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Electron Spin Resonance Spin Trapping Identification of Radicals Formed during Aerobic Forced Aging of Beer

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Using 2-methyl-2-nitrosopropane, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), phenyl-*N*-*tert*-butylnitrone , and 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone as spin traps, spin adducts of free radicals formed in beer under aerobic conditions have been characterized by ESR spectroscopy. The 1-hydroxyethyl radical is found to be the quantitatively most important radical, while the hydroxyl radical is trapped only by DMPO and only at high concentrations (0.5 M). Fenton-like oxidation processes in beer thus involve reaction of the 1-hydroxyethyl radical in an important reaction with oxygen, converting it to hydroperoxyl radicals.

Keywords: *Beer; ESR; spin trapping; oxidation; 1-hydroxyethyl radical; hydroxyl radical*

INTRODUCTION

The role of oxidation processes in the staling of beer flavor has received considerable interest, as it has been recognized that the compounds responsible for the off-flavor most likely are oxidation products of components present in either the wort or the beer (Bamforth et al., 1993). The increase in the concentration of carbonyl compounds during beer aging has been linked to the presence of oxygen and metal ions (Bamforth and Parsons, 1985; Irwin et al., 1991). Free radicals have been identified by electron spin resonance spectroscopy (ESR) to be reaction intermediates in the oxidation processes in beer during aerobic forced aging, and it was demonstrated that addition of iron and hydrogen peroxide accelerated the formation of radicals in beer (Uchida and Ono, 1996). Addition of catalase, which destroys hydrogen peroxide, or addition of chelating agents that bind metal ions was, moreover, found to slow the production of radicals (Uchida and Ono, 1996). These results taken together provide evidence that Fenton-type oxidations in beer can take place in the presence of oxygen serving as a precursor for hydrogen peroxide, which subsequently reacts with iron or copper ions to yield hydroxyl radicals:

$$\mathrm{Fe^{2+}/Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + {}^{\bullet}OH + OH^{-}} \quad (1)$$

Uchida and Ono (1996) used the spin traps phenyl-*N*-*tert*-butylnitrone (PBN) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) to detect radicals in beer as spin adducts and assigned the trapped radical as the hydroxyl radical. Hydroxyl radicals have also been claimed to have been trapped by PBN during γ-irradiation of beer and incubation of beer at 60 °C (Kaneda et al., 1988). However, the half-life of the PBN/$^{\bullet}$OH adduct is only 1 min at room temperature and most likely less than that at the elevated temperatures used in the accelerated experiments (Janzen et al., 1992). Moreover, the reactions in beer occur in a homogeneous aqueous medium containing $\approx$1 M ethanol, and spin trapping studies with model aqueous ethanol solutions, where hydroxyl radicals also were generated by the Fenton reaction, have been found to lead to trapping of the 1-hydroxyethyl radical instead of the hydroxyl radical (Sridhar et al., 1986; Pou et al., 1994).

We have undertaken the present ESR study using four different spin traps to identify the free radicals that are trapped in beer and to investigate the fate of hydroxyl radicals in beer.

EXPERIMENTAL PROCEDURES

Chemicals. 2-Methyl-2-nitrosopropane (MNP) (Sigma, St. Louis, MO), DMPO (Fluka, Buchs, Switzerland), PBN (Sigma), 4-pyridyl-1-oxide-*N*-*tert*-butylnitrone (POBN) (Sigma), hydrogen peroxide 30% (purum p.a., Fluka), $FeSO_4 \cdot 7H_2O$ (p.a., Merck, Darmstadt, Germany), and ethanol (puriss. p.a., Fluka) were used as received. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification train. The beer used was a commercially available lager beer containing 4.6% ethanol.

ESR Experiments. Carbon dioxide was removed from the lager beer by decanting, and the beer was subsequently heated for 100 min at 55 °C in a closed bottle under atmospheric oxygen to exhaust the natural antioxidants present in the beer (Uchida and Ono, 1996). The spin traps were dissolved in 2 mL samples of preheated beer. Hydrogen peroxide and $FeSO_4$ were added as freshly prepared aqueous solutions (29 and 2.0 mM, respectively). The reaction mixtures were kept in test tubes with screw caps at room temperature protected from light. The ESR spectra were obtained with an ECS 106 spectrometer (Bruker, Rheinstetten, Germany) equipped with an ER 4103 TM cavity. The settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; conversion time, 164 ms; and time constant, 82 ms. The beer samples were contained in a quartz flat aqueous cell (Wilmad Glass, Buena, NJ), and all spectra were recorded at room temperature. The response of the ESR instrument was checked daily by recording the spectra of an aqueous solution of 2,2,6,6-tetramethylpiperidine-1-oxyl radical (Tempo) (Aldrich, Steinheim, Germany).

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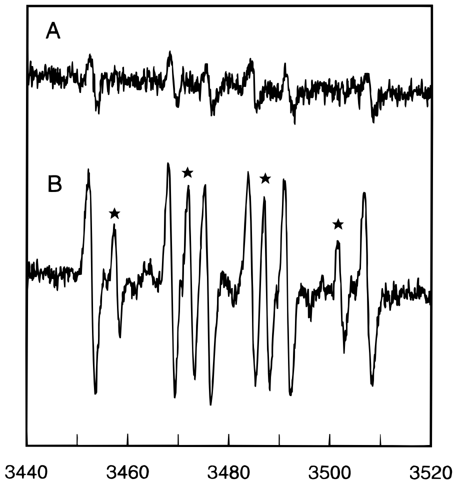


Figure 1. ESR spectra of a preheated lager beer kept at room temperature 45 min after the addition of (A) 50 mM DMPO and (B) 500 mM DMPO. The DMPO/•OH adduct signals are marked by stars.

RESULTS

Spin Trapping with DMPO. The formation of hydroxyl radical spin adducts of DMPO in lager beer in the presence of atmospheric oxygen has previously been demonstrated by Uchida and Ono (1996) using a relatively high concentration of DMPO (1 M) and obtaining the spectra at room temperature. We have conducted similar experiments but with lower concentrations of DMPO (down to 50 mM). The DMPO/•OH adduct is evident in the spectra obtained with 0.5 M DMPO as presented in Figure 1 (marked by stars; hyperfine coupling constants $a_N \approx 14.8$ G and $a_H \approx 14.8$ G). However, an additional six-line spectrum (a triplet of doublets; $a_N = 22.9$ G and $a_H = 15.9$ G) of almost similar intensity is also present. The signal due to the DMPO/•OH adduct is absent when the concentration of DMPO is lowered 10 times to 50 mM. Only the six-line spectrum can be identified, and clearly the intensity depends on the DMPO concentration. The intensity of the DMPO/•OH signals was found to decrease with time both in the presence and in the absence of iron and hydrogen peroxide, whereas the intensity of the six-line spectra increased.

A similar concentration dependence of DMPO was observed when iron(II) sulfate and hydrogen peroxide were added to the beer with dissolved DMPO, as may be seen from Figure 2. However, the intensity of the spectra was much higher than without added iron and hydrogen peroxide. The DMPO/•OH adduct was absent at the low concentration of DMPO (50 mM), where only the six-line spectrum was seen.

The coupling constants of the six-line spectra are identical to values determined for the spin adduct of the 1-hydroxyethyl radical and DMPO in aqueous solution, hence providing direct evidence for the presence of the 1-hydroxyethyl radical in beer under oxidation (Buettner, 1987).

Spin Trapping with MNP. The spin trap MNP forms spin adducts in which the nitroxyl moiety is bonded directly to the added radical, which makes the hyperfine couplings of the spin adducts very sensitive to the structure of the radicals (Perkins, 1980). Accordingly, the number of hydrogen atoms bonded to the atom

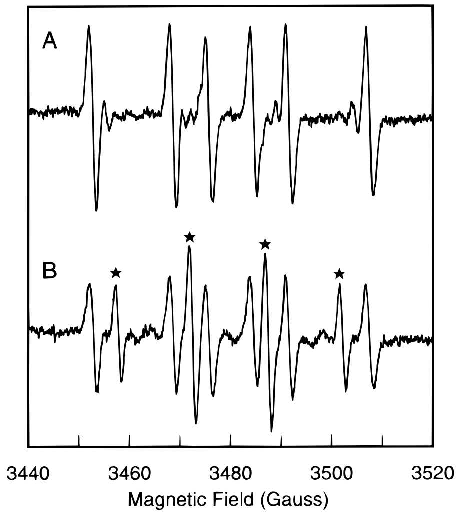


Figure 2. ESR spectra of radical adducts of DMPO formed in a preheated lager beer at room temperature 10 min after the addition of (A) DMPO (50 mM), $FeSO_4$ (2 μM), and H_2O_2 (130 μM) and (B) DMPO (500 mM), $FeSO_4$ (1 μM), and H_2O_2 (65 μM). The DMPO/•OH adduct signals are marked by stars.

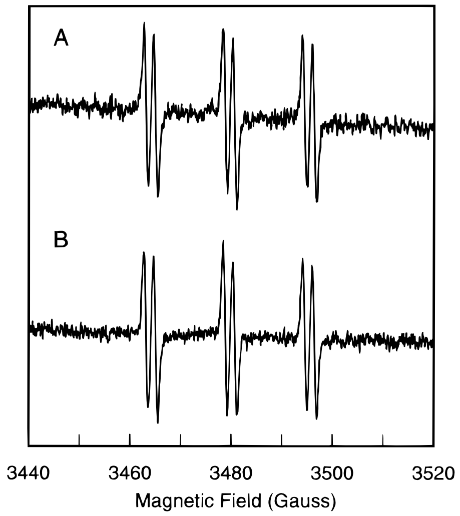


Figure 3. ESR spectra of radical adducts of MNP formed at room temperature following addition of $FeSO_4$ (10 μM), H_2O_2 (580 μM), and MNP (17 mM) to a preheated lager beer (A) and to a solution of ethanol (1 M) in water (B).

with the unpaired spin in the added radical can easily be detected from the pattern of hyperfine splitting. The formation of hydroxyl radicals could also in this experiment be enhanced by adding iron(II) sulfate and hydrogen peroxide to lager beer with dissolved MNP. A single spin adduct with a spectrum consisting of a triplet of doublets ($a_N = 15.7$ G and $a_H = 1.9$ G) was detected (see Figure 3). An identical spin adduct could be formed by substituting the lager beer with a 1 M solution of ethanol in water under similar conditions. The spin adducts were in both cases destroyed by heating the solutions for 5−10 min at 55 °C. Experiments with iron(II) but without hydrogen peroxide gave no detectable ESR signals.

The pattern of splitting in the ESR spectra of the MNP radical adduct clearly indicates that the trapped radical has one hydrogen atom on the radical-bearing atom. In addition, the completely identical spin adduct formed in a 1 M aqueous ethanol solution had coupling constants that were identical to the values expected for the 1-hydroxyethyl spin adduct (Buettner, 1987). These results further confirm that 1-hydroxyethyl radicals are formed in beer under conditions in which Fenton chemistry is important.

Spin Trapping with PBN and POBN. Adducts with six-line spectra (triplets of doublets) were formed when PBN and POBN were used as spin traps in beer. Addition of iron(II) sulfate and hydrogen peroxide led in both cases to an increased amount of formed spin adducts. The coupling constants [$a_N = 16.0$ G, $a_H = 3.3$ G (PBN) and $a_N = 15.6$ G, $a_H = 2.6$ G (POBN)] were identical to the values expected for the spin adducts formed from the 1-hydroxyethyl radical (Buettner, 1987; Pou et al., 1994), and addition of iron(II) sulfate and hydrogen peroxide to 1 M aqueous ethanol in the presence of PBN or POBN gave ESR spectra that were identical to spectra obtained with beer (data not shown).

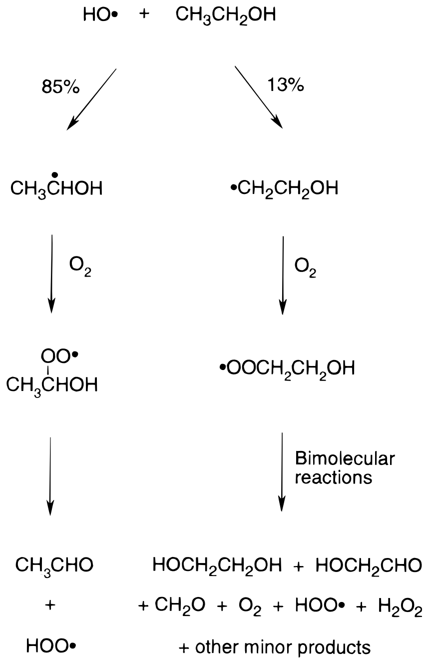
DISCUSSION

The formation of the DMPO/$^\bullet$OH adduct in preheated beer as seen in Figure 1B demonstrates that hydroxyl radicals are formed in beer under aerobic conditions even without addition of Fe^{2+} or hydrogen peroxide. However, long reaction times were needed to obtain the weak signals. We therefore chose to perform the spin trapping experiments by adding Fe^{2+} and hydrogen peroxide to the beer and thereby accelerate the formation of hydroxyl radicals. This increased the intensity of the ESR spectra as seen in Figure 2, but no new lines appeared in the spectra when compared to the spectra in Figure 1. Evidently the same type of radicals were trapped in the two set of experiments. The intensity of the DMPO/$^\bullet$OH quartet is relatively higher in Figure 2B than in Figure 1B, which most likely is a consequence of the higher steady-state concentration of hydroxyl radicals under the Fenton conditions. The experiments with MNP provided strong evidence for the 1-hydroxyethyl radical as the radical that is being trapped. Furthermore, the hyperfine coupling constants for the spin adducts we observe with PBN, POBN, and DMPO (at 50 mM concentration) are all in agreement with adducts with the 1-hydroxyethyl radical.

The hydroxyl radical reacts with essentially diffusion-controlled reaction rates with most organic compounds with almost no selectivity (Buxton et al., 1988). Compounds present in the highest concentration in a biological or food system will accordingly determine the major reaction paths for oxidative deterioration initiated by hydroxyl radicals. A typical lager beer contains $\approx$1 M ethanol, which makes it the component found in the highest concentration apart from water. Carbohydrates (and especially glycerol), amino acids, and proteins are found in total concentrations of 20−50 mM, whereas the concentration of carbon dioxide in bottled beer is $\approx$100 mM (Moll, 1994).

Hydroxyl radicals abstract hydrogen atoms from ethanol with a rate constant of $k = 1.9 \times 10^9$ M^{-1} s^{-1} mainly at the C-1 carbon (85%) and to a minor extent at the C-2 carbon (13%) (Scheme 1) (Asmus et al., 1973; Buxton et al., 1988). In lager beer this leads to a pseudo-first-order rate constant equal to 1.9×10^9 s^{-1} for the reaction between the hydroxyl radical and ethanol. The rate constants for the reactions between hydroxyl radical and the spin traps DMPO, POBN, and PBN are 4.3×10^9, 4.0×10^9, and 6.1×10^9 M^{-1}s^{-1}, respectively, as detected in aqueous solution (Sridhar et al., 1986; Buxton et al., 1988). A 50 mM concentration of the spin traps will consequently result in $<$10% of the hydroxyl radicals being trapped by the spin traps, the highest value obtained with PBN. Furthermore, only PBN and POBN adducts with the hydroxyl radical added to the nitrone moiety are sufficiently long-lived to be detected by ESR (Neta, 1980). Only spin trap concentrations of $\sim$0.5 M will lead to comparable amounts of hydroxyl radicals reacting with ethanol and with the spin trap. This kinetic discrimination explains why the DMPO/$^\bullet$OH adduct is seen only at the high concentrations of DMPO.

Scheme 1



The experiments have been performed with beer, in which the natural antioxidants were exhausted by preheating. This is not expected to affect the reactions of the hydroxyl radicals since ethanol even in the presence of antioxidants will be the major reactant. Sulfite is probably the most important antioxidant, but it is usually found in parts per million levels, and it is therefore kinetically unable to influence the almost diffusion-controlled reaction between ethanol and the hydroxyl radicals. However, the antioxidants in beer may be able to react with long-lived intermediates that are formed during the oxidation reactions, and it is very likely that especially sulfite is able to trap hydrogen peroxide before it can initiate Fenton reactions.

The main fate of the 1-hydroxyethyl radical under aerobic conditions is most likely the diffusion-controlled reaction with dissolved oxygen producing 1-hydroxyethylperoxyl radicals, which decay to hydroperoxyl radicals and acetaldehyde either unimolecularly ($k = 50$ s^{-1}) or by catalysis by OH^- or HPO_4^{2-} (see Scheme 1) (Bothe et al., 1983). This reaction, which leads to the production of hydroperoxyl radicals, is unique for 1-hydroxyalkylperoxyl radicals. Other alkylperoxyl radicals are usually more stable and decompose in bimolecular reactions forming alkoxyl radicals.

In air-saturated aqueous solutions with $\approx$0.3 mM oxygen, the reaction between oxygen and 1-hydroxyethyl radicals has a pseudo-first-order rate constant of 3×10^6 s^{-1}. The second-order rate constants for the addition of 1-hydroxyethyl radicals to DMPO, POBN, and PBN are 1.1 × 10^6, 3.1 × 10^7, and 1.5 × 10^6 M^{-1} s^{-1}, respectively (Pou et al., 1994). At a 50 mM concentration of the spin traps 2% (DMPO), 37% (POBN), and 3% (PBN) of the 1-hydroxyethyl radicals are expected to be trapped. These numbers are maximum values since they are based on the assumption that reactions other than with oxygen and spin traps are negligible for the 1-hydroxyethyl radical. Only a few percent of the 1-hydroxyethyl radicals are trapped by DMPO and PBN, but the increased stability of the spin adducts results in higher concentrations than in the case of the hydroxyl radical adducts, which decompose more rapidly (Pou et al., 1994). The difference in stabilities is even more pronounced at the higher temperatures used in the forced aging experiments with beer (Uchida and Ono, 1996). The relatively small amounts of hydroxyl radicals trapped by DMPO and PBN suggest that the presence of these two spin traps has a negligible effect on the course of the radical chain reactions and that they therefore do not inadvertently act as antioxidants.

The small amount of 2-hydroxyethyl radicals formed by the hydrogen abstraction from ethanol is also expected to react with oxygen, yielding 2-hydroxyethylperoxyl radicals (see Scheme 1). These radicals are known to decay in bimolecular reactions yielding glycolaldehyde, ethylene glycol, formaldehyde, oxygen, hydrogen peroxide, and hydroperoxyl radicals as the major products (Zegota et al., 1986).

The hydroperoxyl radicals are weak acids ($pK_a = 4.8$), and a significant amount is found as the corresponding base superoxide ($O_2^{\bullet -}$) in beer, where the pH is in the range of 4−5. Hydroperoxyl radicals and superoxide react, forming hydrogen peroxide and oxygen ($k \approx 10^8$ M^{-1} s^{-1}):

$$H^+ + HOO^{\bullet} + O_2^{\bullet -} \rightarrow H_2O_2 + O_2 \qquad (2)$$

Oxidation by Fe^{3+} and Cu^{2+} to yield oxygen and reduction by Fe^{2+} and Cu^+ to hydrogen peroxide are other decay routes available for superoxide and hydroperoxyl radicals. However, there is no evidence of spin adducts arising from hydroperoxyl/superoxide radicals and DMPO in Figures 1 and 2, but the absence does not rule out the formation of hydroperoxyl radicals. First of all, DMPO/$^{\bullet}$OOH adducts are not stable (half-life 90 s at pH 5), and it is unlikely that any detectable amount would accumulate during the long reaction time of the experiments in Figure 1 (Buettner and Oberley, 1978). Second, even micromolar concentrations of Fe^{2+} can react with hydroperoxyl radicals and thereby prevent the formation of the DMPO/$^{\bullet}$OOH adduct (Buettner and Oberley, 1978).

It is clear from the mechanism outlined in Scheme 1 that ethanol plays a significant role in the oxidative processes in beer. First of all, it quenches most of the hydroxyl radicals that are formed from the Fenton reaction. Second, the 1-hydroxyethyl radicals bind oxygen and transform it into hydroperoxyl radicals that eventually are reduced to hydrogen peroxide, which can continue the chain of radical reactions by reacting with iron and copper ions.

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