Oxidative Reactions during Early Stages of Beer Brewing Studied by Electron Spin Resonance and Spin Trapping

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An electron spin resonance (ESR)-based method was used for evaluating the levels of radical formation during mashing and in sweet wort. The method included the addition of 5% (v/v) ethanol together with the spin trap α-4-pyridyl(1-oxide)-*N*-*tert*-butylnitrone (POBN) to wort, followed by monitoring the rate of formation of POBN spin adducts during aerobic heating of the wort. The presence of ethanol makes the spin trapping method more selective and sensitive for the detection of highly reactive radicals such as hydroxyl and alkoxyl radicals. Samples of wort that were collected during the early stages of the mashing process gave higher rates of spin adduct formation than wort samples collected during the later stages. The lower oxidative stability of the early wort samples was confirmed by measuring the rate of oxygen consumption during heating of the wort. The addition of Fe(II) to the wort samples increased the rate of spin adduct formation, whereas the addition of Fe(II) during the mashing had no effect on the oxidative stability of the wort samples. Analysis of the iron content in the sweet wort samples demonstrated that iron added during the mashing had no effect on the iron level in the wort. The moderate temperatures during the early steps of mashing allow the endogenous malt enzymes to be active. The potential antioxidative effects of different redox-active enzymes during mashing were tested by measuring the rate of spin adduct formation in samples of wort. Surprisingly, a high catalase dosage caused a significant, 20% reduction of the initial rate of radical formation, whereas superoxide dismutase had no effect on the oxidation rates. This suggests that hydrogen peroxide and superoxide are not the only intermediates that play a role in the oxidative reactions occurring during aerobic oxidation of sweet wort.

KEYWORDS: Beer; sweet wort; electron spin resonance spectroscopy; oxidation; spin trapping; mashing

INTRODUCTION

The storage stability of beer is often impaired by oxidative reactions that affect the flavor and colloidal stability (*1*). Some of the minor constituents of beer, such as transition metals, sulfite, and ascorbate, have significant effects on the oxidative phenomena. The amounts and presence of these minor constituents in the final beer are the combined result of the raw materials and all of the individual steps of the brewing process. Careful control of the brewing process can to some extent control the contents of these important compounds. However, complete control, resulting in a well-defined reproducible oxidative stability of the final beer, is virtually impossible (*2*).

The conditions during mashing, which is one of the initial steps of beer brewing, can potentially favor oxidative reactions that affect the quality of the final beer. The physical mixing of ground malt and water and the continued stirring can efficiently introduce atmospheric oxygen into the mash. The moderate temperatures (from 35 °C up to about 75 °C) ensure a considerable solubility of oxygen. In addition, the moderate temperatures during mashing are optimal for many enzymatic reactions. The products of enzymatic oxidation of polyunsaturated lipids during mashing have been demonstrated to have important impact on the quality of the final beer (*3−5*).

The analysis of oxidative mechanisms in beer has been successfully carried out by detection of radicals by the spin trapping technique and electron spin resonance (ESR) spectroscopy (*6, 7*). This technique has been used to study the effects of different factors, such as metals, phenolic compounds, and Maillard compounds, on the stability of beer (*6, 8, 9*). Furthermore, a lag phase is observed for the formation of radicals, and the lengths of lag phases have been correlated to the flavor stabilities (shelf life) of the beer, making the method a powerful tool for the prediction of the stability of beer.

A few studies have been reported where the spin trapping technique was used to study oxidative reactions in different types of wort (*10−12*). Although the radical formation in wort

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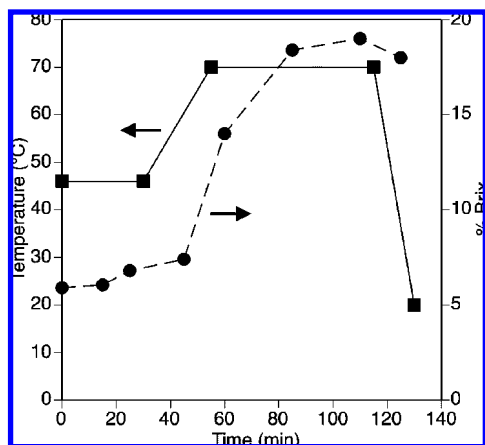† University of Copenhagen.
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Figure 1. Temperature profile and dissolved matter in wort during mashing.

proceeds without a lag phase, the rates of formation of ESR detectable spin adducts are generally low, as compared to beer. Franz and Back (*12*) found that the rate of spin adduct formation in wort could be enhanced by the addition of ethanol. In the present study, the ESR method has been used to examine the oxidative stability of sweet wort sampled during the different steps of the mashing process and to study the role of reactive oxygen species in the mechanisms of oxidation.

EXPERIMENTAL PROCEDURES

Mashing. A congress mashing (*13*) was carried out with modifications by using a MA-001 Mashing Apparatus (Lg-automatic ApS, Frederiksvaerk, Denmark). Finely ground, well-modified malt (Danish Malting group, Vordingborg, Denmark) (100 g) was mashed in at 46 °C in 200 mL of deionized water for 30 min followed by an increase in temperature to 70 °C for 25 min (increase of 1 °C per min). A 100 mL amount of deionized water was added to the mash when the temperature reached 70 °C. The temperature was held at 70 °C for 60 min (saccharification rest) after which the mash was cooled to room temperature (**Figure 1**). To ensure the same dry content of all samples, each beaker was adjusted to the same weight of 450.0 g by the addition of deionized water. The mash was subsequently filtered through an open-folded filter (#597 1/2, Whatman, Schleicher & Schnell, Dassel, Germany), and the sweet wort samples were frozen immediately after filtration. Wort samples that were collected before the end of the mashing program were also adjusted to a total weight of 450 g before filtration by the addition of deionized water to the beaker. Brix % measurements were performed on all samples prior to freezing.

ESR Forced Aging Test of Wort. Wort samples (25.0 mL), containing 5% (v/v) ethanol (96% pure ethanol, Danish Distillers, Aalborg, Denmark) and POBN [α-4-pyridyl(1-oxide)-*N-tert*-butylnitrone, Aldrich, St. Louis, MO] were stirred at room temperature in closed blue-cap bottles (250 mL), with a sufficient headspace of atmospheric air, for 10 min, to get the reactants into solution. The bottles were transferred to a preheated water bath (*T* = 55 °C) (Heto Laboratory Equipment, Allerød, Denmark). Samples (50 μL) were withdrawn at given time intervals. ESR spectra of the wort samples were recorded with a Miniscope MS 200 X-band spectrometer (Magnettech, Berlin, Germany) using 50 μL micropipettes as sample cells (Brand GMBH, Wertheim, Germany). The settings used were as follows: microwave power, 10 mW; sweep width, 95.9 G; modulation frequency, 1000 mG; receiver gain, 900; and sweep time, 60 s. All spectra, consisting of single scans, were recorded at room temperature. The amplitudes of the spectra were measured and are reported as the height of the central doublet relative to the height of the central line in the ESR signal of an aqueous TEMPO solution (2 μM). The TEMPO standard was measured as the first and last sample of the day. All samples were measured in triplicate. The oxidative stability of the wort samples was quantified by the initial rate of spin adduct formation determined by
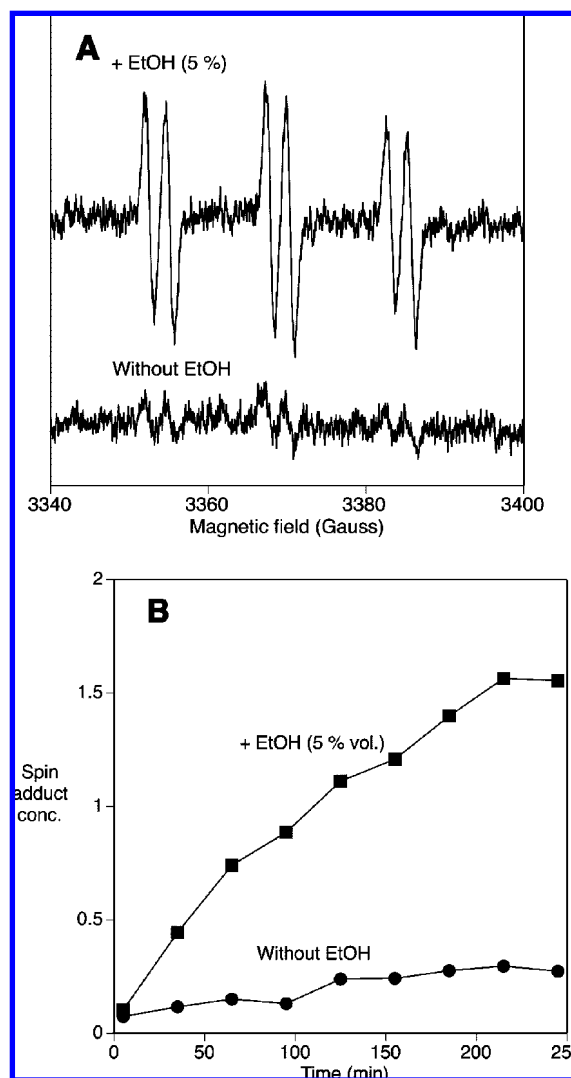

Figure 2. Spin trapping of radicals with POBN during heating of wort. (**A**) ESR spectra of POBN spin adducts formed in wort with added ethanol (5% vol) and without ethanol after heating at 55 °C for 155 min. (**B**) Intensity of ESR signals during heating wort containing POBN (40 mM) with added ethanol (5% vol) and without ethanol. *T* = 55 °C.

linear regression of the initial increase in spin adduct concentrations during forced aging and before the spin adduct concentrations began to level off (**Figure 3**).

Oxygen Consumption of Heated Wort. Room temperature wort samples were transferred to a 4 mL chamber (MR-Chamber, Unisense, Aarhus, Denmark), leaving no headspace of air. The filled chamber was placed in a preheated water bath (*T* = 60 °C) (Heto Laboratory Equipment, Allerød, Denmark). The chamber was equipped with a Clark electrode (Unisense NR-sensor, Aarhus, Denmark), and the measurements started immediately. The oxygen concentration was recorded every 10 s by an oxygen analyzer (Picoammeter PA 2000, Unisense, Aarhus, Denmark). A two-point calibration (air saturated water at 60 °C and 0.1 M sodium ascorbate in 0.1 M NaOH solution at 60 °C) was used to calibrate the electrode and oxygen analyzer prior to the measurement.

ESR Forced Aging Test of Mash and Wort Added Fe. From a freshly prepared aqueous FeSO₄ (Merck, Darmstadt, Germany) stock solution, a final concentration of 50 μM FeSO₄ was added (i) to the mash at the beginning of the mashing, when the malt was being mixed with water, or (ii) to the wort obtained after mashing and filtration. ESR forced aging tests were carried out on the wort samples collected after 25 and 125 min of mashing.

Metal Analysis. The concentrations of Fe and Cu were determined in wort samples collected 25 and 125 min after initiation of mashing
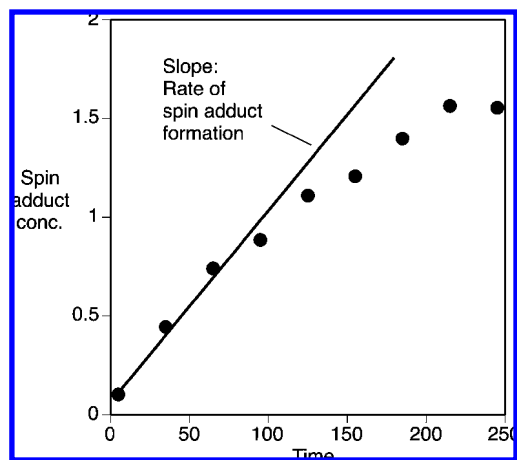
Figure 3. Initial rate of spin adduct formation as a measure of wort oxidative stability. Spin adducts formed in wort with added ethanol (5% vol) and POBN (40 mM). $T = 55$ °C.

using atomic absorption spectrometry (Perkin-Elmer 3300, United States). To the wort samples were added 50 μM FeSO$_4$ either (i) at the beginning of the mashing or (ii) to the wort after mashing and filtration and were compared to a control wort, to which FeSO$_4$ was not added.

Effects of Hydrogen Peroxide, Catalase, and Superoxide Dismutase. Hydrogen peroxide (30%, Sigma-Aldrich, Steinheim, Germany), catalase (E.C. 1.11.1.6), pure *Scytalidium thermophilum* catalase enzyme with no glucose oxidase side activity (Novozymes A/S, Bagsvaerd, Denmark), or bovine superoxide dismutase (E.C. 1.15.1.1) (Sigma-Aldrich, Steinheim, Germany) were added to refrigerated samples of wort immediately before the addition of ethanol and POBN, and the ESR forced aging experiments were performed as described above. One catalase unit (CIU) corresponds to the amount of enzyme that decomposes 1 μmol of hydrogen peroxide per min at pH 7 and 30 °C. CIU was determined by a spectrophotometric method evaluating the removal of H$_2$O$_2$ at 240 nm [$\epsilon = 39.4$ M^{-1} cm^{-1} (*14*)], by catalase at 30 °C in a 50 mM phosphate buffer (pH 7.0). The time (in seconds) necessary for the absorbance at 240 nm to decrease from 0.450 (start concentration of 10.3 mM H$_2$O$_2$) to 0.400 in the presence of catalase is converted to CIU per g of the product of the enzyme. One superoxide dismutase unit (SODU) will inhibit the rate of reduction of cytochrome c by 50% in a coupled system (with xantine and xantine oxidase) at pH 7.8, at 25 °C, in a 3.0 mL reaction volume, using a xantine oxidase concentration that will cause a change in absorbance at the rate of 0.025 per min at 550 nm (*15*).

Statistical Analysis. Statistical analysis was performed using the SAS JMP 6.0.0 package (SAS Institute, Inc., United States). Data were analyzed by the analysis of variance to determine the significance of the main effects. Significant ($p < 0.05$) differences between means were identified by the least significant difference (LSD) test.

RESULTS

Oxidative Stability of Sweet Wort. The oxidative stability of wort was studied by an ESR-based method that was analogous to the well-established method for evaluation of flavor stability of beer (*6*). A spin trap was added to wort collected at different stages of mashing, and the formation of spin adducts during heating with access to atmospheric air was detected by ESR. The use of the spin trap POBN alone gave ESR-detectable signals, which increased with time but had very low amplitudes (**Figure 2**). The addition of ethanol together with the spin trap increased the amplitude of the signals considerably, as was previously shown by Franz and Back (*12*). The POBN spin adducts formed in the presence of ethanol had hyperfine coupling constants ($a_N = 15.5$ G and $a_H = 2.5$ G) that were similar to the values expected for spin adducts formed from 1-hydroxyethyl radicals (*16*). The weak ESR signals arising from
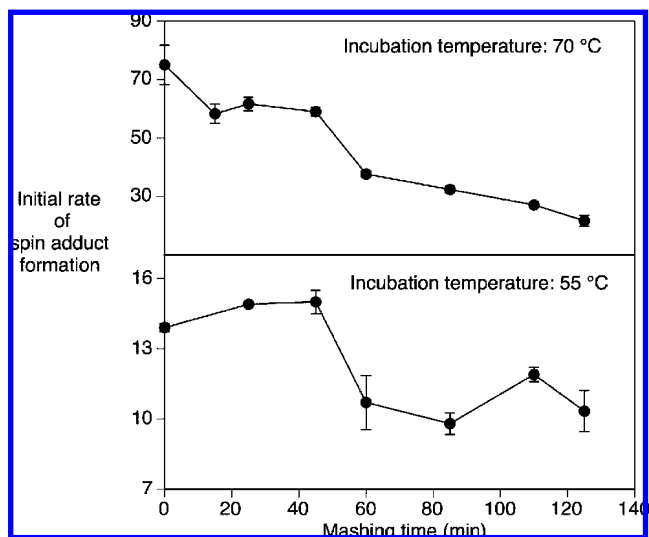

Figure 4. Oxidative stabilities of wort samples collected at different stages of mashing. The wort samples containing ethanol (5% vol) and POBN (40 mM) were incubated at 55 °C (lower panel) or 70 °C (upper panel) during the oxidative stability measurements.

spin adducts formed in the absence of ethanol had very similar hyperfine coupling constants ($a_N = 15.3$ G and $a_H = 2.6$ G), which suggests that carbon-centered radicals were trapped.

In all samples of sweet wort, the formation of spin adducts took place without an initial lag phase, and the amount of spin adducts leveled off during prolonged heating of the samples. However, the rates of formation and the levels of the formed spin adducts were found to vary between the different wort samples. Heating the wort samples under a nitrogen atmosphere almost completely hindered the formation of spin adducts (data not shown). This shows that the formation of spin adducts is strongly linked to oxidative reactions in the wort. The spin adduct formation is expected to be a result of a competition between the pro-oxidative effects leading to the formation of radicals and the antioxidative effects that quench radicals. The oxidative stability of the wort samples was therefore quantified by the initial rate of spin adduct formation, before the onset of the decay of the spin adducts that eventually levels off the amount of ESR-detectable spin adducts (**Figure 3**). A high initial rate of radical formation is expected in wort samples possessing either a low antioxidative defense, which hinders oxidative processes, and/or a high level of pro-oxidative components, which favor radical formation. In either case, the oxidative stability of the wort is expected to be impaired in comparison to a sample that gives a low rate of spin adduct formation.

Oxidative Stability of Sweet Wort during Mashing. Samples of wort were collected at different stages during the mashing program, and their oxidative stabilities were evaluated by the spin trapping method. Generally, the wort samples from the initial low temperature stage of mashing-in gave higher rates of spin adduct formation than samples from the later, high temperature stage (**Figure 4**). A distinct change in the rates of spin adduct formation was observed when the mashing temperature was increased. This change also coincided with a change in the amount of extract in the collected wort samples (**Figure 1**). The effect of the level of extract in the wort samples on the rate of spin adduct formation was therefore examined by comparing the rates of spin adduct formation in wort samples after their content of dissolved organic compounds was increased by the addition of sucrose. The rate of spin adduct formation declined by only 24% when the density of wort, collected after
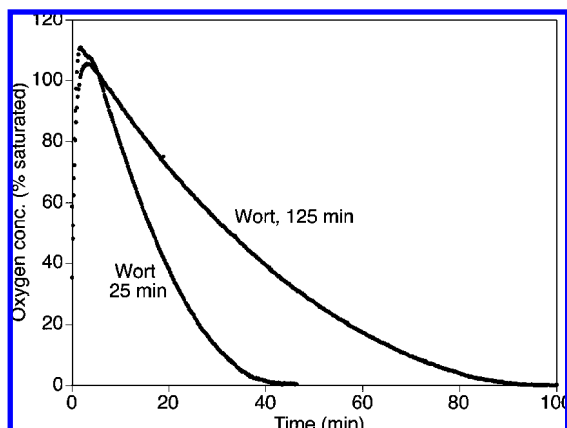
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Figure 5. Consumption of oxygen during heating wort from an early stage of mashing and the final wort. $T = 60\ ^\circ C$.

Table 1. Concentration of Iron and Copper in Wort Samples Collected 25 and 125 min after Initiation of Mashing

	wort (25 min)		wort (125 min)	
	Fe (μM)	Cu (μM)	Fe (μM)	Cu (μM)
no addition of Fe	7.1 ± 0.0	5.9 ± 0.0	4.0 ± 0.2	4.0 ± 0.0
Fe added at beginning of mashing	7.5 ± 0.2	5.9 ± 0.0	4.2 ± 0.0	4.0 ± 0.0
Fe added to wort	45.8 ± 0.5	5.6 ± 0.2	38.1 ± 0.5	4.0 ± 0.0

25 min of mashing (8.4 °Brix), was doubled (17.8 °Brix) to a level comparable to the extract content of the wort at the late stage of mashing. Increasing the density of the wort up to 32 °Brix did not lead to further reductions in the rates of spin adduct formation (data not shown). This indicates that sucrose may, to a limited extent, act as a radical scavenger. The observed increase in radical formation at the early stages of mashing, in comparison to the later stages, can therefore not be fully explained by the differences in °Brix. Then again, many other chemical changes, such as gelatinization and saccharification of the starch, hydrolysis and denaturation of proteins, and numerous oxidative reactions involving lipids, proteins, polyphenols, and carbohydrates do occur in the mash during the increasing temperatures of the mashing process.

The oxidative stability of the wort samples was also examined by measuring their ability to consume dissolved oxygen when heated to 60 °C (**Figure 5**). The early wort sample consumed oxygen at a higher rate than the wort collected at the end of the mashing. This was in agreement with the relative oxidative stabilities that were observed with the ESR-based spin trapping method, and it thus demonstrates that the addition of ethanol and the spin trap POBN does not give rise to major changes in the oxidative behavior of the wort samples. The consumption of oxygen was fast in the wort samples at 60 °C. Preheating the samples before the oxygen consumption measurements resulted in samples with very different levels of oxygen at the start of the oxygen measurements. Therefore, the most reproducible results were obtained when the measurements were started by adding room temperature wort samples into the preheated oxygen measurement chambers. This led, however, to the rapidly changing oxygen concentrations seen during the first 10 min of the measurements, when the temperature of the samples reached 60 °C (**Figure 5**).

It was also tested whether the higher rates of radical formation during the incubation in the early wort samples were due to the presence of endogenous malt redox-active enzymes. Lipoxygenases (LOX-1 and -2) are inactivated at temperatures above 65 °C (*1*) and are expected to become inactivated during the last step of the applied mashing program. Incubating the wort samples at 70 °C during the evaluation of oxidative stability gave approximately four times higher rates of radical formation for all samples. Nevertheless, the wort samples from the early, low temperature mashing stage at both incubation temperatures gave higher rates of radical formation than the later, high temperature mashing samples (**Figure 4**). This demonstrates that the lower oxidative stabilities of the early samples are most likely not caused by the action of malt redox-active enzymes

in the wort samples during the incubation, as otherwise only a small difference would have been expected between the oxidative stabilities of the early and the late wort samples at the high incubation temperature, at which the enzymes are inactivated. It is, however, possible that redox enzymes may, during the early low temperature stages of mashing, produce precursors that generate radicals upon heating of the wort. In such cases, these potential precursors could have been destroyed during the last high-temperature stage of the mashing, thereby producing late wort samples that are less prone to generate radicals upon heating during the ESR evaluation of radical formation.

Addition of Iron during Mashing. Analysis of the levels of iron and copper in the wort samples showed that the final wort contained less iron and copper than the wort collected after 25 min of mashing (**Table 1**). The presence of transition metals, especially iron and copper, can promote metal-catalyzed oxidations and thereby reduce the oxidative stability. The addition of iron at the beginning of the mash did not influence the formation of spin adducts in the wort samples (**Figure 6**), but the addition of iron directly to the wort after the mashing did increase the levels of spin adducts considerably.

Analysis of the iron concentrations in the wort samples showed that the level of iron in the wort was only slightly increased by the addition of iron during the mashing, whereas the direct addition of metals to the wort samples substantially increased the iron concentrations (**Table 1**). These results suggest that iron becomes trapped during the mashing.

Catalase and Superoxide Dismutase. Hydrogen peroxide and superoxide are important intermediates in the oxidative processes that occur in beer (*6, 16, 17*). Catalase efficiently removes hydrogen peroxide, and the addition of catalase to beer has been demonstrated to inhibit the formation of radicals (*6*). The addition of catalase (6000 CIU/L) to the wort samples resulted in a significant reduction, by 20% ($p < 0.05$), of the rate of radical formation (**Figure 7**). As expected, the addition of hydrogen peroxide markedly increased the radical formation (**Figure 7**). The addition of the hydrogen peroxide together with catalase reduced the rate of spin adduct formation, demonstrating that catalase is active at the high temperature (60 °C) used during the incubation of the wort samples. The effect of superoxide dismutase was similarly examined. In spite of this, the addition of superoxide dismutase alone, or in combination with catalase, did not have any significant positive or negative effects ($p > 0.05$) on the rate of radical formation, as compared to the control (**Figure 8**). The lack of effect of catalase when dosed in combination with superoxide dismutase might be due to the markedly lower applied dosage (200 CIU/L) of catalase. Catalase is capable of acting as a peroxidase at low hydrogen peroxide substrate concentrations if a suitable acceptor such as an organic substrate is present (*18*). This is due to the large differences in the affinities of catalase and peroxidase for hydrogen peroxide (*19*). The possibility of catalase to act as a peroxidase at low substrate concentrations might explain why
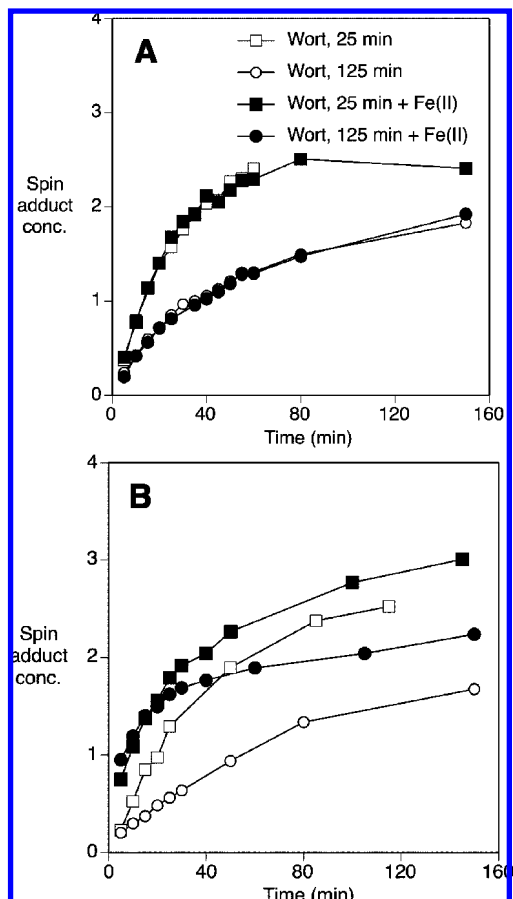
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Figure 6. Effect of the addition of Fe(II) on the formation of spin adducts during heating wort. (**A**) Addition of FeSO$_4$ (50 μM) at the beginning of mashing. (**B**) Addition of FeSO$_4$ (50 μM) to wort. The wort samples with added POBN (40 mM) and EtOH (5%) were heated at 60 °C.
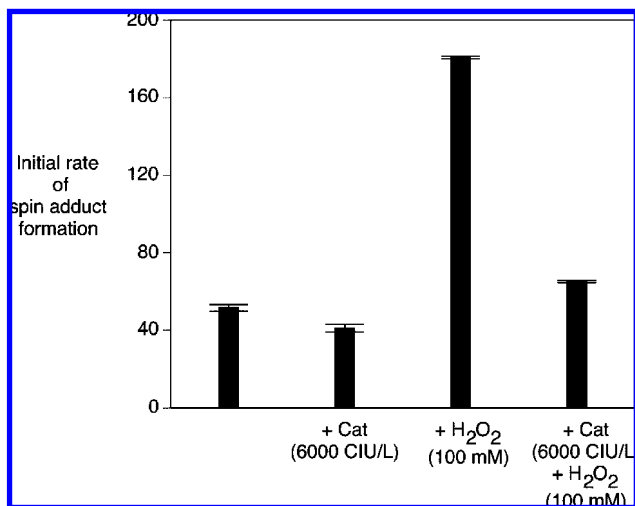

Figure 7. Effect of catalase on the oxidative stability of wort. The wort samples with added POBN (40 mM) and EtOH (5%) were heated at 60 °C. The differences between the samples are all statistically significant ($p < 0.05$).

no effect of catalase was observed when it was dosed at a markedly lower range.

DISCUSSION

The oxidative stability of sweet wort was evaluated by measuring the tendency toward radical formation during heating
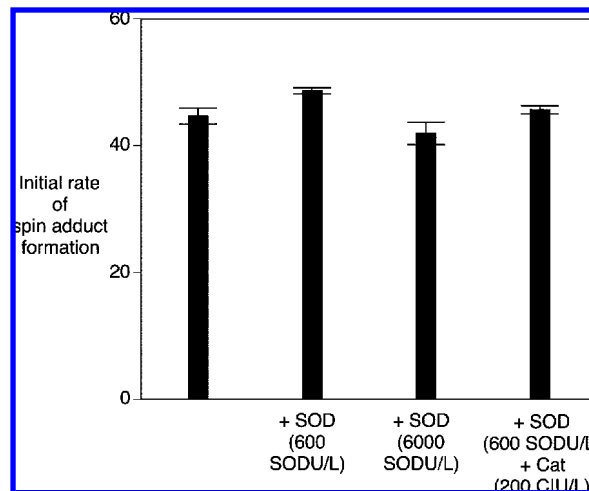

Figure 8. Effect of superoxide dismutase on the oxidative stability of wort. The wort samples with added POBN (40 mM) and EtOH (5%) were heated at 60 °C. The differences between the samples are not statistically significant ($p > 0.05$).

of wort samples with access to atmospheric oxygen. Radicals were detected by the spin trapping technique. The presence of ethanol was, however, necessary to accumulate spin adducts at concentrations that gave ESR signals with sufficient intensities. The effect of ethanol on the formation of spin adducts in wort suggests that highly reactive radicals, such as hydroxyl radicals and alkoxyl radicals, are formed during heating of wort. These radicals are able to react with ethanol and generate the 1-hydroxyl radicals that are trapped by POBN (reactions 1−3) (*16*).

$$CH_3CH_2OH + {}^\bullet OH \rightarrow CH_3{}^\bullet CHOH + H_2O \qquad (1)$$

$$CH_3CH_2OH + RO^\bullet \rightarrow CH_3\cdot CHOH + ROH \qquad (2)$$

$$CH_3\cdot CHOH + POBN \rightarrow {}^\bullet POBN/CH(CH_3)OH \qquad (3)$$

Therefore, the use of ethanol during the spin trapping experiments not only increases the formation of ESR-detectable spin adducts but also makes the detection of highly reactive radicals more selective. Furthermore, the good heat stability of the adduct ${}^\bullet$POBN/CH(CH$_3$)OH allows for the accumulation of relatively high concentrations of this spin adduct during the incubation of wort at elevated temperatures, resulting in ESR spectra with high intensities (*20*). It is unlikely that the low intensities of the ESR spectra that were observed in the absence of ethanol are caused by a lack of formation of radicals in the wort. In the absence of ethanol, the nonselective hydroxyl and alkoxyl radicals react only with carbohydrates, proteins, polyphenols, and other organic components present in the wort, but radicals derived from these compounds apparently do not form spin adducts, or the spin adducts are not as stable as the ethanol-derived adducts. The reactions between hydroxyl radicals and carbohydrates are often not selective and can lead to a wide range of products, including products of depolymerizations and ring openings (*21, 22*).

It was demonstrated that oxygen is needed for the formation of radicals. Enzymatic reactions, such as lipoxygenase activity, are most likely to have a negligible role in the oxidative processes occurring during the heating of wort in this study due to their inactivation at temperatures above 65 °C (*1*). Production of hydroxyl and alkoxyl radicals in biological systems is often closely linked to the presence of iron and copper ions that are

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able to transform peroxides into these radicals by the Fenton reaction (reaction 4).

$$Fe^{2+} + HOOH + H^+ \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O \quad (4)$$
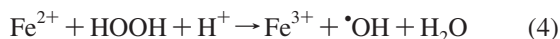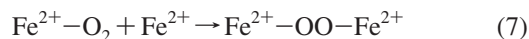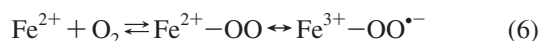
Organic peroxides, ROOH, such as protein peroxides, may react with Fe(II) by a similar reaction, producing alkoxyl radicals (reaction 5).

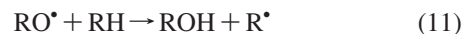$$Fe^{2+} + ROOH + H^+ \rightarrow Fe^{3+} + RO^{\bullet} + H_2O \quad (5)$$
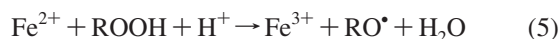
Accordingly, the addition of Fe(II) to the wort samples did increase the formation of spin adducts. It is noteworthy that the addition of iron at the beginning of the mashing did not increase the content of iron in the wort samples. Apparently, high concentrations of iron in the wort are prevented from being detected by their efficient binding to the solids that are removed by filtration or by the lautering in the brew house.

The formation of hydroxyl and alkoxyl radicals indicates that peroxides have been present as precursors in the wort. Consequently, the addition of hydrogen peroxide to the wort increased the formation of radicals, and catalase, which very efficiently removes hydrogen peroxide even at the high temperatures used during the incubation of the wort, was able to hinder the formation of the extra radicals caused by the addition of the hydrogen peroxide. However, the addition of catalase alone during the incubation of the wort had a significant effect on the rate of radical formation in the wort, reducing it by 20%. This suggests that the Fenton reaction, with hydrogen peroxide as the reagent (reaction 4), is not the only reaction mechanism responsible for the oxidative events in the wort. Furthermore, the addition of SOD had no positive or negative effect on the radical formation in the wort, which indicates that superoxide is not an important intermediate during the aerobic oxidation.

Two mechanisms can possibly explain the formation of radicals without free hydrogen peroxide or superoxide as intermediates. It has been suggested that highly reactive ferryl ions, $Fe^{IV}=O$, can be generated by a mechanism where oxygen is bound as a diiron−oxygen complex (reactions 6−8) (*23*). Catalase and SOD have been shown to only partially prevent the formation of oxidizing species during iron autoxidation. Some of these species, which very likely include ferryl ions, $Fe^{IV}=O$, depend on the chelators present, able to oxidize ethanol to 1-hydroxyethyl radicals, which have been detected as spin adducts (*24, 25*).

$$Fe^{2+} + O_2 \rightleftarrows Fe^{2+}-OO \leftrightarrow Fe^{3+}-OO^{\bullet-} \quad (6)$$

$$Fe^{2+}-O_2 + Fe^{2+} \rightarrow Fe^{2+}-OO-Fe^{2+} \quad (7)$$

$$Fe^{2+}-OO-Fe^{2+} \rightarrow 2Fe^{IV}=O \quad (8)$$

Another mechanism can be put forward, where alkyl radicals derived from organic compounds, such as carbohydrates or proteins, trap oxygen as peroxyl radicals (reaction 9). The presence of reducing compounds, such as polyphenols, in the wort leads to the fast transformation of peroxyl radicals into hydroperoxides (reaction 10), which are precursors for the formation of alkoxyl radicals (reaction 5). The alkoxyl radicals subsequently react with carbohydrates or proteins, forming new alkyl radicals (reaction 11).

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (9)$$

$$ROO^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet} \quad (10)$$

$$RO^{\bullet} + RH \rightarrow ROH + R^{\bullet} \quad (11)$$

It is not possible to discern between these mechanisms based on the current results. However, both mechanisms may very likely occur in parallel, where ferryl ions generated by reactions 6−8 react with carbohydrates or proteins forming the alkyl radicals that trap oxygen (reaction 9).

The immediate formation of radicals upon heating the wort samples is in contrast to lager beer, where a lag phase is usually observed before the onset of spin adduct formation (*6*). Good correlations have been demonstrated between the stability of the flavor of lager beer and the lengths of these lag phases of radical formation. The lag phase has been suggested to be due to the competing effects of pro-oxidative components and antioxidants, such as sulfite. Sulfite in beer can efficiently quench peroxides, which are important precursors for the formation of radicals (*9*). The absence of a lag phase for radical formation in wort suggests that the endogenous antioxidants in wort are only able to quench a fraction of radicals, leaving a substantial amount of radicals able to take part in deleterious oxidative reactions. A lag phase of radical formation was not observed when sulfite was added to the wort during mashing-off, indicating that sulfite decays quickly in wort (*11*).

In this study, the initial rate of spin adduct formation in sweet wort was used to quantify and study the extent of occurrence of oxidative reactions during mashing. However, further studies are needed to evaluate how the effects of oxidation during the early steps of the beer brewing process are modulated by the subsequent brewing steps and, most importantly, how they affect the quality of the final beer product. This includes further studies of the impact of the catalase-facilitated reduction of the initial rate of spin adduct formation in sweet wort on the stability of flavor of the final beer.

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