Aging, Free Radicals, and Antioxidants in Wheat Seeds

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Free radical oxidative attack is considered a major cause of disruption and deteriorative changes observed in aged seeds. Antioxidant defense mechanisms may remove potentially damaging molecular species, and carotenoids may act as radical scavengers. The content of lutein, the major carotenoid in wheat seeds, was determined in the flours. It showed a rapid decrease during seed aging. In addition, the content of free radicals in glutens made from flours of wheat seeds after long-term storage was studied. The concentration of radicals appeared to be age dependent, because the highest content of radicals was detected between 13 and 15 years of aging over 36 years of storage. Specific spin labeling of the sulfhydryl groups of gluten proteins enabled comparative EPR studies of the rigidity of the protein chains. A progressive stiffening of polymeric gluten with seed storage was found.

Keywords: *Wheat seeds; seed aging; free radicals; EPR; carotenoids; lutein*

INTRODUCTION

Seeds, like other organisms, age and die, although their longevity varies greatly among species and within a species because of differences in genotype and origin. Influences of origin on longevity result from the cumulative effect of environment during seed maturation, harvesting, drying, and prestorage conditions and the time of seed harvest (Hong and Ellis, 1996). Seed longevity will then depend on the subsequent conditions of storage, which could maintain the seed viability in the medium or long term.

Aged seeds show a variety of symptoms ranging from reduced viability to more or less full viability but with abnormal development of the seedling. The reduction in the rate of germination may be an expression of aging of the embryo, but changes in the remainder of the seed could also contribute. Important processes during seed germination, such as the establishment of respiration, ATP production, and protein synthesis, are often perturbed by seed aging (Bewley and Black, 1994). However, it is generally accepted that loss of viability with seed aging is mainly connected with the loss of plasma membrane integrity (Senaratna et al., 1988). In wheat the changes in plasma membrane permeability appear to involve not the whole seed, but only the embryo axis and aleurone layer, because the scutellum cells do not show an increased permeability of the plasma membrane as the above-reported seed tissues (Golovina et al., 1997a). Lipid peroxidation caused by free radicals appears to be the main cause of plasma membrane deterioration during seed aging (Wilson and MacDonald, 1986). Among the other causes involved in the loss of seed viability, protein modifications might play an important role. These modifications can occur by nonenzymatic glycation with reducing sugars (Sun and Leopold, 1995) and/or by reaction with aldehydes produced by the oxidation of fatty acids mediated by free radicals (Priestley and Leopold, 1983; Priestley et al., 1985). These aldehydes seem to react with sulfhydryl groups of proteins (Stadtman, 1992; Mudgett and Clark, 1993). Nevertheless, an infrared microspectroscopy study failed to demonstrate protein aggregation and denaturation in naturally aged seeds of different species (Golovina et al., 1997b).

Increasingly evident is that carotenoids may act as radical scavengers both as singlet oxygen quenchers and as quenchers of triplet excited states of molecules (Krinsky, 1994; Palozza and Krinsky, 1994). Two possibilities have emerged (Mortensen and Skibsted, 1997): according to the first mechanism, carotenoids can react with radicals (one for all, the phenoxyl radical) to form an adduct; in the second mechanism, they can transfer one electron to the radical, giving rise to a stable carotenoidic radical cation and regenerating the original molecule.

In the present work, we investigate the natural aging of wheat seeds from two points of view. First, we measured both the free radical content by electron paramagnetic resonance (EPR) and the amount of antioxidants (carotenoids) by HPLC in flour and gluten obtained from naturally aged wheat seeds. This was made in the attempt to correlate the content of free radicals with that of antioxidants and with seed age. Second, we labeled the sulfhydryl groups of glutens from

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aged seeds with *N*-[3-(2,2,5,5-tetramethyl-1-pyrrolidinyloxyl)]maleimide (3-MAL), an "unnatural" radical (Keana, 1978). The EPR spin-labeling of cysteine residues has been already used to study the dynamics of the gluten protein on a molecular basis (Hargreaves et al., 1994a) in order to characterize the molecular organization of the gluten protein aggregates. This application was complementary to rheological and microscopic measurements, which characterize the gluten network organization. Afterward, Hargreaves et al. (1994b), investigating the segmental flexibility of polypeptides in spin-labeled gluten subfraction, found that the population of fast moving/immobilized spin label is correlated to the proportion of large glutein polymers and to the height of the rubbery plateau, G_N^0, obtained from a rheological study of the viscoelastic behavior. Therefore, looking at the gluten organization at a macroscopic and molecular level, it is seen that rheological data relate to the flexibility of the polypeptides as observed by EPR spectroscopy (Hargreaves et al., 1996).

Our aim was to study the dynamic behavior of 3-MAL in the protein matrix of gluten, to describe some of the effects of aging in terms of flexibility, elasticity, and loss of integrity, and to correlate them with the germination rate and capacity of aged wheat seeds.

EXPERIMENTAL PROCEDURES

Materials. All experiments were performed on wheat seeds (*Triticum durum* cv. Cappelli). We will refer to these grains as seeds, although botanically they are considered fruits, named caryopses. The wheat utilized in the present work was cultivated in the experimental fields of the Dipartimento di Scienze Botaniche (Pisa). The environmental conditions during seed maturation and harvesting were registered for each crop. Harvested seeds were dried for 20 days at ambient temperature and then stored in a gene bank in sufficiently big lots (to reduce to a minimum the differences within the seed population). The storage room was maintained in the dark at 10 °C and up to 35% relative humidity. The crops utilized were harvested in the years 1961, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1973, 1976, 1980, 1981, 1983, 1984, 1987, 1991, 1993, and 1995. They were chosen because of the similarities in environmental conditions during maturation and at the moment of harvest.

The 3-MAL spin label (Sigma Chemical, St. Louis, MO) was used to label the sulfhydryl groups of cysteine residues in the gluten samples. Zeaxanthine and lutein were purified by semipreparative HPLC separation of methanol extracts from *Zea mays* flour. β-Carotene was obtained from Fluka. Solvents were of HPLC quality. All reagents were of analytical grade and used without further purification.

Instruments. The HPLC apparatus used was a Jasco 880 Pu pump equipped with a Jasco 875-UV or a Shimadzu SPD-10-A spectrophotometric detector. The latter was able to measure the vis−UV absorption spectrum (200−600 nm) of the chromatographic peak entrapped in the detector spectrophotometric cell. HPLC analyses were carried out by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Spherisorb S5 ODS2 column (250 × 4.6 mm) with an RP-C18 precolumn (10 × 4.6 mm) and a 5 μm filter. A Spherisorb S5 ODS2 column (250 × 10 mm) was used in semipreparative separations.

For vis−UV spectrophotometry, a Cary 219 instrument was used; CD measurements were carried out on a Jasco J40 AS machine.

EPR measurements were carried out on a Varian X-band (9.2 GHz) E112 spectrometer (Palo Alto, CA), equipped with a Varian variable temperature controller and interfaced to a personal computer 486/100 by means of a homemade data acquisition system. This consists of an acquisition board (Ambrosetti and Ricci, 1991) and a software package especially designed for EPR and electron nuclear double-resonance experiments (Pinzino and Forte, 1992). Spectra were recorded at each temperature, using a standard EPR cavity, at a field set of 3265 G, a scan range of 100 G, a microwave power of 10 mW, a time constant of 0.125 s, and a modulation amplitude of 1.25 G.

Germination Tests. Seeds from each crop, separated by sieving to obtain caryopses of uniform size, were surface-sterilized in 1% NaClO for 4 min and washed 10 times in sterile distilled water. Sixty seeds (three replicates) were spread uniformly within Petri dishes and placed inside a large polythene box on top of three very moist paper towels. The box was closed with a lid and placed in an incubator in the dark at 23 °C for 24, 48, and 72 h.

Preparation of Flours and Glutens. The flour was routinely obtained by using the following procedure. After excision of the embryos, 100 g (five replicates corresponding to ∼13000 seeds) of wheat endosperms was ground in a break roller-mill (Labormill 4RB, Italy), and the white flour was stored at 4 °C.

For some determinations, whole seeds were ground in a hammer mill (Retsch GmbH & Co. KG, Germany), and the flour was separated through a 50 mesh filter; the residual material (∼5%) was discarded. Whole meal was produced to measure the amount of free radicals and antioxidants present in the whole seeds. We proved that the milling does not significantly change the free radical content. In addition, the whole meal and the white flour showed, within experimental error, similar contents of free radicals per gram.

Gluten was extracted mechanically from white flour, previously defatted at room temperature (MacRitchie and Gras, 1973), by using the Glutomatic 2200 apparatus (Perten Instruments, Sweden). Ten grams of flour was placed in each of the two wash chambers assembled with the fine 88 μm polyester filters, and 4.8 mL of 2% (w/v) sodium chloride solution was added. Each wash chamber, gently shaken so that the salt solution spread evenly over the flour, was fixed to the Glutomatic, and the dough mixing program was started for ∼20 s. After this time, the dough was left to rest for 1 min and then the washing program was restarted for 3 min. The washing procedure was continued for 2 min by transferring the doughs into two wash chambers with large hole sieves. The gluten thus obtained was frozen, lyophilized, homogenized in dry conditions with a pestle and mortar, and stored at −20 °C. It had a moisture content of ∼6% and reconstituted readily with water at room temperature to form a cohesive elastic mass.

Extraction of Carotenoids. Approximately 300 mg of fresh flour from aged wheat seeds was added with 6 mL of MeOH. After 2 h, under vigorous stirring, the mixture was centrifuged for 10 min in a bench centrifuge. The extraction was repeated three timess and the supernatants were collected and reduced to 5 mL by evaporation into vacuum. No additional colored material (445 nm) could be extracted from the precipitate with 5 mL of water-saturated *n*-butanol or $CHCl_3$. In fact, negligible light absorption of the extracted phase was measured in the 350−700 nm spectral region, even after concentration of the solution to 1 mL by evaporation. The HPLC determination of the carotenoid content of the flours was made by direct injection of MeOH extracts. The amount of carotenoids obtained with the methanol procedure was identical, within experimental error (<5%), to that obtained with exhaustive extraction of flours with the other solvents. The methanol extraction had some advantages with respect to the use of other solvents: separation of phases was easier than with chlorurated solvents, volume reduction was much easier than with the far less volatile butanol, and, finally, the resulting solute dissolved in MeOH, that is, the mobile phase used in subsequent HPLC analyses, avoiding some HPLC problems such as those described for carotenoids determination (Khachik et al., 1988). In addition, MeOH is able to dissolve only minute amounts of lipidic uncolored materials.

Analysis of Extracted Carotenoids. The determination of carotenoid content in the MeOH extracted solutions was

performed by isocratic RP-HPLC. After injection (25 μL), the carotenoids were eluted at room temperature and at a flow rate of 0.5 mL/min with CH_3OH (100%) and detected at 445 nm. In these conditions, reasonable retention times for lutein, ~15 min, and other carotenoids were obtained with corresponding well measurable and reproducible elongations. In addition, absorption spectra relative to some chromatographic peaks were registered in the wide 200−600 nm spectral region for characterization purposes.

Other mobile phases were also used isocratically to determine the presence of more hydrophobic molecules, such as α- and β-carotene, mono- and diesters of alcoholic xanthophylls, tocopherols, and vitamin K, which have retention times with methanol that are too long (up to some hours). The mobile phases used were a mixture of CH_3CN/ethyl acetate [62:38 v/v or 88:12 (Subagio et al., 1996)] and ternary mixtures of $CH_3CN/CH_3CHOHCH_3$/THF [64:18:18 or 56:22:22 (Kaneko et al., 1995)]. The identification of the peaks corresponding to some substances, such as lutein, β-carotene, zeaxanthine, and isoluteine, was performed by comparison of the retention times and the absorption spectra, in the 200−550 nm spectral range, of the most important peaks (as detected at 445 nm) with those reported in the literature (Sims and Lepage, 1968; Müller, 1997; Mercadante et al., 1997; Rouseff et al., 1996) or with those of standard samples and by co-injection of the same with the samples under investigation.

EPR Measurements. The content of free radicals was measured at room temperature on dry gluten powder, inserted in a quartz tube with a diameter of 4 mm and pressed to improve detection of weak signals. A well measurable signal was found about an order of magnitude higher than with equal amounts of flours.

Spin labeling was performed following the method of Hargreaves et al. (1995), slightly modified. Typically, an aqueous solution (137 μL) of nitroxide radical 3-MAL (1 mg/mL) was added to 46 mg of gluten powder, to obtain a 300% (w/v) excess. The sample was stirred gently for 30 min until uniformly hydrated and left to rest for ~24 h in the dark at room temperature. The nitroxide radical excess was removed by up to eight subsequent washings, under stirring, with distilled water; centrifugation for 10 min at 4000*g* was carried out after each washing. The last washing contained no detectable amounts of the label, as revealed by HPLC analysis (RP-C18 column; 6 min 40 sec retention time of 3-MAL at 0.6 mL flow rate; mobile phase $CH_3CN/H_2O/CF_3COOH$, 63/37/0.05; $\lambda = 230$ nm). About 15 mg of the labeled and hydrated gluten was first inserted into a quartz capillary (1 mm diameter) and then into a quartz tube (4 mm diameter) that was closed at both ends to avoid sample loss. EPR spectra were recorded between 5 and 90 °C with 5 °C increments. After heating, the samples were cooled to 20 °C and EPR spectra were recorded to check the reversibility of the heating.

RESULTS AND DISCUSSION

Xanthophyll. The xanthophylls are not the sole antioxidants or free radical scavengers present in seeds. One could mention, for example, the groups of vitamins A, E, and K. Anyway, xanthophylls proved to be completely extractable with MeOH (i.e., the HPLC mobile phase), to be identifiable, and to have concentrations measurable with relatively high accuracy by RP-HPLC, thanks to their characteristic intense absorption band at 450 nm. Among the xanthophylls, lutein was chosen as the most representative for the following reasons:

(1) It is the xanthophyll present in the highest quantity in wheat seeds (Sims and Lepage, 1968) and is particularly abundant in cv. Cappelli; (2) its HPLC characteristics are such that precise determination is possible [high elongations (>50 mm), no tailing, reasonable retention time]; (3) its vis−UV absorption spectrum is well-known (Sims and Lepage, 1968); (4) the ratios of the HPLC elongation of lutein to that of other substances with absorption peaks at 445 nm was found to be almost constant with seed aging, so we can choose lutein as reference for all xanthophylls. For example, the ratio of lutein to zeaxanthine concentrations is ~0.03 in all of the examined samples.

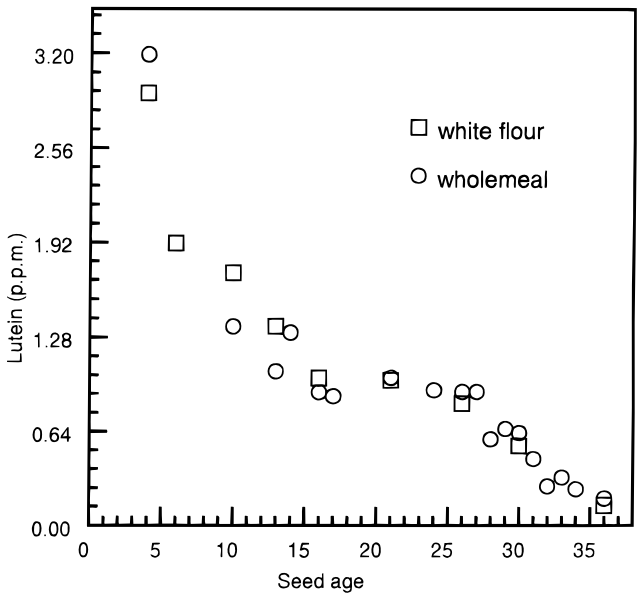

Figure 1. Lutein content versus seed age in whole meal and white flour obtained from aged wheat seeds.

Kaneko et al. (1995) showed that endosperm lutein of wheat (cv. Kanto) was esterified with fatty acids, mainly linoleic and palmitic, following accelerated aging of seeds. Because the esterification of lutein did not occur when the seeds had been heated in an autoclave before storage, the authors conclude that the reaction was enzymatically catalyzed. However, in naturally aged seeds of *T. durum* cv. Cappelli, extremely small quantities (<1%) of these esters were found. The difference between cv. Cappelli and cv. Kanto wheat seeds might depend on the cultivar used or on the differences between natural (cv. Cappelli) and artificial aging (cv. Kanto). In a following paper, Kaneko and Oyanagi (1995) did not find esterification in 9 wheat cultivars of the 138 examined.

Figure 1 shows the amount of lutein as a function of the natural aging of the wheat seeds cv. Cappelli. The quantities of lutein in the white flours and in the whole meals are reported. No appreciable differences between the two types of flours are evident. This indicates that the distribution of lutein in germ, bran, and endosperm is nearly uniform. More interesting is the fact that the lutein content decreases with seed aging. In fact, a rapid decrease in lutein content is observed during the first 6−12 years of aging: the content varies from >3 ppm to ~1 ppm. During the following 10−12 years of storage, lutein content remains almost constant and finally decreases rapidly in the subsequent 10 years. In the most aged seeds, the lutein content was close to zero. These data suggest a possible correlation between seed deterioration and lutein content. Because oxidative reactions are assumed to be responsible for the deteriorative changes observed in aged seeds (Bernal-Lugo and Leopold, 1998), the progressive disappearance of lutein might be due to oxidative processes during seed dry storage.

Germination. Two germination parameters have been used: (a) the germination capacity, which is the

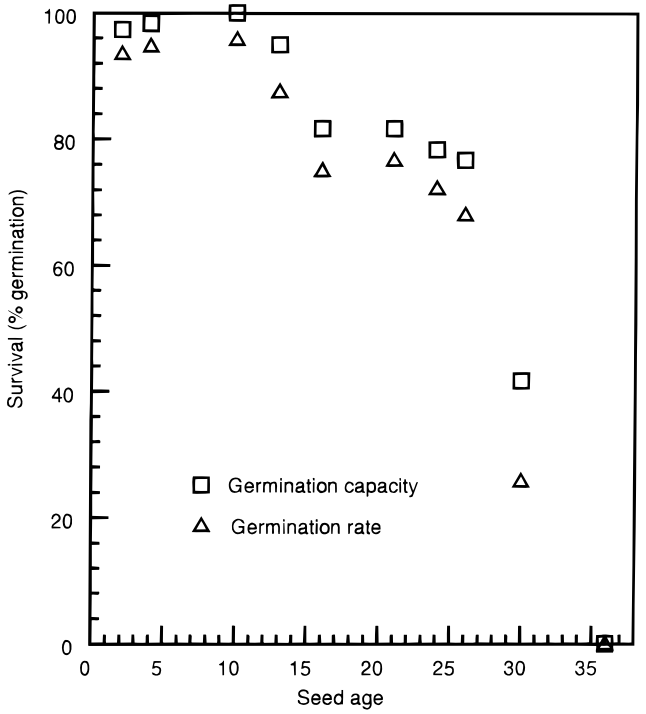

Figure 2. Survival curves versus seed age for *T. durum* cv. Cappelli aged seeds. The germination was expressed as germination capacity and rate.

percentage of seeds completing the germination at 72 h; and (b) the germination rate.

The germination capacity curve (Figure 2) appears to follow the classical two-phase pattern described for wheat seeds in the literature (Bernal-Lugo and Leopold, 1998). Such a mortality curve emphasizes the existence of an initial period of low mortality, which corresponds to ~10 years for the wheat seeds.

The germination rate of wheat seeds has been determined according to the equation

$$\text{germination rate} = (nt_1 \times 100 + nt_2 \times 50 + nt_3 \times 33.3)/N_{tot} \quad (1)$$

where nt_1 are the seeds germinated at 24 h, nt_2 the seeds germinated between 24 and 48 h, and nt_3 the seeds germinated between 48 and 72 h. The germination rate gives additional information about seed aging (Figure 2). In fact, it is known that the aging retards the rate at which the germination process occurs before it affects viability appreciably. Germination rate can also give information on the uniformity of a seed population, which is uniform if individual germination rates are close to the mean rate of germination for the population as a whole. In the case of the *T. durum* cv. Cappelli seeds, the germination rate data obtained with the crops utilized in this work allow us to conclude that the seed populations have similar uniformity. Finally, it should also be emphasized that the reduction in the rate of germination might also be an expression of aging of the embryo, even though changes in the remainder of the seed might also contribute.

Free Radicals. An evaluation of the free radical content in 11 glutens from aged wheat seeds was obtained from the maximum amplitude of EPR spectra on dry gluten powders. These spectra are characterized by a predominant line centered at $g = 2.006$ (Figure 3); from the intensity of this line we could obtain the total

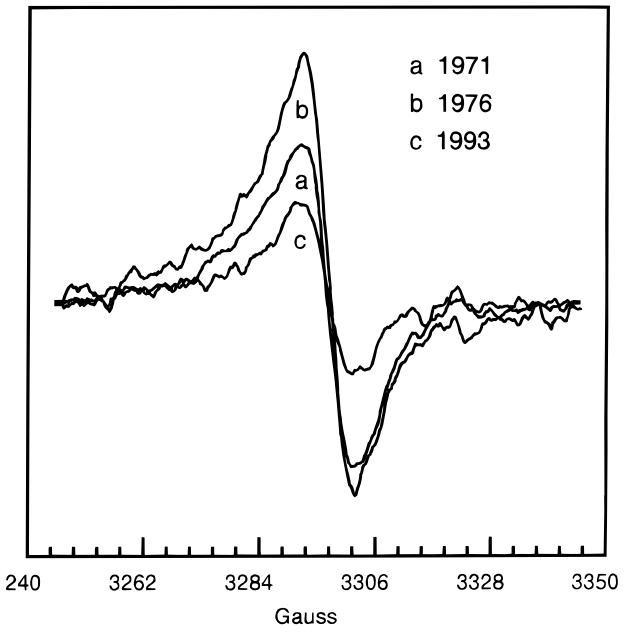

Figure 3. Examples of free radical EPR spectra at $g = 2.006$ for glutens obtained from aged wheat seeds.

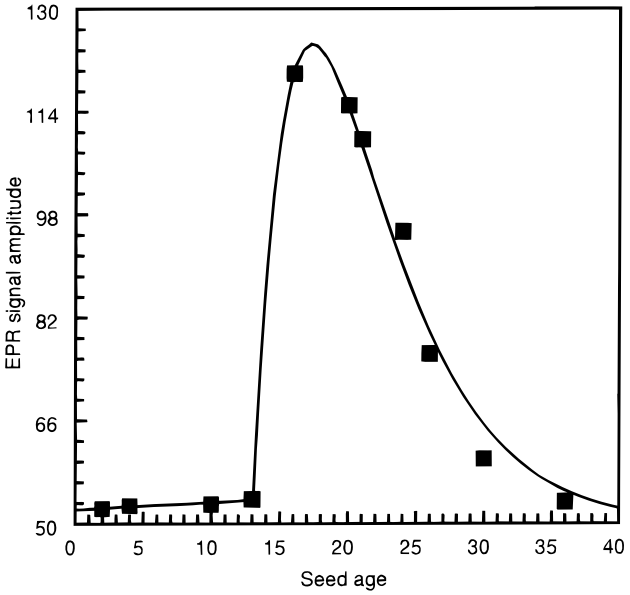

Figure 4. Amplitude of free radical EPR signal versus seed age for 100 mg of gluten powder obtained from aged wheat seeds.

content of organic radicals present. The resulting plot of EPR signal against aging, normalized to 100 mg of powder (Figure 4), does not show a direct relationship between organic radical content and aging, in agreement with literature data (Hepburn et al., 1986; Girard and Le Meste, 1992). Three trends are clearly put in evidence: the EPR signal is almost constant for glutens from seeds aged for up to 13 years, indicating that the concentration of radicals is age independent in this time lapse; a relevant increase of radicals is indeed observed from 13 to 15 years of aging, and a corresponding decrease follows from 15 to 36 years of aging. This behavior may be consistent with the presence of two pseudo-first-order simultaneous reactions corresponding to the production and disappearance of carbon-centered radicals, respectively.

Any molecule in the gluten network undergoes the transformation to the corresponding radical, with a constant rate $k_1 = 0.233$ (year)$^{-1}$, because of various factors (O_2, transition metal ions, presence of the highly reactive hydroxyl radicals HO$^\bullet$ produced by Fenton reactions or other highly reactive radicals); in turn, the radical species may decay, with a constant rate $k_2 = 0.232$ (year)$^{-1}$, both because of antioxidants and because of disproportioning or by interaction with other organic species. The production and the disappearance of radical species are pseudo-first-order reactions because the concentration of the radicals is always less than the concentration of the reagents inducing the formation and the decay.

Comparison of the radical with the antioxidant trend showed a loss of protection and a significant change to the way free radicals are formed.

Spin-Label Studies. EPR spectra of spin-labeled glutens were of a composite line shape type, indicating the presence of at least two populations of nitroxide radicals differing in molecular mobility (Figure 5). Before and after heating, the spectra showed similar line shapes, with only a slight reduction of intensity due to a reduction of spin label.

The line shape of EPR spectra reflects the value of the correlation time τ_c, which represents the time during which the label stays in a given orientation. The value of the correlation time is determined by the flexibility of the spin-labeled protein segment, solvent viscosity, and steric hindrance due to the chains of the neighboring proteins.

An R value, reflecting the ratio of slow to fast moving spin labels, was calculated from the spectra, according to the equation (Hargreaves et al., 1994a)

$$R = i/m \tag{2}$$

where i and m are the amplitudes of the low-field peaks corresponding to slow moving (immobile) and to fast moving (mobile) labels, respectively.

The rotational correlation time τ_c of the mobile population of spin labels was calculated from the spectra according to the Freed and Fraenkel equation (Freed and Fraenkel, 1963)

$$\tau_c = (6.65 \times 10^{-10}) \times \Delta h_{+1} \times [(I_{+1}/I_{-1})^{1/2} - 1] \tag{3}$$

where Δh_{+1} is the peak to peak width in Gauss of the low-field line and I_{+1} and I_{-1} are the amplitudes of the low and high lines, respectively. From τ_c (Figure 6) and R (Figure 7), it is possible to determine the activation energy E and the difference in enthalpy ΔH and entropy ΔS, according to the Arrhenius and Gibbs equations, respectively

$$\tau_c = \tau_\infty \exp(E/R_g T) \tag{4}$$

$$R = R_0 \exp(-\Delta S/R_g) \exp(\Delta H/R_g T) \tag{5}$$

where τ_∞ is the pre-exponential factor, T the absolute temperature, R_g the ideal gas constant, and R_0 the proportional factor between the label concentration ratio and the spectrum amplitude ratio.

The rotational correlation time of the fast moving component decreases, at all temperatures, with seed age. The values (≈0.4ns) indicate that the spin labels are bound to the sulfhydryl groups, which allows the labels to retain a high degree of rotational freedom, increasing with aging, with respect to the whole protein matrix of gluten. The values of the magnetic parameters g_0 (isotropic g factor) and A_0 (isotropic hyperfine coupling constant) of this spectrum component equal, at all ages, the values ($g_0 = 2.0056$, $A_0 = 16.2$ G) of 3-MAL in aqueous solution. This is consistent with spin labels bound to shallow, solvent-exposed protein groups.

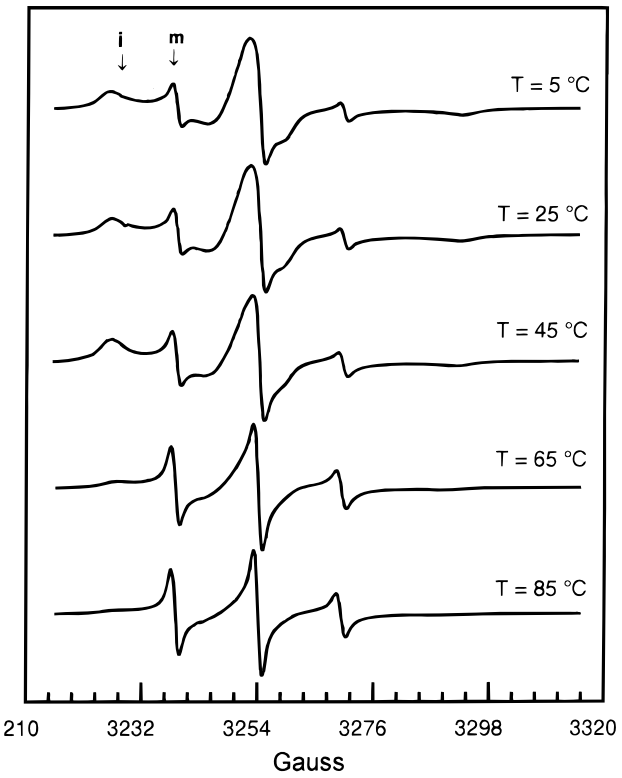

Figure 5. Examples of EPR spectra of 3-MAL spin-labeled gluten obtained from wheat seeds harvested in 1976. The spectra were recorded at different temperatures. The lines marked "i" and "m" are the low-field lines corresponding to the low-mobile and mobile spin radicals, respectively.

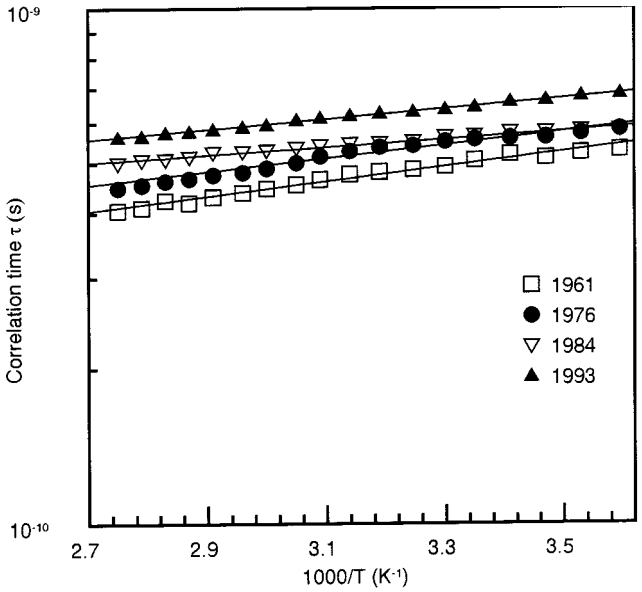

Figure 6. Examples of Arrhenius plot of the rotational correlation time (τ_c) against the inverse of absolute temperature (T) of 3-MAL labeled glutens obtained from aged wheat seeds of different crops.

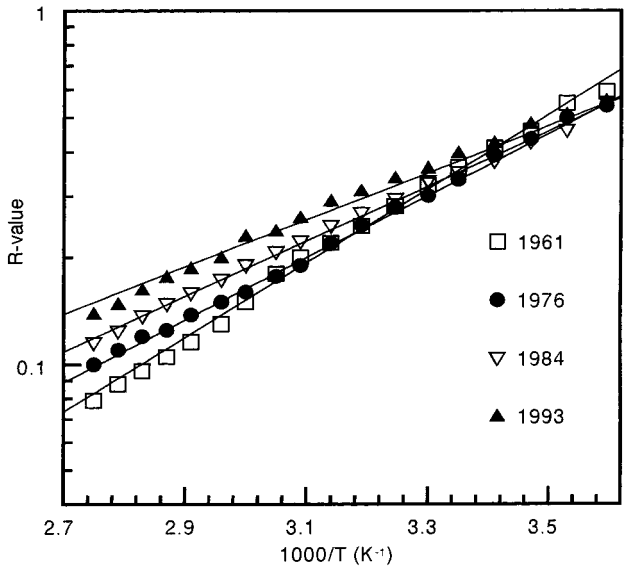

Figure 7. Examples of Gibbs plot of the *R* value against the inverse of absolute temperature (*T*) of 3-MAL labeled glutens obtained from aged wheat seeds of different crops.

The slow moving component of the gluten spectrum is due to the label bound to groups localized in somewhat restrictive, crevice-like regions of the protein matrix.

With increasing temperature, we can see in Figure 7 a progressive transfer of less mobile radicals to a more mobile population, pointed out from the decrement in the *R* values. This trend agrees with the viscoelastic behavior of gluten, where the height of the rubbery plateau (G_N^0) has been reported to decrease upon heating from 10 to 50 °C, due to the disruption of low-energy intermolecular bonds (Hargreaves et al., 1995). Above this temperature, the trend of *R* disagrees with the viscoelastic behavior, as the height of G_N^0 is reported to strongly increase. This increase has been explained by assuming an increase in the size of gluten polymers and a reorganization of intermolecular bonds, caused by the sulfhydryl−disulfide interchange between the proteins (Scofield et al., 1983). On the other hand, because the spin label used in the EPR experiments is a sulfhydryl blocking agent, we believe that the label interferes with the sulfhydryl−disulfide interchange and prevents the consequent enlargement of the gluten polymers.

Plots of *R* show also appreciable differences in the *R* values of glutens at the same temperature from different aged wheats: the younger were the seeds, the less was the fast moving population of spin labels in the glutens. This decrease in *R* with increase in seed age can be explained by assuming an entropy increase due to a reduction in the content of large gluten polymers. The cross-linking breakage of gluten subunits destroys the polymers and increases the mobility of the cysteine environment of gluten proteins. Furthermore, in Figure 8, there is evidence of an increase of the difference in enthalpy ΔH with seed age from 102 to 167 kJ/mol, indicating that the transfer of slow moving to fast moving population is more impeded. On the other hand, an activation energy of 90 kJ/mol has been reported for the retardation time (τ_0) associated with the loss compliance peak of the mechanical spectra (Ferry, 1980). Hargreaves et al. (1995) have reported that this time could be related to the displacement of theoretic unit

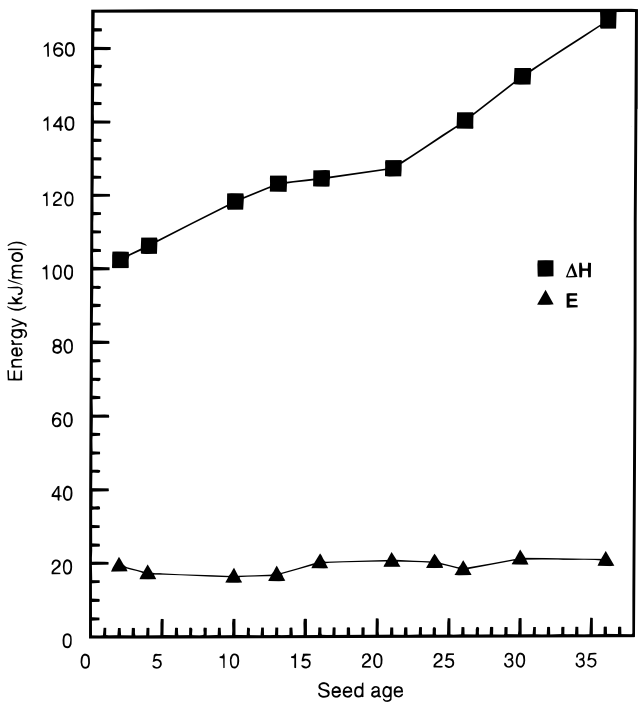

Figure 8. Plot of activation energy *E* and difference in enthalpy ΔH versus seed age determined with 3-MAL labeled glutens obtained from aged wheat seeds.

polymer length, corresponding to the peptide length between two covalent bonds. As the unit polymer length is not modified, being the total number of disulfide bonds constant in the gluten upon heating (Schofield et al., 1983), this activation energy would be related to low-energy interactions (hydrogen bonds, hydrophobic interaction, and entanglement points) between two covalent bonds.

The rotational correlation time τ_c of the mobile fraction decreases both with seed age and with increasing temperature (Figure 6), even though the activation energy for this process (*E*) (Figure 8), related to low-energy bonds in the immediate surrounding of the spin label, is substantially constant (≈20 kJ/mol) for all glutens. These results are in agreement with the hypothesis that aging is responsible for the deterioration of the polymeric gluten network, caused by breakage in the network structure and by alteration of protein conformation.

CONCLUSIONS

HPLC analysis of lutein indicates a possible correlation between the age of the wheat seeds and lutein content. In particular, after 24 years of aging, the germination capacity and the amount of antioxidants notably decrease. On the contrary, there seems to be a complex dependence between seed aging, antioxidants, and organic radical content, showing an exponential trend in the intermediate years. This is probably due to two simultaneous processes: the increase of production of radicals and their decay according to pseudo-first-order reactions.

EPR measurements on spin-labeled glutens show a change in dynamic properties depending on temperature and aging. In particular, we can see a change with age in the linear decrease of *R* and τ_c with increasing temperature according to a Gibbs and Arrhenius type

equations, respectively, indicating a progressive stiffening of polymeric gluten.

These results are in agreement with the hypothesis that the deterioration of proteins in dry systems, although slow, might significantly contribute to seed aging.

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