Modification of the Levels of Polyphenols in Wort and Beer by Addition of Hexamethylenetetramine or Sulfite during Mashing

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The effects of addition of hexamethylenetetramine (HMT) or sulfite during mashing on the polyphenol content and oxidative stability of wort and beer have been evaluated in a series of laboratory mashings and pilot brews. HMT reduced the concentration of catechin, prodelphinidin B-3, and procyanidin B-3 in wort and beer, whereas the concentration of ferulic acid was unaffected. Sulfite had only a minor effect on the concentration of phenolics in wort and beer. Addition of HMT or sulfite during mashing increased the oxidative stability of the beer slightly as judged by the tendency of formation of radicals (ESR spin trapping technique), although sensory analysis gave identical flavor acceptance scores to beers produced from untreated and HMT-treated wort and lower scores to beer from sulfite-treated wort. No difference in the oxidative stability of the differently treated sweet worts could be detected as judged by the rate of formation of radicals. HMT addition during mashing has thus been demonstrated to be a valuable experimental tool to control the level of polyphenols in wort and for producing brews with various levels of polyphenols from a single malt.

Keywords: *Beer; hexamethylenetetramine; mashing; polyphenols; sulfite; wort*

INTRODUCTION

Polyphenols have been shown to have a negligible effect on the oxidative stability of beer (*1*, *2*). Beer is a homogeneous liquid wherein all components are evenly distributed throughout the medium. The efficiency of potential antioxidants therefore depends on how well these compounds compete with other components in beer as reaction partners with deleterious reactive species (radicals and reactive oxygen species). Polyphenols are present only in very low concentrations, which makes it kinetically impossible for polyphenols to efficiently trap highly reactive radicals and thus act as efficient antioxidants before these radicals react with other and much more abundant compounds in beer (especially ethanol) (*2*).

On the other hand, the heterogeneous medium during mashing and lautering makes it possible that high local concentrations of polyphenols can exert an efficient antioxidative effect. The high temperatures during mashing and handling of hot wort together with access to oxygen are perfect conditions for oxidation processes, and it is possible that the products of oxidation during the early stages of brewing can influence the stability of the final beer. In fact, these conditions are very similar to the conditions of "ESR lagtime" measurements in beer during which the formation of radicals has been clearly demonstrated by spin trapping (*3*, *4*). It would therefore be of interest to have a method to control the concentration of polyphenols during the brewing process in order to study the role of polyphenolic compounds during the early steps of brewing.

Methods are available for partial removal of polyphenols from beer by using stabilizing agents such as PVPP and to some degree fining with gelatin (*5*−*7*). Modulating the levels of polyphenols in wort is less straightforward. Wort with low level of polyphenols can be made by using varieties of barley with low levels of polyphenols such as proanthocyanidin-free varieties (*8*). However, this approach is complicated because it is necessary to compare brews made by using malts from different barley varieties or harvests. Minor differences in the enzymatic behavior of the malts during mashing can have profound effects on the extraction of compounds that in the end affect the behavior of wort and beer, thereby making it difficult to discern the possible effects caused by the different levels of polyphenols.

Macey et al. have demonstrated that addition of formaldehyde during mashing can remove anthocyanogens from wort and beer (*9*). It is possible to obtain the same effect by using hexamethylenetetramine (HMT), which is easier to handle than solutions of formaldehyde (*10*, *11*). HMT is a solid adduct of ammonia and formaldehyde that is slowly hydrolyzed in aqueous solutions, thereby liberating formaldehyde (*12*). This approach would make it possible to produce wort and beer with various concentrations of phenolic compounds by using only a single type of malt. It was therefore of interest to investigate in detail the effect of adding HMT during mashing by studying the effect on the levels of specific polyphenolic compounds in wort and beer by HPLC in contrast to Macey et al., who studied the effect of formaldehyde on the total level of polyphenols in wort by nonspecific analytical methods (*9*). Furthermore, the effect of changing the level of polyphenols on the oxidative stability of the wort and beer has been examined by the ESR spin trapping technique.

The effect of adding sulfite during the mashing has also been examined. Addition of sulfite could potentially give a wort with a high level of polyphenols in different ways. First, it may act as an antioxidant at the early stages of production of beer, thereby preventing oxida-

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Table 1. Effect of Addition of HMT to Laboratory Mashings on the Concentrations of Polyphenolic Compounds in Sweet Wort

time of addition of HMT	added HMT (mg/kg of malt)	catechin (mg/L)	ferulic acid (mg/L)	PDB-3 (mg/L)	PCB-3 (mg/L)
control (no addition)	0	2.3	4.3	9.6	9.2
start of mashing-in	250	0.4	4.5	0.0	0.0
start of protein rest	250	0.2	4.2	0.0	0.0
start of saccharification rest	250	0.0	4.8	0.0	0.0
	500	0.0	4.5	0.0	0.0
during saccharification rest	250	0.1	4.9	0.0	0.0
	500	0.0	4.2	0.0	0.0
start of mashing-off	250	0.3	4.6	0.0	0.0
	500	0.0	4.5	0.0	0.0
finely ground malt					
start of mashing-in	50	1.8	5.4	4.0	5.0
start of mashing-in	100	1.1	5.2	0.5	1.1
start of mashing-in	250	0.0	5.7	0.0	0.0
coarsely ground malt					
start of mashing-in	50	1.4	4.9	0.7	2.4
start of mashing-in	100	0.7	4.9	0.0	0.5
start of mashing-in	250	0.0	5.3	0.0	0.0

tion of polyphenols during wort handling, resulting in wort and beer with a high level of proanthocyanidins. Second, sulfite has been shown to reduce and thereby regenerate oxidized flavonoids in model experiments (*13*).

EXPERIMENTAL PROCEDURES

N-tert-Butyl-α-phenylnitrone (PBN) was obtained from Molecular Probes (Leiden, The Netherlands), and *N-tert*-butyl-α-(4-pyridyl)nitrone-*N′*-oxide (POBN) and hexamethylenetetramine (HMT; 1,3,5,7-tetraazatricyclo[3.3.1.1^{3,7}]decane) were obtained from Fluka (Buchs, Switzerland).

Laboratory Mashing. An infusion mashing was used whereby ground production malt (100 g) was mashed-in at 37 °C in 400 mL of water. The mashing program included a proteolytic rest (52 °C, 10 min), a saccharification rest (68 °C, 30 min), and mashing-off (77 °C, 10 min). The mash was subsequently filtered through an open-folded filter, and the sweet wort was frozen immediately (*1*).

Beer Brewing. Beers A−E were brewed essentially as previously described (*2*). Brew A was a control. To brews B and C was added HMT at mashing-in corresponding to 200 mg/kg of malt. In brew C tannin-free hop extract was replaced by hop pellets as bittering agent to elucidate the influence of hop tannins on possible radical formation in wort and beer. To brews D and E was added sodium pyrosulfite at mashing-off, 150 and 300 mg/kg malt, respectively.

HPLC Analysis. The content of the polyphenols catechin, prodelphinidin B-3 (PDB-3), procyanidin B-3 (PCB-3), and ferulic acid in wort and beer was analyzed by HPLC with electrochemical detection using authentic samples as standards as previuosly described (*1*, *2*). Beer samples were degassed and filtered, whereas wort samples were diluted with an equal volume of water and filtered before injection.

Measurement of Haze. The beer was stored at 60 °C for 5 days followed by 24 h at 0 °C. Total haze was measured at 0 °C using an Lg-Automatic Hz 013 hazemeter (Lg-Automatic, Frederiksvaerk, Denmark).

Sensory Analysis of Beer. The flavor acceptance score of the fresh beer was evaluated by a trained panel with eight tasters. A score of 0 is equivalent to dislike, whereas 10 is equivalent to excellent. Three beers were tested during each session.

ESR Experiments. Wort containing 40 mM POBN or degassed beer containing 30 mM PBN was heated to 55 °C in closed blue-cap bottles with a headspace of atmospheric air (20% oxygen). Samples were withdrawn at given intervals. ESR spectra were recorded with a JES-FR 30 spectrometer (JEOL, Tachikawa, Japan) using a flat quartz aqueous cell (Wilmad, Buena, NJ). The following settings were used: microwave power, 4 mW; and modulation amplitude, 1 G. A sweep time equal to 4 min was used for beer samples, whereas a sweep time equal to 15 min was used for wort samples. The amplitude of the spectra was measured as the height of the central doublet relative to the height of the ESR signal of a Mn^{2+} marker.

RESULTS

Sweet Wort. *Addition of HMT during Mashing.*The efficiency of HMT in removing polyphenols in sweet wort was evaluated in a series of laboratory mashings; HMT was added at different steps during the mashing. The effect of adding HMT was evaluated by HPLC analysis of the concentration of the major phenolic compounds in the sweet wort (Table 1). The gravities of all sweet worts were ∼16.5 °P.

The dimeric proanthocyanidins procyanidin B-3 and prodelphinidin B-3 were removed from the sweet wort irrespective of the step during mashing at which the HMT was added. The amount of the flavanol catechin was also diminished by the addition of HMT, and only negligible amounts were present when 250 mg of HMT/kg of malt was added. Increasing the amount of added HMT to 500 mg/kg of malt removed all of the catechin from the sweet wort. The phenolic acid ferulic acid was essentially unaffected by the addition of HMT, and only minor reductions in the concentration of ferulic acid were observed when the amount of HMT was increased to 500 mg/kg of malt.

The amount of HMT that is necessary for complete removal of the proanthocyanidins was determined by addition of various amounts of HMT. The malt was used either as finely or coarsely ground in order to examine the effect of different rates of extraction of phenolic compounds. Adding 250 mg of HMT/kg of malt at the mashing-in removed all of the dimeric proanthocyanidins and catechin, whereas ferulic acid was unaffected. Differences in concentrations of catechin, PDB-3, and PCB-3 in wort were, however, observed when using only 50 mg of HMT/kg of malt; finely ground malt gave the highest concentrations of proanthocyanidins, presumably because of the higher rate of release of these phenolic compounds.

Addition of Sulfite during Mashing. The potential protective effect of sulfite on the level of phenolic compounds in wort was examined by adding sulfite before the mashing-off. The addition of sulfite had only a minor effect on the concentration of phenolic com-

Table 2. Effect of Addition of Sulfite to Laboratory Mashings at the Start of the Mashing-off on Polyphenols in Sweet Wort

added $Na_2S_2O_5$ (mg/kg of malt)	catechin (mg/L)	ferulic acid (mg/L)	PDB-3 (mg/L)	PCB-3 (mg/L)
0	1.9	4.7	10.6	13.3
150	2.0	4.8	8.3	14.7
300	2.2	4.8	9.7	15.6

pounds in the final sweet wort (Table 2). Minor increases in the concentrations of catechin and PCB-3 were observed when the amount of sulfite was increased from 150 to 300 mg/kg of malt, suggesting a small protective effect of sulfite.

Hopped Wort and Beer. Five pilot brews were prepared in order to study the effect of HMT or sulfite on the level of polyphenols in hopped wort and the final beer compared to that in an untreated control brew (brew A). HMT was added after the mashing-in (brews B and C), and sodium pyrosulfite was added before the mashing-off (brews D and E). All brews were hopped by using tannin-free hop extract except brew C, for which hop pellets were used in order to study the role of hops as a source of phenolic compounds in beer.

Concentration of Phenolic Compounds in Hopped Wort and Beer. HPLC analysis of the hopped wort (14.5 °P) and the resulting final beer showed that adding HMT during the mashing removed the polyphenols almost completely from the hopped wort and the final beer, whereas ferulic acid was unaffected by the HMT treatment (Tables 3 and 4). The removal of the polyphenols led as expected to a considerable decrease in the formation of haze in beer stored at 60 °C for 5 days (Table 4).

Bittering the HMT-treated beer with hop pellets instead of extract increased the amount of phenols in wort and beer, which indicates that hop pellets contribute to the amount of polyphenols in wort and beer. A similar effect has previously been demonstrated by using proanthocyanidin-free malt (*14*). Adding sulfite to the wort during mashing increased the level of phenolic compounds above the control in the hopped wort, but in the beer these were at similar concentrations to the control. Despite this, less haze was formed in the sulfite-treated beer than in the control after 5 days of storage at 60 °C (Table 4).

The gravities of the worts from the five brews were identical, indicating that neither HMT nor sulfite had any effect on the enzymatic activity of the amylases during the mashing (Table 3), in agreement with the previous results by Macey et al. (*9*). A similar conclusion was reached by inspecting the levels of ferulic acid in sweet and hopped wort (Tables 1 and 3). Ferulic acid is covalently bound to barley cell-wall polysaccharides, and the extraction of ferulic acid into the wort depends mainly on the enzymatic activity of esterases during mashing-in (*15*). The level of ferulic acid in wort was independent of the treatment with HMT, showing that HMT had no effect on the enzymatic activity. The gravities and the levels of ferulic acid were also unaffected by the treatment with sulfite.

Sensory Analysis of Beer. The effect of the HMT and sulfite treatments on the flavor of the fresh beer was evaluated by a taste panel during two sessions. The first session included the two HMT-treated brews, brews B and C, and the control, brew A, whereas the two brews treated with sulfite at two levels, brews D and E, were compared to brew A in the second session (Table 5). The flavor acceptance scores of HMT-treated brews B and C were not significantly different from the scores of brew A ($P > 0.1$), whereas the scores of sulfite-treated brews D and E were significantly lower than the scores of brew A ($P < 0.005$ and $P < 0.00005$, respectively).

The taste panel found that the five beers had very similar flavor profiles. The differences were mainly associated with the sulfury characteristics (sulfitic, cooked vegetable, yeasty) and especially the sulfidic characteristics (lightstruck, burnt rubber, onion). Brews C and D had a slightly sulfidic flavor characteristic, whereas brew E had a stronger, more noticeable sulfidic flavor and a strongly sulfury flavor. The other four brews had a just noticeable sulfury flavor.

Assessment of the Oxidative Stability of Wort and Beer by ESR. The effect of addition of HMT or sulfite during mashing on the oxidative stability of wort was examined by the spin-trapping technique. A nitrone spin trap, POBN, was added to the wort, and the formation of spin adducts upon heating the sample was monitored by ESR. The nitrone spin trap PBN, which has been used in a similar manner with beer samples (*2*, *3*, *16*, *17*), was also tested as spin trap in wort; however, no PBN spin adducts could be detected by ESR.

The ESR spectra of the POBN spin adducts in sweet and hopped wort were identical in all of the tested samples. Only the intensity of the signals varied. The hyperfine splitting constants were equal to $a_N = 15.5$ G and $a_H = 2.6$ G, which are typical values for POBN spin adducts formed from carbon-centered radicals (*4*, *18*) (Figure 1). The magnitude of a_H excludes the possibility that the observed adducts arise from trapping hydroxyl radicals. POBN adducts with hydroxyl radicals have $a_H = 1.7$ G, which is significantly lower than the observed value (*18*). It is noteworthy that removing the polyphenolic compounds with HMT had no effect on the ESR spectra of the spin adducts, which confirms that the polyphenols play a minor/secondary role during the radical reactions that take place upon heating wort. The HMT treatment most likely transforms the polyphenolic compounds into high molecular weight compounds (polymers) that would give spin adducts with rather large ESR line widths because of their expected lower diffusional mobility, if they were oxidized to radicals that could be trapped by POBN (*19*). A minor signal (marked by × in Figure 1) was observed in all of the samples. This signal is assigned to a steady state

Table 3. Effect of Adding HMT or Sulfite during Mashing on the Gravity and Polyphenolic Compounds in Hopped Wort from Pilot Brews

brew	treatment	hopping agent	gravity (°P)	catechin (mg/L)	ferulic acid (mg/L)	PDB-3 (mg/L)	PCB-3 (mg/L)
A	none	extract	14.43	5.3	4.1	11.3	7.6
B	200 mg of HMT/kg of malt	extract	14.47	0.3	3.7	0.1	0.0
C	200 mg of HMT/kg of malt	pellets	14.82	1.9	3.8	0.5	0.6
D	150 mg of $Na_2S_2O_5$/kg of malt	extract	14.53	5.9	4.0	11.5	7.6
E	300 mg of $Na_2S_2O_5$/kg of malt	extract	14.42	6.1	4.7	13.8	9.1

Table 4. Effect of Adding HMT or Sulfite during Mashing on Formation of Haze and the Concentration of Polyphenolic Compounds in Beer

brew	haze after 5 days at 60 °C (EBC units)	catechin (mg/L)	ferulic acid (mg/L)	PDB-3 (mg/L)	PCB-3 (mg/L)
A	5.2	3.2	2.7	5.4	3.7
B	0.8	0.2	2.6	0.4	0.0
C	1.1	1.1	2.7	0.5	0.3
D	3.7	3.3	2.6	4.8	3.7
E	3.8	3.6	2.8	5.5	3.8

Table 5. Flavor Acceptance Scores[a] of Fresh Beer

	av flavor acceptance score
session 1	
brew A	4.4
brew B	4.6
brew C	3.1
session 2	
brew A	5.1
brew D	3.6
brew E	2.5

[a] A score of 0 is equivalent to dislike, whereas 10 is equivalent to excellent.

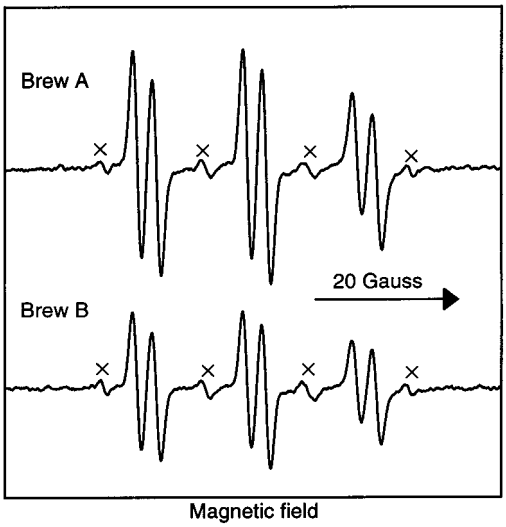

Figure 1. ESR spectra of spin adducts detected in hopped wort containing 40 mM POBN. HMT was added to brew B immediately after the mashing-in. Peaks marked by × are assigned to the *t*-BuNHO• radical and the triplet of doublets to a spin-trapped carbon-centered radical. Spectra were recorded after heating at 55 °C for 250 min.

concentration of the *tert*-butylaminoxyl radical (*t*-BuNHO•), which is formed by oxidation of *tert*-butylhydroxylamine that is formed by hydrolysis of the spin trap POBN or the spin adducts during the heating.

The effect of adding HMT or sulfite during mashing on the ability of sweet and hopped wort to generate radicals during heating was examined by ESR (Figures 2 and 3). Wort containing the spin trap POBN was heated to 55 °C in closed bottles under atmospheric oxygen. The formation of spin adducts began immediately after the heating was initiated. The level of detected spin adducts was ~4 times higher in hopped wort than in sweet wort, as has also previously been reported by Uchida et al. (*16*, *17*). The addition of HMT or sulfite during the mashing had no significant effect on the rate of formation of radicals in the sweet worts compared to the control (Figure 2). The two hopped worts that had been brewed with addition of HMT (brews B and C) gave lower levels of spin adducts,

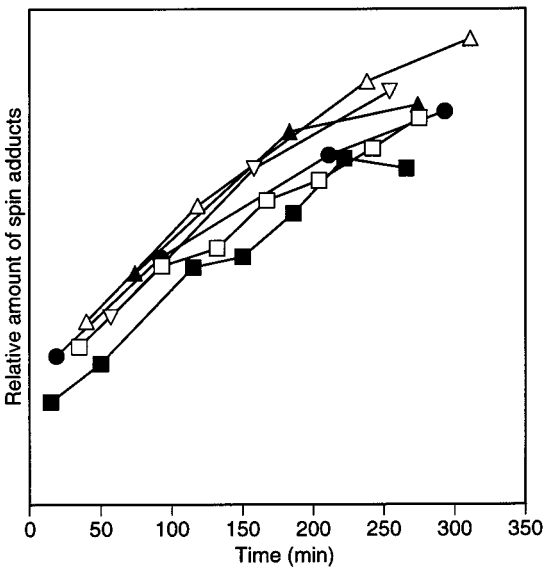

Figure 2. Intensity of ESR spectra of spin adducts formed in sweet wort treated with HMT or sulfite during mashing: control (●); HMT (250 mg/kg of malt) added at the start of mashing-in (△), at the start of saccharification rest (▲), or at the start of mashing-off (▽); sodium pyrosulfite (150 mg/kg of malt) added at the start of mashing-off; coarsely ground malt (■); finely ground malt (□). The worts containing POBN (40 mM) were kept at 55 °C.

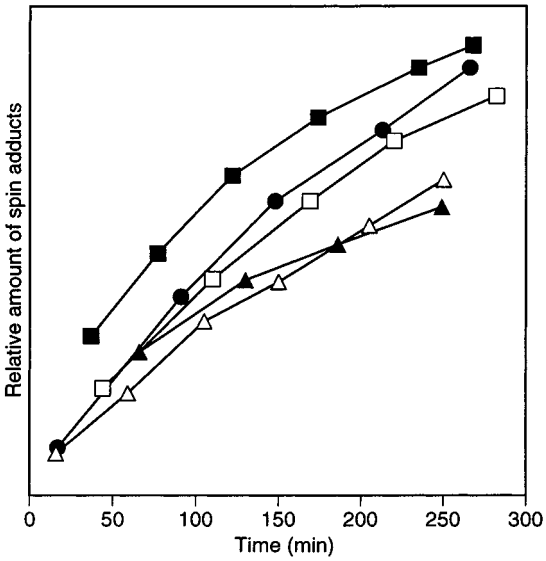

Figure 3. Intensity of ESR spectra of spin adducts formed in pilot-brewed hopped wort treated with HMT or sulfite during mashing: brew A, control (●); brew B, HMT (200 mg/kg of malt) added after the mashing-in, hopped with extract (▲); brew C, HMT (200 mg/kg of malt) added after the mashing-in, hopped with pellets (△); brew D, sodium pyrosulfite (150 mg/kg of malt) added at start of mashing-off (■); brew E, sodium pyrosulfite (300 mg/kg of malt) added at start of mashing-off (□). The worts containing POBN (40 mM) were kept at 55 °C.

whereas the two worts to which sulfite had been added (brews D and E) gave levels of spin adducts that were similar to those of the control or higher (Figure 3). It is noteworthy that no lagtime was observed with wort to which sulfite had been added. Addition of sulfite has been found to inhibit the generation of spin adducts in beer, thereby generating a lagtime. The absence of a lagtime in wort indicates that sulfite most likely was consumed (oxidized or bound irreversibly) during the mashing-off.

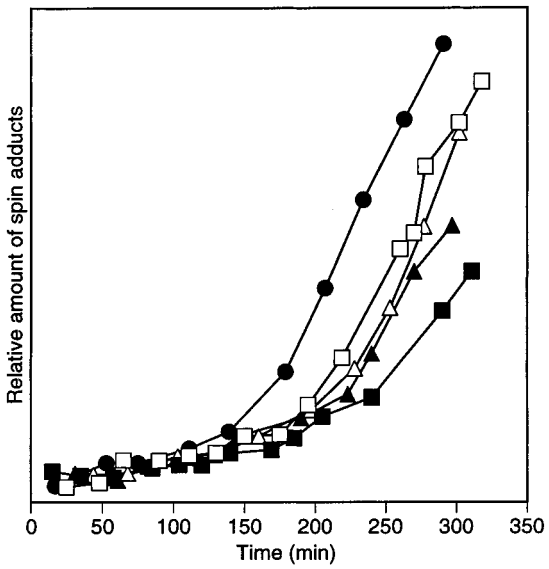

Figure 4. Intensity of ESR spectra of spin adducts formed in pilot-brewed beer treated with HMT or sulfite during mashing: brew A, control (●); brew B, HMT (200 mg/kg of malt) added after the mashing-in, hopped with extract (▲); brew C, HMT (200 mg/kg of malt) added after the mashing-in, hopped with pellets (△); brew D, sodium pyrosulfite (150 mg/kg of malt) added at start of mashing-off (■); brew E, sodium pyrosulfite (300 mg/kg malt) added at start of mashing-off (□). The beer contained PBN (30 mM) and was kept at 55 °C.

The oxidative stability of pilot-brewed beer was examined by ESR using the spin trap PBN (Figure 4). Identical spin adducts were detected in all of the beers (data not shown), indicating that radicals from polyphenolic compounds are not trapped. Addition of HMT or sulfite during the mashing led in both cases to an increase in the lag phase of the beer compared to the untreated control. All of the beers gave similar rates of formation of spin adducts after the lag phase had ended, suggesting that the differences in the length of the lag phases must be caused by differences in the amount of antioxidants in the final beer and not by different rates of formation of radicals.

DISCUSSION

The addition of HMT during mashing can be used as a method to control the levels of polyphenols in wort and beer and is thus an alternative approach to using proanthocyanidin-free varieties of barley or stabilization of the final beer with, for example, PVPP. The treatment of wort with HMT seems only to affect the level of polyphenolic compounds. Neither the enzymatic activity during mashing nor the flavor of the final beer was affected. The present results agree very well with the previous findings of Macey et al. (*9*) and Stowell (*10*), who studied the effect of formaldehyde and HMT on the total level of polyphenols. However, we find that HMT is able to remove catechin, PDB-3, and PCB-3 completely from wort even when it is added late in the mashing process. This is in contrast to Macey et al., who found that early addition of formaldehyde was necessary for efficient removal of proanthocyanidins form wort (*9*). Our results also show that it is not necessary to prehydrolyze HMT before use; apparently, the hydrolysis during the mashing is fast enough to ensure that all proanthocyanidins are removed (*10*).

Polyphenolic compounds have been demonstrated to have no effect on the oxidative stability of beer (*1*, *2*). The present results, which are based on comparison of normal wort and beer with proanthocyanidin-free HMT-treated wort and beer, further demonstrate that polyphenolic compounds play a negligible role as antioxidants during brewing and storage of beer. Furthermore, it was demonstrated that polyphenols originating from hop pellets had no effect on the oxidative stability of wort, in agreement with previous results of Takaoka et al. (*20*).

The absence of POBN/OH adducts during wort heating is caused by two factors. First, the short lifetime of hydroxyl radical adducts with PBN and POBN makes it unlikely that spin adducts are detected on the time scale of the ESR measurements. Second, the major source of radicals in wort and beer under aerobic conditions is most likely the Fenton reaction, by which trace levels of reduced metal ions such as Fe^{2+} and Cu^{+} react with peroxides to form hydroxyl radicals. The high concentration of ethanol in beer prevents the direct spin trapping of hydroxyl radicals, and the observed spin adducts are formed by trapping 1-hydroxyethyl radicals, which arise from hydrogen abstraction from ethanol by hydroxyl radicals (*4*). A similar situation is likely in wort, in which carbohydrates are the major components. The high concentration of carbohydrates in wort quenches hydroxyl radicals formed by the Fenton reaction by donating hydrogen atoms. The formed carbohydrate α-hydroxyalkyl radicals can be trapped by oxygen-forming peroxyl radicals that decompose to form hydroperoxyl radicals and carbonyl groups (*21*). Radicals could be trapped during the heating of wort containing the spin trap POBN, whereas no ESR signals were observed when PBN was used as spin trap. This difference is most likely a consequence of the very short lifetime of carbohydrate radicals when oxygen is present. Furthermore, PBN generally traps carbon-centered radicals more slowly than POBN (*22*). The low intensity of the ESR spectra observed with wort compared with similar experiments with beer does not rule out a high degree of radical processes during heating, but may simply be a consequence of a lower rate of reaction between the spin traps and carbohydrate radicals.

The absence of a lag phase during heating of sulfite-treated sweet wort may be a consequence of either fast oxidation of sulfite during the mashing-off or reaction with compounds in the wort. Sulfite has been shown to bind irreversibly to the carbon−carbon double bond in α,β-unsaturated aldehydes (*23*, *24*). The resulting irreversibly bound sulfonic acid groups are not expected to exert any antioxidative effect; however, the reaction with staling aldehydes, for example, *trans*-2-nonenal, could contribute to an improvement of the final beer by removing these aldehydes. The flavor acceptance scores of the two sulfite-treated beers were nevertheless lower than that of the control, together with stronger sulfury and sulfidic flavor notes than found for the control.

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