Potential Antioxidants in Beer Assessed by ESR Spin Trapping

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A number of potential antioxidants have been evaluated for their effect on formation of radicals in beer using the electron spin resonance (ESR) lag phase method. Sulfite was found to be the only compound that was able to delay the formation of radicals, whereas phenolic compounds such as phenolic acids, catechin, epicatechin, and proanthocyanidin dimers had no effect on the formation of radicals. Ascorbate, cystein, and cysteamin were on the other hand found to be prooxidants. It is suggested that antioxidants must be able to either scavenge peroxides or trap metal ions in order to be effective in beer. The effectiveness of sulfite is suggested to be a consequence of its two-electron nonradical producing reaction with peroxides.

Keywords: *Beer; ESR spin trapping; radicals; antioxidants*

INTRODUCTION

The shelf life of packaged pasteurized beer is essentially determined by either the appearance of haze or the deterioration of the flavor. Both of these phenomena are the result of nonbiological oxidation processes that involve active oxygen species, such as H_2O_2, $HO^{\bullet}$, and $HOO^{\bullet}/O_2^{\bullet-}$ (Dadic, 1984; Bamforth et al., 1993; Uchida and Ono, 1996). The colloidal instability of beer (i.e., the formation of haze) is mainly caused by the formation of insoluble complexes between proteins and oxidized polyphenols. Lowering the concentration of the phenolic proanthocyanidins in beer, e.g., by cold filtration or treatment with polyvinylpolypyrrolidone (PVPP), can efficiently delay the formation of haze during storage (McMurrough et al., 1996; McMurrough et al., 1997). The oxidation reactions involve Fenton reactions which are dependent on oxygen and iron or copper ions, and minimizing the content of these compounds in the packaged beer, have a positive effect on the stability of the flavor (Irwin et al, 1991; Narziss et al., 1993).

Delaying the oxidation processes by addition of antioxidants is less straightforward. Proanthocyanidins and other phenolic compounds are potential antioxidants, but contradicting results exist on their effectiveness in beer. Barley and malt have been demonstrated to contain phenolic compounds that after extraction were effective as antioxidants during the accelerated autoxidation of methyl linolate (Maillard et al., 1996), and addition of the flavonoid 2″-*O*-glycosylisovitexin to beer decreased the rate of formation of acetaldehyde in beer stored at 50 °C for 10 days (Nakajima et al., 1998). However, neither the removal of flavonoids by PVPP-stabilization nor the addition of simple flavanols to beer have been found to affect the sensory score and the concentration of *trans*-2-nonenal compared to a control after forced aging at 60 °C for 7 days (McMurrough et al., 1996), and addition of catechin or ferulic acid to beer had no effect on the formation of carbonyl compounds during an extended storage trial (Walters et al., 1997).

The formation of radicals in lager beer can be monitored by trapping the short-lived reactive radicals with spin traps and detection of the long-lived spin adducts that is formed with electron spin resonance spectroscopy (ESR) (Kaneda et al., 1988; Uchida and Ono, 1996; Andersen and Skibsted, 1998). Initially a negligible amount of spin adducts is detected when beer is heated (≈50 °C) under access to atmospheric oxygen. After a certain period of time, called the lag phase, the amount of spin adducts begins to increase linearly with time. The length of the lag phase of fresh beer has been shown to correlate with the flavor stability of the beer, and the ESR method is thus a promising accelerated method for predicting the stability of beer (Uchida et al., 1996). The two unique advantages of this method is (1) it monitors the formation of primary intermediates produced during the oxidation processes, and (2) the lag phase is a result of the competition between the actions of the prooxidative and antioxidative components in beer. The method consequently provides an excellent way to examine potential antioxidants in beer. This assay furthermore has the advantage that it is possible to observe both the time it takes before the natural antioxidants are exhausted (the lag phase) and, after that, the rate at which radicals are formed in the absence of antioxidants. In this study, we have used the ESR method to test a number of different compounds that potentially could act as antioxidants in beer. The purpose of this study is to examine the potential antioxidative strategies that can be used to increase the shelf life of beer.

EXPERIMENTAL PROCEDURES

Chemicals. *N-tert*-Butyl-α-phenylnitrone (PBN) (Molecular Probes, Leiden, The Netherlands), L-cysteine and potassium disulfite (Merck, Darmstadt, Germany), (+)-catechin, cysteamine, L-methionine, sodium ascorbate, and vanillic acid (Fluka, Buchs, Switzerland), hypotaurine (Sigma, St. Louis, MO), caffeic acid, (−)-epicatechin, ferulic acid, gallic acid, quercetin, rutin, and sinapic acid (Aldrich, St. Louis, MO), and 3,3′-thiodipropionic acid (Theodor Schuchardt & Co, Hohen-

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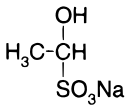
brunn, Germany) were used as received. The natural dimeric proanthocyanidins, prodelphinidin B-3 (PDB-3) and procyanidin B-3 (PCB-3), were synthesized from catechin and the dihydroflavonols dihydromyricetin and dihydroquercetin, respectively, isolated from bark of *Pinus contorta* (Andersen et al., 1999). Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification train.

Acetaldehyde−Sulfite Adduct (Sodium 1-Hydroxyethanesulfonate (1)). Acetaldehyde (2 g, 46 mmol, Merck, Darmstadt, Germany) was dissolved in 50 mL of ethanol (96%) at 5 °C. A solution of 40% aqueous $Na_2S_2O_5$ (7.5 mL, 41 mmol HSO_3^-) was added to the stirred cold solution, initially dropwise and later at a faster rate. Precipitation began after approximately 2.5 mL of the sulfite solution had been added. Ethanol (50 mL) was added to the reaction mixture, and it was kept at 5 °C overnight. The precipitate was collected by cold filtration on a glass filter and was subsequently washed with ethanol. The structure and purity were confirmed by NMR spectroscopy.



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Beer. Four different lager beers A−D were examined. Beers A and B were all-malt beers brewed in the 50-L Pilot brewery with Caminant malt and Alexis malt (control), respectively, using a conventional infusion mashing procedure. Beer C was made by fermentation of a production wort with a modified yeast strain unable to produce sulfite. All fermentations were carried out at 14 °C. The beer was maturated at 7 °C to remove diacetyl. Before filtration, the beer was cooled to −1 °C for at least 24 h to ensure a good haze stability. No measure to further reduce the polyphenol content was taken. Finally, beer D was a commercial lager beer, containing 2 ppm sulfite. The concentration of sulfite in beer was determined by headspace gas chromatography (Lowe and Dreyer, 1997).

HPLC. A slightly modified version of the method by Madigan et al. (1994) was used for the analysis of proanthocyanidins, catechin, and phenolic acids in beer (Andersen et al., 1999). The beer was degassed and filtered prior to injection on the HPLC system that was equipped with a Macherey-Nagel ET Nucleosil 100−10 C18 column and an ESA (ESA Inc., MA) model 5011 electrochemical detector operating at a cell potential equal to +450 mV. A solvent gradient was used with solvents A (2.5% acetic acid in water) and B (2.5% acetic acid and 2.5% water in methanol). The amount of solvent B was increased from 0% at $t = 0$ min to 5% at $t = 25$ min, 40% at $t = 50$ min, and finally to 100% at $t = 60$ min.

ESR Experiments. Carbon dioxide was removed from the bottled lager beer by stirring for 5 min. The lag phase experiments were performed by heating 25 mL of degassed beer containing 30 mM PBN in a 100-mL glass bottle closed with a screw cap. The beer samples were kept at 55 °C in a water bath. Samples (≈2 mL) of the heated beer were transferred directly to a flat ESR aqueous cell (Wilmad, Buena, NJ) and were allowed to cool to room temperature before the ESR spectrum was obtained on either a Bruker ECS106 (Bruker, Rheinstetten, Germany) or a JEOL JES−FR30 (JEOL Ltd., Tokyo, Japan) ESR spectrometer, both operating in X-band mode, with a 100 kHz modulation frequency and 1 G modulation amplitude.

RESULTS

Phenolic Compounds. *Beer Lacking Catechin and Proanthocyanidins.* Caminant is a pigmented malting barley with a negligible content of catechin and proanthocyanidins, which are usually found in the testa layer of the barley kernel (Jende-Strid, 1997). HPLC analysis of the beer brewed with Caminant malt (beer A) showed that it was free from proanthocyanidin and that the concentration of the flavanol catechin was negligible (Table 1). A corresponding beer brewed with the conventional Alexis malt (beer B) contained considerable amounts of catechin and the two dimeric proanthocyanidins. The concentrations of ferulic acid, which is mainly found in the cell walls of the aleurone layer of the barley kernel, were almost identical in the two beers. Both beers were brewed without using adjuncts in order to enhance any possible effects caused by the difference in the contents of polyphenolic compounds in the two varieties of barley.

Table 1. Phenolic Compounds in Lager Beers

beer	catechin[a] mg/L	ferulic acid[a] mg/L	prodelphinidin B-3[b] mg/L	procyanidin B-3[b] mg/L
A	0.2	3.4	0	0
B	3.7	3.3	4.5	3.6
C	1.9	2.3	2.2	3.0
D	1.3	1.6	1.5	1.3

[a] ±10%. [b] ±20%.

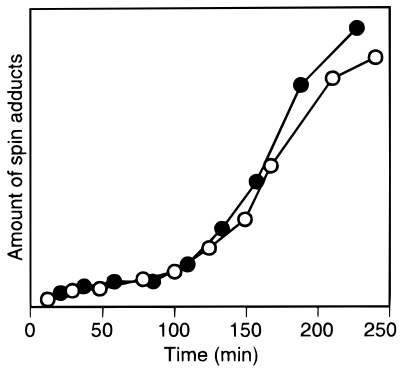

Figure 1. Amount of spin adducts measured by ESR in all-malt lager beer brewed with Caminant malt (●, beer A) or Alexis malt (○, beer B). The beers contained PBN (30 mM) and were heated to 55 °C.

The oxidative stabilities of beers A and B were tested by the ESR spin trapping technique. The spin trap PBN was added to the beers, and they were subsequently heated to 55 °C in the presence of atmospheric oxygen. The intensity of the ESR spectra of the samples was recorded at time intervals (Figure 1). Only small ESR signals were detected during the first 100 min of the experiment (the lag phase), and after this initial period, the intensity of the signals began to increase. The observed PBN spin adducts in beer have been shown to arise from the trapping of 1-hydroxyethyl radicals that are formed from ethanol by hydrogen abstraction by hydroxyl radicals (Andersen and Skibsted, 1998). The lengths of the lag phases were identical for the two beers, indicating their antioxidative capacities were identical. Furthermore, the identical slopes after the lag phase suggest that the two beers had similar capacities to generate radicals. The lack of polyphenolic compounds in beer A seems consequently to affect neither the antioxidative capacity of the beer nor the ability to form radicals.

Beer with Added Phenolic Compounds. Great care was taken in order to ensure that beers A and B were brewed in exactly the same way so that any difference between the two beers was only caused by the use of different malts. However, even minor variances during the brewing and fermentation can affect factors such as pH, the amount of metal ions, and sulfite content, all of these

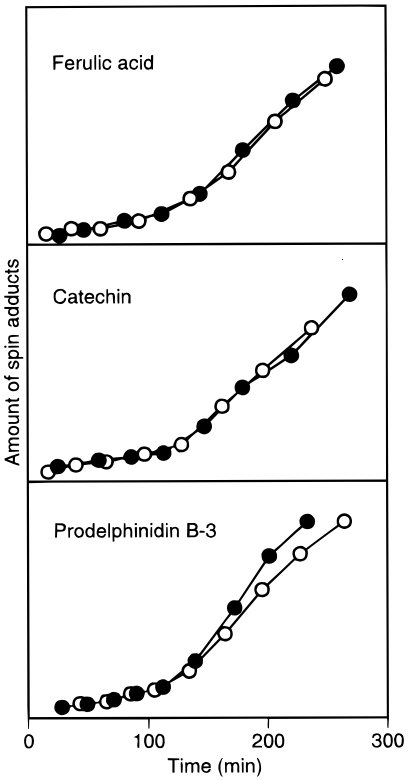

Figure 2. Effect of added phenolic compounds on the formation of spin adducts in lager beer. The intensity of ESR signals from PBN spin adducts in beer D (○, control) and with 0.2 mM of phenolic compounds added (●). The beer samples contained PBN (30 mM) and were heated to 55 °C.

variables being important for the oxidative stability of the final beer. The potential antioxidative role of phenolic compounds were therefore also tested by addition of phenolic compounds to lager beer from a single batch of brewing in order to eliminate the effect of possible variances between different brews.

A number of phenolic compounds such as gallic acid, caffeic acid, ferulic acid, sinapic acid, catechin, epicatechin, quercetin, rutin, vanillic acid, and prodelphidin B-3 were tested as potential antioxidants by the ESR spin trapping assay. A lager beer (beer D) with a lag phase around 120 min and containing 2 ppm sulfite was used for all of these experiments. The concentration of added phenolic compound was 0.2 mM in all the experiments, a concentration which is considerable higher than the natural concentrations found in lager beer. However, none of the tested phenolic compounds affected the length of the lag phase or the rate at which the PBN spin adduct was formed once the lag phase had ended, despite the high concentrations of phenolic compounds that were used (Figure 2). It is therefore doubtful whether the phenolic compounds should act as antioxidants or prooxidants during the aerobic oxidation of lager beer when present at lower concentrations. Similarly, we have previously shown that addition of procyanidin B-3 and prodelphinidin B-3 to an all-malt lager beer brewed with Caminant malt did not have any effect on the course of formation of radical adducts (Andersen et al., 1999).

Sulfur-Containing Compounds. *Beer with a Low Level of Sulfite.* Uchida and Ono (1996) have demonstrated that addition of sulfite to lager beer increases the length of the lag phase, and other studies have likewise indicated that the length of the lag phase

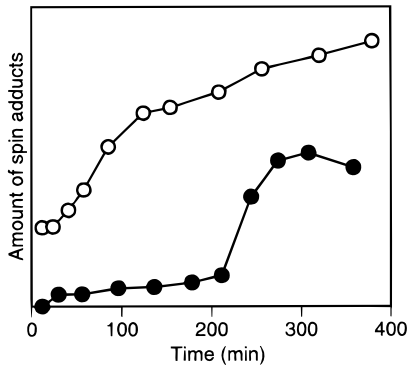

Figure 3. Formation of spin adducts in lager beer lacking sulfite. The height of the ESR signals from spin adducts in beer C where the concentration of sulfite was ≤1 ppm (○), and in a sample of the same beer where 4 ppm SO_2 had been added (●). Both samples contained 30 mM PBN and were heated to 55 °C.

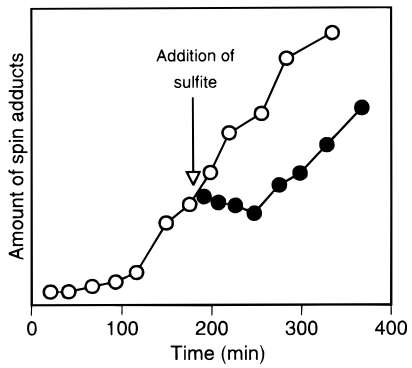

Figure 4. Effect of adding sulfur compounds to lager beer after the end of the initial lag phase. The intensity of ESR signals from PBN spin adducts in beer D (○). The beer sample was divided after 190 min and sulfite (40 μM) was added to one of the two portions (●). The beer contained PBN (30 mM) and was heated to 55 °C.

correlates with the amount of sulfite in the lager beer (Uchida et al., 1996; Forster et al., 1999). The role of sulfite as antioxidant was tested by using a lager beer, beer C, which had been brewed with a strain of yeast that is unable to produce sulfite. The concentration of sulfite was less than 1 ppm in the final beer. The amount of spin adducts was found to increase instantaneously when beer C were heated (Figure 3). Adding 60 μM of sulfite (≈4 ppm of SO_2) to the beer resulted in a lag phase equal to 200 min before the formation of spin adducts began. Beer C was brewed with a conventional malting barley, which is reflected in the typical concentrations of catechin, PDB-3, and PCB-3 (Table 1). However, the presence of these phenolic compounds did not prevent the formation of radicals and thereby induce a lag phase in beer C, which further demonstrates that these compounds do not act as antioxidants in lager beer. The absence of the lag phase in beer C, on the other hand, underscores the unique role that sulfite plays as antioxidant in beer.

The ability of sulfite to induce a lag phase was further tested in an experiment where sulfite was added after the lag phase had ended and the formation of spin adducts had started (Figure 4). A new lag phase was observed after the addition of sulfite where the formation of new spin adducts stopped. However, only a minor decrease in the level of spin adducts that was formed at the time of addition of sulfite was observed, indicating that the PBN spin adducts are stable in the presence

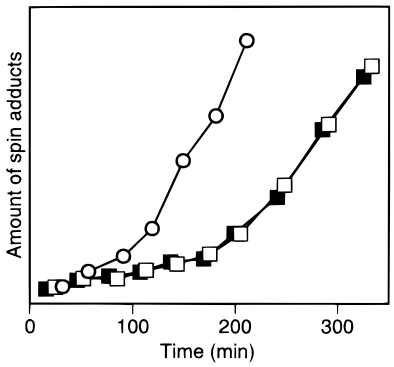

Figure 5. Effect of adding sulfite or aldehyde−sulfite adduct to lager beer on radical formation. The intensity of the ESR signals from spin adducts formed in beer D with added sulfite (0.1 mM) (□) or aldehyde−sulfite adduct **1** (0.1 mM) (■) compared to control (○) without addition. PBN (30 mM) was added, and the beer was heated to 55 °C.

of sulfite on the time scale of the experiment. The amount of spin adducts began to increase again with a rate that was almost similar to that of the untreated control after the end of the second lag phase.

Beer with Addition of Sulfite Bound as an Acetaldehyde Adduct. Sulfite reversibly forms adducts with carbonyl compounds (Dufour et al., 1999). Thus, acetaldehyde and bisulfite form the adduct **1** in water (reaction 1) with an equilibrium constant, *K*, equal to 800 M^{-1} (0 °C) (Lowry and Richardson, 1987).

$$CH_3CHO + HSO_3^- \leftrightharpoons CH_3CH(OH)SO_3^- \qquad (1)$$

Addition of 0.1 mM acetaldehyde−sulfite adduct **1** to beer D gave the same result as adding 0.1 mM sulfite as disulfite as judged from the ESR spin trapping experiment (Figure 5). The length of the lag phases was in both cases increased by 100 minutes relative to the nonspiked beer D. The similar behavior of the sulfite acetaldehyde adduct **1** and sulfite suggests that the adduct **1** is rather labile and is able to liberate sulfite that acts as the active antioxidant. The aldehyde−sulfite adducts that may be formed from saturated aldehydes in beer are most likely not active antioxidants but may act simply as reservoirs of sulfite, whereas α,*β*-unsaturated aldehydes are able to bind sulfite irreversibly (Dufour et al., 1999; Nyborg et al., 1999).

Beer with Other Sulfur Compounds Added. The sulfur compounds cysteine, methionine, and hypotaurine were tested as potential antioxidants. The oxidation of cysteine in malt has been demonstrated to be linked to the formation of hydrogen peroxide (Irwin et al., 1991; Muller, 1997), and it may consequently act as a prooxidant. Addition of 0.1 mM of cysteine to lager beer resulted in a 40% increase in the rate of formation of spin adducts after the lag phase had ended (Figure 6), but the length of the lag phase was unaffected. Addition of cysteamine to beer D resulted in both a shortening of the lag phase and an increased rate of formation of spin-adducts after the end of the lag phase (data not shown). Cysteamine is thus a prooxidant in beer despite the reported ability of cysteamine to scavenge both hydroxyl radicals and hydrogen peroxide (Aruoma et al., 1988).

Spiking beer D with methionin had no effect on either the lag phase or the rate of spin-adduct formation (Figure 6). Similar results were obtained with the

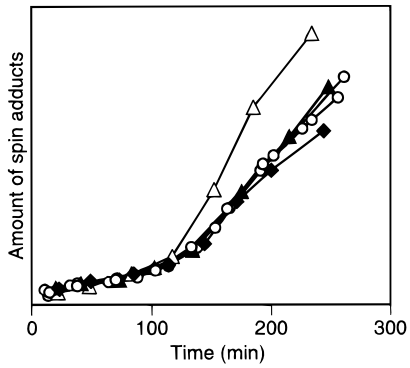

Figure 6. Effect of adding sulfur compounds to lager beer on radical formation. The intensity of ESR signals from PBN spin adducts in beer D spiked with 0.1 mM hypotaurin (◆), 0.1 mM methionin (▲), 0.1 mM cystein (△), and control (○). PBN (30 mM) was added, and the beer was heated to 55 °C.

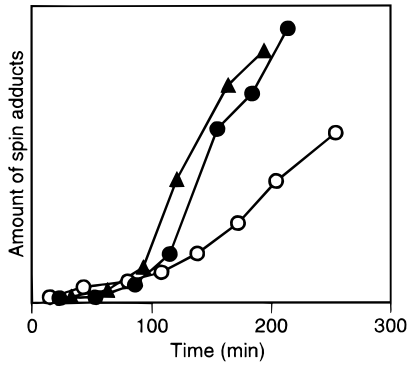

Figure 7. Prooxidative effect of ascorbate in lager beer. The intensity of ESR signals from PBN spin adducts in beer D spiked with 0.2 mM ascorbate (●), 0.5 mM ascorbate (▲), and control (○). PBN (30 mM) was added, and the beer was heated to 55 °C.

sulfide 3,3′-thiodipropionic acid which acts as an antioxidant in lipid systems by reacting with peroxides and especially with peracids originating from oxidized lipids (Lindsay, 1996). However, spiking beer D with 3,3′-thiodipropionic acid had no effect on the formation of radical adducts (data not shown).

Hypotaurine, a compound where sulfur is present in an intermediate oxidation state as a sulfinic acid, showed only an insignificant antioxidative effect by lowering the rate of formation of spin adducts by less than 10% and had no effect on the length of the lag phase (Figure 6). Hypotaurine has been shown to scavenge hydroxyl radicals, but not hydrogen peroxide (Aruoma et al., 1988).

Ascorbic Acid. Addition of ascorbic acid to beer as an antioxidant has been a widely used practice. The effect of ascorbic acid was therefore tested by spiking beer D with ascorbate. Addition of sodium ascorbate accelerated the rate of formation of spin adducts, which demonstrates that it acts as a prooxidant during the oxidation of beer (Figure 7). Increasing the amount of ascorbate from 0.2 to 0.5 mM led to a decrease in the length of the lag phase, whereas the rate of formation of spin adducts after the end of the lag phase was unaffected. It is noteworthy that PBN spin adducts were formed in these experiments since ascorbic acid has been shown to be able to reduce paramagnetic spin adducts to diamagnetic and hence ESR-invisible products (Stoyanovsky et al., 1998).

DISCUSSION

Beer is characterized by being a homogeneous aqueous solution. The aerobic forced aging of beer that takes place during the ESR experiments involve radical chain reactions of the Fenton-type with ethanol as the major quencher of hydroxyl radicals (see eqs 2−5) (Andersen and Skibsted, 1998; Qian and Buettner, 1999). The spin trap PBN traps 1-hydroxyethyl radicals; however, it has been estimated that less than 3% of these radicals react with PBN, ensuring that presence of PBN has a negligible effect on the course of the oxidative reactions (Andersen and Skibsted, 1998).

$$H_2O_2 + Fe^{2+}/Cu^{+} + H^{+} \rightarrow HO^{\bullet} + H_2O + Fe^{3+}/Cu^{2+} \quad (2)$$

$$HO^{\bullet} + CH_3CH_2OH \rightarrow H_2O + CH_3CH(OH)^{\bullet} \quad (3)$$

$$CH_3CH(OH)^{\bullet} + O_2 \rightarrow CH_3CH(OH)OO^{\bullet} \rightarrow CH_3CHO + HOO^{\bullet} \quad (4)$$

$$HOO^{\bullet} \rightleftarrows O_2^{\bullet -} + H^{+} \quad (5)$$

Hydrogen peroxide may be formed by slow reduction of oxygen by metal ions such as Fe^{2+} or Cu^{+} or by oxidation of sulfur compounds such as cystein by oxygen.

The efficiency of potential antioxidants depends not only on the rate of reaction between the antioxidant and the reactive intermediates in the radical chain mechanism but also on the reactivity between the intermediates and other compounds that are present in the actual product. The reaction between the hydroxyl radical and ethanol in beer clearly illustrates the latter point. The almost diffusion-controlled reactivity of hydroxyl radicals toward most organic substrates leads to a loss of selectivity, and the compounds that are present in the highest concentrations become the preferred reaction partners. Ethanol is the organic component that is present in the highest concentration in lager beer (≈1 M), and the major fraction of hydroxyl radicals that are formed in beer are therefore quenched by ethanol via eq 3 (Andersen and Skibsted, 1998). The low selectivity of the hydroxyl radical and the high amounts of potential reactants make it virtually impossible to quench this intermediate in beer by adding antioxidants that would react specifically with hydroxyl radicals.

Hydrogen peroxide and organic hydroperoxides are the only reactive oxygen species that are so long lived that they can be trapped efficiently by antioxidants which normally only are present in micromolar concentrations. Sulfite reacts with hydrogen peroxide via a general acid-catalyzed mechanism that does *not* involve radical intermediates (Hoffmann and Edwards, 1975). Viable strategies for selecting antioxidants for beer should therefore focus on either compounds such as sulfite that quench peroxides, or compounds that are able to inactivate the trace amounts of metals that otherwise may generate hydroxyl or alkoxyl radicals from the peroxides. It is noteworthy in this respect that the compounds that were found to act as prooxidants are also the compounds that are known to be able to generate hydrogen peroxide (e.g., cysteine) or are known to be able to reduce metal ions to the oxidation states that are active for the Fenton reactions with peroxides (e.g., ascorbate). The ESR lag phase experiments as those described in the present study are performed under conditions with a relatively high oxygen concentration. The rate-limiting step is most likely the activation of oxygen by reduced metal ions under these conditions. The availability of reduced metal ions to reduce oxygen to superoxide is the limiting factor, and any compound that is capable of reducing metal ions such as ascorbate and thiols will act as prooxidants (Buettner and Jurkiewicz, 1996).

Sulfite is a clearly a unique antioxidant in beer. It is formed naturally by the yeast during the fermentation, and according to the present study, it is the most efficient antioxidant that is naturally present in beer (Ilett, 1995; Kaneda et al., 1996). Beer lacking sulfite was found to have no lag phase for formation of radicals and accordingly no defense against the oxidative radical chain reactions. Sulfite either added as such or bound by carbonyl compounds as 1-hydroxysulfonates resulted in a lag phase. The lability of such carbonyl adducts ensure that the antioxidative effect of sulfite is not reduced.

The phenolic compounds were on the other hand found not to have any effect on the length of the lag phase. Caminant beer, where the proanthocyanidins and catechin are absent, gave the same behavior as beer with natural amounts of these compounds. Furthermore, addition of these phenols had no effect on the lag phase. These results are in contrast to other forced aging experiments where phenolic compounds have been shown to act as antioxidants (Nakajima et al., 1998). However, these experiments were performed with beer heated for a rather long time, and the conclusions were based on oxidative processes following the lag phase. In ESR lag phase experiments with beer in general, antioxidants are consumed once the lag phase finishes, but it is the length of the lag phase that has been shown to correlate with the flavor stability of beer. It is therefore not relevant, and it may even be misleading to use too harsh conditions (such as a very long time or too high temperatures) for forced aging experiments.

The ESR lag phase method is a powerful way to study active antioxidants in beer and should be used during forced aging experiments of beer. Experiments that continue after all sulfite has been consumed are meaningless and may lead to completely unrealistic results, since the radical chain reactions that take place after the lag phase has ended may overestimate the importance of other compounds as active antioxidants for realistic storage conditions.

CONCLUSION

Sulfite is the most efficient naturally occurring antioxidant in beer. Phenolic compounds such as catechin, phenolic acids, and dimeric proanthocyanidins are neither antioxidants nor prooxidants. Thiols and ascorbic acid were on the other hand shown to be prooxidants.

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