallic acid at concentrations ranging between 50 and 500 mg/L was used as a standard ($y=0.0009\times+0.0127$; $R^2=0.9987$).

Determination of total carbohydrate, glucose, and fructose The total sugar content of high molecular weight material extracted from different food samples was assayed using the phenol-sulphuric acid method after mild acid hydrolysis (15). Samples (5 mg/mL) were hydrolyzed in 1 N HCl for 2 h at 105ºC. After rapid cooling, the

hydrolyzed solutions were diluted 10× using distilled water, then filtered. These mixtures were then used for determination of total sugars. A total of 100 μL of hydrolyzed solution was added to 100 μL of 5% phenol in water. After mixing, 1 mL of concentrated H_2SO_4 was added and the solution was immediately vortexed with a vortex mixer (Fisher Scientific, Illkirch-Graffenstaden, France) for a few seconds before reading at 500 nm. Mannose was used as a standard since it is the principal sugar present in coffee melanoidins (20). Glucose was used as a standard for other high molecular weight fractions. The D-glucose and D-fructose contents in the hydrolyzed solutions were assayed using a D-glucose/D-fructose enzymatic assay kit (Megazyme, Wicklow, Ireland).

Spectroscopic analysis Spectroscopic analyses were performed by measuring the specific extinction coefficient (K) of high molecular weight material at 280 and 420 nm (20).

Use of the specific extinction coefficient (K) was preferred over the molar extinction coefficient (ε) since the molecular weight of melanoidins is unknown. The high molecular weight fractions are a mixture of different melanoidin populations that may have different molecular weights. By using K, the concentration parameter used in the Lambert-Beer Law is expressed as L/g cm, which makes it applicable for comparisons of different foods.

Fe^{2+}-chelation ability Retentates were diluted in 0.1 M Na-acetate buffer at pH 3 to simulate gastric conditions, then incubated with 50 mg/L of $FeSO_4$ (dissolved in 0.1 M Na-acetate buffer at pH 3) at room temperature. After vortexing with a vortex mixer (Fisher Scientific) for 2 h, samples were allowed to stand for 22 h at room temperature, then they were ultrafiltered using a Microcon YM-10 (Millipore), at 14,000×*g* for 30 min at 22ºC. The quantity of chelated iron was determined by measuring the content of Fe^{2+} in the filtrate (unbound Fe^{2+}) and subtracting this value from the initial quantity of Fe^{2+} incubated with melanoidins (8). The assay to measure the content of Fe^{2+} used TPTZ, as described in Tagliazucchi *et al.* (15).

Heme binding ability Hemin at a concentration of 10 μM was dissolved in a 20% dimethyl sulfoxide (DMSO) solution to avoid monomer aggregation. The hemin solution was brought to pH 3 to simulate gastric conditions. Aliquots (5 μL each) of retentates diluted in 0.1 M Na-acetate buffer at pH 3 to simulate gastric conditions were added to the samples (995 μL of 10 μM hemin in 20% DMSO, pH 3) and to a reference (995 μL of 20% DMSO, pH 3) cuvette. The amount of bound hemin was assayed by measuring the absorbance at 400 nm before and after the addition of retentates (15). The difference between the absorbance values at 400 nm before and after addition of melanoidins is referred to as Δ Abs. In order to calculate the Hill coefficient and the value of the equilibrium binding constant (Kd), a non-linear regression analysis method was applied (GraphPad Prism 5.0; GraphPad Software, San Diego, CA, USA).

Radical scavenging activity The antioxidant activity of melanoidins was measured as the radical scavenging activity using an ABTS assay, as described by Re *et al.* (21). ABTS was dissolved in distilled water to a 14 mM concentration. The ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting an ABTS stock solution with 4.9 mM potassium persulphate at a ratio of 1:1 and leaving the mixture to stand in the dark at room temperature for 12-16 h before use. The resulting blue-green ABTS radical solution was diluted using 0.1 M Na-acetate buffer at pH 3 to simulate gastric conditions with an absorbance value of 0.700±0.050 at 734 nm. An amount of 40 μL of high molecular weight melanoidins diluted in the same acetate buffer at pH 3 was added to 1,960 μL of the resulting blue-green $ABTS^{\cdot+}$. The mixture, protected from the light, was incubated in a Jasco V-550 spectrophotometer at 37ºC for 10 min. The decrease in absorbance at 734 nm was measured after 10 min. A vitamin C standard curve that correlated the concentration of vitamin C (ranging from 1 to 150 mg/L) with the amount of absorbance reduction was obtained. Results were expressed as the vitamin C equivalent antioxidant capacity (VCEAC) value defined as mg of vitamin C per gram of melanoidins.

Statistical analysis All data are presented as mean± standard deviation (SD) for at least three replications for each prepared sample. Statistical analyses were performed using Graph Pad Prism 5.0 software (GraphPad Software). Pearson's correlation test was used for determination of correlations between the chemical composition and the biological activity. A *p*-value of less than 0.05 ($p<0.05$) was considered statistically significant. Correlations were expressed as r^2 (the coefficient of determination), which represents the fraction of the variance in 2 variables that is shared.

Results and Discussion

The dry matter contents of food samples and respective high molecular weight fractions are shown in Table 1. On the basis of total dry matter, barley coffee exhibited the highest recovery of high molecular weight material, followed by coffee, dark beer, and TBV. The high absorption value at 420 nm (Table 1), the presence of nitrogen (Table 2), and the high molecular weight allowed definition of these

fractions as rich in melanoidins, indicated hereafter as the melanoidin fraction (MF).

Spectroscopic analysis of the melanoidin fractions of foods The most obvious characteristic of melanoidins is the color. The K_{420} index (Table 1) can be used to compare the concentration of melanoidins in food MFs. The coffee MF and the TBV MF had the highest K_{420} values. Therefore, they were richer in melanoidins than dark beer and barley coffee MFs. Extracted MFs also showed a high absorption value at 280 nm (Table 1), indicating that proteins and/or phenolic groups were present in the MFs.

Analysis of dark beer and barley coffee melanoidin fractions The results of chemical analysis carried out on dark beer and barley coffee MFs are shown in Table 2. In barley, the most abundant carbohydrate was starch, constituting up to 65% of the dry weight (22). During malting and barley coffee production, starch is partially degraded in soluble dextrins and low molecular weight sugars (22,23). Glucose (Table 2) was identified as the most common carbohydrate released after hydrolysis of barley coffee and dark beer MFs, where it accounted for 71.8 and 78.4%, respectively, of total carbohydrates. The released glucose probably derived both from un-modified and modified glucose polysaccharide chains, perhaps the result of incorporation of glucose polysaccharide chains in melanoidins by reactions with amino acids/peptides at the reducing end. Cämmerer *et al.* (24) showed that maltodextrin can react with amino acids in a water-free model system and that glucose, representing 95% of melanoidins in dry weight, is released from maltodextrin/glucose model melanoidins due to mild acid-catalyzed hydrolysis.

After separation of high molecular weight materials from hydrolyzed samples, the resulting low molecular weight solutions retained some absorption capability at 420 nm (3 and 9% of the product absorption capabilities at 420 nm of barley coffee and dark beer, respectively), suggesting that glucose polysaccharide chains are partially incorporated into melanoidins. Thus, hydrolysis of glucose polysaccharide chains caused a reduction in the molecular weight of brown molecules that were then able to pass thorough the ultrafiltration units with a 10 kDa cut-off. The protein and nitrogen contents of dark beer and barley coffee MFs are shown in Table 2. The total nitrogen content is higher than the protein nitrogen content (obtained by dividing the protein content by a factor of 6.25) suggesting that most of the nitrogen could not be ascribed to proteins in the MFs of dark beer and barley coffee. Some of the amino groups probably reacted during production of dark beer and barley coffee MFs, ending up in the melanoidin structure.

Barley coffee and dark beer MFs showed similar phenolic group contents (Table 2), probably because they originated from the same raw material. The presence of these phenolic groups in the MFs of barley coffee and dark beer might be a result of incorporation of phenolics in proteins, and/or formation of new phenolic structures

Table 1. Dry matter contents of samples and melanoidin fractions (MF) and spectroscopic data for MF extracted from food samples

Food	Dry matter content (mg/mL)		Recovery (% w/w)[1)]	K_{420}[2)]	K_{280}[2)]
	Total	MF			
Coffee	20.0±0.1	7.6±0.4	38.0	1.1289	5.9616
Barley coffee	20.0±0.1	14.4±0.5	72.0	0.3672	1.2446
Dark beer	61.6±2.2	12.3±0.6	20.0	0.5838	2.1848
TBV	755.2±14.5	73.7±3.2	9.8	1.1038	3.8260

[1)]On a total dry matter basis
[2)]K=specific extinction coefficient expressed as L/g cm

Table 2. Total carbohydrate, glucose, fructose, protein, total nitrogen (TN), protein nitrogen (PN), non-protein nitrogen (NPN) and phenolic group contents of the MF extracted from barley coffee (BC), dark beer (DB), traditional balsamic vinegar (TBV), and coffee[1)]

Melanoidin fraction	Carbohydrate (%, w/w)	Glucose (%, w/w)	Fructose (%, w/w)	Protein (%, w/w)	TN (%, w/w)	PN (%, w/w)	NPN (%, w/w)	Phenolic group (%, w/w)
BC MF	77.14±0.63	55.40±3.01	ND[2)]	1.13±0.02	0.88±0.09	0.18	0.69	2.76±0.26
DB MF	91.58±6.46	71.78±4.46	ND[2)]	5.02±0.03	2.62±0.26	0.80	1.82	2.91±0.36
TBV MF	66.92±2.18	44.29±2.00	10.46±0.45	1.61±0.13	0.88±0.08	0.26	0.62	7.38±0.46
Coffee MF	69.81±1.58	0.83±0.01	0.55±0.01	8.83±0.46	3.04±0.31	1.41	1.63	16.00±1.15

[1)]Results are expressed as g in 100 g of MF.
[2)]ND, not detected

during production of dark beer and barley coffee.

The high molecular weight materials of dark beer and barley coffee are mainly composed of glucose polysaccharide chains that either did or did not react with amino compounds to synthesize melanoidins (Table 2). Although the existence of covalent interactions between melanoidins and polysaccharides were not proven in this study, findings suggest that glucose polysaccharide chains in barley are involved in melanoidin formation and structure.

Analysis of the traditional balsamic vinegar melanoidin fractions In white grape must (the starting material for production of TBV), the most abundant sugars are the monosaccharides glucose and fructose. Concentrations can reach up to 200 g/kg (25,26). During must cooking and TBV ageing, there is a progressive consumption of these sugars through Maillard reactions (4) and sugar degradation (26) pathways. These reactions are favored by the reduction of water content that occurs during must cooking and TBV ageing. The intact glucose and fructose molecules that are released from the TBV MF after mild acidic hydrolysis accounted for 82% of the total carbohydrate content (Table 2). Because the concentration of glucose and fructose-containing polysaccharides in must is low with respect to the amount of free monosaccharides, it is unlikely that the glucose and fructose released from the TBV MF are derived from polysaccharides. More probably they are incorporated as they are in the melanoidin structure. Cämmerer *et al.* (24) found that glucose can be released by acid-catalyzed hydrolysis from model melanoidins that are built-up by reactions between glucose and amino acids, suggesting that glucose reacts with a carbohydrate structure via formation of a glycosyl cation incorporated as a side chain in the melanoidin skeleton (24). It is also possible that, during TBV production, some glucose and fructose molecules bind to the melanoidin skeleton via trans-glycosylation reactions and are easily subsequently removed by hydrolysis. Furthermore, transglycosylation reactions are catalyzed under acidic pH conditions and proceed through water elimination and, therefore, could occur during TBV production. It is also possible that some oligomerization or polymerization reactions involving glucose occur under TBV production conditions and that these oligomers or polymers can be incorporated as they are in the melanoidin structure by transglycosylation reactions, or by reacting with amino acids/peptides at the reducing end. Fructose is found in the TBV MF at a lower concentration than glucose, despite being in an equimolar concentration with glucose in must (26). This is explicable considering that fructose is more reactive in Maillard (4) and sugar degradation (26) pathways.

Hydrolyzed samples were then separated using ultrafiltration with a 10 kDa cut-off, and absorption at 420 nm in the low molecular weight fraction was detected. The low molecular weight solution retained some absorption at 420 nm (9% of the product absorption at 420 nm). Thus, hydrolysis of oligomeric/polymeric sugar chains causes a reduction in the molecular weight of brown molecules, suggesting that fructose and/or glucose are partially incorporated into the brown melanoidin skeleton. The TBV MF also contains phenolic groups, proteins, and non-protein nitrogen (Table 2).

Analysis of coffee melanoidin fractions The chemical composition and characterization of the coffee MF has already been reported (20,27). Chemical analyses have been also been carried out on the coffee MF with the aim to use these data for analysis of the biological activities and the relationships between the chemical compositions and biological activities of the melanoidin fractions in foods.

The total carbohydrate (69.81±1.58% w/w) and the glucose and fructose (0.83±0.01 and 0.55±0.01% w/w, respectively) contents of the coffee MF agree with previously reported data (20,27). Bekedam *et al.* (20) found that mannose, galactose, and arabinose are the most abundant sugars in coffee high molecular weight melanoidins. These results support the hypothesis that polymeric galactomannans and arabinogalactans are incorporated into the melanoidin structure (12).

The protein (8.83±0.46% w/w), the total nitrogen (3.04±0.31% w/w), the protein nitrogen (1.41% w/w), and the non-protein nitrogen (1.63% w/w) contents of the coffee MF also agree with previously reported results (20,27).

The coffee MF was rich in phenolic groups (approximately 16%, w/w) in agreement with previous results (20). The high content of phenolic groups in the coffee MF can be attributed to incorporation of chlorogenic acids in the melanoidin skeleton (28).

Biological activities of the melanoidin fractions in foods The biological activities of food melanoidins were investigated. All analyses were carried out under gastric conditions (pH 3), since the stomach is an important location for the biological activity of dietary melanoidins. The coffee MF had the highest radical scavenging activity (Fig. 1) and all food MFs tested retained some radical scavenging activity under gastric conditions, suggesting that dietary melanoidins protect stomach tissues from oxidative stress. The metal ion chelating ability may also be important for the antioxidant activities of melanoidins. All food MFs tested exhibited Fe^{2+}-chelating abilities under gastric conditions with the coffee MF as the highest (Fig. 2).

The gastrointestinal tract is constantly exposed to reactive radical species that can be present in foods and beverages,

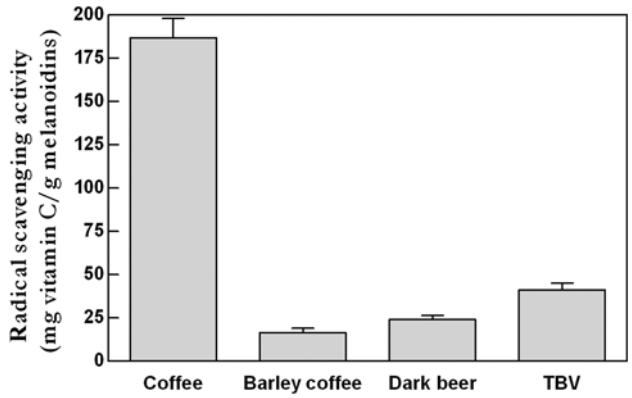

Fig. 1. Radical scavenging activity of the food melanoidin fractions (MF) measured using an ABTS assay under gastric conditions. Data are expressed as VCEAC (mg vitamin C/g of the melanoidin fraction) and are presented as mean±SD of three replications for each prepared sample.

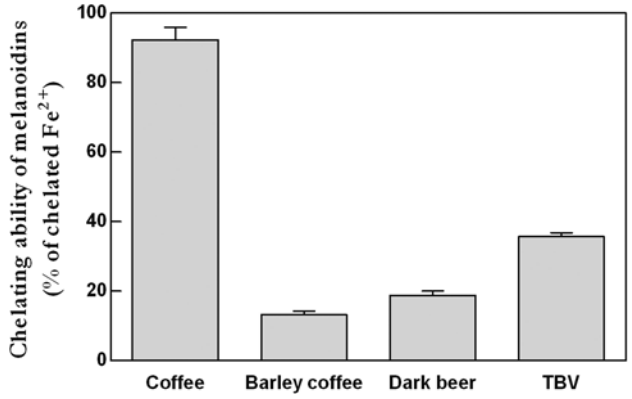

Fig. 2. Iron binding ability of the food melanoidin fractions (MF) under gastric conditions. Data are expressed as percentage of Fe^{2+} chelated by 3 mg/mL of different food MFs and are presented as mean±SD of three replications for each prepared sample.

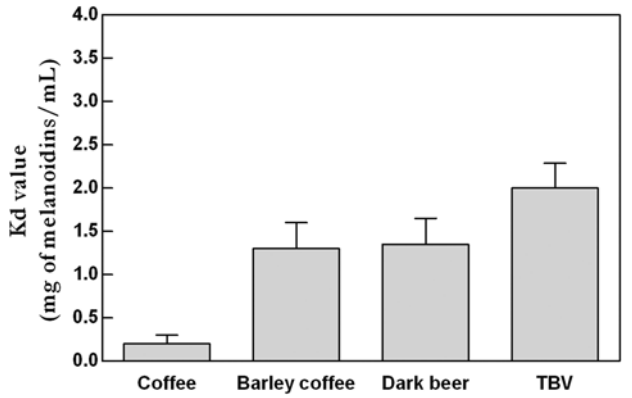

Fig. 3. Heme binding capacity of the food melanoidin fractions (MF). The assay was carried out under gastric conditions. Data are expressed as Kd values (the dissociation constant expressed as mg/mL of the MF) and are presented as mean±SD of three replications for each prepared sample.

Table 3. The r^2 values (coefficient of determination) between biological activities and chemical compositions of food melanoidin fractions

Chemical composition	Radical scavenging activity	Fe^{2+}-Chelating activity	Heme binding activity
Phenolic group	0.9469[1)]	0.9888[2)]	-0.4920
Protein	0.8513	0.9277[3)]	-0.3555
Total nitrogen	0.4463	0.3789	-0.5837
Non-protein nitrogen	0.1906	0.1421	-0.3725
Total carbohydrate	-0.2148	-0.2921	0.0031
Glucose	-0.9000	-0.9184[4)]	0.5150
Fructose	-0.0279	-0.0008	0.4439

[1)-4)]Statistically significant ($p<0.05$)

or can be generated by chemical reactions between dietary components within the stomach (29). Food melanoidins might exert direct protective effects in the gastrointestinal tract by scavenging reactive species and/or preventing their formation. Food melanoidins can inhibit heme/Fe^{2+}-induced peroxidation in the stomach during digestion of meat (15) and chelate dietary metal ions (30) that can act as pro-oxidants in the gastrointestinal tract. Another important biological activity of high molecular weight melanoidins is an ability to bind heme (15). Recent studies have demonstrated the prooxidant and cytotoxic effects of circulating heme and involvement in cardiovascular pathologies (31). Moreover, it has been suggested that the heme in consumed red meat can act as a catalyst for oxidative damage and can initiate several kinds of cancer, cardiovascular diseases, and other diseases (32). Therefore, the binding of heme and prevention of heme absorption may be an important mechanism of cardiovascular disease prevention by melanoidins. All the food MFs tested had heme-binding abilities under gastric conditions (Fig. 3). The Kd values showed that the coffee MF had a greater affinity towards heme, in comparison with the other MFs tested. The calculated Hill coefficient of the food MFs was higher than 1, indicating positive cooperation in the binding of heme (data not shown). These results suggest the presence of more than one binding site for heme in food melanoidins.

Relationship between the chemical compositions and biological activities of the melanoidin fractions of foods

Relationships between the chemical compositions and biological activities of food MFs are shown in Table 3. A statistically significant positive correlation ($p<0.05$) was found between the phenolic group content of the food MFs and the radical scavenging activity (Fig. 4A). Polyphenols exhibit radical scavenging activities (29) and these results suggest that, under gastric conditions, phenolic groups incorporated into high molecular weight materials are responsible for the radical scavenging activities of these

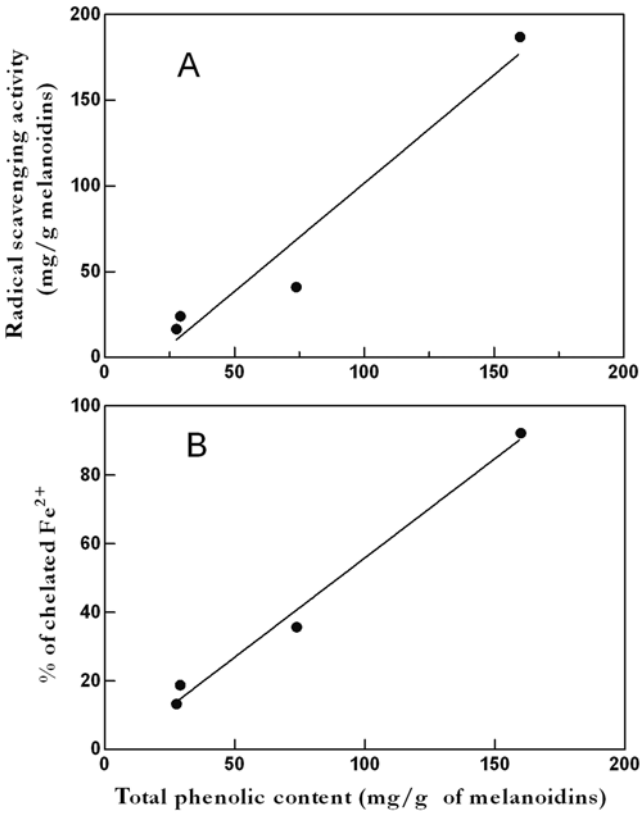

Fig. 4. (A) Correlations between the total phenolic contents and the radical scavenging activities of food melanoidin-rich extracts. Correlation coefficient r=0.9731, linear regression y=12.62x-24.47. Coefficient of determination r^2=0.9469. (B) Correlations between the total phenolic contents and the iron chelating abilities of food melanoidin-rich extracts. Correlation coefficient r=0.9944, linear regression y=5.79x-2.01. Coefficient of determination r^2=0.9888

fractions. Apart from their radical scavenging activity, polyphenols are able to chelate metal ions (30) and are responsible for the iron chelating ability of the food MFs. A strong and statistically significant positive correlation was found between the iron chelating ability and the phenolic group content of MFs (p<0.05) (Table 3 and Fig. 4B).

The protein content of MFs was significantly positively related with the iron binding ability (p<0.05), consistent with the results of Cosovic *et al.* (33) in which the nitrogen part of model melanoidins was proposed to be responsible for the chelation of copper ions. No statistically significant correlations were found between the heme binding abilities and the chemical compositions of the food high molecular weight fractions.

In this work, the radical scavenging activities and the iron chelating abilities of food melanoidins is attributed to phenolic groups as a consequence of the presence of phenolic compounds in the raw starting material, and because of phenolic compound participation in Maillard reactions and incorporation into melanoidins (27,28). However, other structures present in melanoidins could contribute to the biological activities of these compounds. It is known that melanoidins formed from model systems also have metal chelating and antioxidant biological activities (34). Some recent studies (9,10,35) support the hypothesis that ionically bound melanoidin compounds, for instance, polyphenols and low molecular weight Maillard reaction products, contribute to antioxidant activities. Ionically bound melanoidin compounds of coffee (34) and glucose-amino acid Maillard reaction products (10) showed higher antiradical activities than pure melanoidins. In any case, the mechanism of the antioxidant effect of melanoidins is still unclear because the chemical structure of melanoidins is unknown.

In conclusion, the high molecular weight fractions of food consist mainly of carbohydrates, phenolic compounds, and proteins. All the MFs studied contained nitrogen that could not be ascribed to proteins. In barley coffee and dark beer, the main sugar released intact after hydrolysis of the MF is glucose. In TBV, both glucose and fructose are the main carbohydrates released after mild hydrolysis of the MF. In the coffee MF, mannose, galactose, and arabinose are the most abundant sugars. The presence of these constituents produces MFs with strong biological activities. Phenolic groups are mainly responsible for the radical scavenging activities and iron chelating abilities of MFs. The heme binding ability of food melanoidins could not be assigned to specific substituents. Since high molecular weight melanoidins are poorly absorbed in the gastrointestinal tract, but retain high biological activities under gastric conditions, the stomach might be the main biological site of action of these antioxidant compounds.

References

1. Friedman M. Food browning and its prevention: An overview. J. Agr. Food Chem. 44: 631-653 (1996)
2. Martins SIFS, Jongen WMF, van Boekel MAJS. A review of Maillard reaction in food and implications to kinetic modeling. Trends Food Sci. Tech. 11: 364-373 (2001)
3. Rufián-Henares JA, Morales FJ. Angiotensin-I converting enzyme inhibitory activity of coffee melanoidins. J. Agr. Food Chem. 55: 1480-1485 (2007)
4. Tagliazucchi D, Verzelloni E, Conte A. Antioxidant properties of traditional balsamic vinegar and boiled must model systems. Eur. Food Res. Technol. 227: 835-843 (2008)
5. Borrelli RC, Fogliano V. Bread crust melanoidins as potential prebiotic ingredients. Mol. Nutr. Food Res. 49: 673-678 (2005)
6. Morales FJ, Somoza V, Fogliano V. Physiological relevance of dietary melanoidins. Amino Acids 42: 1097-1109 (2012)
7. Morales FJ, Jimenez-Perez S. Peroxyl radical scavenging activity of melanoidins in aqueous systems. Eur. Food Res. Technol. 218: 515-520 (2004)
8. Morales FJ, Fernandez-Fraguas C, Jimenez-Perez S. Iron-binding ability of melanoidins from food and model systems. Food Chem. 90: 821-827 (2005)

9. Tagliazucchi D, Verzelloni E, Conte A. Contribution of melanoidins to the antioxidant activity of traditional balsamic vinegar during ageing. J. Food Biochem. 34: 1061-1078 (2010)
10. Rufián-Henares JA, Morales FJ. Functional properties of melanoidins: *In vitro* antioxidant, antimicrobial and antihypertensive activities. Food Res. Int. 40: 995-1002 (2007)
11. Verzelloni E, Tagliazucchi D, del Rio D, Calani L, Conte A. Antiglycative and antioxidative properties of coffee fractions. Food Chem. 124: 1430-1435 (2011)
12. Moreira ASP, Nunes FM, Domingues R, Coimbra MA. Coffee melanoidins: Structures, mechanisms of formation and potential health impact. Food Funct. 3: 903-915 (2012)
13. Delgado-Andrade C, Morales FJ. Unraveling the contribution of melanoidins to the antioxidant activity of coffee brew. J. Agr. Food Chem. 53: 1403-1407 (2005)
14. Faist V, Erbersdobler H. Metabolic transit and *in vivo* effects of melanoidins and precursor compounds deriving from the Maillard reaction. Ann. Nutr. Metab. 45: 1-12 (2001)
15. Tagliazucchi D, Verzelloni E, Conte A. Effect of dietary melanoidins on lipid peroxidation during simulated gastro-intestinal digestion: their possible role in the prevention of oxidative damage. J. Agr. Food Chem. 58: 2513-2519 (2010)
16. Verzelloni E, Tagliazucchi D, Conte A. From balsamic to healthy: traditional balsamic vinegar melanoidins inhibit lipid peroxidation during simulated gastric digestion of meat. Food Chem. Toxicol. 48: 2097-2102 (2010)
17. Sirota R, Gorelik S, Harris R, Kohen R, Kanner J. Coffee polyphenols protect human plasma from postprandial carbonyl modifications. Mol. Nutr. Food Res. 57: 916-919 (2013)
18. Bradford M. A rapid and sensitive method for the quantition of microgram quantities of protein utilizing the principle of dye binding. Anal. Biochem. 72: 248-254 (1976)
19. Borrelli RC, Visconti A, Mennella C, Anese M, Fogliano, V. Chemical characterization and antioxidant properties of coffee melanoidins. J. Agr. Food Chem. 50: 6527-6533 (2002)
20. Bekedam EK, Schols HA, van Boekel MAJS, Smit G. High molecular weight melanoidins from coffee brew. J. Agr. Food Chem. 54: 7658-7666 (2006)
21. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26: 1231-1237 (1999)
22. Briggs DE, Hough JS, Stevens R, Young TW. Malting and Brewing Science. Kluwer Academic Publisher, Dordrecht, the Netherlands. pp. 57-109 (1981)
23. Clarke RJ, Macrae R. Coffee. Related Beverages. Elsevier Applied Science Publisher, Essex, UK. pp. 63-89 (1987)
24. Cämmerer B, Jalyschko W, Kroh LW. Intact carbohydrate structures as part of the melanoidins skeleton. J. Agr. Food Chem. 50: 2083-2087 (2002)
25. Verzelloni E, Tagliazucchi D, Conte A. Changes in major antioxidant compounds during ageing of traditional balsamic vinegar. J. Food Biochem. 34: 152-171 (2010)
26. Falcone PM, Tagliazucchi D, Verzelloni E, Giudici P. Sugar conversion induced by the application of heat to grape must. J. Agr. Food Chem. 58: 8680-8691 (2010)
27. Bekedam EK, Loots MJ, Schols HA, van Boekel MAJS, Smit G. Roasting effects on the formation mechanisms of coffee brew melanoidins. J. Agr. Food Chem. 56: 7138-7145 (2008)
28. Bekedam EK, Schols HA, van Boekel MAJS, Smit G. Incorporation of chlorogenic acids in coffee brew melanoidins. J. Agr. Food Chem. 56: 2055-2063 (2008)
29. Halliwell B, Zhao K, Whiteman M. The gastrointestinal tract: A major site of antioxidant action? Free Radic. Res. 33: 819-830 (2000)
30. Ronca G, Palmieri L, Maltinti S, Tagliazucchi D, Conte A. Relationship between iron and protein content of dishes and polyphenol content in accompanying wines. Drugs Exp. Clin. Res. 29: 271-286 (2003)
31. Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, Eaton JW, Balla G. Pro-oxidant and cytotoxic effects of circulating heme. Blood 100: 879-887 (2002)
32. Tappel A. Heme of consumed red meat can act as catalyst of oxidative damage and could initiate colon, breast and prostate cancers, heart disease and other diseases. Med. Hypotheses 68: 562-564 (2007)
33. Cosovic B, Vojvodic V, Boskovic N, Plavsic M, Lee C. Characterization of natural and synthetic humic substances (melanoidins) by chemical composition and adsorption measurements. Org. Geochem. 41: 200-205 (2010)
34. Wang HY, Qian H, Yao WR. Melanoidins produced by the Maillard reaction: Structure and biological activity. Food Chem. 128: 573-584 (2011)
35. Delgado-Andrade C, Rufian-Henares JA, Morales FJ. Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. J. Agr. Food Chem. 53: 7832-7836 (2005)