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Free Radicals in Wheat Flour Change During Storage in Air and are Influenced by the Presence of Ozone During the Growing Season

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Electron paramagnetic resonance (EPR) spectra of wheat flour show components from Fe(III), Mn(II) and free radicals (FR). The metal signals were higher in the samples from the stressed plants, and reflected the higher total levels of these elements determined analytically. They remained essentially constant throughout the experiment, but the FR signal increased progressively with time over a period of 4–6 months after milling, after which it reached a maximum. The rate of increase in the FR signal during this period was considerably higher in the flour from plants that had been exposed to elevated ozone levels.

Keywords: Free radicals; Wheat flour; Electron paramagnetic resonance (EPR); Ozone

INTRODUCTION

Elevated levels of ozone are recognised as an abiotic stress, which affects the yields of cereals, such as wheat.[1,2] Exposure to elevated levels of ozone has also been shown to increase the rates of unstable free radical generation in leaves of pea and bean[3] and perennial ryegrass,[4,5] and the levels of stable free radicals (FR) in freeze-dried wheat leaves.[6] There is, however, currently little information on the effects of exposure to elevated ozone levels during the growing season on the properties of harvested food products. In this paper, we report the results of an investigation of flour from wheat grown under ambient and elevated ozone levels using electron paramagnetic resonance (EPR) spectroscopy to measure directly the signals from stable free radicals and associated paramagnetic transition metal ions. After milling the grain, measurements were made over a period of approximately 10 months, which was considered to cover a typical timescale over which the product might be stored during domestic use.

MATERIALS AND METHODS

Sample Production and Preparation

Seeds of winter wheat (*Triticum aestivum* L. cv. Perlo) were germinated at 20°C for 24 h and vernalised at 1°C for two months (October–December); seedlings were then planted in eight 12-l pots filled with a standard soil mix (Frux ED 73). For the first three weeks after emergence, the day/night temperature was kept at 15/10°C; it was then changed to 20/12°C for the remainder of the experiment, which was performed in a temperature-controlled greenhouse. Plants were thinned to 15 per pot, and from growth stage 21,[7] they were grown either in an atmosphere with ambient ozone (22 ± 6 nmol mol^{-1}) or exposed to 80 nmol mol^{-1} ozone (7 days/week, 09.00–17.00). Ozone was produced from pure oxygen by an ozone generator (Fischer Model 502, Meckenheim, Germany).

Fully mature wheat plants from each pot were harvested manually at the beginning of June and

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ears were threshed using a threshing-machine (F. Walter & H. Wintersteiger KG, Ried/Innkreis, Austria). Grains were then stored for 42 months in closed plastic boxes in a cold room (4°C) until milling using a ball mill (MM2, Retsch GmbH & Co KG, Germany).

After milling, all samples were transferred to the EPR laboratory, where they were kept at ambient temperature in an air-conditioned room (20 ± 2°C). EPR measurements were commenced 30 days after milling. Specimens (approximately 200 mg) were weighed into 4 mm i.d. quartz tubes, where they were kept for the duration of the experiment. Tubes were filled to ~1.5 cm of their 25 cm length and sealed with a plastic cap; the caps were removed for a short period after each measurement, then replaced. The samples can, therefore, be considered to have been stored in air with ambient composition. In addition, three separate sets of sample were taken from the original batches, which were also stored in air, at different time intervals to check whether any observed changes were the result of a previous spectral measurement history.

All determinations were made on eight replicate samples from each treatment.

EPR Spectroscopy

EPR measurements were performed with a Bruker ESP300E (Bruker UK Ltd., Coventry) computer-controlled spectrometer operating at X-band frequencies and using an ER4103TM cylindrical cavity. Microwave generation was by means of a klystron (ER041MR) and the frequency was measured with a built-in frequency counter. All spectra were recorded using 100 kHz modulation frequency in 1024 points at −196°C (77 K) with the sample immersed in liquid nitrogen in a quartz "finger dewar" (Wilmad WG816B). Other parameters used for acquisition of the various spectral components are shown in Table I.

Signal heights for the spectral components were measured using the WIN-EPR software on the spectrometer, and then normalised for receiver gain and unit weight of sample. Since the Mn and Fe spectra did not change during the course of the experiment, the relative heights of the signals were taken as proportional to their intensities. For the free radical signal, the intensities were taken as proportional to $y(\Delta H_{pp})^2$, where y is the amplitude and ΔH_{pp} the field separation of the 1st derivative peaks.[8] g-Values were calculated by reference to diphenylpicrylhydrazyl (DPPH) ($g = 2.0036$), which was used as an external standard.

Chemical Analysis

Analyses for total manganese and iron were performed by digesting the flour in (5:1 by volume) nitric acid/perchloric acid in an automatic heating block (Behrotest TBS 200, Behr Labortechnik, Germany) with the following temperature cycle: 1 h at 50°C, 0.5 h at 80°C, 0.5 h at 150°C, 1 h at 180°C, 0.5 h at 210°C. The samples were made up to a final volume of 25 ml with deionised water, and the concentrations of Fe and Mn measured with a plasma emission spectrometer (Vista AX, Varian Inc. Australia).

RESULTS AND DISCUSSION

Typical EPR spectra obtained from the flour samples are shown in Fig. 1. Four distinct components can be identified, and these correspond to free radical, Mn(II) and Fe(III) species. Such spectra are common in plants and plant-derived food products.[9–11]

The free radical signal (Fig. 1d) has a g-value of 2.0043 and a 1st derivative peak-to-peak linewidth in the range 0.75–0.82 mT. This g-value, which remained essentially constant throughout the experiment, is somewhat lower than the value of 2.0053–2.0059 reported for extruded flour.[12] The plot of the relationship between signal intensity and microwave power (Fig. 2c) shows that the free radical saturates easily at 77 K. Some saturation can be seen for microwave powers >0.1 mW, whereas Schaich and Rebello[12] reported no saturation for microwave powers <10 mW for the radical in extruded flour at this temperature. These results indicate, therefore, that the chemical nature of the radical(s) seen in the present work is distinctly different from those seen in extruded wheat flour. Schaich and Rebello[12] produced strong arguments for the assignment of the radicals in extruded flour to N- and S-centred species trapped in protein molecules, and these suggest that we are not dealing with a protein-based radical in the present work. This is not conclusive,

TABLE I EPR spectral acquisition parameters used for flour samples

Signal	Centre field (mT)	Field scan (mT)	Microwave power (mW)	Modulation amplitude (mT)	Conversion time (ms)	Time constant (ms)
Whole spectrum	250	400.0	5.0	1.0	40.94	10.24
Fe(III)	160	50.0	10.0	1.0	40.96	40.96
Mn(II)	336	100.0	0.16	0.5	20.48	10.24
FR	336	6.0	0.10	0.16	40.96	40.96

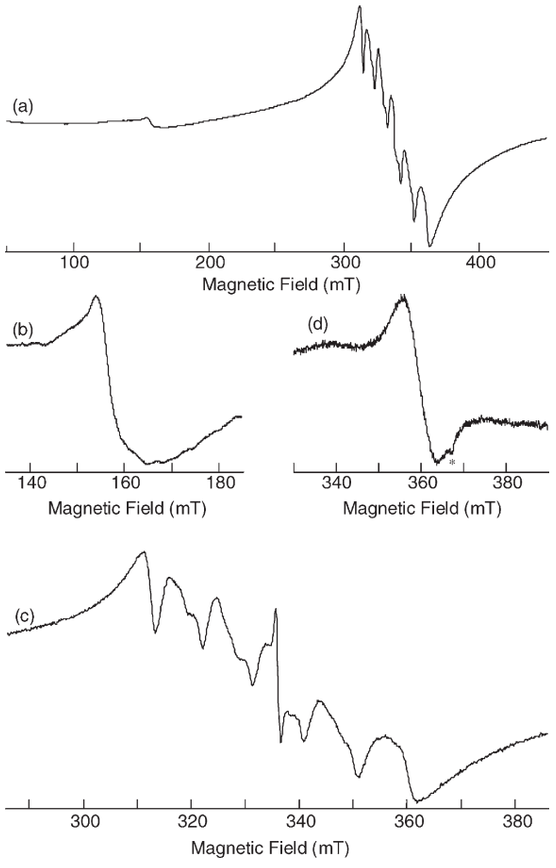

FIGURE 1 Typical EPR spectra of wheat flour at 77 K; (a) overall spectrum, (b) the Fe(III) $g = 4.27$ signal, (c) the Mn(II) signal, and (d) the free radical signal. Spectral acquisition parameters are given in Table I. The small peak marked by * in spectrum (d) arises from a radical centre in the "finger dewar".

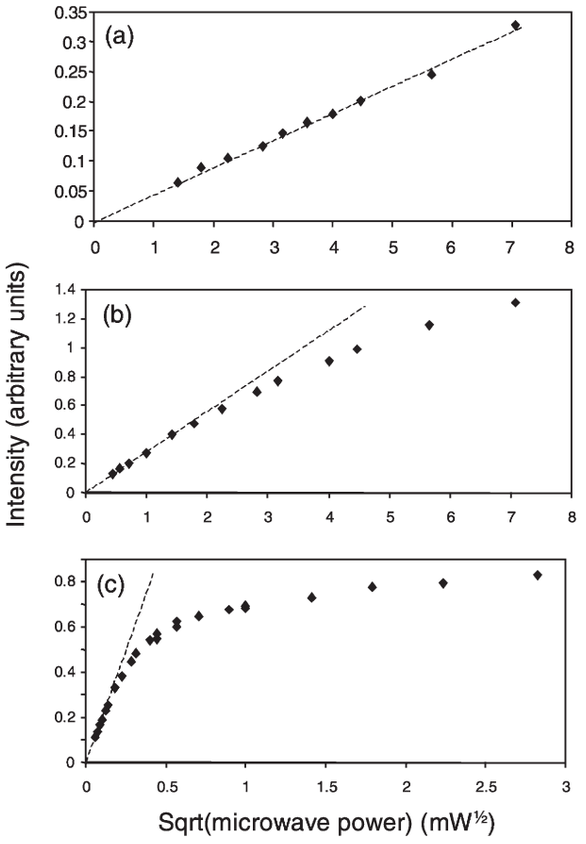

FIGURE 2 Microwave power saturation curves for (a) the Fe(III) $g = 4.27$, (b) the Mn(II), and (c) the free radical signals in wheat flour samples. The dashed lines indicate the signal intensities in the absence of saturation.

however, because the tyrosine-based radical in the thylakoid protein complex, known as Photosystem II, in leaf tissue has similar g-values[13] and saturation characteristics (our unpublished results) to those observed here. Nevertheless, we are able to eliminate a number of possibilities for the identity of the radical(s) in our flour samples. The radical is stable over long periods of time (even though the spectral characteristics are similar to those observed to be turning over rapidly in living tissue,[14] it is inconceivable that rapid radical turnover will occur in a stable product such as milled flour). Thus unstable radicals, such as hydroxyl or superoxide can be eliminated as being responsible for our spectra. The presence of a peroxyl species can also be eliminated, because the g-value is too low.[15] The g-value is, however, within the range observed for phenoxyl radicals,[15] and it is conceivable that it could correspond to a flavonoid or semiquinone. The stability of such phenoxyl radicals could be enhanced by their incorporation into cell wall material,[16] and such insoluble forms account for ~75% of the phenolic content of wheat grains.[17] Also, we cannot exclude the possibility of a C-centred radical, since the g-value is within the range reported for these species.[15]

There was a small but progressive increase in linewidth with increasing age of both sets of sample, suggesting that the radical formed slowly on storage was not identical to that present initially. The rate of this increase was greater for the samples from the plants grown under elevated ozone levels (Fig. 3). In both sets of sample, plots of the 1st derivative peak-to-peak linewidth, Γ (mT), against time (days) from milling, D, were essentially linear. The coefficients a and b in the relationship $\Gamma = a + bD$ were, respectively, 0.754 and 10×10^{-5} for the control samples and 0.770 and 16×10^{-5} for the samples from the plants which had received elevated ozone levels.

EPR spectra from Mn(II) ions are characterised by sextet hyperfine structures, arising from interactions of unpaired electrons with the ^{55}Mn nucleus, which has spin, I, equal to 5/2. The g-value was approximately 2.00 and the hyperfine splitting approximately 9.55 mT for both groups of flour sample; this value for the hyperfine splitting indicates that the manganese is in a largely ionic environment, such as occurs on coordination to

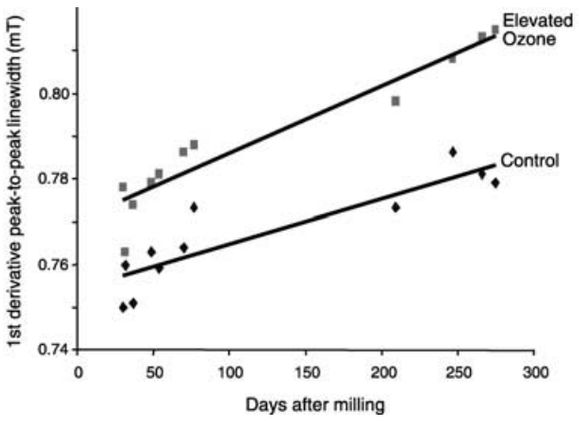

FIGURE 3 Variation with time from milling of the linewidths of the free radical signal in flour from plants grown under control and elevated (80 nmol mol^{-1}, 8 h d^{-1}) ozone concentrations.

oxygen nuclei.[18] In plant tissues, such spectra have often been assigned to the solvated Mn(II) ion (e.g. Monk *et al*.[19]). The spectrum in Fig. 1c, however, shows additional peaks, with approximately 20% of the intensities of the main peaks, equally spaced between the hyperfine peaks. These additional "forbidden" peaks arise as a result of an interaction between the ^{55}Mn quadrupole moment and the electric field gradient at the manganese nucleus. Since the intensity of these peaks is proportional to the square of the ratio (effective quadrupole splitting)/(effective hyperfine splitting),[20] there must be an appreciable electric field gradient at the manganese nucleus. The ligand environment cannot, therefore, be symmetrical, and hence the spectrum does not correspond simply to the solvated Mn(II) ion. Furthermore, the quadrupole interaction affects the positions of the "allowed" transitions, and introduces inaccuracies into the determination of the g- and hyperfine values by conventional measurement. For this reason, the quoted values for these parameters are stated to be approximate.

The signal at *c*. 155 mT ($g = 4.27$) is commonly assigned to mononuclear Fe(III) complexes with (near) rhombic symmetry,[21] although a signal in this position can also be observed with tetragonal symmetry in a strong crystal field.[22] Such signals are very common in nature,[23] and occur because of the isotropic nature of the relevant transition. Transitions between other electron spin levels are generally highly anisotropic,[24] and consequently their intensities approach zero in powder samples. The broad resonance with $g \approx 2.0$ is associated with magnetically-interacting ions.[23,25] These are typical of Fe(III) oxyhydroxide species, as occur in the cores of iron storage proteins, such as ferritin,[26] but could also involve Mn(II) species. This latter signal has not been considered further in this paper.

The Fe(III) $g = 4.27$ signal showed no tendency to saturate at 77 K with microwave powers up to 50 mW, the maximum used in the present experiments (Fig. 2a). The Mn(II) signal also showed little tendency to saturate at this temperature, but a small degree of saturation of the signal was evident at microwave powers >3 mW (Fig. 2b).

There was no significant change in the intensity of the Mn(II) signal in the two types of flour sample during the 9 months of the experiment. It was, however, $\sim$10% higher in the samples from plants grown under elevated ozone compared to the controls. The Mn(II) signal was thus used as an internal reference for determining variations in the Fe(III) and free radical intensities, since this decreased some of the errors in the measurements. The Fe(III) signal was approximately 25% higher in the samples that had been exposed to elevated ozone than in the control samples (Fig. 4b).

The differences in the intensities of the Mn and Fe signals from the control and stressed plants reflect the different total levels of these elements (Table II). Thus growth of the plants for long periods in elevated ozone levels increased the levels of these metals (especially iron) in the grains, but there is no evidence for ozone-induced changes in their speciation. We do not at present know whether these observations represent ozone-induced increases in uptake of these metals from the soil, or if they are the consequence of altered partitioning within the plant.

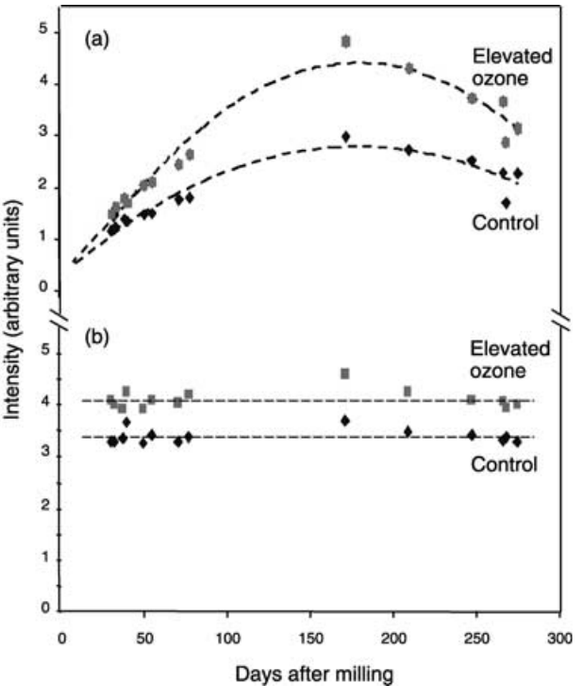

FIGURE 4 Variation with time of the intensity of (a) the free radical and (b) the Fe(III) $g = 4.27$ signals of flour from wheat grown under ambient and elevated (80 nmol mol^{-1}, 8 h d^{-1}) ozone concentrations.

TABLE II Fe and Mn contents of the flour samples

	Fe ($mg\,kg^{-1}$)	Mn ($mg\,kg^{-1}$)
Control	55 ± 21	61 ± 28
80 ppb ozone	99 ± 39*	75 ± 20

* Indicates a significant differences between treatments (Student's *t*-test), $p < 0.05$.

They are, however, relevant to the relationship between food quality and environmental conditions, which will be the subject of a future research programme.

The free radical signal intensities (Fig. 4a), in contrast, changed appreciably as a function of time after milling and reached a maximum at around 180 days. At this time the free radical signal was ~60% higher in the flour from the plants that had experienced elevated ozone levels than in the control samples. Both curves converged, however, on extrapolation back to zero time (i.e. the time of milling). These results are shown in Fig. 4. Although it was not possible to check retrospectively with these samples for any changes in the free radical signal in the short-term after milling, such measurements were made with similar samples from a different experiment. These showed that there were no significant differences in the free radical signal intensities from control and stressed plants of the same variety in the two weeks after milling. There were, however, significant differences between different varieties (T.G. Reichenauer & B.A. Goodman, unpublished results). There were no significant changes in the *g*-value of the free radical signal during the course of the experiment, suggesting that there was no fundamental change in the chemical nature of the free radical, even though the progressive increase in linewidth (see above) indicates that the radical generated on storage was not identical to that present initially.

Seeds typically contain appreciable levels of stable free radicals.[27] A proportion of these are associated with the testa, but others are present in the cotyledons and embryonic axes.[28,29] Additional radicals will have been generated, however, during the milling process. This represents the situation at the beginning of our measurements. The changes we see, therefore, represent the result of reaction of the milled samples with atmospheric oxygen (i.e. product oxidation). The observation that the free radical EPR signal in the samples from the plants grown under elevated ozone changed more rapidly than in the controls, indicates that ozone exposure during the growing season results in a decreased stability towards oxidation in the resulting flour.

The reasons for the different stabilities of the flour samples must originate from subtle differences in the compositions of the original grains. In living non-photosynthetic plant tissues, such as seeds, the mitochondria are regarded as the main source of radical generation,[30] where reactive oxygen species (ROS) are byproducts of aerobic metabolism. Under stress conditions (such as elevated ozone concentrations), generation of ROS is increased as a result of an imbalance in concentrations of substrates in the electron transport chain. The main initial ROS is the superoxide radical, which is converted to hydrogen peroxide by the enzyme superoxide dismutase (MnSOD).[31] Thus the slight increase in Mn levels in flour from ozone-stressed plants might be the result of increased generation of MnSOD in response to elevated ozone levels. Although hydrogen peroxide is detoxified by a number of enzymes in plant mitochondria,[30] it can produce the highly reactive hydroxyl radical in the presence of oxidisable substrates (the Fenton reaction[32]). Thus elevated hydrogen peroxide levels leads to reductions in the levels of antioxidant free radical scavengers, such as ascorbic acid and glutathione[30,33] through both direct reaction and indirectly by their reaction with products from reactions of the hydroxyl radical. It seems likely, therefore, that the higher rate of free radical generation in the flour from ozone-stressed plants is the consequence of lower levels of free radical scavengers in these samples. Although a similar increase in free radical levels from ozone-stressed wheat plants has been observed with freeze-dried leaf samples, this hypothesis remains to be tested.

In a previous study of flour samples using EPR spectroscopy, Schaich and Rebello[12] reported that the spectra at 77 K contained transition metal and free radical signals that were too weak for quantitative measurement. The objective of that work, however, was the investigation of free radicals that were generated during extrusion in the temperature range 160–185°C. It was appropriate, therefore, to record spectra with experimental conditions that were optimised for the radicals formed during the extrusion treatment. The microwave power of 10 mW used in that work would have given a high level of saturation of the free radical signal in the untreated flour and rendered it difficult to separate from the Mn(II) signal. By using a much lower microwave power, we have found that the free radical signal in flour could, not only be clearly resolved from the Mn(II) component, but also measured quantitatively.

CONCLUSIONS

Exposure of wheat plants to elevated ozone levels during the growing season resulted in changes in total Mn and Fe levels, and in the EPR-detected free radical signal in flour obtained from the grains.

The EPR signals from the metals did not change during ageing over a period of 9 months, but the free radical signal underwent significant changes in intensity during this period. These changes were more marked in the samples from the plants grown under elevated ozone, suggesting that the environmental conditions during the growing period have an appreciable impact on the storage properties of the resulting food product. Although further work is needed to determine the relevance of these observations to food quality criteria, this work has established that EPR spectroscopy can provide a fairly rapid, non-destructive approach to the measurement of changes in a chemical property (FR) during storage of relatively stable food products.

Acknowledgements

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