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Unraveling the Contribution of Melanoidins to the Antioxidant Activity of Coffee Brews

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Instant coffees produced from the same green coffee beans were supplied from a company in different roasting degrees, light, medium, and dark. Melanoidins were obtained by ultrafiltration (10 kDa) and subsequent diafiltration. Pure melanoidins were isolated from melanoidins after overnight incubation in 2 M NaCl. The antioxidant activities of instant coffees, melanoidins, and pure melanoidins were tested using the conjugated diene formation from a 2,2′-azobis(2-amidinopropane) dihydrochloride-induced linoleic acid oxidation in an aqueous system. No significant differences were found between melanoidins and pure melanoidins with different roasting degrees. Therefore, the contribution of the pure melanoidin fraction to the total antioxidant activity of melanoidins was significantly lower. More than 50% of the antioxidant activity of melanoidins is due to low molecular weight compounds linked non-covalently to the melanoidin skeleton. A new concept of the overall antioxidant properties of food melanoidins is described, where chelating ability toward low molecular weight antioxidant compounds is connected to the stabilization of these compounds involved in the shelf life of the product.

KEYWORDS: Coffee brew; roasting degree; melanoidin; lipid oxidation; AAPH

INTRODUCTION

Thermal processes utilized in the food industry (extrusion, baking, canning, pasteurizing, roasting, etc.) often add value to raw materials and are applied to produce color, texture, and flavor and to sterilize the materials, providing longer shelf life and enhancing product safety (*1*). Processed foods are widely consumed in the human diet, and one of the most common reactions related to heat treatment of foods is the Maillard reaction (MR), which involves the condensation of the carbonyl group of reducing sugars with the amino group of amino acids and proteins. The generation of a series of products (Schiff bases, premelanoidins, melanoidins, etc.) known as Maillard reaction products (MRP) takes place, and their characteristics and molecular weights depend both on the source of reactants and on the reaction conditions (*2*).

Melanoidins have been studied in recent years due to their nutritional, biological, and health implications; they have become a subject of growing interest for the scientific community, as the creation of a specific Co-operation in Science and Technology (COST) action titled "Melanoidins in Food and Health" shows (*3*). Their antioxidant activity results are especially interesting because these products are naturally formed during food processing and storage (*4*).

One of the major sources of daily melanoidin intake is coffee beverages, in which they play an important role in the development of aromas during the roasting process (*5*). The antioxidant properties of coffee have frequently been attributed to certain phenolic compounds present in green coffee, such as chlorogenic acid (CGA), caffeic acid, ferulic acid, or *p*-coumaric acid, all of them with a still unclear contribution to the peroxyl radical scavenging activity (*6*). The roasting process affects the final composition of coffee (*7*); thus, levels of phenolic acids vary depending on the treatment of beans. Whereas compounds with antioxidant properties (i.e., CGA) are lost to some extent during thermal processing, the development of other antioxidant compounds, such as MRP (*8*−*11*), maintains or even improves the antioxidant properties of coffee brew (*12*). Some authors have confirmed that this effect is due to the ability of a donated hydrogen to break the radical chain (*13*), its effectiveness as a metal-chelating agent, and its capacity to reduce hydroperoxide to nonradical products (*14*). According to these researchers the oxygen scavenging activity was higher for the intermediate MRP formed throughout the reaction and for those produced in alkaline conditions (*9*).

The aim of the present work is to evaluate the antiperoxyl radical scavenging properties of melanoidins in coffee brews of different roasting degrees by applying a 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation of linoleic acid (LH) in aqueous dispersion. Correlation between this antioxidant activity and the CGA content in coffees was also studied. These findings will help to determine the real

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contribution of melanoidins to the overall antioxidant effect of coffee brew and subsequently their possible health implications.

MATERIALS AND METHODS

Chemicals. Chlorogenic acid (3-caffeoylquinic acid, CGA), ferulic acid, vanillic acid, caffeic acid, benzoic acid, Tween 20 (polyoxyethylenesorbitan monolaurate), and linoleic acid (99%) were from Sigma. NaCl, NaOH, and glacial acetic acid were purchased from Panreac. Acetonitrile was from Lab-Scan. AAPH and benzyl alcohol were from Aldrich.

Samples. Instant coffees produced from roasted coffee beans were supplied by the Nestlé Research Centre (Lausanne, Switzerland) in three different roasting degrees (roasting color, roasting loss): light (CTn 110, 14.5%), medium (CTn 85, 16.2%), and dark (CTn 60, 18.9%).

Preparation of Coffee Brews. One gram of the different instant coffees was resuspended in 100 mL of hot water (50−60 °C). The aqueous solutions obtained were then filtered (Whatman filter paper no. 40, ashless, Whatman, Maidstone, U.K.) and stored at 4 °C until analyses were shortly performed (CTn 110, CTn 85, and CTn 60 samples, respectively).

Preparation of Melanoidins Extract and Pure Melanoidins Extract from Coffee Brews. An aliquot of each above-described sample was subjected to ultrafiltration, using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA), equipped with a 10000 Da nominal molecular mass cutoff membrane. The retentate was filled up to 200 mL with water and washed again. This washing procedure (diafiltration) was repeated at least three times. The high molecular weight fraction corresponding to melanoidins was freeze-dried and stored in a desiccator until analysis. Melanoidins (M) isolated from these systems were identified as M110, M85, and M60, respectively.

Obtention of pure melanoidins (PM) was performed by preparing solutions containing 5 mg (to obtain a representative amount of product) of different melanoidins per milliliter in 2 M NaCl. NaCl was used to release low molecular weight (LMW) compounds ionically attached to the melanoidin skeleton, such as CGA. After overnight incubation, solutions were again ultrafiltered (Microcon YM-10, regenerated cellulose 10000 Da, Bedford, MA) at 14000*g* for 50 min. Retentates, containing PM, were resuspended in water and then freeze-dried and stored in a desiccator at 4 °C until analysis. PM obtained were named PM110, PM85, and PM60, respectively.

Analytical Techniques. *High-Performance Liquid Chromatography (HPLC) Analysis.* CGA extracted from the melanoidin was quantified by reversed phase HPLC. Degassed elution phases were prepared: (A) glacial acetic acid−Milli-Q water (5%, v/v) and (B) acetonitrile. A Spherisorb ODS2 analytical column (25 × 0.40 cm, 5 μm particle size, Analytical Tracer, Barcelona, Spain) was used at 32 °C. The injection volume was 10 μL, and detection at 340 nm (0.1 AUFS sensitivity and 0.5 s response time) was selected. An external standard method was used for quantification within the range of 0.1−0.005 mg·mL^{-1} NaCl and 2 M CGA.

Capillary Zone Electrophoresis (CZE) Analysis. Electromigration was carried out with an HP^{3D} system equipped with a built-in diode array detector and an HP ChemStation for system control, data collection, and data analysis from Hewlett-Packard (Madrid, Spain). CZE was performed on an uncoated fused silica capillary with a 48.5 cm total length (40 cm effective length), a 50 μm internal diameter, and an extended light path (bubble factor × 3) supplied by Hewlett-Packard. The capillary was conditioned by washing with 1 M NaOH for 20 min, a 5 min wait, washing with water for 5 min, and then washing with buffer for 20 min. CTn 110, CTn 85, and CTn 60 samples were hydrodynamically injected by applying 50 mbar of pressure for 2 s at the anionic end of the capillary. After every run, the capillary was high-flushed (7 bar) with 0.1 M NaOH (0.2 min) and 50 mM sodium tetraborate, pH 9.3, for 0.4 min. Electrode buffers were renewed every five runs with 0.5 mL per pot. The temperature of the cartridge was maintained at 25 °C, and total separation time was set at 15 min. A standard electrophoretic run was performed at a constant voltage of 25 kV with the anode at the inlet side. Electropherograms were monitored at 200 and 340 nm wavelengths, with raw spectral data collection between 190 and 700 nm. Benzyl alcohol (3.2 min) was used as a marker of the electroosmotic flow (EOF) and a benzoic acid solution (1 mg/mL, 9.8 min) as internal standard.

Antioxidant Assay. (1) Substrate. An aqueous solution of linoleic acid (LH, 16 mM) was prepared as was described in Morales and Jiménez-Pérez (*15*). The dispersion was checked for autoxidation before use. AAPH decomposes slightly, and its products increase the absorbance at 234 nm. A net increase of 0.4020 absorbance unit 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 toward AAPH was not detected. It was applied to the data validation steps described by Morales and Jimenez-Perez (*15*).

(2) AAPH Solution. AAPH (40 mM) 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.

(3) Antioxidant Activity. The procedure of Morales and Jimenez-Perez (*15*) was applied. Sample (CTn, M, PM) 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 equipped with a thermostated automatic sample positioner was used. Measurements were recorded every 60 s. Antioxidant efficiency (AE_{50}, mL·min·mg^{-1}) was calculated as the slope of the time of inhibition at 50% (T_{inh}; min) against concentration (mg·mL^{-1}).

Statistical Treatment. All of the analyses were performed at least in triplicate. The Statgraphics v 5.1 statistical procedures were performed at a significance level of 95%.

RESULTS AND DISCUSSION

The method used for evaluating the peroxyl radical scavenging properties of melanoidins is based on the rate of oxidation of linoleic acid (LH) to its conjugated diene hydroperoxide (LOOH) in aqueous media in the presence or absence of antioxidant activity compounds. This approach has been previously applied for MRP (*15*). The increase in the inhibition phase was proportional to the coffee, melanoidin, or pure melanoidin concentration, which denotes a process of oxidation inhibition. In contrast, no pro-oxidant effect was observed for coffee brews, melanoidins, and pure melanoidins. Once the inhibition time is over, the oxidation proceeds at the same rate as in the absence of inhibitor. Although these products themselves absorb at 234 nm, which could be a drawback because this could reduce the range of analysis, concentrations employed did not show significant interferences.

AE_{50} was calculated for each sample (CTn, M, PM) in an appropriate concentration range. The upper limit of the range was related to the possible interference of the color of the sample with the analytical procedure. Analyses were performed in a defined and realistic concentration range to avoid bias in the interpretation of results, because it has been described that MRP could exert antioxidative or pro-oxidative activities as well (*16*, *17*). CGA was used as reference because it is one of the most active phenolic compound involved in the overall antioxidant activity of coffee (*6*). **Figure 1** shows a classical representation of conjugated diene formation from AAPH-induced oxidation of LH alone and in the presence of CGA. It also illustrates the significance of the inhibition time (T_{inh}), corresponding to the point at which 50% of inhibition take place. **Figure 2** shows the effect of CGA concentration on AAPH-induced linoleic acid oxidation, expressed as inhibition time (T_{inh}) versus CGA concentration. Several concentrations (0.1−0.00094 mg·mL^{-1}) were assayed, and the corresponding T_{inh} values were calculated. An AE_{50} value of 1125.55 ± 32.74 min·mL·mg^{-1} was estimated.

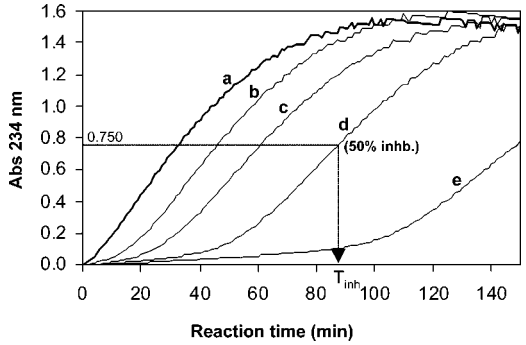

Figure 1. Classical time course of conjugated diene formation from control (**a**, LH in absense of CGA) and 0.0094 (**b**), 0.0188 (**c**), 0.0375 (**d**), and 0.0750 mg·mL^{-1} (**e**) of CGA. T_{inh} (0.0375 mg·mL^{-1}) is 41.5 min. Residual absorbance from the metabolites of AAPH decomposition is already subtracted.

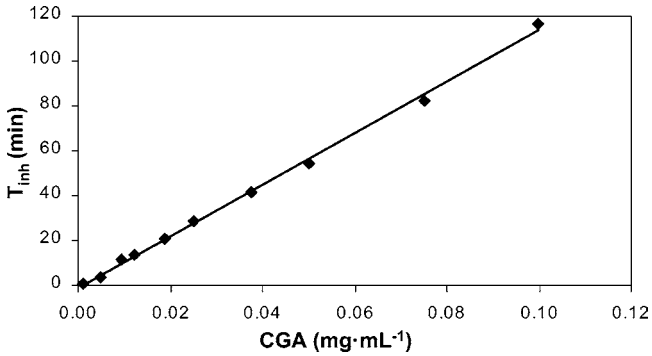

Figure 2. Effect of CGA concentration on AAPH-induced linoleic acid oxidation, expressed as inhibition time (T_{inh}) versus CGA concentration (0.1–0.00094 mg·mL^{-1}) ($y = 1145.6x + 0.8612$; $n = 10$; $r^2 = 0.9982$).

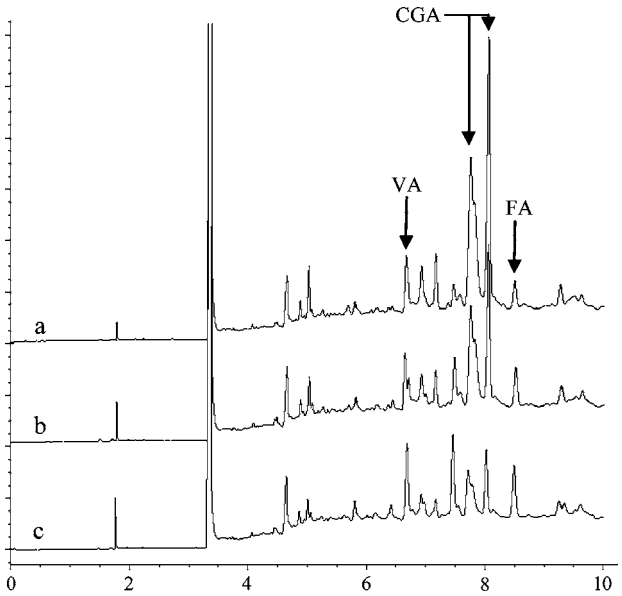

Figure 3. E-grams (200 nm) of aqueous solutions of instant coffee (1% w/v) from light (**a**), medium (**b**), and dark (**c**) roasting degrees. CGA, chlorogenic acid isomers; FA, ferulic acid; VA, vanillic acid.

Figure 3 shows the e-grams obtained for coffee brews at 200 nm. Several phenolic compounds can be recorded, such as CGA, vanillic acid, and ferrulic acid, in a short time of analysis. CGA isomer peaks decreased significantly according to the degree of roasting. The main peak of CGA, 3-caffeoylquinic acid (standard reference), migrated at 7.8 min. Average values of CGA present in coffee brews were 0.023, 0.053, and 0.097 mg·mL^{-1} for CTn 60, CTn 85, and CTn 110, respectively.

To make data comparisons, a reference concentration of 1 mg·mL^{-1} was used for the different melanoidins, pure melanoidins, and coffee brews. CZE measured levels of CGA for each coffee brew were replaced in the calibration curve of CGA peroxyl radical scavenging activity (standard) to obtain the theoretical values for time of inhibition, named CGA equivalents. This is the estimation of the contribution of CGA to the total antioxidant activity related to coffee brew. In the same way, the exact value of inhibition time for melanoidins contained in coffees (1 mg·mL^{-1}) was calculated using the calibration curve of each melanoidin isolated and by taking into account the percentage of melanoidins present in each coffee sample. Variable amounts of melanoidins were obtained according to the degree of roasting, 16.4, 18.4, and 19.5% for CTn 110, CTn 85, and CTn 60 coffee brews, respectively. A higher proportion of melanoidin was obtained at severe roasting conditions. The contribution of CGA and melanoidins to the total antioxidant activity of coffee brew was calculated (**Table 1**). Data of total inhibition time measured in different coffee brews showed no significant differences. Thus, no variations were found in their antioxidant activities. The peroxyl radical scavenging activities of the melanoidins fraction contained in them did not vary either, although statistical analysis was not possible in these samples due to the existence of a unique percentage of melanoidins present in each coffee.

Analysis demonstrated that the contribution of CGA to the antioxidant properties of different coffees was more important as roasting level decreased, which was supported by the statistical increment of T_{inh} of CGA from CTn 60 to CTn 110. In this sense, Steinhart et al. (*18*) compared the antioxidant activity of coffee and chlorogenic acid-free coffee, showing that stronger roasting conditions result in higher losses of CGA. However, these researchers affirmed that the loss of natural antioxidants was not accompanied by a decrease in the total antioxidative capacity, which is in accordance with the absence of statistical difference found between the total antioxidant activities of studied coffees. As has been mentioned by other authors (*7*), although compounds with antioxidant properties (i.e., CGA) can be lost during the roasting process, the overall peroxyl radical scavenging activity can be maintained or even enhanced by the formation of new products possessing this same characteristic, mainly Maillard reaction products (*8*−*10*). Other studies have manifested a higher antioxidant activity in water for light and medium roasted coffees than that shown by the green or the dark coffee, using the ABTS$^{\bullet+}$ method (*12*). The percentage of unknown compounds accounting for the total antioxidant activity of the coffee brews is higher as the roasting degree increases, being 40.2, 58.1, and 63.1% for CTn 110, CTn 85, and CT n60, respectively. This observation is in line with results from Anese et al. (*16*), who demonstrated that LMW compounds of the Maillard reaction are responsible for the balance of antioxidant activity of highly roasted coffees. Development of browning is associated with an increase in the antioxidant properties in systems where Maillard reaction is the prevalent reaction (*19*, *20*). In coffee, it is not clear because phenolic compounds play an important role. Although we have evaluated the antioxidant action from only the melanoidin fraction of coffee (**Table 1**), earlier products of the reaction might be contributing to the final activity of coffee brews and could even become the prevailing contributors (*9*).

Otherwise, the specific purpose of the present work was to assess the implication of CGA in the total antioxidant activity

Table 1. Contribution of CGA and Melanoidins to the Peroxyl Radical Scavenging Activity of Coffee Brews (1 mg·mL^{-1}) Expressed as Time of Inhibition

	CTn 60		CTn 85		CTn 110	
coffee component	T_{inh}[a] (min)	CV[b] (%)	T_{inh}[a] (min)	CV[b] (%)	T_{inh}[a] (min)	CV[b] (%)
total	172.26 ± 16.02a	8.30	208.05 ± 10.08a	4.20	217.99 ± 8.70a	4.01
CGA	25.29 ± 0.15a	0.59	57.72 ± 0.62b	1.07	105.79 ± 0.73c	0.69
melanoidin	38.37c		29.54c		26.85c	

[a] Time of inhibition of the conjugated diene formation. [b] Interassay coefficient of variation. [c] Data obtained from the percentage of melanoidins present in each coffee (1 mg·mL^{-1}) and the lineal regression of peroxyl radical scavenging activity of each melanoidins. Different letters in the same row indicate significant differences between different coffee brews (one-way ANOVA and Duncan's test, $P < 0.05$).

Table 2. Contribution of Different Components of Coffee Melanoidins (1 mg·mL^{-1}) to Total Peroxyl Radical Scavenging Activity of Melanoidins Expressed as Time of Inhibition

	M60		M85		M110	
melanoidin component	T_{inh}[a] (min)	CV[b] (%)	T_{inh}[a] (min)	CV[b] (%)	T_{inh}[a] (min)	CV[b] (%)
total	197.18 ± 9.26a	4.70	157.76 ± 10.72a	6.81	170.12 ± 19.05a	11.20
bound CGA	4.13 ± 0.08a	1.94	12.24 ± 0.01b	0.08	22.70 ± 0.01c	0.04
pure melanoidin	44.94 ± 1.25a	2.80	52.00 ± 11.38a	21.91	55.88 ± 8.49a	15.20

[a] Time of inhibition of the conjugated diene formation. [b] Interassay coefficient of variation. All data are referred to coffee brews prepared at 1 mg·mL^{-1}. Different letters in the same row indicate significant differences between different melanoidins (one-way ANOVA and Duncan's test, $P < 0.05$).

of melanoidin extracted from coffee brews. The melanoidin fraction was treated with NaCl to remove non-covalently bound LMW compounds attached to the skeleton. This approach was previously outlined by Fogliano et al. (*21*), who treated melanoidins with 1 M NaCl before gel filtration separation to evaluate their ionic affinities. It is well-known that melanoidins have the ability to bind micronutrients (i.e. refs *2* and *22*) or aroma compounds (i.e., refs *23* and *24*). The LMW fraction (<10 kDa) from NaCl-treated melanoidins was analyzed by HPLC, CGA being identified as the main peak. Data of CGA extracted from different melanoidins were as follows: 3.15 μg/mL for M60, 10.60 μg/mL for M85, and 20.20 μg/mL for M110. CGA was linked in a non-covalent way to the melanoidin core and released after high ionic strength treatment. These concentrations of CGA bound to melanoidin were also replaced in the calibration curve of CGA peroxyl radical scavenging activity (standard) to obtain the theoretical values for time of inhibition. **Table 2** shows the contribution of CGA non-covalently bound to the melanoidin to its total peroxyl radical scavenging activity.

Then, the antioxidant properties of melanoidins from the roasted coffee brews cannot be attributed exclusively to the pure melanoidin itself or to the CGA linked in a non-covalent way to the melanoidin core. Once again, for the highest intensity heating procedure the lowest CGA contribution to total antioxidant activity of M was found. Pure melanoidins contributed at the same level in the three coffee brews, and the values were significantly different from those obtained for the corresponding melanoidin (**Figure 4**). Likewise, it seems obvious that there must be other compounds linked by ionic bonds to the melanoidin structure contributing to the total peroxyl radical scavenging activity. In this respect, some investigators have suggested the ability of some residual sugars still without to participate in the Maillard reaction, which could be linked to the melanoidin core (*25*). Accepting this hypothesis, in the same way LMW Maillard reaction products could also have been chelated and contributed to the total effect, because LMW products have been attributed higher antioxidant capacity than high molecular weight products (*12*). The production of enediol structure reductones throughout the Maillard reaction has been related with the slowing of the oxidation rate of fats in sugar cookies (*26*). Some other authors state that the production of

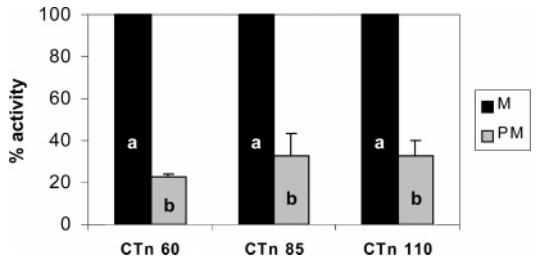

Figure 4. Contribution of pure melanoidin to the total peroxyl radical scavenging activity of melanoidin. Different letters within each coffee indicate significant differences melanoidin–pure melanoidin (one-way ANOVA and Duncan's test, $P < 0.05$).

pyrolysis compounds that takes place successively to the formation of Maillard reaction products when more severe thermal conditions are applied could also explain the maintenance of the antioxidant activity (*27*).

In summary, no significant differences were found among the different roasting degrees for melanoidins and pure melanoidins concerning the antiperoxyl radical activity. The peroxyl radical scavenging activity of melanoidins from coffee brew is mainly due to the chelated compounds of the high molecular weight fraction. Probably, these kinds of compounds have been generated throughout the roasting process, and they are related with the development and progress of the Maillard reaction. These results are in line with Anese et al. (*16*), who stated that heat-induced antioxidants would balance the thermal loss of naturally occurring phenolic compounds. However, the redox potential values indicate that the antioxidant efficiencies of MRP are higher than those of the natural ones. This observation also agrees with results from Del Castillo et al. (*12*), who demonstrated that the LMW fraction of coffee brews possesses higher antioxidant activity than the high molecular weight fraction. On the other hand, it has been described previously that melanoidins have a direct impact on the reduction of odor compounds in coffee (*28*) and sweet wine (*24*). It could be plausible that melanoidin exists in a dynamic equilibrium in the food matrix, where LMW compounds are non-covalently linked to the core structure (named, pure melanoidin). It is concluded for the first time that food melanoidins play a role

in the stabilization of compounds involved in the shelf life of foods, such as CGA. In this model, CGA could be released from the melanoidin core according to ionic changes in the food matrix. A new research line is open where previous results on the functional properties of melanoidins have to elucidate the activity by the melanoidin itself and the accompanying LMW compounds. Many of the functional activities attributed to the melanoidins could be ascribed to non-covalently bound compounds. Further studies are underway to understand the mechanisms of binding and the impact on food stability.

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