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Electron Spin Resonance (ESR) Studies on the Formation of Roasting-Induced Antioxidative Structures in Coffee Brews at Different Degrees of Roast

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The antioxidative properties of coffee brew fractions were studied using electron spin resonance spectroscopy using 2,2,6,6-tetramethyl-1-piperidin-1-oxyl (TEMPO) and Fremy's salt (nitrosodisulfonate) as stabilized radicals. TEMPO was scavenged by antioxidants formed during roasting and not by chlorogenic acid, whereas Fremy's salt was scavenged by all antioxidants tested including chlorogenic acid. The stabilized radical TEMPO allowed the exclusive measurement of roasting-induced antioxidants. The roasting-induced antioxidant activity of coffee brews increased with increasing degree of roast, and most of these antioxidants were formed during the initial roasting stage. The majority of these roasting-induced antioxidants were present in the high molecular weight fractions, indicating that the formation of these antioxidants preferably occurs at specific high molecular weight structures, likely being arabinogalactan and/or protein moieties which might be part of the melanoidin complex. It was found that chlorogenic acids most probably do not lose their antioxidant activity and phenolic characteristics upon incorporation in coffee melanoidins. The parameter fast reacting antioxidants (FRA) was introduced as an alternative for the antioxidative potential. FRA levels showed that coffee fractions rich in roasting-induced antioxidants exposed their antioxidant activity relatively slowly, which must be a consequence of its complex structure. Finally, the melanoidin content and the roasting-induced antioxidant activity showed a positive and linear correlation for the coffee brew fractions, showing that roasting-induced antioxidants are present within melanoidins. This is the first time that the formation of roasting-induced antioxidants could be directly correlated with the extent of Maillard reaction and melanoidin formation in a complex product such as coffee.

KEYWORDS: Coffee; melanoidins; antioxidant activity; ESR; Maillard reaction; polyphenolics

INTRODUCTION

It has been known for a long time that both green and roasted coffee beans and coffee brews contain compounds that exert antioxidant activity (*1–8*). The presence of large amounts of chlorogenic acid, a polyphenolic compound, in coffee contributes significantly to this antioxidant activity (*4, 9–11*). Upon roasting, the chlorogenic acid level decreases from 5−8% in green Arabica beans to levels as low as 0.2% for dark-roasted coffees (*12*). The fate of this disappearing chlorogenic acid is not totally clear, although several explanations can be found in the literature, including acyl migration, hydrolysis, oxidation, fragmentation, polymerization, and association with denatured/degraded proteins (*12*). More specifically, it was reported that chlorogenic acids could be converted into flavor compounds upon roasting (*13, 14*) or that they might be incorporated in coffee melanoidins (*15, 16*). Heinrich and Baltes showed that Curie point pyrolysis of coffee melanoidins yielded phenolic degradation products (*16*). Adams et al. indicated that chlorogenic acids might be involved in melanoidin formation (*15*). More recently, it was shown that caffeic acid (*17*) and quinic acid, the building blocks of chlorogenic acids, are chemically incorporated in melanoidins upon roasting (*18*).

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Melanoidins form an abundant group of complex molecules in coffee brew (*8*). Melanoidins are generally referred to as high molecular weight (Mw), nitrogenous, brown-colored Maillard reaction end-products. Despite all efforts, the molecular structures of melanoidins are still unknown due to the extreme complexity of these molecules. One of the functional properties of coffee melanoidins is its antioxidant activity (*5, 19*). This antioxidant activity could be due to incorporated chlorogenic acids in melanoidins. Additionally, the Maillard reaction may also result in the formation of new antioxidative structures in coffee melanoidins. The latter was shown by Nicoli et al. (*19*), who showed that coffee brews exhibited oxygen-scavenging properties compared to a brew prepared from green coffee beans. Furthermore, Charurin et al. (*20*), and Cämmerer and Kroh (*4*) showed that a model reaction between sugars and amino compounds resulted in the formation of antioxidative compounds.

A problem that is faced when the antioxidative properties of coffee are studied is the difficulty of distinguishing polyphenolic antioxidants from antioxidants formed by the Maillard reaction. This problem is most pronounced when Maillard reaction antioxidants are studied because the high levels of polyphenolic antioxidants are expected to dominate total antioxidant activity (*21*). Therefore, there is a need for a detection method that specifically measures nonphenolic antioxidants in coffee. An electron spin resonance (ESR) spectroscopy method using stabilized radicals seems to be capable of making this distinction (*4*). ESR is a rapidly evolving technique that is highly suitable for measuring antioxidant activity (*22*). The usage of different stabilized radicals allows differentiating between antioxidants that differ in structural properties. A wide range of antioxidants can be detected when nitrosodisulfonate (Fremy's salt) is used as stabilized radical, whereas 2,2,6,6-tetramethyl-1-piperidin-1-oxyl (TEMPO) could be scavenged by model system Maillard reaction products, although it was not or only negligibly sensitive toward antioxidants with a phenolic nature (*4*). Thus, the use of TEMPO should allow the detection of antioxidative structures in coffee brew melanoidins formed by the Maillard reaction itself, the roasting-induced antioxidants, whereas incorporated chlorogenic acids should not be detected.

The objective of the present study was to investigate the total antioxidant and roasting-induced antioxidant properties of coffee brew melanoidins. Coffee brew melanoidin populations, isolated from coffee brews having four degrees of roast, were investigated for their antioxidative properties.

MATERIALS AND METHODS

Materials. Green and roasted Colombian coffee beans (*Coffea arabica*) were provided by a local factory. The degrees of roast, which is the total weight loss upon roasting, of the light-, medium-, and dark-roasted beans were 14.7, 16.4, and 19.2% (w/w), respectively, and were 6.1, 8.0, and 11.1% (w/w) on a dry matter basis, respectively. The colors of the light-, medium-, and dark-roasted beans were 60, 50, and 40, respectively, according to the color test Neuhaus (CTN). The denominations GB, LR, MR, and DR were added to the fraction name to indicate that the fraction was isolated from green or light-roasted, medium-roasted, or dark-roasted coffee beans, respectively.

Preparation of Coffee Brew. Green (frozen with liquid nitrogen) and roasted coffee beans were ground, and a brew was prepared as described previously (*23*). Briefly, coffee brew was obtained by adding 200 g of ground coffee beans to 1200 g of filtered and demineralized water (Millipore Corp, Billerica, MA) at 90 °C. Subsequently, this coffee suspension was kept at 90 °C for 15 min with constant stirring. The extract was filtered over a Büchner funnel using an S&S 595 filter (Whatman, Maidstone, U.K.). For characterization purposes, part of the brew was lyophilized, yielding "brew". The major part of the brew was used for further isolation.

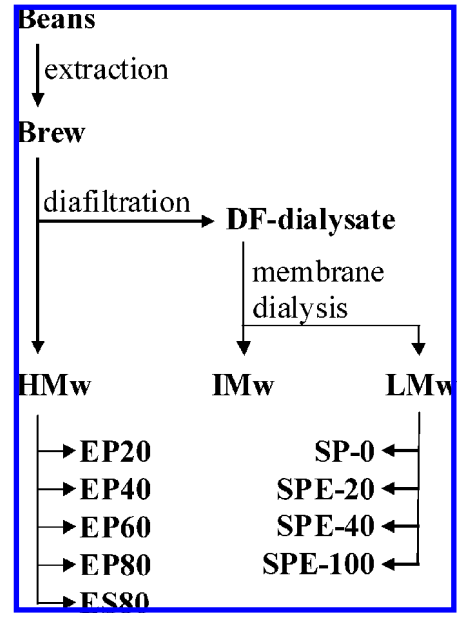

Figure 1. Isolation scheme of various melanoidin coffee brew populations from roasted Arabica coffee beans.

Defatting of Coffee Samples. Lyophilized coffee brew samples were defatted by Soxhlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany), as described previously (*24*). The solvent used for extraction was dichloromethane.

Isolation of High Molecular Weight Coffee Material. High Mw material was obtained from brew by diafiltration (cutoff of 3 kDa) according to the procedure previously described (*23*). The retentate and dialysate were lyophilized, yielding a high molecular weight fraction (HMw) and a fraction with a lower molecular weight (DF-dialysate), respectively (**Figure 1**).

Isolation of Intermediate and Low Molecular Weight Coffee Material. Intermediate and low Mw material was obtained from defatted DF-dialysate sample by membrane dialysis based on a procedure previously described (*24*). The DF-dialysate sample (500 mL, 100 mg/mL) was dialyzed using a Visking size 9 dialysis membrane with a cutoff of 12−14 kDa (Medicell International Ltd., London, U.K.) for 3 days at 4 °C against 5 L of demineralized water with four water renewals. The retentate was lyophilized, yielding the intermediate Mw fraction (IMw) (**Figure 1**). The first two dialysate fractions were pooled and lyophilized, yielding the low Mw fraction (LMw) (**Figure 1**), whereas the last three dialysate fractions were discarded.

Ethanol Precipitation of the Brew HMw. The HMw fraction obtained after diafiltration was subjected to ethanol precipitation, as described previously (*23*). Absolute ethanol was added to the HMw solution until the desired concentration. The solution was left for precipitation and was subsequently centrifuged. The supernatant was subjected to further precipitation steps. Coffee fractions that precipitated at 20, 40, 60, and 80% ethanol were coded EP20, EP40, EP60, and EP80, respectively (**Figure 1**). The supernatant of 80% ethanol was coded ES80 (**Figure 1**).

Reversed-Phase Solid-Phase Extraction of the LMw Coffee Material. Defatted LMw fractions were fractionated by reversed-phase solid-phase extraction as described previously (*25*). Briefly, 10 mL of aqueous LMw fraction (10 mg/mL) was applied on a 5 g Sep-Pak Vac 20 cm^3 C18 cartridge (Waters, Milford, MA). Elution was conducted in four steps: (I) 50 mL of water, (II) 50 mL of 20% (v/v) aqueous methanol, (III) 50 mL of 40% (v/v) aqueous methanol, and (IV) 50 mL of 100% methanol. This procedure was conducted in 20-fold, fractions were pooled, and methanol was evaporated by a rotating evaporator. The fractions were lyophilized, yielding SPE-0, SPE-20, SPE-40, and SPE-100 for the fractions that eluted at 0, 20, 40, and 100% methanol (**Figure 1**), respectively.

Determination of 5-Caffeoylquinic Acid by Reversed-Phase Chromatography. The 5-caffeoylquinic acid level was determined as described previously (*18*). Briefly, coffee sample was analyzed by reversed-phase high-performance liquid chromatography (HPLC) on an XTerra MS C18 column in combination with an XTerra MS C18 guard column (Waters) using 0.1% acetic acid in water and 0.1% acetic acid in methanol as eluents. The absorbance of the eluate was measured at 325 nm using a Spectra System UV3000 (Thermo Electron Co., Waltham, MA). The sample was dissolved in the eluent (1 mg/mL) and was centrifuged prior to injection. Measurements were conducted at least in duplicate. The average coefficient of variation was 1%.

Total Phenolic Groups Content. The total phenolic groups content of the coffee samples was determined with the Folin−Ciocalteu assay as described previously (*23*). The used reference compound was 5-caffeoylquinic acid. Measurements were conducted at least in duplicate. The average coefficient of variation was 2%.

Specific Extinction Coefficient of Coffee Material. The absorption of aqueous sample solutions (1 g/L) was determined at 405 nm using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). The specific extinction coefficient $K_{mix,405nm}$ (L/g/cm) was calculated as described previously (*23*).

Determination of the Antioxidant Activity by ESR Spectroscopy. ESR spectroscopic measurements were performed on a Miniscope MS 100 spectrometer (Magnettech, Berlin, Germany) with Fremy's salt (Sigma Chemical Co., St. Louis, MO) and TEMPO (Sigma) as stabilized radicals. For investigation with Fremy's salt, Fremy's salt (1 mM) was dissolved in 50 mM phosphate buffer, pH 7.4. Aliquots of coffee sample (100 μL) were allowed to react with an equal volume of an aqueous 1 mM Fremy's salt solution. ESR spectra were recorded every 35 s for 10 min, after which time the reaction speed had stabilized. For investigation with TEMPO, TEMPO (10 mM) was dissolved in methanol and subsequently diluted to 1 mM with water. Aliquots of coffee sample (300 μL) were allowed to react with 100 μL of 1 mM TEMPO solution. ESR spectra were obtained after 120 min, by which time the reaction speed had stabilized. To guarantee linearity, the sample concentration was chosen in such a way that >10% and <90% of the radicals were scavenged. In practice, sample concentrations were between 0.25 and 1.5 mg/mL and between 1 and 40 mg/mL for measurements with Fremy's salt and TEMPO, respectively. Unless mentioned otherwise, both Fremy's salt and TEMPO antioxidant activity were calculated as Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma) equivalents. The Fremy's salt antioxidant activity was calculated using the following equation: [(% Fremy's salt scavenged by sample)$_{t = 10min}$/(μg/mL sample)]/[(% Fremy's salt scavenged by Trolox)$_{t = 10min}$/(μg/mL Trolox)] × 1000‰. The TEMPO antioxidant activity was calculated using the following equation: [(% TEMPO scavenged by sample)$_{t = 120min}$/(mg/mL sample)]/[(% TEMPO scavenged by Trolox)$_{t = 120min}$/(mg/mL Trolox)] × 1000‰. Microwave power and signal phase were set at 10 dB and 0°, respectively. Modulation amplitude, center field, and sweep width were set at 1.5, 3397, and 71 G, respectively. Measurements were conducted at least in duplicate. The average coefficients of variation were 3 and 5% for measurements with Fremy's salt and TEMPO, respectively. Trolox, chlorogenic acid (5-caffeoylquinic acid) (Sigma), and ascorbic acid (Serva, Heidelberg, Germany) were used as reference compounds.

RESULTS AND DISCUSSION

Scavenging Specificities of Fremy's Salt and TEMPO. It was previously reported that Fremy's salt reacts rather unspecifically as it is scavenged by different types of antioxidants, including the polyphenolic chlorogenic acid (*4*). In this research, this was confirmed because Fremy's salt was indeed scavenged by chlorogenic acid, as well as by Trolox and ascorbic acid with similar reaction velocities. The stabilized radical TEMPO was effectively scavenged by Trolox and ascorbic acid, but it was not scavenged by chlorogenic acid, being in line with earlier findings (*4*). Thus, by using both Fremy's salt and TEMPO as stabilized radicals, a distinction could be made between phenolic and nonphenolic antioxidants (*4*). It is desirable to make this distinction when antioxidant properties of coffees are studied because coffee brew is expected to exhibit antioxidant activity due to the presence of chlorogenic acids as well as roasting-induced structures that are formed upon roasting.

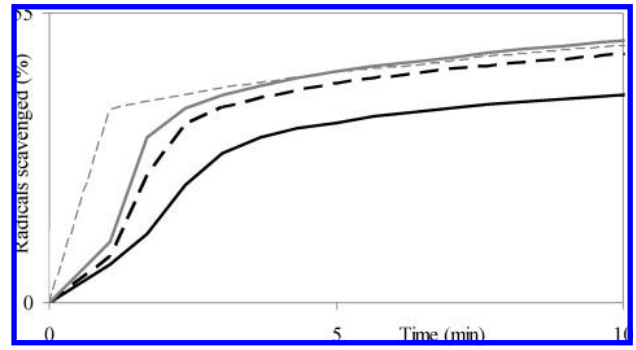

Figure 2. Reaction kinetics of Fremy's salt scavenging by coffee brews (150 μg/mL) made from green (gray dashed line), light- (gray solid line), medium- (black dashed line), and dark (black solid line)-roasted beans.

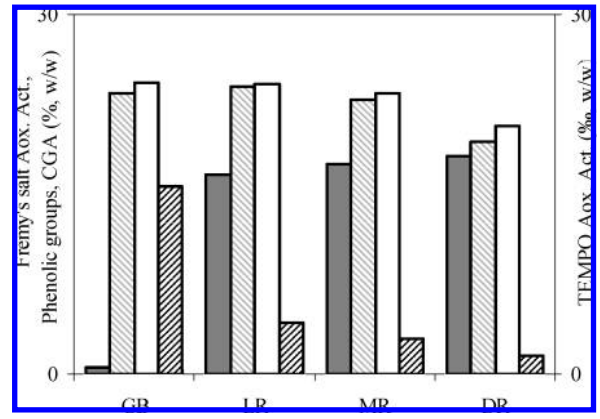

Figure 3. TEMPO (Trolox equivalents, gray bars) and Fremy's salt (5-caffeoylquinic acid equivalents, gray striped bars) antioxidant activity levels, the phenolic groups level by Folin−Ciocalteu (5-caffeoylquinic acid equivalents, white bars), and chlorogenic acids level by HPLC (5-caffeoylquinic acid equivalents, black striped bars) of coffee brews made from green (GB) and light- (LR), medium- (MR), and dark (DR)-roasted beans.

At first, kinetic ESR experiments with Fremy's salt were conducted for 2 h to explore the antioxidant properties of coffee brews in time. It was found that Fremy's salt radicals were rapidly consumed by coffee brew antioxidants during the first 10 min (**Figure 2**). After that, the radical consumption leveled off and no major differences between different coffee samples were observed. As the first 10 min provided the most valuable information, it was decided to conduct kinetic ESR measurements with Fremy's salt for 10 min for all coffee samples. Kinetic ESR experiments with TEMPO (not shown) were also conducted for 2 h, and these measurements revealed that coffee antioxidants scavenged the radical TEMPO much more slowly. The radical consumption leveled off only after >1 h, and therefore it was decided to maintain the timeframe of 2 h for ESR measurements with TEMPO.

Antioxidant Properties of Coffee Brews with Various Degrees of Roast. The Fremy's salt and TEMPO antioxidant activity levels, the phenolic groups level, and the 5-caffeoylquinic acid levels for coffee brew from beans with different degrees of roast are shown in **Figure 3**. The green bean brew had a Fremy's salt antioxidant activity level of 23% and a phenolic groups level of 24%, both expressed as 5-caffeoylquinic acid

Table 1. Fremy's Salt and TEMPO Antioxidant Activity Levels of Coffees with Various Degrees of Roast[a,b]

	green bean		light roast		medium roast		dark roast	
	Fremy's (‰, w/w)	TEMPO (‰, w/w)	Fremy's (‰, w/w)	TEMPO (‰, w/w)	Fremy's (‰, w/w)	TEMPO (‰, w/w)	Fremy's (‰, w/w)	TEMPO (‰, w/w)
brew	170	<1	174	16	166	17	141	18
HMw	5	0	21	19	30	31	36	41
IMw	167	<1	113	10	92	11	85	11
LMw	207	0	204	3	196	3	155	3

[a] Antioxidant activity levels were measured as Trolox equivalents. [b] The average coefficients of variation were 3 and 5% for the Fremy's salt and TEMPO antioxidant activities, respectively.

equivalents. The actual 5-caffeoylquinic acid content was 16% instead of 23%. This difference was ascribed to the fact that 5-caffeoylquinic acid was the only chlorogenic acid determined by HPLC, whereas other chlorogenic acids are also present in green beans (*26*). Likewise, Perrone et al. reported that 5-caffeoylquinic acid was around 60% of the total chlorogenic acids present in green Arabica beans (*27*). Additionally, a small part of the phenolics might originate from other phenolic components, such as proteins. It was estimated that phenolic amino acids in coffee brew corresponded to ~0.4% 5-caffeoylquinic acid equiv (*23*). Still, the majority of the antioxidant activity and phenolics in green bean brew could be ascribed to chlorogenic acids.

Upon roasting, the 5-caffeoylquinic acid content decreased from 16% in green bean brew to levels as low as 2% in dark-roast brew. This drop in chlorogenic acid level was expected as chlorogenic acid degradation is known to occur upon roasting (*12*). Whereas the chlorogenic acids level drastically decreased, the Fremy's salt antioxidant activity only slightly decreased from 24% in light-roast brew to 19% in dark-roast brew (**Figure 3**). These relatively stable Fremy's salt antioxidant activity levels were in line with the literature, too (*4, 21*), but were also rather remarkable because the chlorogenic acid level had decreased so drastically. These findings imply that the antioxidant activity from chlorogenic acids is not destroyed upon roasting but survives these extreme heating conditions, even though the chemical structure alters. It is expected that the chlorogenic acids are incorporated in melanoidins without losing their antioxidative properties. Alternatively, it might also be that formation of new roasting-induced antioxidants compensates for the loss of antioxidant activity due to chlorogenic acid degradation. The latter is not likely, though, for two reasons. First, it will be shown below that the contribution of newly formed roasting-induced antioxidants to the total (Fremy's salt) antioxidant activity is rather limited. Second, it was found that the phenolic groups levels as determined by the Folin−Ciocalteu assay showed the same trend and had about the same values as the Fremy's salt antioxidant activity levels, with phenolic groups levels of 24% for light-roast brew to 21% for dark -roast brew (**Figure 3**). The latter implies that phenolic groups dominate the Fremy's salt antioxidant activity in coffee brews and that the relatively constant antioxidant activity should be ascribed mainly to the presence of phenolics. Therefore, it is expected that chlorogenic acids do not lose their antioxidant activity or phenolic characteristics upon incorporation in coffee melanoidins during roasting. This moiety is likely not present as free chlorogenic acids but might be bound to other molecules via ionic bonds (*1*), via ester bonds (*17, 18*), and possibly via other types of bonds such as ether linkages (*18*).

With respect to the TEMPO antioxidant activity levels (**Figure 3**), it stands out that these levels were totally different from Fremy's salt antioxidant activity levels. The TEMPO antioxidant activity level was very low, not even 1‰, for green bean brew. This was expected because the radical TEMPO should be scavenged only by roasting-induced antioxidants (*4*), and these structures were of course not present in green coffee beans. The TEMPO antioxidant activity level of the brew prepared from light roasted beans was 16‰. The steep increase from <1‰ in green bean coffee to 16‰ in light-roast coffee brew showed that roasting led to the formation of relatively many roasting-induced antioxidants. A similar increase was observed by Nicoli et al. (*19*), who determined the antioxidant activity of Maillard reaction products in coffee by measuring their oxygen-scavenging properties. The TEMPO antioxidant activity increased when coffee beans were roasted more intensely (**Figure 3**), indicating that prolonged roasting leads to the formation of more roasting-induced antioxidants. It was shown that TEMPO indeed is capable of exclusively measuring roasting-induced antioxidants in a complex foodstuff such as coffee brew. Furthermore, these roasting-induced antioxidants were especially formed during the initial stage of roasting even though somewhat more roasting-induced antioxidants are formed upon prolonged roasting.

When both the Fremy's salt and TEMPO antioxidant activity levels are compared, it stands out that the levels with TEMPO were much lower than the levels with Fremy's salt (**Table 1**), even though the timeframe for scavenging with the radical TEMPO was 12 times longer. For example, for brew from medium-roasted coffee beans, the TEMPO antioxidant activity level was found to be only 10% of the Fremy's salt antioxidant activity level. These results showed that the contribution of the newly formed roasting-induced antioxidants upon roasting to the overall antioxidant activity is rather limited and that phenolic antioxidants dominate the overall antioxidant activity of coffee brews.

With respect to the kinetics, it was observed that coffee brews with different degrees of roast showed differences in reaction kinetics (**Figure 2**). For the brew of green beans, the percentage of radicals scavenged increased rapidly within the first minutes, showing that the antioxidants present in green beans scavenge radicals readily. When looking to the brews from roasted beans, it stands out that the reaction velocity within this first minute decreased with increasing degree of roast. This suggested that the converted or formed antioxidants present in roasted coffee brews have a lower mobility than green coffee antioxidants or that these antioxidants do not react as readily as do the antioxidants present in the brew from green beans.

Antioxidant Activity of High, Intermediate, and Low Mw Coffee Fractions. It can be seen in **Table 1** that the LMw fractions from coffee brew had the highest Fremy's salt antioxidant activities and that the HMw fractions had the lowest values. The fact that most of the Fremy's salt antioxidants of the green bean brew were recovered in the LMw fraction was expected because chlorogenic acids are low Mw molecules. This observation was also in line with the literature (*6*). However, the roasting process seems to cause a change in distribution of this Fremy's salt antioxidant activity. Upon roasting, the activity of the HMw fraction increased from 5 to 36‰, whereas the

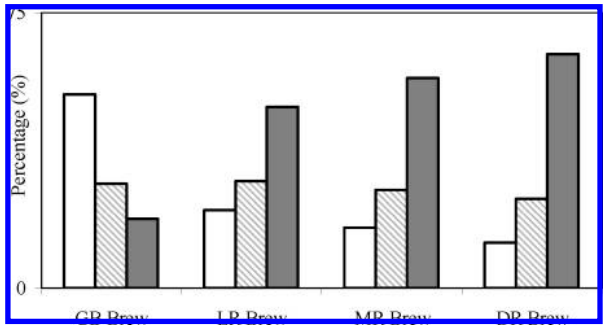

Figure 4. Distribution of TEMPO antioxidant activity from coffee brews with various degrees of roast over LMw (white bars), IMw (striped bars), and HMw (gray bars). (GB, LR, MR, and DR are green and light-, medium-, and dark-roasted beans, respectively.)

activity of the IMw and LMw fractions decreased from 167 to 85‰ and from 207 to 155‰, respectively. These findings can be explained by the fact that LMw and/or IMw antioxidative molecules such as chlorogenic acids undergo reaction and might be incorporated into HMw material during prolonged roasting, as was reported previously (*18*).

With respect to the TEMPO antioxidant activities, the HMw coffee brew fractions were opposite to the Fremy's salt antioxidant activities because the TEMPO antioxidant activities of the HMw fractions were now higher, instead of lower, compared to the IMw and LMw fractions (**Table 1**). These findings suggest that the formation of roasting-induced antioxidants preferably occurs at specific types of molecules that are present in HMw material, which consists of galactomannans, arabinogalactan proteins, protein, and melanoidins (*23*). The increase in roasting-induced antioxidants in HMw upon further roasting is far more pronounced than was observed for the brews. When the yields of the fractions are taken into account (**Figure 4**), it becomes even more evident that high Mw material contributes most to the observed roasting-induced antioxidants in coffee brews and that the contribution of this HMw material to the total roasting-induced antioxidants present in the brew continuously increases upon prolonged roasting. The observed Fremy's salt antioxidant activities, representing the total antioxidant level, for the HMw coffee fractions are similar to the TEMPO antioxidant activities, indicating that a significant part of the total antioxidant activity is caused by roasting-induced antioxidative structures. However, the contribution of phenolic antioxidants to high Mw antioxidant activity should not be excluded or underestimated because the Fremy's salt antioxidant activity levels would have been higher when the timeframe for these measurements was 2 h as for the TEMPO measurements as well. Thus, the observed antioxidant activity in high Mw coffee material should be caused both by incorporated phenolics (*18*) and by newly formed roasting-induced antioxidative structures.

Antioxidative Potential of Coffee Brew Fractions. It is well-known that antioxidants can differ with respect to their reaction kinetics; some antioxidants react slowly, whereas other antioxidants are fast-reacting, such as many phenols (*28*). A parameter that provides insight into the reaction kinetics of antioxidants is the antioxidative potential, which is the initial reaction velocity by which the radical is scavenged by the antioxidant (*22*). Due to the complex composition of coffee and coffee fractions, which may contain different antioxidants in different concentrations, it is extremely difficult if not impossible to obtain comparable antioxidative potential values. However, differences in reaction kinetics can be observed for different coffee brews (**Figure 2**). In this figure, it can be seen that the

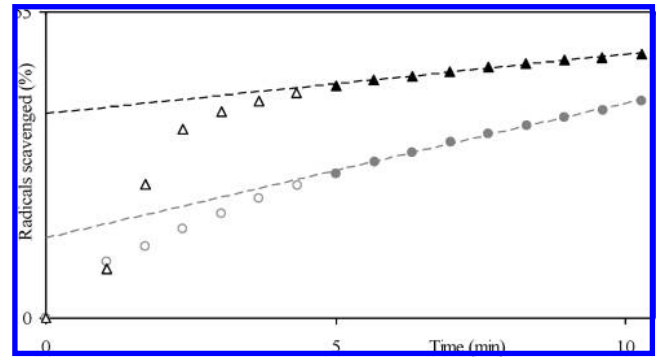

Figure 5. Reaction kinetics of Fremy's salt scavenging by medium-roast HMw (750 μg/mL, gray circles) and brew (150 μg/mL, black triangles). Trend line equations using solid data points: $2.4 \times t + 14.2$ for HMw (gray) and $1.1 \times t + 36.7$ for brew (black).

Table 2. Fast-Reacting Antioxidant (FRA) Levels of Coffees with Various Degrees of Roast

	FRA level (%)			
	green bean	light roast	medium roast	dark roast
brew	80	77	78	76
HMw	51	30	37	39
IMw	77	51	34	35
LMw	79	77	77	77

radical-scavenging curve for green bean brew showed a very steep increase during the first seconds. This was not the case for the curves of coffee brews, of which the scavenging curve became more and more S-shaped upon prolonged roasting (**Figure 2**). Differences in reaction kinetics can also be observed for coffee brew fractions, even though the determined antioxidative potential can be rather similar (**Figure 5**). From the latter figure, it is evident that the brew reaches an equilibrium phase more quickly than the HMw fraction. Initially (0−5 min), the reaction velocity for brew was higher than that for HMw. However, the reaction velocity for the brew leveled off after 5 min, whereas the reaction velocity for HMw decreased much less. This resulted in a higher reaction velocity for the HMw fraction than for the brew between 5 and 10 min. Obviously, antioxidants in HMw coffee material expose their antioxidant activity over a wider range of time. This clearly showed that the antioxidants present in different coffee brew fractions might have different properties. An alternative for the antioxidative potential, which also provides insight into the reaction kinetics, was introduced and was defined as the percentage "fast reacting antioxidants" (FRA). This value was calculated using the trend line of Fremy's salt scavenging data between 5 and 10 min of reaction (**Figure 5**). The percentage FRA was calculated as $(\text{value trend line})_{t=0\text{min}}/(\text{value trend line})_{t=10\text{min}} \times 100\%$. This approach was found to yield reproducible results as the average variation was only 3% of the value, and results for various coffee fractions are shown in **Table 2**. The percentage FRA for the chlorogenic acid rich, melanoidin free green bean brew was 80%, which was similar to the 82% obtained for the reference compound, chlorogenic acid. The FRA values for the brews from roasted beans were somewhat lower, which must be ascribed to the presence of antioxidants that react more slowly. The FRA levels were lowest in the HMw fractions, with FRA levels as low as 30% for the HMw fraction from light-roasted beans. As the FRA levels were lowest for the HMw fractions and as their TEMPO antioxidant activity levels were highest for these fractions, it can be reasoned that roasting-induced antioxidants are antioxidants that react slowly initially but

Table 3. Fremy's Salt Antioxidant Activity, TEMPO Antioxidant Activity, and Fast-Reacting Antioxidant (FRA) Levels of Medium-Roast High Molecular Weight Ethanol Precipitation Fractions[a,b]

	antioxidant activity		
	Fremy's (‰,w/w)	TEMPO (‰,w/w)	FRAlevel (%)
HMw	30	31	37
EP20	18	12	39
EP40	21	11	38
EP60	22	11	37
EP80	27	19	43
ES80	49	33	46

[a] Antioxidant activity levels were measured as Trolox equivalents. [b] The average coefficients of variation were 3 and 5% for the Fremy's salt and TEMPO antioxidant activities, respectively.

continue scavenging radicals for a relatively long time. The presence of "slow" but highly efficient roasting-induced antioxidants that represent the antioxidant reservoir of food products was also mentioned by Nicoli et al. (*29*). This phenomenon of slow-reacting antioxidants might be explained by the fact that roasting-induced antioxidants likely possess structural characteristics that are not as optimal as the structural characteristics of well-known and fast-acting antioxidants. Additionally, the initial reaction velocity of the roasting-induced antioxidants might also be slowed by a limited mobility of the HMw molecule and by steric hindrance by the non-antioxidant part.

Antioxidant Activity of Ethanol Precipitation Fractions from Medium-Roast HMw Material. The medium-roast HMw fraction was separated by ethanol precipitation as described previously (*23*). It was previously found that galactomannans already precipitated at low ethanol concentrations and were mainly present in the fractions EP20 and EP40 (*23*). Proteins and arabinogalactans were soluble at these low ethanol concentrations and were mainly recovered in the fractions EP80 and ES80 (*23*). Now, the antioxidant properties of these HMw fractions were determined (**Table 3**). It was found that the TEMPO antioxidant activity increased with increasing ethanol solubility. The fact that EP80 and ES80 had the highest TEMPO antioxidant activity levels implies that roasting-induced antioxidants are preferably formed at structures that are rich in arabinogalactans and/or proteins. As arabinogalactan proteins (AGPs) are involved in melanoidin formation (*24*) and as arabinose is susceptible to degradation upon roasting (*30*), it is to be expected that arabinose from AGPs is involved in the formation of roasting-induced antioxidants upon roasting. Furthermore, melanoidins that are rich in galactomannans may also be involved in the formation of roasting-induced antioxidants because the fractions rich in galactomannans, EP20 and EP40, still had TEMPO antioxidant activity values of 12 and 11‰, respectively. The formation of roasting-induced antioxidants on galactomannan−melanoidin complexes seems to be plausible because galactomannans were shown to be involved in the Maillard reaction upon roasting of coffee beans (*31*).

The level of Fremy's salt antioxidant activity was only slightly (1.4−2 times) higher than for TEMPO (**Table 1**). This indicates that the roasting-induced antioxidants are the main contributor to the overall antioxidant activity of HMw material from roasted coffee brew. It should be mentioned, though, that the Fremy's salt antioxidant activity levels would have been somewhat higher when the timeframe of these measurements would have been lengthened to 2 h as well. The FRA level was highest for the two fractions containing the most ethanol-soluble material (EP80 and ES80). This is probably again due to the presence of relatively more fast-reacting polyphenolic compounds in these fractions, as shown previously (*23*).

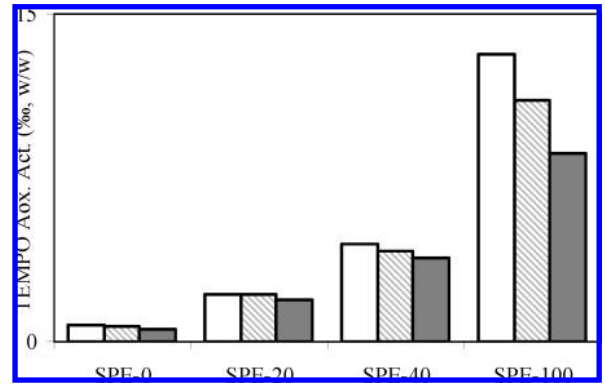

Figure 6. TEMPO antioxidant activity levels in low molecular weight coffee brew fractions isolated by reversed-phase solid-phase extraction. The white, striped, and gray bars represent the light-, medium-, and dark-roasted coffee brew fractions, respectively.

Table 4. Fremy's Salt Antioxidant Activity and Fast Reacting Antioxidant (FRA) Levels of Low Molecular Weight Coffee Brew Fractions with Various Degrees of Roast[a,b]

	light roast		medium roast		dark roast	
	Fremy's (‰, w/w)	FRA level (%)	Fremy's (‰, w/w)	FRA level (%)	Fremy's (‰, w/w)	FRA level (%)
LMw	204	77	196	77	155	77
SPE-0	119	80	103	79	70	76
SPE-20	404	79	380	78	350	81
SPE-40	301	79	335	81	285	78
SPE-100	108	70	132	67	155	71

[a] Antioxidant activity levels were measured as Trolox equivalents. [b] The average coefficient of variation was 3% for the Fremy's salt antioxidant activities.

Antioxidant Activity of Solid-Phase Extraction Fractions from Medium-Roast LMw Material. Previously, low Mw coffee material was fractionated on the basis of polarity using reversed-phase solid-phase extraction (*25*). It was found that most of the nonmelanoidins, for example, minerals, sugars, and chlorogenic acids, had a rather polar character and ended up in the fractions SPE-0 and SPE-20. In contrast, most of the melanoidins were quite apolar and ended up in the fractions SPE-40 and SPE-100. This allowed separation of most of the melanoidins from the majority of the non-melanoidin components using reversed-phase solid-phase extraction. Because the isolated apolar fractions were rich in melanoidins, it was expected that the roasting-induced antioxidant activity would be especially high in these apolar fractions. **Figure 6** shows that the TEMPO antioxidant activity was indeed high for fractions that had an apolar character (SPE-40 and SPE-100). This proved that also low Mw Maillard reaction products possess a relative high TEMPO antioxidant activity. It is noteworthy that the TEMPO antioxidant activity in these apolar fractions decreased with increasing degree of roast. This suggested that the formed low Mw roasting-induced antioxidants react and might be incorporated in HMw molecules upon prolonged roasting. The results of the Fremy's salt antioxidant activity and FRA levels are shown in **Table 4**. The Fremy's salt activity level in SPE-0 was relatively low, which was ascribed to the presence of many non-antioxidative compounds such as sugars and minerals, as was shown previously (*25*). The low Fremy's salt antioxidative activity in SPE-0 should be due to polyphenols because the TEMPO antioxidant activity levels were low (**Figure 6**) and the FRA levels (76−80%) were high and similar to the FRA level of chlorogenic acid (82%) (**Table 4**). The SPE-20 fractions showed the highest activity levels due to the

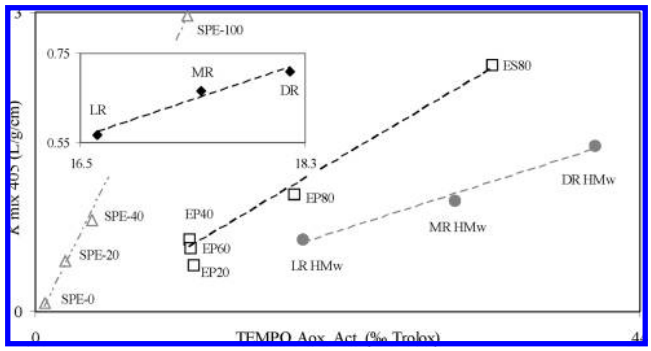

Figure 7. $K_{mix,405}$ values of light (LR)-, medium (MR)-, and dark (DR)-roast coffee fractions plotted as a function of the TEMPO antioxidant activity: HMw fractions (circles), ethanol precipitation fractions (squares), MR solid-phase extraction fractions (triangles), and whole brews (inset, diamonds).

presence of high concentrations of chlorogenic acids (*25*), which resulted in high FRA levels as well. Some of the chlorogenic acids might end up in the SPE-40 fraction, resulting in a relatively high Fremy's salt antioxidant activity and high FRA levels. Another explanation might be that caffeine scavenges Fremy's salt, too, as caffeine is also capable of scavenging hydroxyl radicals (*32*). SPE-100 had low Fremy's salt antioxidant activities and low FRA levels, which is in line with the presence of relatively many roasting-induced antioxidants.

Relationship between Antioxidant Activity and Melanoidins. To the best of our knowledge, a positive and linear correlation between antioxidant activity and color was found only in model systems and in foods where the Maillard reaction was the sole or prevalent event (*10, 33*). In coffee, this correlation is not clear because phenolic compounds play an important role in antioxidant activity as well (*10, 11*). In this research, it was shown that the TEMPO antioxidant activity provides insight into the extent of roasting-induced antioxidants formed upon roasting of coffee beans, whereas polyphenolics are not detected. The melanoidin level can be quantified by determination of the brownness, which is represented by the parameter $K_{mix,405}$ (*23*). As both the TEMPO antioxidant activity and $K_{mix,405}$ values are related to the extent of Maillard reaction, it was expected that there would be a positive correlation between these two parameters. The $K_{mix,405}$ values of the coffee brews, HMw fractions, ethanol precipitation fractions, and solid-phase extraction fractions are plotted as a function of the TEMPO antioxidant activity in **Figure 7**. Starting with the coffee brews, it is clear that the light-, medium-, and dark-roast brews showed a positive and linear correlation ($R^2 = 0.97$) between the $K_{mix.405}$ value and TEMPO antioxidant activity. A similar positive and linear correlation was found for the light-, medium-, and dark-roast HMw fractions ($R^2 = 0.98$). It should be noted that the samples that were isolated at different stages or under different conditions do not result in a single line in which the $K_{mix,405}$ correlates with the antioxidant activity. This might be due to a loss of antioxidant activity during each isolation step, whereas the recovery of the $K_{mix,405}$ level was good. Therefore, only samples isolated under similar conditions should be compared. The IMw and LMw fractions were not plotted because all of these fractions had similar TEMPO antioxidant activity and $K_{mix,405}$ values at different degrees of roast, which left little space for observing linearity. However, the solid-phase extraction fractions isolated from LMw showed differences in TEMPO antioxidant activity and $K_{mix,405}$ values; the values for these fractions are plotted in **Figure 7** as well. Also for these low Mw subfractions, a positive and linear correlation ($R^2 = 0.99$) was found. Finally, the ethanol precipitation subfractions of the medium-roast HMw fraction showed a positive and linear correlation ($R^2 = 0.99$) between the $K_{mix,405}$ value and TEMPO antioxidant activity. The fraction EP20 was excluded due to its limited solubility, causing an underestimation of $K_{mix,405}$ (*23*). A positive and linear correlation was found for the coffee brews, HMw fractions, and the subfractions of both the HMw material and LMw material. From these results, it could be concluded that there is indeed a positive and linear correlation between the $K_{mix,405}$ values and TEMPO antioxidant activity for all series, proving that the extent of roasting-induced antioxidant formation is directly linked to the extent of melanoidin formation. From the latter, it can be concluded that the roasting-induced antioxidants formed upon roasting of coffee beans are present within coffee melanoidins. To the best of our knowledge, this is the first time that a positive and linear correlation between roasting-induced antioxidants and melanoidin level has been reported in a complex product such as coffee.

General Discussion. At present, not much information is available on the bioavailability of coffee melanoidin antioxidants. Rufián-Henares and Morales (*34*) reported on the degradability of coffee melanoidins by human digestive enzymes present in the gastrointestinal (GI) tract and its effect on the antioxidant activity. Gniechwitz et al. recently reported on the degradability of coffee brew material that was rich in dietary fiber and melanoidins by human fecal microbiota (*35*). However, research on the digestibility and bioavailability of coffee melanoidins is still in its infancy. In general, there is a consensus that melanoidins, of any source, have a low digestibility and bioavailability (*36*). There is also evidence that melanoidins are not digested in the upper part of the GI tract and are mainly recovered in the feces (*37*). As most of the roasting-induced antioxidants are present in high Mw melanoidins and as melanoidins are complex structures, these molecules are probably not digested and not taken up in the bloodstream at all. As a result, it is then likely that coffee melanoidins will pass through the GI tract and that the roasting-induced antioxidants will show antioxidant activity in the intestine if these antioxidants survive gastric conditions. Coffee melanoidins, containing both phenolic antioxidants (*18*) and roasting-induced antioxidants, might then provide protection against a wide range of radicals throughout the GI tract. It was calculated that the high Mw melanoidins present in one cup of coffee (150 mL) will show antioxidant activity against the radical TEMPO equivalent to 30 mg of Trolox, a derivative of vitamin E. This is twice as much as the daily recommended intake for vitamin E (*38*), showing that coffee melanoidins may significantly contribute to the desired daily antioxidant consumption. Of course, much research needs to be conducted to elucidate the reaction mechanism and radical specificity of antioxidants formed in coffee beans upon roasting.

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