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Influence of Roasting on the Antioxidant Activity and HMF Formation of a Cocoa Bean Model Systems

TERESA OLIVIERO,[†] EDOARDO CAPUANO,[†] BETTINA CÄMMERER,[§] AND VINCENZO FOGLIANO*,[†]

Department of Food Science, University of Napoli "Federico II", Napoli, Italy, and Institut fur Lebensmitteltechnologie and Lebensmittelchemie, Technische Universität Berlin, Berlin, Germany

During the roasting of cocoa beans chemical reactions lead to the formation of Maillard reaction (MR) products and to the degradation of catechin-containing compounds, which are very abundant in these seeds. To study the modifications occurring during thermal treatment of fat and antioxidant rich foods, such as cocoa, a dry model system was set up and roasted at 180 °C for different times. The role played in the formation of MR products and in the antioxidant activity of the system by proteins, catechin, and cocoa butter was investigated by varying the model system formulation. Results showed that the antioxidant activity decreased during roasting, paralleling catechin concentration, thus suggesting that this compound is mainly responsible for the antioxidant activity of roasted cocoa beans. Model system browning was significantly higher in the presence of catechin, which contributed to the formation of water-insoluble melanoidins, which are mainly responsible for browning. HMF concentration was higher in casein-containing systems, and its formation was strongly inhibited in the presence of catechin. No effects related to the degree of lipid oxidation could be observed. Data from model systems obtained by replacing fat with water showed a much lower rate of MR development and catechin degradation but the same inhibitory effect of catechin on HMF formation.

KEYWORDS: Antioxidant activity; cocoa; model system; Maillard reaction

INTRODUCTION

Thermal treatment of solid foods having low water content rapidly leads to color and aroma development as well as to degradation of bioactive compounds present in the raw materials (*1*). In this respect, coffee roasting represents a deeply investigated process, but also cocoa roasting is of great interest, and the consequences of the presence of about 50% of fat on the chemical modifications during the process are still poorly investigated.

Beside fats, cocoa is also enormously rich in polyphenols, which are stored in the pigmented cells of the cotyledons. These polyphenolic substances have gained much attention due to their antioxidant activity (reducing agents, free radical scavengers, metal chelators), but their beneficial implications in human health are related not to just the antioxidant activity (*2*). Three groups of polyphenols can be distinguished in cocoa: anthocyanins (about 4%), catechins or flavan-3-ols (about 37%), and proanthocyanidins (about 58%). The main catechin is (−)-epicatechin, accounting for up to 35% of the polyphenol content (*3*).

* Address correspondence to this author at the Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli "Federico II", Via Università 100, Parco Gussone, Portici, Italy (telephone +39 81 2539356; fax +39 81 7754942; e-mail fogliano@unina.it).

[†] University of Napoli "Federico II".

[§] Technische Universität Berlin.

Roasting is the most important technological operation of cocoa bean processing. During heat treatment, nonenzymatic browning is developed through the Maillard reaction (MR), accompanied by the formation of a variety of MR products (MRPs). This reaction involves not only reducing sugars and amino acids but also carbonyl compounds resulting from lipid oxidation. Together with oxidation, condensation, and complexation of polyphenol compounds and following protein and starch hydrolysis, MR is responsible for the formation of the characteristic brown color, pleasant aroma, and texture of roasted beans. It was established that MR is responsible for the decrease of reducing sugar and amino acid concentrations observed during roasting of cocoa beans (*4*) as well as for the formation of alkylpyrazines (*5*).

Phenolic compounds with antioxidant properties are rapidly lost during the roasting of cocoa beans; however, the antioxidant capacity of cocoa is partially restored by the development of compounds possessing antioxidant activity. These compounds are mainly MRPs and, in particular, the melanoidins, the final product of the MR (*6*, *7*). It has been shown that this nonenzymatic browning reaction produces strongly reducing substances, which are responsible for their free radical scavenging activity (*8*). To monitor MR development, 5-(hydroxymethyl)-2-furaldehyde (HMF) has been widely used as a marker compound both in foods and in model systems (*9*). HMF is an intermediate product of MR (*10*, *11*), but it is also formed as a

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Table 1. Cocoa Model Systems and Native Cocoa Beans Composition

complete model system	%	one-component-missing model system (i.e., catechin-missing model system)	%	butter-free model system	%	fermented and dried cocoa seed composition	%
H_2O	11	H_2O	11	H_2O	51	H_2O	5
cocoa butter	50	cocoa butter	50	(+)-catechin hydrate	6	fat	54
(+)-catechin hydrate	6	SiO_2	6	SiO_2	10	polyhydroxyphenols	6
starch	10	starch	10	starch	10	starch	6
cellulose	10	cellulose	10	cellulose	10	cellulose	9
soy proteins	12	soy proteins	12	soy proteins	12	protein	11.5
sucrose	1	sucrose	1	sucrose	1	pentosans	1.5
						carboxylic acids	1.5
						ash	2.6
						other compounds	0.5

degradation product of sugars at high temperatures (*12*). Also, color formation was used to monitor the development of MR, but the methods based on color measurement should be tailored for each food item (*13*).

Most of the MR studies have been performed in aqueous solutions of single sugar−amino acid combinations (*14*−*16*). The development of MR in these simple model systems is often quite far from that occurring in foods as they are not aqueous solutions; therefore, the availability of models closer to real food is an important challenge in food science. Hofmann et al. (*17*) used "in bean" model roast experiments, prepared as coffee bean reconstitutes to investigate the influence of coffee roasting on the thiol binding activity of coffee beverages. Fallico et al. (*18*) investigated the roles of oil, hexanal, and sucrose in color development and HMF formation during roasting of hazelnuts by using a model system formed by defatted or partially defatted crushed hazelnuts.

The aim of this study was to investigate the effect of roasting on the antioxidant properties of a dry model system rich in antioxidants and fat, as well as to determine the influence of the different components on their antioxidant activity by means of solid cocoa bean model systems. The model system was composed of water, cocoa butter, starch, cellulose, soy proteins, catechin, and sucrose. Two different types of proteins as well as three different catechin concentrations were used. The antioxidant activity was evaluated by ABTS assay and by electron spin resonance (ESR). Furthermore, the effect of the formulation on HMF formation and browning development during the roasting of the cocoa bean model system was investigated.

MATERIALS AND METHODS

Chemicals and Reagents. All of the chemicals and reagents were obtained from Merck (Darmstadt, Germany), Carlo Erba (Milano, Italy), and Fluka AG (Buchs, Switzerland).

Preparation of Cocoa Bean Model System. To prepare the cocoa bean model systems the following reagents were used: (+)-catechin hydrate from Sigma (St. Louis, MO); casein hydrolysate, sucrose, and cellulose from Fluka; soybean proteins from Chimpex (Caivano, Italy); and cocoa butter from Mennella SpA (Torre del Greco, Italy). The composition of the model systems is reported in **Table 1**. Roasting experiments were carried out in an oven (Memmert, Schwabach, Germany) at 180 °C for different times.

Determination of Antioxidant Activity by ABTS Assay. To evaluate the water-soluble antioxidant activity (AA), the ABTS method described by Re et al. (*19*) was used. A 200 mg portion of the powdered model system sample was suspended in 10 mL of water, and the mixture was vortexed for 2 min and centrifuged at 4000 rpm for 5 min at 4 °C. The supernatant was separated and filtered (cutoff = 0.45 μm) to eliminate the cocoa butter from the surface. A 100 mL portion of the clear supernatant sample was transferred in a centrifuge tube, and the reaction was started by adding 1.7 mL of ABTS reagent. The mixture was vortexed for 10 s, and after 2.5 min (including 10 s of vortexing), the absorbance was measured at 734 nm using a Shimadzu model 2100 variable-wavelength UV−vis spectrophotometer.

The antioxidant activity was expressed as millimoles of Trolox equivalent antioxidant capacity (TEAC) per kilogram of sample by means of a Trolox dose−response curve. The water-extracted samples were diluted prior to measurement if measured absorbance values were outside the linear response range of the radical discoloration solution. Dilution was performed with distilled water.

Determination of Antioxidant Action by Electron Spin Resonance. One gram of powdered sample was suspended in 10 mL of water, and the mixture was vortexed for 2 min and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was separated and filtered (cutoff = 0.45 μm) to eliminate cocoa butter from the surface. The ESR spectroscopic measurements with stabilized radicals were performed with Fremy's salt using a previously described procedure (*20*).

The reaction time was 30 min and the water solutions were diluted with water. Spectra were measured on a Miniscope MS 100 spectrometer (Magnetech, Berlin, Germany). Microwave power and modulation amplitude were set at 10 dB and 1500 mG, respectively, center field = 3397 G.

(+)-Catechin Hydrate Determination. The ground sample was defatted using 2 mL of hexane, and the mixture was vortexed for 2 min and centrifuged at 4000 rpm for 10 min at 4 °C. The hexane was removed, and 2 mL of a mixture of water and methanol (70:30) and 5 μL of BHT were added to the pellet. The mixture was vortexed for 1 min and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was collected in a 10 mL volumetric flask, and one further extraction was performed using 2 mL of a mixture of water and methanol (70:30) and 5 μL of BHT. The extracts were analyzed by HPLC (Shimadzu, Tokyo, Japan). The HPLC system consisted of LC-10AD class VP pumps, an SPD-M10A diode array detector, and an SCL-10A class VP controller. HPLC phases were acetonitrile/methanol (60:40) (A) and water (0.2% formic acid) (B). The following gradient elution was applied: 0−6 min, 20% A; 6−16 min, 40% B; 16−24 min, 50% A; 24−32 min, 90% A; 32−35 min, 90% A; 35−38 min, 20% A. The flow rate was 1 mL min^{-1}. A Prodigy 5 μm ODS3 100A, 25 × 4.6 cm (Phenomenex), column was used. The UV detector was set at 280 nm, and the catechin was quantified using the external standard method. The linearity of the detection was within the range of 3.75−750 mg L^{-1}.

HMF Determination. HMF determination was based on the method of Garcia-Villanova et al. (*9*) with slight modifications. The ground sample was suspended in 5 mL of deionized water in a 10 mL centrifuge tube. The tube was vortexed vigorously for 1 min and clarified with 0.25 mL each of potassium ferrocyanide (15% w/v) and zinc acetate (30% w/v) solutions. The resulting mixture was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was collected in a 10 mL volumetric flask, and two further extractions were performed using 2 mL of deionized water. The extracts were filtered (0.45 μm), and 50 μL was analyzed by HPLC using the same apparatus as described above.

The mobile phase was a mixture of acetonitrile in water (5% v/v) at a flow rate of 1 mL min^{-1} under isocratic conditions and a Synergy

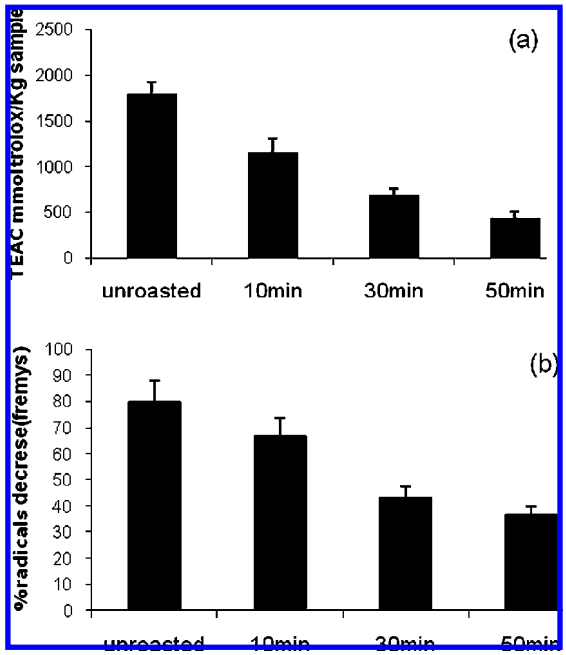

Figure 1. Antioxidant activity of complete cocoa bean model system (**Table 1**) roasted at 180 °C: (**a**) measured by the ABTS assay; (**b**) measured by ESR.

4 μ Hydro-RP 80A, 25 × 4.6 cm (Phenomenex), column. The UV detector was set at 280 nm, and HMF was quantified using the external standard method. The linearity of the detection was in the range of 0.025−75 mg L^{-1}. The limit of detection of the method was 0.010 mg L^{-1}, whereas the limit of quantification was 0.025 mg L^{-1}.

Color Determination. Color parameters *L**, *a**, and *b** were measured using a colorimeter (Minolta, Italy) on a layer of 0.5 cm of powdered sample. Duplicate analyses were performed on the ground samples.

Oxidation Product Determination. Cocoa butter was thermoxidized by heating 15 mL of butter in an uncovered beaker under stirring for 24 h at 180 °C.

Peroxide Number. Peroxide number was determined by using conventional iodometric titration with thiosulfate (*21*). The sample was suspended in 25 mL of a mixture acetic acid/chloroform (3:2) and 0.5 mL of saturated KI. The resulting mixture was kept in the dark for 5 min, and then 70 mL of distilled water and 1 mL of starch paste were added. The mixture was titered with 0.01 N sodium thiosulfate. The peroxide number was determined according to the formula *N*(thiosulfate) × mL of thiosulfate used × 1000 g of sample and expressed as milliequivalents of oxygen per kilogram of sample.

TBARS. Lipid peroxidation was measured directly in the medium by the thiobarbituric acid-reactive substances (TBARS) assay. Malondialdehyde (MDA) was used for the preparation of a standard curve. The absorbance was detected at 532 nm using a Shimadzu model 2100 variable-wavelength UV−vis spectrophotometer.

TBARS number was expressed as milligrams of MDA per milligram of fat (*22*).

Statistical Analysis. All data were subjected to analysis of variance (ANOVA). The general linear model SPSS statistical package was used for the evaluation of statistical significance of the differences between mean values by Tukey's test and for the calculation of Pearson correlation coefficients between different variables.

RESULTS AND DISCUSSION

The modification occurring in the fat- and antioxidant-rich model system during roasting was investigated after treatment at 180 °C for 10, 30, and 50 min. The antioxidant activity was measured by using two methods: the ABTS assay and ESR, both measuring the free radical scavenging ability.

Results summarized in **Figure 1** showed the trend of AA in this system during roasting. A strong decrease of AA was recorded: interestingly, a more rapid decrease was detected when AA was measured by ABTS than by ESR. Catechins are rapidly degraded upon roasting (see **Figure 4**), thus explaining the fast decrease of AA measured by ABTS assay. On the other hand, MRPs and particularly melanoidins having high antioxidant activity are produced during roasting. It is likely that these compounds have a good radical scavenger ability toward Fremy's salt radical. Therefore, this result can be explained by the different radical species used by the two methods and by the different antioxidant compounds present in the systems. It is well-known that the polyphenols have a good radical scavenger ability toward $ABTS^{+}$ (*23*). Serpen et al. (*23*) showed that the ABTS radical cation is more sensitive to phenolic-containing compounds than to MRPs. This is because polyphenols are much more efficient than MRPs in donating the hydrogen atom to quench the colored radical cation of ABTS, thanks to the ability of polyhydroxylated benzene rings to stabilize the forms without one hydrogen atom. On the other hand, a significant part of MRPs' AA could be ascribed to the activity of nondonating hydrogen atoms, such as reductones.

The decrease of AA upon roasting was already described by Arlorio et al. (*24*), who measured the AA of methanol extracts from roasted cocoa beans from different geographic origins. It is worth noting that a similar decrease of AA during roasting was already reported by Gentile et al. (*25*) during roasting of pistachio beans; however, considering chemical composition and roasting conditions, data on cocoa beans should be above all compared with those obtained of coffee beans. A marked increase of AA upon roasting, particularly in the medium-roasted coffee, has been reported (*20, 26*). In fact, roasting determines not only chlorogenic acid degradation but also a contemporary massive formation of MRPs, particularly melanoidins (*27, 28*). In the case of coffee the AA due to the formation of MRPs overcomes the loss of natural antioxidant also because coffee melanoidins represent 25−30% of coffee brew dry weight (*29*).

In the following set of experiments the complete cocoa model system was compared to model systems obtained replacing each time one component with an inert material (SiO_2) as illustrated in **Table 1**. All of these one-missing-component systems were roasted at 180 °C for 30 min, and the AA was measured by ESR and ABTS. Usually cocoa seeds are roasted at 150 °C for 15/20 min (*3*). However, as this heat treatment was insufficient to achieve an adequate browning of model systems, a roasting temperature of 180 °C was selected.

Results obtained with the one-missing-component systems by ESR measurements are shown in **Figure 2**. The catechin-missing model system showed the highest drop of AA when compared to the complete model system, thus confirming that phenol compounds are mainly responsible for the antioxidant activity of roasted cocoa beans. On the other hand, it is worth noting that also carbohydrates gave a significant contribution; in fact, in the starch-missing system a decrease of 50% of AA was found. Also, in the sucrose-missing model system a decrease in AA can be observed, although it was lower than that found in the starch-missing system. This difference could be explained considering that the amount of sucrose used in the formulation of our model systems is much lower than the amount of starch. All in all, these findings confirmed that the formation of MRPs could be responsible for a significant moiety of the overall AA of roasted cocoa beans. Arlorio et al. (*24*) reported no direct correlation ($r^2 = 0.75$) between total phenolic content and the AA of roasted cocoa beans, highlighting the importance of

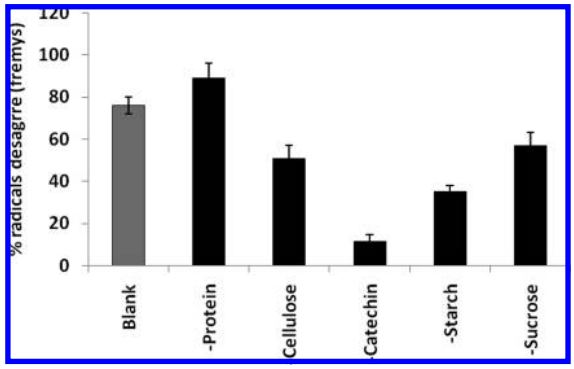

Figure 2. Antioxidant activity of one-component-missing model systems measured by ESR: Blank, complete model system; -protein, protein-missing model system; -cellulose, cellulose-missing model system; -starch, starch-missing model system; -catechin, catechin-missing model system; -sucrose, sucrose-missing model system.

Table 2. Browning Development of Cocoa Bean Model Systems after Roasting Measured by CIE *L* a* b** Color Scale

		hydrolyzed casein samples			soy protein samples		
model system	(+)-catechin content (%)	*L**	*a**	*b**	*L**	*a**	*b**
with cocoa butter	0	53.3	23.7	27.3	72.0	7.3	27.3
	3	32.7	21.3	13.7	42.0	17.3	14.7
	6	28.3	19.0	26.0	38.1	18.7	16.7
without cocoa butter	0	67.7	17.0	33.0	89.0	1.0	16.0
	6	61.7	19.7	42.7	72.7	14.7	26.0

MRPs for their total AA. These authors pointed out that also the clovamide content (clovamide is a powerful antioxidant compound present in cocoa beans) is not directly correlated with the overall AA. Summa et al. (*30*), performing a water extraction of roasted cocoa beans, demonstrated that the high molecular weight (HMW) fractions (5−10 and 10−30 kDa) are mainly responsible for total radical scavenging activity, thus confirming the role of MR in the roasted cocoa antioxidant activity.

To investigate the effect on the AA of the type of protein and catechin concentration, a set of samples including hydrolyzed casein were prepared and compared with those including soybean proteins. For each of these model samples, three different catechin concentrations were tested: 0, 3, and 6%. Browning of the systems was measured using the CIE *L* a* b** color scale, and data are reported in **Table 2**. Data demonstrated that color development increased as catechin concentration increased, and it was higher in casein-containing samples with respect to soybean-containing samples. The absorbance measurements at 360 and 420 nm of the water extracts of the samples indicated no significant differences among them, thus suggesting that catechins are involved in the formation of insoluble HMW compounds that, in turn, are mainly responsible for system browning. It has been suggested that oxidation of polyphenols contributes to the formation of melanoidins during the roasting of coffee beans (*31*). It is likely that also in our model systems oxidized catechin is incorporated into HMW melanoidins, which are mainly responsible for browning.

Data about AA and catechin content on this set of roasted model systems are summarized in **Figures 3** and **4**, respectively. Altogether these data indicate that the AA of model systems mainly depended on residual catechin concentration. The AA

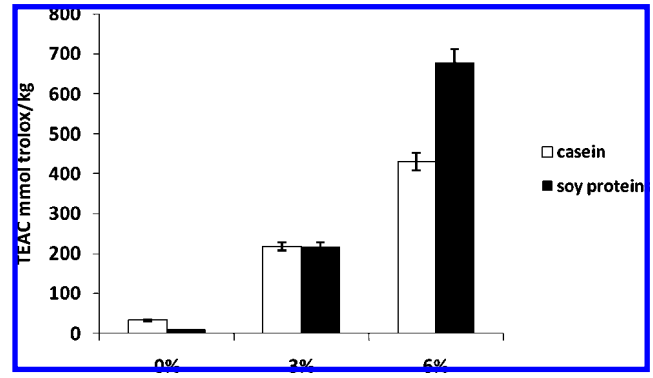

Figure 3. Antioxidant activity of cocoa model systems after roasting. The percentage values on the *x*-axis indicate the catechin concentration in the model systems.

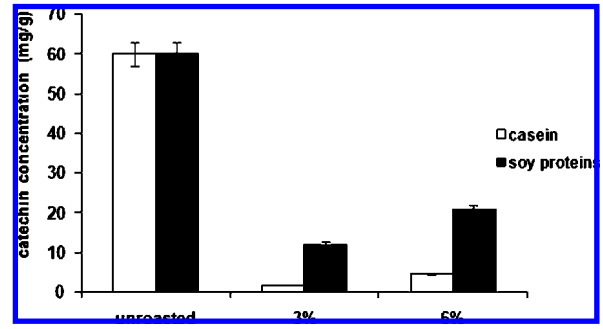

Figure 4. Catechin concentration of cocoa model systems after roasting. The percentage values on the *x*-axis indicate the catechin concentration in the model systems.

measured in catechin-free model systems arose from MRPs formation. This AA might appear to be low when compared to those measured in catechin-containing samples; however, the absolute value of the AA in the catechin-missing system is comparable with that reported for bakery products, such as bread crisps (*1*)

Data reported in **Figure 4** about catechin concentration are in good agreement with those of Kealey et al. (*32*), who demonstrated that the proanthocyanidin content of cocoa beans undergoes a 60% decrement after roasting at 127 °C for 30 min and an 80% decrement after roasting at 181 °C. De Brito et al. (*4*) also found a 57% decrease of total phenol content after toasting of the cocoa beans at 150 °C for 30 min. Moreover, in the casein-containing model system a further reduction of detectable catechin was probably due to the reaction between caseins and catechin (*33*), leading to the formation of casein−catechin complexes. In this respect, it is worth noting that hydrolyzed caseins are much more reactive than soybean proteins. It is likely that casein−catechin complexes still exert a significant antioxidant activity because TEAC values measured in casein-containing samples are comparable with those measured in soy protein-containing samples, despite the clear difference in residual catechin concentration detected by HPLC.

The development of the MR in this set of sample was investigated by measuring HMF concentration as shown in **Figure 5**. HMF formation was higher in model systems with cocoa butter as well as in casein-containing model systems. In all cases, HMF concentration paralleled browning development. It was higher in casein-containing samples, thus confirming that this protein is more reactive than soybean protein in MR. This finding is not surprising as hydrolyzed caseins have a higher lysine content with respect to soybean proteins and also free α-amino groups due to protein hydrolysis.

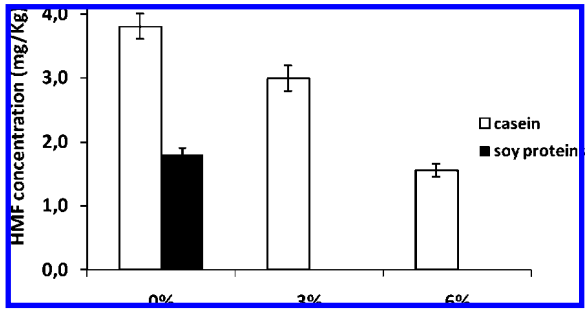

Figure 5. HMF concentrations in cocoa model systems after roasting. The percentage values on the *x*-axis represent catechin concentration.

Table 3. Browning Development and 5-(Hydroxymethyl)-2-furaldehyde (HMF) Content Measured of Cocoa Bean Model System after Roasting

cocoa bean model system	*L**	*a**	*b**	HMF[a] (mg/kg)
oxidized cocoa butter	37	23	25	43.5 ± 16.2a
nonoxidized cocoa butter	36	24	27	51.0 ± 15.5a

[a] Values represent mean ± SE. Different letters within the same factor indicate statistical differences (one-way ANOVA and Duncan's test, $p < 0.05$).

Data also indicated that HMF formation was inhibited by the presence of catechin whatever the composition of the model system. This effect could be ascribed to the "carbonyl trapping" effect of catechin, the concentration of which in this model system, as well as in cocoa, was comparable to that of carbohydrates. This reaction would lead to the formation of catechin−carbohydrate compounds as described by Totlani and Peterson (*34*), who showed that catechin could react with deoxyhexosuloses to generate C−C6 sugar moiety adducts. Stark et al. (*35*) reported that model reactions between amylose and (−)-epicatechin led to the detection of mono- and oligo-C-glucosylated flavan-3-ols. This reaction would explain the inhibitory effect of catechin on the generation of MRPs under roasting conditions: the reaction with catechin subtracted carbohydrates and/or deoxyosuloses to the pathways leading to HMF formation. 3-Deoxyosulose is a well-known Amadori degradation product generated in the intermediate stage of the Maillard reaction, and it is a precursor of HMF (*36*). An alternative explanation could be related to the radical scavenger activity of catechin, but this is not convincing as HMF formation should not involve oxidative steps or formation of radicals.

It is worth noting that in these model systems HMF concentration was quite lower than expected considering the heat treatment applied. This limited formation can be explained by the use of sucrose in the model systems: sucrose is not a reducing sugar and thus not reactive in MR unless it is hydrolyzed. Probably, in the roasting conditions applied, sucrose was also poorly hydrolyzed.

It has been hypothesized that a higher rate of MR development in a fat-rich system could be related to the formation of carbonyl compounds produced during lipid oxidation. Several studies reported that lipid oxidation products could increase the browning as well as HMF formation in fat-rich model systems and foods (*18*, *37*). To test the effect of cocoa butter oxidation on MR development, two model systems were prepared: one with thermoxidized cocoa butter (peroxide number, 8.7 mequiv of O_2 kg^{-1}; TBARs, 3 nmol of MDA g^{-1}) and one with nonoxidized cocoa butter. As reported in **Table 3**, HMF formation and browning development were not significantly affected by lipid oxidation, thus ruling out the possibility that the lipid oxidation products are responsible for the higher browning development.

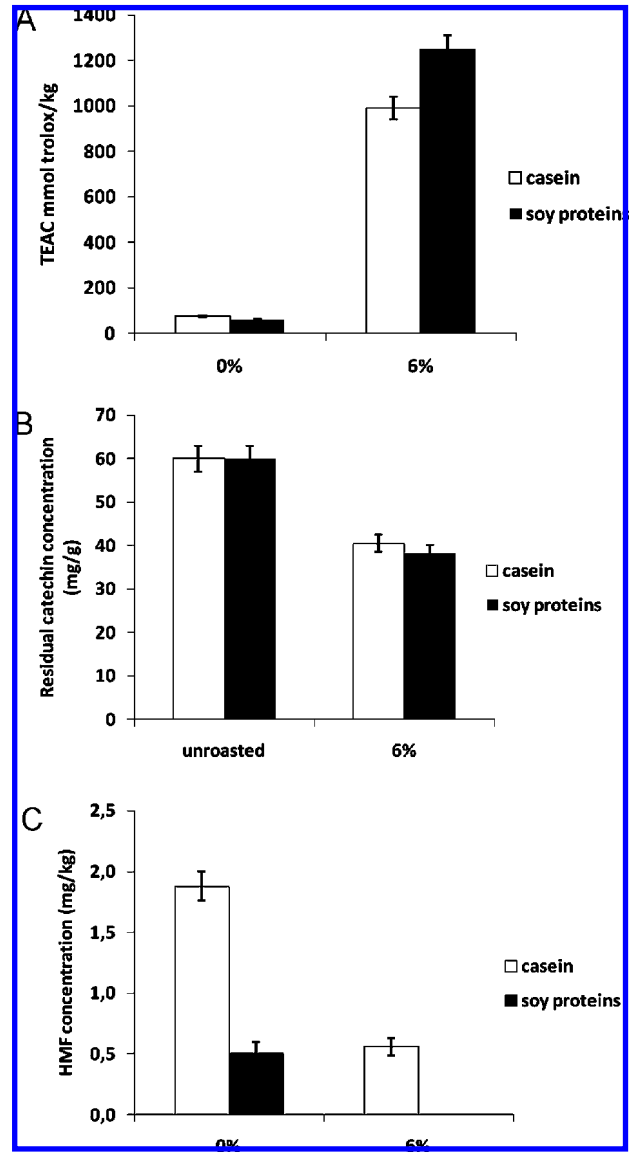

Figure 6. (**a**) Antioxidant activity, (**b**) residual catechin concentration, and (**c**) HMF formation in butter-free model systems. The percentage values on the *x*-axis indicate the catechin concentration in the model systems.

Finally, in **Figure 6** and **Table 2** the results obtained with a set of butter-free model systems, prepared as reported in **Table 1**, are shown. Casein hydrolysate or soy proteins and two different catechin concentrations were used: 0 and 6%. In the catechin-free samples, catechin was replaced by silica gel. In this system, due to the higher water content, both the MR and the catechin degradation reaction were strongly delayed with respect to the butter-containing systems.

However, also in this high-water system, about 30% of catechin was degraded during roasting and a significant AA was present also in the absence of catechin. However, in both cases no difference was detected between the systems with soybean proteins and that containing the hydrolyzed caseins. On the other hand, HMF formation, although at much lower concentration, had the same trend previously observed in butter-containing system, being strongly inhibited by the presence of catechin.

In conclusion, the cocoa model system described in this study proved to be a suitable tool to study MR development and antioxidant activity variation in dry antioxidant- and fat-rich systems. This study demonstrated the inhibitory effect of (+)-

catechin on HMF formation and proposed the role of catechin in browning development through the formation of water-insoluble melanoidins. Further studies are thus necessary to clarify the influence of lipid oxidation products on MR, for example, by using unsaturated fats, which more easily undergo thermoxidation.

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