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Characterization of Free Radicals in Soluble Coffee by Electron Paramagnetic Resonance Spectroscopy

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EPR spectra of soluble coffee display single-line free radical signals in both the solid state and aqueous solution, along with signals from the paramagnetic ions Fe(III) and Mn(II). The intensity of the free radical signal in the pure solid was estimated to be ca. 7.5×10^{16} unpaired electrons/g, and there was no significant change on dissolution in water. In aqueous solutions, however, the free radical signal declined rapidly over ca. 10−15 min in the temperature range 20−65 °C, after which only slow changes were observed. This decline, which was essentially independent of atmosphere, was greatest for the lowest temperatures used, and the intensity after 1 h fitted well to an exponential curve with respect to temperature. The free radicals responsible for the single-peak EPR signal did not react with any of the spin traps tested in the present experiments, but unstable free radicals with parameters consistent with adducts of C-centered radicals were detected in coffee solutions in the presence of PBN and 4-POBN spin traps. The presence of oxygen in the solutions increased the initial rate of formation of these free radical adducts. No adducts were detected when DEPMPO was used as spin trap. However, •OH adducts of DEPMPO were shown to be unstable in the presence of coffee, a fact which illustrates the strong free radical scavenging ability of coffee solutions.

KEYWORDS: Coffee; oxidation; EPR spectroscopy; free radicals; transition metal ions

INTRODUCTION

Oxidative processes are responsible in a major way for the instability of coffee flavor during storage (*1*−*6*). The electron-transfer reactions, which are characteristic of oxidative processes, often involve the generation of free radicals. Although many free radicals have very short half-lives at room temperature, especially in the fluid environment, some are stable and can be measured directly by EPR spectroscopy, a technique which selectively detects free radicals and other paramagnetic species. Several previous workers have used EPR spectroscopy to reveal a strong and stable radical signal in R&G, soluble, and liquid coffee (e.g., *7*−*12*), and there has been a considerable amount of speculation as to the origin and chemical nature of the signal. Recently, Hofmann et al. (*13*) have shown that a free radical with EPR spectral characteristics similar to those of the 1,4-bis(5-amino-5-carboxy-1-pentyl)pyrazinium radical cation (*14*, *15*) is formed in a range of thermally treated food products, including coffee. Hofmann et al. (*16*) also provided evidence for the involvement of protein-bound lysine in the radical formation, but it is by no means clear what fraction of the total free radical activity this represents.

The objective of the present study was two-fold. First, we wanted to characterize fully the EPR spectral characteristics of the free radical and transition metal components in soluble coffee and to investigate changes that occur with time during storage in solution. Some preliminary results of this work have been presented at the 19th International Conference on Coffee Science (*17*), where the presence of Fe(III) and Mn(II) metal signals was reported along with those from free radicals.

It is generally assumed that the free radicals in coffee are extremely long-lived. We have, however, shown previously that ascorbic acid is destabilized in coffee solutions above ca. 320 K (47 °C) (*12*), and that over a period of several days there is a noticeable effect of oxygen on the free radical content of coffee solutions (*17*). These results suggest, therefore, that free radical reactions occur in the beverage.

The second objective of the present work was, therefore, to investigate the formation of (unstable) free radicals in coffee solutions. These experiments have made use of chemicals known as "spin traps", which react with unstable free radicals to produce (more) stable radicals (radical adducts). The magnitudes of the hyperfine splittings of radical adducts are dependent (to some extent) on the chemical nature of the trapped radical (*18*), so it is often possible to identify the initial radical. In the present work, we have used three different nitrone spin traps, PBN, 4-POBN, and DEPMPO, which generate nitroxides after reaction with free radicals. EPR spectra of PBN and 4-POBN adducts in fluid solutions consist of six peaks from the ^{14}N of the nitroxide group and the ^{1}H on the α-carbon atom (i.e., that adjacent to the nitroxide). With DEPMPO, an additional doublet

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hyperfine structure is seen from the ^{31}P nucleus. Specifically, spin traps were used to study the generation of free radicals in coffee solutions during oxidation at 60 °C, which is a fairly typical domestic storage temperature after preparation. Some preliminary results from this work have been presented in ref *17*.

MATERIALS AND METHODS

Origin and Preparation of Samples. Several jars of a popular soluble coffee were purchased from a local supermarket, and the same brand and batch were used for all measurements in order to avoid effects which might be attributable to variations in bean genetics, production, postharvest storage, roasting conditions, extraction process, etc. The spin traps PBN and 4-POBN, and the stable free radical DPPH, were purchased from Sigma-Aldrich (Poole, UK), DEPMPO from Calbiochem (Beeston, Notts., UK), and ethanol from Fluka (Gillingham, UK). Deionized water was prepared with a Purite Select Analyst HP25 water purifier (Thame, Oxon, UK).

The coffee was studied directly as a pure solid or a 5% aqueous solution in air or nitrogen. In addition, spin-trapping measurements were made on 5% coffee solutions; these closely followed the procedure of Uchida and Ono (*19*) for determining the oxidative stability of beer. In initial experiments, 0.2 mL of a 2.55 M solution of PBN in aqueous ethanol (1:1 v:v) was added to 10 mL of a 5% solution in water of soluble coffee, giving a final concentration of 0.05 M PBN. The coffee solution was made up in deionized water that either had natural oxygen content (~8 ppm) or was deoxygenated by flushing with nitrogen gas for 15 min. The spin trap solution was added to the coffee solution either immediately after preparation or after the coffee solution had been aged at 60 °C for 18.5 h, and these solutions were then incubated in air at 60 °C. The incubation procedure involved transferring 1.4-mL aliquots to 4-mL amber vials (66% headspace), which were placed in a heating block (Pierce and Warriner, Chester, UK) at 60 °C. Vials were removed at intervals during a 4-h period, and 0.4-mL aliquots were inserted into a quartz flat cell for measurement of their EPR spectra at room temperature (20 ± 1 °C). For work with 4-POBN, 466 mg of the spin trap was dissolved in 10 mL of 5% coffee solutions to give a final concentration of 240 mM 4-POBN. As with PBN, the spin trap was added either to freshly made coffee solutions or to coffee solutions that had been aged at 60 °C for 18.5 h. EPR spectra of 0.4-mL aliquots, taken after various periods of incubation at 60 °C, were recorded in a quartz flat cell at room temperature as in the PBN-based experiments, or at 77 K on 0.4-mL aliquots in 4-mm-i.d. quartz tubes. The experiments with DEPMPO were identical to those with 4-POBN at room temperature, except that the concentration of the spin trap was 100 mM. All experiments were performed at least in duplicate on separate days.

EPR Spectroscopic Measurements. EPR spectra were all recorded on a Bruker ESP300E computer-controlled spectrometer (Bruker (UK) Ltd., Coventry) operating at X-band frequencies (~9.5 GHz) and using a TM/9202 cylindrical cavity. Microwave generation was by means of a klystron (ER041MR), and the frequency was measured with a built-in frequency counter. Spectra were recorded at various temperatures in the range 20−65 °C (293−338 K) or 77 K in 1024 points as first derivatives of the absorption using 100 kHz modulation frequency. Measurements at temperatures above ambient (20 °C) were made using a Bruker variable-temperature (VT) unit, which passed air at controlled temperatures across the sample in the spectrometer. Low-temperature measurements were made by immersing the sample in liquid nitrogen in a quartz "finger dewar" which was inserted into the microwave cavity.

For measurements at room temperature of solid coffee samples and solutions without spin traps, sweep widths of 5 and 200 mT centered on 348 mT were used to optimize the spectra of the free radical and Mn(II), respectively. Frozen solution measurements were also made with a 500-mT scan centered on 250 mT and a 50-mT scan centered on 160 mT in order to observe the Fe(III) component with $g = 4.27$. The modulation amplitude for Mn(II) and Fe(III) measurements was set to 1 mT; that for free radical measurements was 0.5 mT (after ascertaining that no decrease in line width was obtained with lower values). For solutions containing spin traps, room-temperature measurements were made with 10 mW microwave power and 0.1 mT modulation amplitude over scan ranges of 6 mT for experiments using PBN and 4-POBN spin traps and 15 mT for those with DEPMPO. Low-temperature measurements with 4-POBN adducts used 0.1 mW microwave power, 1 mT modulation amplitude, and a scan range of 15 mT. With all samples, the receiver gain, conversion time, time constant, and number of scans over which spectra were accumulated were optimized for individual spectra.

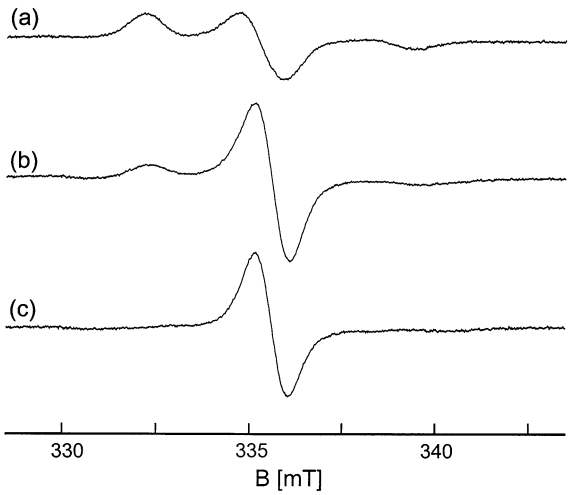

Figure 1. EPR spectra at 77 K of free radical adducts of POBN obtained during the oxidation at 60 °C of (a) beer and (b) a 5% soluble coffee solution. (c) Spectrum of the coffee free radical, which was obtained by subtracting spectrum a from spectrum b.

For all low-temperature spectra, the weak signal from the quartz "finger dewar" was subtracted from the experimental spectra before numerical analyses were performed.

Analysis of EPR Data. For monitoring variations in the single-peak EPR spectra as a function of time, intensities were measured as the product of the height and width squared of the distance between inflection points of the first derivative recording. Quantitative assessments of free radical concentrations were, however, made on double integrals of the first derivative recordings (see the following section). To minimize the contribution of instrumental noise to the intensity measurements, all spectra were smoothed by applying a 15-point second-order polynomial function (×2) after cubic background correction. The shapes of isotropic spectra were determined using the WinSIM least-squares fitting program from Public EPR Software Tools (P.E.S.T.), National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC 27709 (http://EPR.niehs.nih.gov). Simulations using an anisotropic spin Hamiltonian were made with the Bruker SimFonia software package. For solutions containing spin traps, intensity measurements were made on the low-field doublet using the Bruker WINEPR software package after smoothing as described above.

Intensity measurements on frozen solutions of spin trap (4-POBN) adducts were complicated by the superimposition of signals. Typical adduct spectra consist of three features (see, e.g., **Figure 1a**). The two outermost peaks correspond to the first and third peaks of the $A_{||}(^{14}N)$ triplet, while the central peak represents a combination of the peaks associated with $g_{\perp}$ and the central peak of the $A_{||}(^{14}N)$ feature; no ^{1}H hyperfine structure is resolved in these spectra. In samples from coffee (e.g., **Figure 1b**), the central peak also contains a contribution from the single-peak signal from the stable coffee radical (see above). Furthermore, the third peak of these anisotropic spectra overlaps the fourth peak of the Mn(II) spectrum, which is a regular feature of spectra from coffee solutions. The intensities of the adduct spectra at 77 K were, therefore, measured from the first peak of the anisotropic signal. An estimate of the intensity of the coffee radical signal was obtained by subtracting an appropriate fraction of the spectrum obtained with beer (**Figure 1a**), which has no stable free radical signal, from those of the coffee solutions, so that the intensities of the first peak became

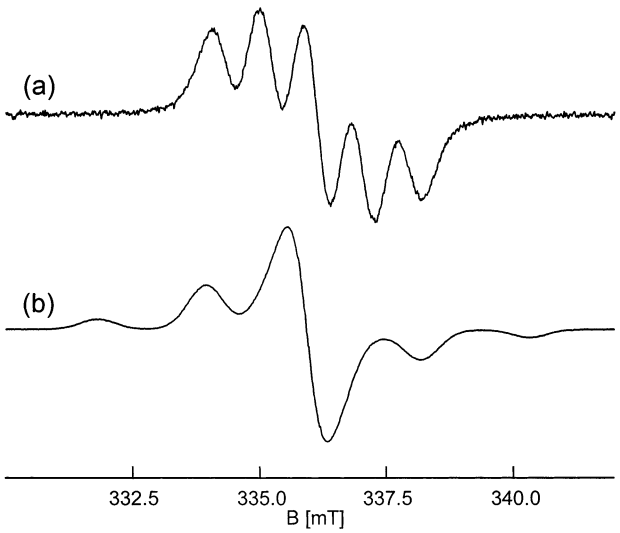

Figure 2. EPR spectra of a 1.0 mM solution of DPPH in toluene at (a) 20 °C and (b) 77 K.

zero. The resulting difference spectrum (**Figure 1c**) was then considered to be that of the coffee radical.

Estimation of Free Radical Concentrations. Although, the intensity of an EPR spectrum is proportional to the number of unpaired electrons in the specimen, the accurate determination of free radical concentrations is extremely difficult (see, e.g., *20*). We have used the following method to estimate approximate free radical concentrations in coffee.

The method is based on the use of the stable free radical, DPPH, as a standard reference material. In fluid solution, the EPR spectrum consists of five isotropic peaks (intensity ratio 1:2:3:2:1), resulting from the interaction of the unpaired electron with the two ^{14}N nuclei ($I = 1$) (**Figure 2a**), whereas at 77 K the spectrum is anisotropic (**Figure 2b**) as a result of immobilization of the molecules. In both cases, intensities were measured by double integration of the first derivative spectra recorded in 1024 data points over a 12 mT scan range. Curves showing the relationship between spectral intensity and microwave power (power saturation curves) for 1.0 mM solutions in toluene at 293 and 77 K are shown in **Figure 3a**,**b**, and the relationships between intensity and concentration are shown in **Figure 4**.

For our experimental samples, it was necessary first to determine the microwave power saturation curves for the spectra, to ensure that essentially nonsaturating conditions were being used. Free radical concentrations were then estimated according to the following methods while ensuring that essentially nonsaturating conditions existed for both DPPH and coffee spectra.

For solid coffee samples, the concentration (C) of free radicals in unpaired electrons per gram is given by the relationship

$$C = \frac{(\mathrm{Int_C})C_\mathrm{D}V_\mathrm{D}PA}{2.4(\mathrm{Int_D})\mathrm{M_C}}$$

where A is Avogadro's number (6.022×10^{23}), $\mathrm{Int_C}$ and $\mathrm{Int_D}$ are the intensities (normalized for receiver gain and number of scans in the acquisition) of the coffee and DPPH spectra, respectively, P is the ratio of the square roots of the microwave powers used for recording the coffee and DPPH spectra ($\sqrt{\mathrm{MP_D}}/\sqrt{\mathrm{MP_C}}$), M_C is the mass (grams) of coffee, C_D is the concentration of DPPH (moles per liter), V_D is the volume (in liters) of DPPH solution in the sample tube, and the factor 2.4 arises from the different scan ranges used for recording the DPPH and coffee spectra.

For coffee solutions the relationship is

$$C = \frac{(\mathrm{Int_C})C_\mathrm{D}PA}{2.4(\mathrm{Int_D})C_\mathrm{C}}$$

where C_C is the concentration of the coffee solution (grams per liter).

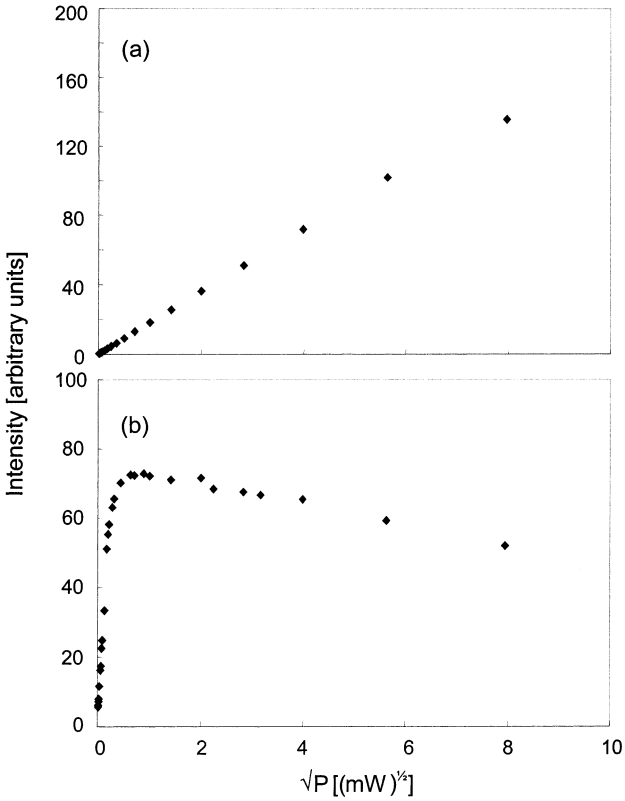

Figure 3. Saturation curves for a 1.0 mM solution of DPPH in toluene at (a) 20 °C and (b) 77 K.

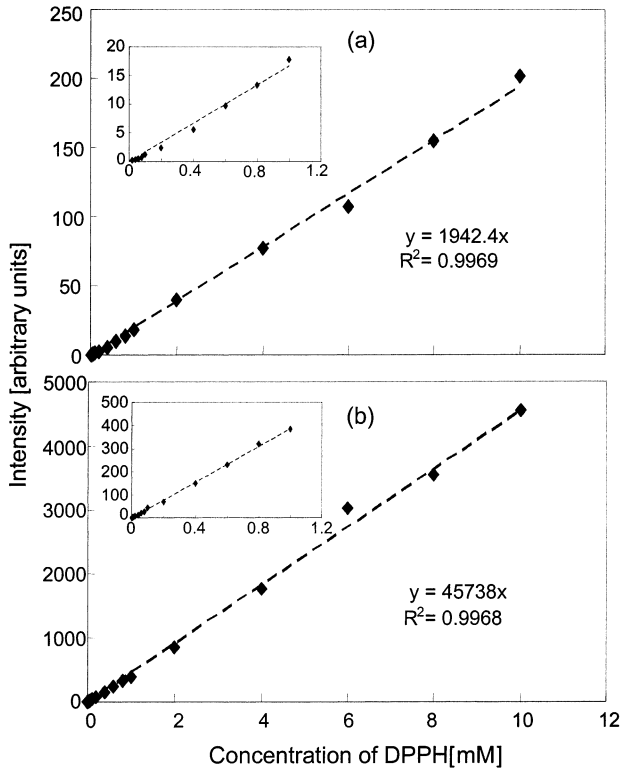

Figure 4. EPR spectral intensity versus concentration for DPPH in toluene at (a) 20 °C in a flat cell and (b) 77 K in a 4-mm-i.d. tube. Both sets of spectra were acquired using 0.01 mW microwave power and 0.5 mT modulation amplitude.

It should be noted that at room temperature, spectra of coffee and DPPH solutions were measured in the same flat cell, and at 77 K, equal volumes (200 μL) were measured in 4-mm-i.d. quartz tubes.

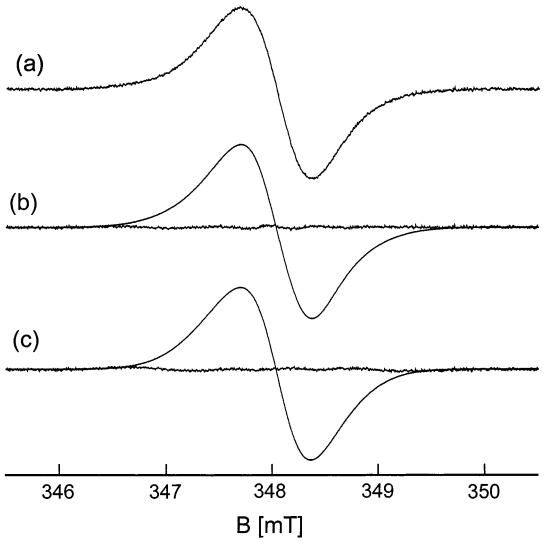

Figure 5. (a) EPR spectrum of soluble coffee powder, (b) a computer fit to three isotropic components with Gaussian shape ($g = 2.0044$, $\Gamma = 0.98$ mT, 61%; $g = 2.0043$, $\Gamma = 0.57$ mT, 34%; $g = 2.0039$, $\Gamma = 0.31$ mT, 5%), and (c) simulation with one anisotropic component having Gaussian line shape and $g_{xx} = 2.0045$, $\Gamma = 0.98$ mT; $g_{yy} = 2.0042$, $\Gamma = 0.80$ mT; $g_{zz} = 2.0040$, $\Gamma = 0.40$ mT. Traces b and c also show the differences between the experimental and simulated data.

RESULTS

Free Radical in the Solid Coffee Powder. The EPR spectrum of solid coffee powder consisted of a single peak with $g \approx 2.0043$ and a first derivative peak-to-peak line width of ~0.6 mT under nonbroadening conditions (**Figure 5a**). This is similar to results reported in the literature (e.g., *10*, *11*, *21*). Such peaks are commonly seen in roasted beans and grains and have been assigned to either charred polysaccharides (*11*) or Maillard reaction products (*13*, *22*). The spectrum could not be fitted satisfactorily to a single line with Lorentzian or Gaussian line shapes, or any admixture of the two. A good fit was obtained when three Gaussian peaks were used (**Figure 5b**), but this is not a unique fit and the spectrum could also be fitted to a single anisotropic component (**Figure 5c**).

The saturation curves for coffee powder at room temperature and 77 K are presented in **Figure 6**. As expected from the Boltzmann populations of the electron spin energy states, the signal is more intense at the lower temperature, but it saturates at a lower microwave power. Free radical concentrations were calculated as 6.4×10^{16} and 8.6×10^{16} unpaired electrons per gram for a typical commercial specimen at 77 K and room temperature, respectively. The difference between these numbers is not regarded as statistically significant, because of the various unquantifiable errors involved in their calculation.

Free Radical in a 5% Aqueous Coffee Solution. The EPR spectrum of a 5% aqueous solution of coffee in air gave a single-peak free radical signal, which could be fitted to a single isotropic component with a 50:50 Lorentzian:Gaussian line shape and a peak-to-peak line width of 0.58 mT (**Figure 7**).

Measurements were also made on frozen coffee solutions (at 77 K) to examine whether there was evidence for any anisotropy in the free radical signal. After subtraction of the peak from the "finger dewar", the spectrum in **Figure 8a** was obtained. Its shape is intermediate between that of the solution spectrum at room temperature (**Figure 7a**) and that of the solid coffee powder (**Figure 5a**). It could be fitted to a single component with a small degree of anisotropy (**Figure 8b**). Saturation of this signal commenced at much lower microwave power (between 0.016 and 0.032 mW) than in fluid solutions at room temperature.

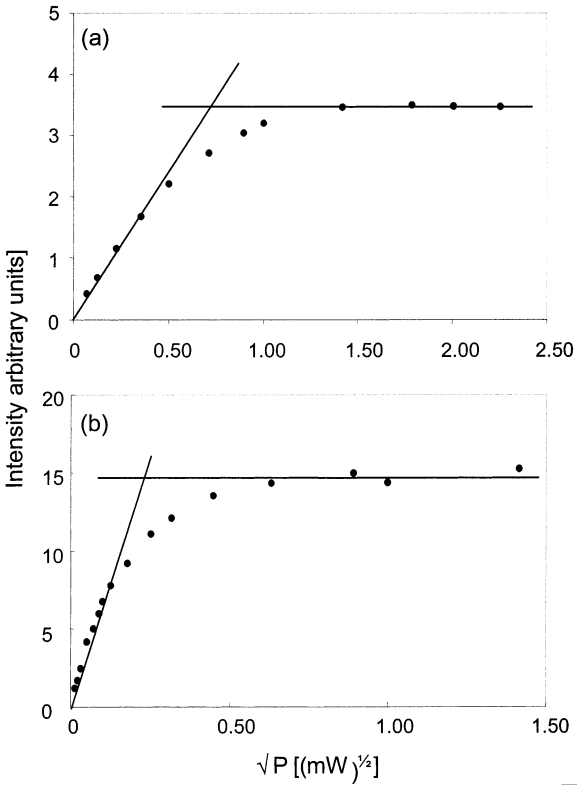

Figure 6. Power saturation curve for soluble coffee powder at (a) room temperature and (b) 77 K.

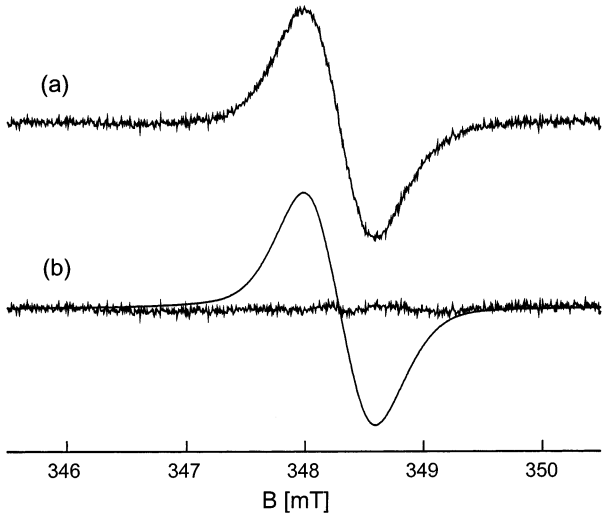

Figure 7. (a) EPR spectrum of 5% aqueous solution of soluble coffee in air at room temperature, and (b) computer fit (plus difference trace) with mixed Lorentzian/Gaussian (1:1) line shape, $g = 2.0044$ and $\Gamma = 0.58$ mT.

The intensity of the free radical signal in aqueous solution decreased progressively over a period of 10−15 min, after which it stabilized. The magnitude of this decrease was, however, temperature-dependent (**Figure 9**). The smallest decreases were observed with the highest temperatures, although the time scale was essentially temperature-independent. Determination of the free radical content of a rapidly frozen solution gave a value of 7.6×10^{16} unpaired electrons per gram of coffee, i.e., similar to the values calculated for the coffee powder. The correspond-

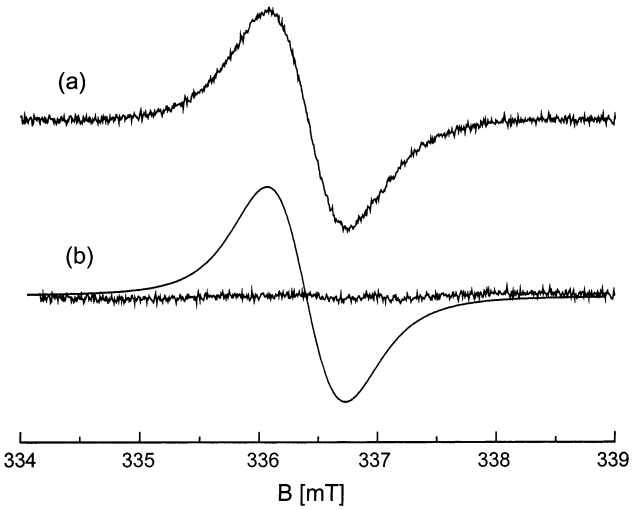

Figure 8. (a) Free radical EPR signal from 5% aqueous solution of soluble coffee at 77 K, and (b) computer simulation (plus difference trace) with Gaussian line shape and $g_{xx} = 2.0044$, $\Gamma = 0.57$ mT; $g_{yy} = 2.0041$, $\Gamma = 0.50$ mT; $g_{zz} = 2.0041$, $\Gamma = 1.20$ mT.

ing value for the fluid solution (after stabilizing at room temperature) of the same sample was 2.6×10^{16} unpaired electrons per gram. This decrease compared to the value at 77 K can be only partly explained by the short-term decrease shown in **Figure 9**. Absorption of microwaves by water decreases the sensitivity of the spectrometer, even though this effect is kept to a minimum by use of a flat cell; such variations in spectrometer sensitivity are a serious problem for accurate determination of free radical concentrations in samples containing water.

The influence of oxygen on the changes in free radical intensity in the soluble coffee solutions was also investigated by comparing the evolution of the free radical signal at room temperature in solutions made up under air or nitrogen atmospheres. The results (not shown) gave similar patterns for intensity versus time over a 30-min period, although there was a slightly greater decrease for the sample under anaerobic conditions.

Metal Signals in 5% Aqueous Coffee Solution. In addition to the free radical signals, EPR spectra of coffee solutions at ambient temperature contain an isotropic sextet component with a hyperfine coupling constant of 9.5 mT and a line width of ~3.2 mT (**Figure 10**). This is consistent with the solvated Mn(II) ion and probably originated in the unroasted coffee bean, since similar spectra are commonly seen in plant tissues (e.g., *23*−*26*). This signal did not saturate in the microwave power range that was used for the free radical measurements described above.

A wide-scan spectrum of 5% aqueous coffee at 77 K (**Figure 11**) shows the presence of four distinct features, a low-field component with $g = 4.27$ (at 160 mT) and three separate components with $g \approx 2.0$ (i.e., centered on 336 mT). These latter signals consist of a narrow component from the free radical(s) discussed in the previous section, a complex component from Mn(II), analogous to that seen in the room-temperature spectra, and a broad single peak, which arises from high-spin Fe(III) in magnetically interacting (i.e., polynuclear) environments (e.g., *27*). The $g = 4.27$ signal also arises from high-spin Fe(III), but in mononuclear complexes with "rhombic" symmetry (*28*). Other Fe(III) species may be present, but their EPR transitions are often highly anisotropic (and hence have zero height) in frozen solution measurements (*29*). None of the

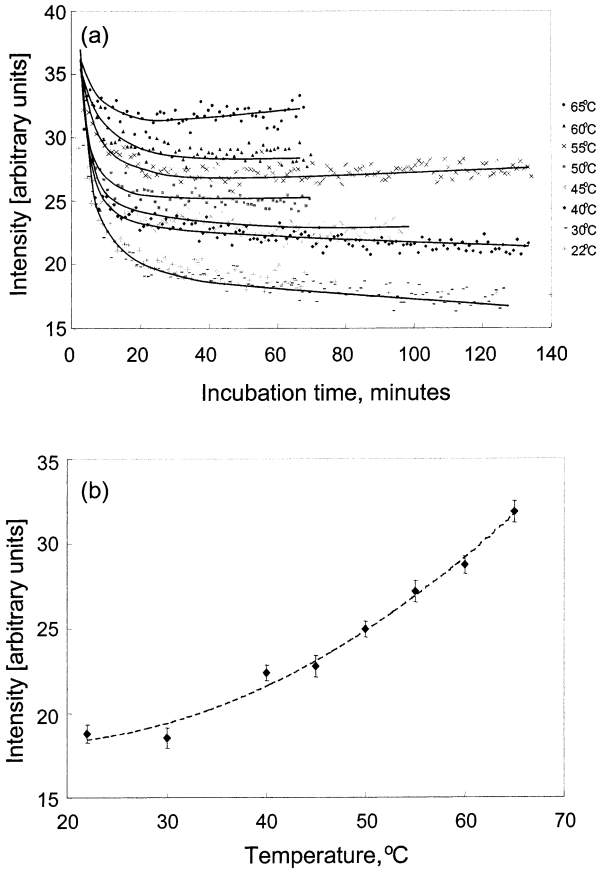

Figure 9. Intensity of the free radical EPR spectrum from 5% aqueous coffee solutions: (a) variation with time and temperature and (b) as a function of temperature after 60 min.

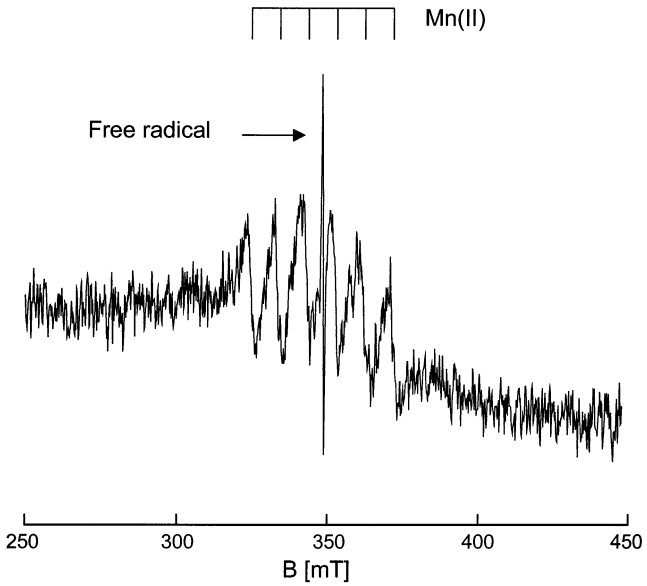

Figure 10. Wide-scan EPR spectrum of 5% aqueous coffee solution at room temperature.

Fe(III) signals showed any significant changes with aging of the solutions over a 5-day period.

Aqueous Coffee Solution at 60 °C in the Presence of PBN. The EPR spectra that were obtained when spin-trapping measurements were carried out during incubation at 60 °C of fresh solutions of 5% soluble coffee and PBN consisted of sextets arising from coupling of the unpaired electron with ^{14}N and ^{1}H nuclei on the α-carbon (**Figure 12a**). For short

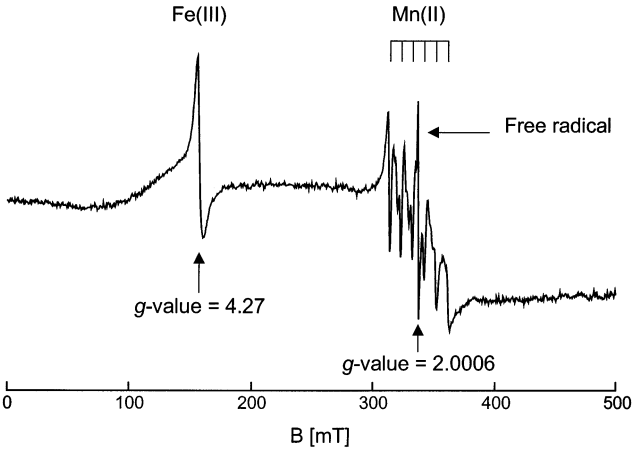

Figure 11. Wide-scan EPR spectrum of 5% aqueous solution of soluble coffee at 77 K.

incubation times, the single-peak coffee free radical was seen in the spectra in addition to the PBN adduct signal, but after about 45 min the spectra were dominated by the adduct component. The hyperfine splittings of the radical adduct are 1.61 mT for $a(^{14}N)$ and 0.33 mT for $a(^{1}H)$, which are consistent both with the •OH adduct of PBN, as assumed by Uchida and Ono (*19*) in their work on the oxidation of beer, and with an adduct of a C-centered radical (*18*). The intensity of the adduct signal increased progressively with time at 60 °C and showed no lag period comparable to that seen during the forced oxidation of beer (*19*). The intensity reached a maximum at around 150 min (**Figure 12b**) and then remained stable for at least 20 h (results not shown).

When fresh coffee solutions made with water that had been thoroughly deoxygenated were incubated at 60 °C in air, the rate of increase in the PBN radical adduct signal intensity (**Figure 12c**) was slower than that in solutions containing natural oxygen contents. Measurements made with similar coffee solutions that had been aged for 18.5 h at 60 °C before addition of the spin trap gave results that were independent of the oxygen content of the initial solution (**Figure 12d**,**e**) and were similar to those of the fresh sample made up in deoxygenated water. These results suggest that the oxygen content of the initial solution may be the most important factor determining the rate of generation of the EPR signal during subsequent oxidation, since the aged coffee solutions would have been severely depleted in oxygen as a result of its reaction with coffee.

Aging of 5% Aqueous Coffee Solution at 60 °C in the Presence of 4-POBN. To avoid the use of ethanol (or other organic solvent), further measurements were made using the water-soluble spin trap 4-POBN instead of PBN. The radical adduct of 4-POBN had hyperfine splittings, $a(^{14}N) = 1.56$ mT and $a(^{1}H) = 0.26$ mT, which correspond to a C-centered radical; these parameters are significantly different from those of the •OH adduct (*18*). Blank et al. (*30*, *31*) were able to observe the formation of both •OH and C-centered radical adducts with 4-POBN in model coffee solutions. The nonobservation of •OH adducts in the real coffee system may, therefore, reflect the greater concentration of organic molecules, which compete successfully with the spin trap for reaction with the •OH radicals. On the basis of this argument, it is likely that the spectra of the PBN adducts also correspond to C-centered radical adducts.

The pattern of behavior of spectral intensity during incubation at 60 °C was similar to that seen with PBN. For freshly made-up solutions, the rate of generation of the EPR signal was appreciably slower in the deoxygenated sample than in the

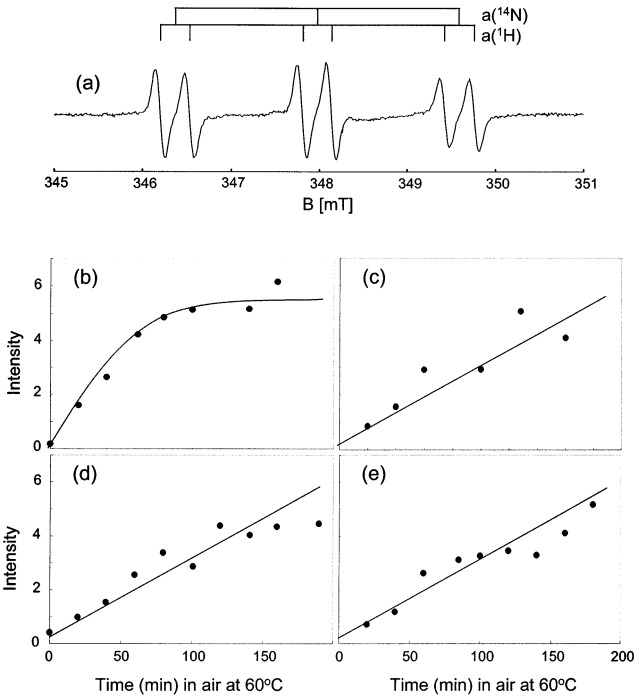

Figure 12. (a) Typical EPR spectrum at room temperature of the free radical adduct of PBN generated during incubation with 5% aqueous solutions of soluble coffee, along with plots of intensity versus time of incubation at 60 °C for freshly made solutions in water with natural oxygen content (b) and deoxygenated water (c), and solutions that had been aged for 18.5 h at 60 °C in air (d) and nitrogen (e) before addition of the spin trap.

equivalent sample with natural oxygen content. When similar measurements were made with coffee solutions that had been aged for 18.5 h at 60 °C before addition of the spin trap, the curves of EPR intensity against time of incubation with 4-POBN were essentially identical and very similar to that of the deoxygenated fresh coffee solution.

To obtain measurements of adduct intensity after short periods of incubation at 60 °C, samples were quenched in liquid nitrogen after various incubation times and their spectra recorded at 77 K. Spectral intensities were measured from the low-field peak, because of interference in the center and high-field peaks from the free radical and Mn(II) signals in the coffee. A plot of signal intensity versus time for the incubation of a fresh deoxygenated solution at 60 °C is shown in **Figure 13**. It is clear that free radical production commences with dissolution of the coffee powder and there is no lag time for EPR signal generation comparable to that seen in beer (*19*).

Behavior of the Coffee Free Radical Signal in the Presence of 4-POBN. The variation with time in intensity of the single-peak coffee free radical signal was also studied in these spectra by deconvoluting the central peak into its spin adduct and free radical components. A plot of the residual free radical signal as a function of time is similar to that from a similar solution incubated at 60 °C without the spin trap (**Figure 14**). Both decreased by about 35% during the first 10 min of incubation, after which they stabilized and then maybe increased slowly. This result illustrates that the coffee free radical does not interact with the spin trap.

5% Aqueous Coffee Solution at 60 °C in the Presence of DEPMPO. Measurements of EPR spectra of soluble coffee solutions in the presence of DEPMPO as spin trap showed no adduct signal under any of the experimental conditions investigated. This could be because the free radicals are not trapped,

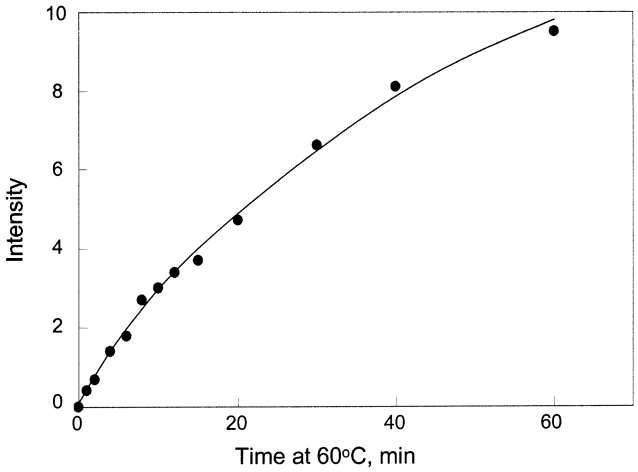

Figure 13. Variation with time of the intensity of the first peak of the EPR spectrum at 77 K of the 4-POBN adduct generated during oxidation at 60 °C of a freshly made 5% soluble coffee solutions in deoxygenated water.

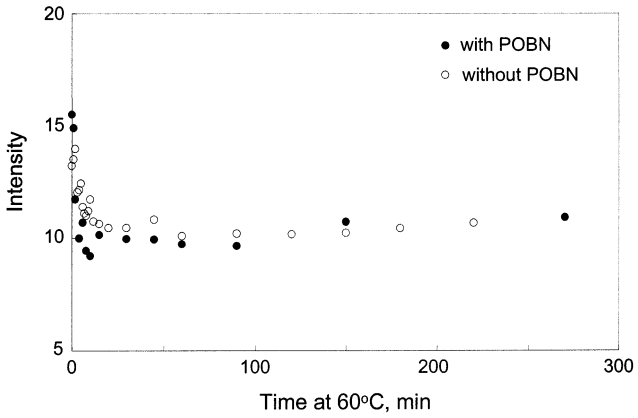

Figure 14. Variation with time of aging in air at 60 °C of the intensities of the EPR spectra at 77 K from the single peak free radical in 5% soluble coffee solutions incubated with and without the spin trap 4-POBN.

or because DEPMPO adducts are unstable in the presence of coffee. The latter hypothesis was tested by first forming the •OH adduct of DEPMPO from a solution containing 5 mM $FeSO_4 \cdot 7H_2O$, 88 mM H_2O_2, and 2 M DEPMPO; its EPR spectrum is shown in **Figure 15a**. This solution was then added (1:1 v:v) to a fresh 5% solution of soluble coffee at room temperature. The adduct signal disappeared immediately, leaving just the single-peak signal that is typical of coffee solutions (**Figure 15b**).

DISCUSSION

The EPR spectra of soluble coffee in the solid state and in aqueous solutions display single-line free radical signals along with signals from the paramagnetic ions Fe(III) and Mn(II). The spectrum from fluid solutions could be fitted to a single isotropic component, but the appreciable Gaussian contribution to the line shape suggests that there may be a distribution of closely related free radical species. The spectrum from frozen solutions was superficially similar to that from fluid solutions, but it required some anisotropy in the *g*-values in order to obtain a satisfactory simulation. The spectrum of the solid powder could also be simulated with a single anisotropic component, but it contained appreciably greater broadening in the wings than the frozen solution spectrum. The most likely explanation for the difference in overall spectral shape is an increase in dipolar

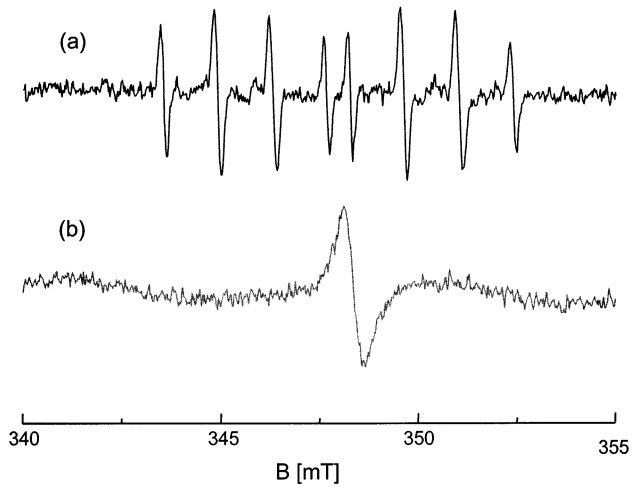

Figure 15. EPR spectra at room temperature of the •OH adduct of DEPMPO (a), generated from the reaction of Fe(II) and H_2O_2 in the presence of DEPMPO (see text for details), and (b) after addition of an equal volume of 5% soluble coffee solution.

interactions between neighboring free radical centers in the powder sample.

The variation with time of the intensity of the free radical signal in solution is remarkably similar to that observed for solutions of diethylpyrazinium diquat that had been heated to 95 °C for various periods of time (*16*). Hofmann et al. (*16*) found an inverse correlation between the free radical signal intensity and the color intensity of their solutions and interpreted their results in terms of the color development being associated with the decomposition of the radical cations. This is not likely to be the explanation for our present results, because the color of soluble coffee is well-developed before dissolution.

The effects of temperature on the free radical signal intensity show that in soluble coffee solutions, free radicals are being generated as well as destroyed, and the steady-state concentration fits well to an exponential curve in the temperature range 20−65 °C, which was investigated here. The decrease in free radical concentration immediately after dissolution was not caused by the presence of oxygen, because a similar and possibly greater decrease was observed when measurements were made under anaerobic conditions. Spin-trapping measurements, however, indicate that oxygen is involved in the generation of unstable free radicals in coffee solutions. These results, therefore, strongly suggest that two separate phenomena occur during the aging of soluble coffee solutions. First, there is an atmosphere-independent (i.e., it does not require oxygen) decrease in free radical concentrations over about 15 min. At the same time, unstable free radicals are generated, and this generation continues over extended periods under aerobic conditions. The initial rate of free radical generation is, however, strongly influenced by the oxygen content of the water used to prepare the coffee solution. It has been shown previously that the saturation characteristics of the free radical signal in coffee solutions that had been stored under air are slightly different from those of the initial solutions (*17*). Thus, the free radical processes which occur in coffee solutions lead to the generation of radicals, which are chemically distinct from those present initially. The initial radicals are probably related to the 1,4-bis-(5-amino-5-carboxy-1-pentyl)pyrazinium radical cation (*13*−*15*), but the identity of the radicals formed in the coffee solutions remains unidentified.

EPR spectral intensity increased progressively with time during incubation of coffee solutions in the presence of the spin

traps PBN and 4-POBN, and there was no lag period before the adduct signals were detected. This is in contrast to the observations reported with fresh beer samples (*19*), where there was a significant lag period prior to the detection of the adduct signals in fresh samples. One possible explanation is that the generation of •OH radicals via a Fenton reaction involving H_2O_2 is a fundamental step in the spin-trapping process. In beer, H_2O_2 is formed during the oxidative spoiling of the beverage, and this is mediated by the presence of antioxidants. As a consequence, free radical adduct formation with a spin trap is inhibited until these antioxidants have been severely depleted. In contrast, H_2O_2 is present from the start in soluble coffee solutions (*32*), and there is, therefore, no delay in the initiation of the Fenton reaction.

The measurements made with 4-POBN as spin trap indicate that the trapped free radical is a C-centered entity and is not the •OH radical. Although a fundamental role has been postulated for •OH in the previous paragraph, and was strongly indicated by biochemical measurements in the work of Uchida and Ono (*19*) on beer, its nonobservation is not altogether surprising. The •OH radical is able to extract H atoms from a wide range of organic molecules, generating C-centered radicals in the process. These latter radicals are generally more stable. It is likely, therefore, that such reactions occur in the beer and coffee solutions, because the spin trap molecules are present at much lower levels than the organic molecules of the beverages. Indeed, with beer, it is probable that the $•CH_2CH_2OH$ radical is formed and trapped in the spin-trapping assay.

The absence of any EPR signal from coffee in the presence of DEPMPO is in contrast to findings reported by Pascual et al. (*33*) for model coffee solutions, where •OH and C-centered radical adducts with DEPMPO were stable for appreciable periods of time. Their absence in the real coffee system thus provides further evidence for the presence of strong free radical scavengers in coffee.

The antioxidative activities of the coffee components, caffeic and chlorogenic acids, have been studied in vitro in various model systems (*34*). In coffee, the degree of roasting and formation of Maillard reaction products correlate positively with hydrogen peroxide formation, but rate of formation of the Maillard reaction products depends on numerous effects, including oxygen tension, pH, temperature, and transition metal availability (*35*, *36*). Some of this activity could be linked to degradation products of caffeic and chlorogenic acids. In vitro mutagenic and oxidative effects have been linked to polyphenols under certain conditions and in the presence of H_2O_2, yet strong evidence also exists for antioxidant and antimutagenic activity under biologically relevant conditions (*37*−*39*). In a FRAP assay for antioxidant activity (*40*), a 1% solution of soluble coffee (same brand, but different batch, from that used for the EPR measurements), gave a value of ca. 13 000 μM (E. C. Pascual, unpublished results), which is comparable to those of many fruit juices (*41*).

In any discussion of health effects, it must be borne in mind that coffee is a traditional food, with a long history of safe use for humans. Coffee and coffee components have been subjected to extensive investigations in animal and human model systems (*39*). Reviews of this information indicate that evidence for a direct link between coffee intake and adverse health effects is limited and inconsistent. On the contrary, human epidemiology strongly suggests the possibility of beneficial health effects (*39*), and overall, the available scientific information supports the safety of moderate coffee consumption (*42*).

ABBREVIATIONS USED

DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DPPH, diphenylpicrylhydrazyl; EPR, electron paramagnetic resonance; *I*, nuclear spin; PBN, phenyl-*N*-*tert*-butylnitrone; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; R&G coffee, roast and ground coffee; Γ, spectral line width (first derivative peak-to-peak).

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