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Beer Thiol-Containing Compounds and Redox Stability: Kinetic Study of 1-Hydroxyethyl Radical Scavenging Ability

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Supporting Information

ABSTRACT: The 1-hydroxyethyl radical is a central intermediate in oxidative reactions occurring in beer. The reactivity of thiol-containing compounds toward 1-hydroxyethyl radical was evaluated in beer model solutions using a competitive kinetic approach, employing the spin-trap 4-POBN as a probe and by using electron paramagnetic resonance to detect the generated 1-hydroxyethyl/4-POBN spin adduct. Thiol-containing compounds were highly reactive toward the 1-hydroxyethyl radical with apparent second-order rate constants close to the diffusion limit in water and ranging from 0.5×10^9 L mol^{-1} s^{-1} for the His-Cys-Lys-Phe-Trp-Trp peptide to 6.1×10^9 L mol^{-1} s^{-1} for the reduced lipid transfer protein 1 (LTP1) isolated from beer. The reactions gave rise to a moderate kinetic isotope effect (k_H/k_D = 2.3) suggesting that reduction of the 1-hydroxyethyl radical by thiol-containing compounds takes place by hydrogen atom abstraction from the RSH group rather than electron transfer. The content of reduced thiols in different beers was determined using a previously established method based on ThioGlo-1 as the thiol derivatization reagent and detection of the derivatized thiols by reverse-phase liquid chromatography coupled to a fluorescence detector. The total level of thiol in beer (oxidized and reduced) was determined after a reduction step employing 3,3′,3″-phosphanetriyltripropanoic acid (TCEP) as the disulfide reductant. A good correlation among total protein and total thiol content in different beers was observed. The results suggest a similar ratio between reduced thiols and disulfides in all of the tested beers, which indicates a similar redox state.

KEYWORDS: *beer, thiol-containing compounds, 1-hydroxyethyl radical, redox balance*

INTRODUCTION

Beer is the most widely consumed alcoholic beverage in the world and known to be sensitive to quality changes during storage, especially with respect to its aroma and taste.[1] Nonenzymatic oxidation is one of the major causes of development of off-flavors during aging.[2,3] Thus, the chemical changes in beer during storage should be controlled to ensure the sensory quality of the product and avoid consumer rejection that will compromise the economic value of the product. The 1-hydroxyethyl radical has been recognized as the key radical intermediate in the oxidative reactions in beer.[4] The 1-hydroxyethyl radical has a moderate reduction potential ($E^{0\prime}$ = +0.98 V vs NHE).[5] de Almeida et al.[6,7] have reported oxidative reactions of hop-derived iso-α-acids and β-acids in the presence of the 1-hydroxyethyl radical, which contribute to the loss of flavor quality of beer.[3] The reaction is initiated by the 1-hydroxyethyl radical abstracting hydrogen atoms from the substrates with rate constants close to the diffusion limit in water, for example, $k = 8.6 \times 10^9$ L mol^{-1} s^{-1} for H-atom abstraction from *trans*-isohumulone in aqueous solution at 25 °C.[6,7] In contrast, polyphenolic compounds such as quercetin were only slightly reactive toward the 1-hydroxyethyl radical ($k = 4.0 \times 10^4$ L mol^{-1} s^{-1})[8] with the exception of phenolic compounds having a cinnamic group or an α,β-unsaturated side chain, which are able to donate allylic H atoms to the 1-hydroxyethyl radical with rate constants approaching $\approx 10^7$ L mol^{-1} s^{-1}.[6,9] Among the compounds in beer that potentially can inhibit oxidative reactions in beer, thiol-containing compounds (RSH) have received increasing attention in recent years.[10,11] Stoyanovsky et al.[12] reported that glutathione reacts with the 1-hydroxyethyl radical, leading to disulfide formation (GSSG). Recently, Kreitman et al.[13] reported the antioxidant capacity of glutathione and cysteine toward the 1-hydroxyethyl radical, which inhibit the formation of the POBN−1-hydroxyethyl radical adduct by 87 and 88% at a thiol concentration of 5 mM, respectively. In beer, it has been shown that reduced thiol-containing peptides and proteins are consumed during beer storage and aging, and this concentration time profile is correlated to the beer redox stability.[11]

We here report a study of the reactivity of thiol-containing compounds (such as amino acids, peptides, and the beer protein lipid transfer protein 1 (LTP1)) toward the 1-hydroxyethyl radical in a beer model system. The role of thiols as antioxidants in beer is additionally assessed by quantifying the amounts of reduced and oxidized thiols in beer. This provides direct information about the redox state of the beer. Furthermore,

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the thiol levels in different beers have been related to their content of proteins.

■ MATERIALS AND METHODS

Chemicals and Materials. Six commercial Danish lager beers and one German lager beer were purchased from a local supermarket. Beer samples were pilsner or stronger pilsner type with alcohol content by volume varying from 4.6 to 7.2% and produced with pure malt. Acetonitrile, methanol, ethanol, deuterated ethanol-d_1(CH_3CH_2OD), deuterium oxide (D_2O), trifluoroacetic acid, formic acid, ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p′*-disulfonic acid), ferric chloride ($FeCl_3$), sodium chloride, glycerol, tris(2-carboxyethyl)phosphine (TCEP), α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), 5,5-dimethyl-1,3-cyclohexanedione, cystine, glutathione, L-homocysteine, *S*-methyl-L-cysteine, L-histidine, Cys-Gly, His-Cys-Lys-Phe-Trp-Trp, and Asn-Arg-Cys-Ser-Gln-Gly-Gly-Ser-Cys-Trp-Asn were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide 30% (H_2O_2), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), tris(hydroxymethyl)aminomethane (Tris), L-cysteine, *N*-acetyl-L-cysteine, L-serine, L-tryptophan, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). ThioGlo-1 fluorescent thiol reagent was obtained from Calbiochem (Darmstadt, Germany); oxidized glutathione, L-methionine, cysteine methyl ester hydrochloride, and L-lysine were purchased from Fluka (Stenheim, Germany); DL-1,4-dithiothreitol (DTT), acetic acid, and 1-octanol were from AppliChem (Darmstadt, Germany). Sodium sulfite (Na_2SO_3) and hydrogen chloride were from J. T. Baker (Deventer, The Netherlands); protein assay dye reagent, acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, and bromophenol blue were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine serum albumin (BSA) was obtained from Thermo Scientific (Rockford, IL, USA). High-purity nitrogen (5.0) was from Air Liquid Danmark A/S (Ballerup, Denmark). Water was purified (18 MΩ·cm) by means of a Milli-Q purification system from Millipore (Billerica, MA, USA).

Isolation and Purification of the LTP1 Protein. Commercial Brazilian lager beer samples (pilsner type, 5.5% of alcohol by volume, pure malt) purchased from a local supermarket were degassed by sonication and then freeze-dried for 3 days (12 bottles). The resulting powder was suspended in 30 mL of Tris-HCl buffer (25.0×10^{-3} mol L^{-1}, pH 7.4)/NaCl (20.0×10^{-3} mol L^{-1}) and the resulting solution centrifuged at 15000 rpm for 30 min at 4 °C. The supernatant was then submitted to dialysis (molecular weight cutoff (MWCO) 3500 Da; Spectra/Por Membrane) overnight against Tris-HCl buffer (20.0×10^{-3} mol L^{-1}, pH 7.4) containing NaCl (20.0×10^{-3} mol L^{-1}). The resulting dialyzed solution was submitted to cationic chromatography in a Macro-prep High S Support resin (Bio-Rad) using an ÄKTA Prime plus HPLC device (GE Healthcare). The resin was previously equilibrated with Tris-HCl buffer (25.0×10^{-3} mol L^{-1}, pH 7.4)/NaCl (20.0×10^{-3}), and the target protein was eluted by a NaCl linear gradient from 20.0×10^{-3} to 0.5 mol L^{-1} at a flow rate of 5 mL min^{-1}. Fractions were collected for each 5 mL of elution volume, and the protein fraction was further purified by chromatography on a Hiload Superdex 75pg prep-grade 16/60 size exclusion column (GE Healthcare) using an ÄKTA Prime plus HPLC device (GE Healthcare). For this last purification step, the resin was previously equilibrated with Tris-HCl buffer (25.0×10^{-3} mol L^{-1}, pH 7.4) containing NaCl (0.1 mol L^{-1}), and the elution was carried out in isocratic mode at a flow rate of 1 mL min^{-1}. The efficacy of each purification step was checked by SDS-PAGE (18% of acrylamide). Finally, the solution containing the target protein was dialyzed overnight against buffer Tris-HCl buffer (5.0×10^{-3} mol L^{-1}, pH 7.4) and further characterized by MALDI-TOF mass spectrometry.

Competitive Kinetics Studies. The reactivity of the investigated compounds toward the 1-hydroxyethyl radical was investigated using a competitive kinetics approach employing the spin trap 4-POBN. The apparent second-rate constants were determined following the procedure previously reported by de Almeida et al.[6] with some adaptations. The reactions were conducted by addition of 80 μL of H_2O_2 (15.0×10^{-3} mol L^{-1}; in water) in a nitrogen-saturated solution containing 60 μL of $FeCl_2 \cdot 4H_2O$ (10.0×10^{-3} mol L^{-1}; in acetate buffer, pH 4.5), 1 mL of 4-POBN (3.2×10^{-3} mol L^{-1}; in acetate buffer, pH 4.5, containing 6% (v/v) of ethanol), and different concentrations of the substrates. After 1 min at 25.0 ± 0.2 °C, the spin adduct 1-hydroxyethyl/4-POBN was monitored by EPR using a JES-FR30 EPR, JEOL spectrometer and a quartz capillary sample cell (0.75 mm i.d., Wilmand Glass, Buena, NJ, USA). The Mn(II) signal from a MgO crystal doped with Mn(II) internally mounted in the EPR cavity was used as an internal standard. The investigated substrates were cysteine, glutathione, Cys-Gly, His-Cys-Lys-Phe-Trp-Trp, Asn-Arg-Cys-Ser-Gln-Gly-Gly-Ser-Cys-Trp-Asn, homocysteine, cysteine methyl ester, *N*-acetyl-L-cysteine, and DTT, as well as compounds containing blocked thiol groups, such as cystine, oxidized glutathione, methionine, and *S*-methyl-L-cysteine. Furthermore, the kinetics was conducted by the presence of the isolated protein LTP1. The reactivities of oxidizable amino acids, such as histidine, serine, tryptophan, and lysine, were evaluated for comparison. The kinetic isotope effect was determined by carrying out the competitive kinetics in deuterated medium (D_2O and CH_3CH_2OD) employing glutathione and cysteine as thiol-containing substrates.

Electron Paramagnetic Resonance (EPR) Spectroscopy. The analyses were carried out employing a microwave power of 4 mW, a sweep width of 50.0 G, a sweep time of 2 min, a modulation amplitude of 1.25 G, and a time constant of 0.3 s.

Identification of Products from the Reaction of Thiol-Containing Compounds with the 1-Hydroxyethyl Radical. To determine the major degradation products from the reaction of thiol compounds and the investigated radical, the reaction solution was directly infused into a high-resolution accurate mass spectrometer equipped with an electrospray ionization interface. The reaction was carried out by adding 100 μL of H_2O_2 (15.0×10^{-3} mol L^{-1}; in water) in a degassed solution containing glutathione (1.2×10^{-4} mol L^{-1}; in acidified solution with formic acid, pH 4.5, containing 6% (v/v) of ethanol) and 80 μL of $FeCl_2 \cdot 4H_2O$ (10.0×10^{-3} mol L^{-1}; in acidified solution with formic acid, pH 4.5). After 1 min of reaction at 25.0 ± 0.2 °C, the final composition was analyzed by direct infusion into an HESI-FT-MS instrument (Thermo Scientific Orbitrap Velos Pro). To verify the possible formation of sulfenic acid as intermediate, 5,5-dimethyl-1,3-cyclohexanedione (dimedone) was added to the reaction solution and then analyzed in the same apparatus.

Direct Infusion Ultrahigh-Resolution Accurate Mass Spectrometry. Identification of the reaction products was carried out using direct infusion at a flow of 5 μL min^{-1} into an LTQ Orbitrap Velos FT-MS mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) equipped with an electrospray interface (HESI-II) and operating in the negative detection mode.

Quantification of the Level of Total and Reduced Thiol Compounds in Beer. Quantification of the reduced thiol concentration in different beers was performed by employing a previously established methodology by Abrahamsson et al.[14] and Hoff et al.,[15] whereby thiols are separated from sulfite by HPLC, and the interfering contribution from sulfite to thiol quantification is overcome. Aliquots of 100 mL of beer samples were degassed by adding 10 μL of 1-octanol and stirred for 5 min under aerobic conditions. After that, 100 μL of each degassed beer sample was diluted to 1 mL using a Tris buffer (0.25 mol L^{-1}, pH 7.5), and 20 μL of the diluted solutions was then transferred to different vials containing glutathione ranging from 0.2×10^{-6} to 1.0×10^{-6} mol L^{-1}. Subsequently, 100 μL of ThiolGlo-1 (26.0×10^{-6} mol L^{-1}; in Tris buffer 0.25 mol L^{-1}, pH 7.5) derivatization reagent was added to the vials and incubated for 5 min. The derivatization reaction was then chemically quenched by adding 10 μL of HCl (12 mol L^{-1}). The derivatized thiols were quantified using a chromatography system equipped with a fluorescence detector and using a C18 reverse-phase column employing an elution gradient (gradient 1).

Liquid Chromatographic Analysis. All chromatographic analyses were performed using an Agilent 1100 series liquid chromatography system equipped with a model G1312A binary pump, a G1379A vacuum degasser, a G1313A autosampler, a G1321A fluorescence detector, and an Agilent ChemStation data handling program (Agilent Technologies Inc., Palo Alto, CA, USA). The separation was performed using a Jupiter C18 column (150 by 2.0 mm, 5 μm particle size, 300 Å pore size; Phenomenex, Torrance, CA, USA) employing a binary mobile phase:

water (mobile phase A) and methanol (mobile phase B), both acidified with TFA (pH 2.0, 10.0×10^{-3} mol L^{-1}). The injection volume was 20 μL, and the mobile phase flow rate was set to 0.5 mL min^{-1}. The detection was performed using excitation and emission wavelengths at 242 and 492 nm, respectively.

Gradient system 1 was carried out at 25% of mobile phase B in A for 8 min (isocratic) and instantly increased to 95% of B in A, then kept isocratic for 6 min. After that, the initial condition (25% of B in A) was returned immediately, spending 7 min to reequilibrate the column.

The quantification of total thiols in beer was carried out by employing TCEP as a disulfide reductant.[16] The reduction reaction was conducted for 5 min by adding various concentrations of TCEP in the degassed beer sample, and the thiol content was quantified using the methodology previously established by Abrahamsson et al.[14] and Hoff et al.[15] and employing elution gradient 2. Gradient system 2 was held at 25% of mobile phase B in A for 3 min (isocratic), then linearly increased to 30% of B in A (17 min) and subsequently to 95% of B in A (2 min), maintaining isocratic for 6 min. After that, the initial condition (25% of B in A) was instantly re-established and kept for 2 min.

Total Protein Quantification in Beer. The total protein content in beer was determined according to the Bradford method using BSA as the protein standard for the calibration curve.[17]

Fenton Reaction in the Presence of TCEP. The effect of TCEP addition was evaluated in the Fenton reaction by adding 50 μL of TCEP solution (concentration ranging from 1.0×10^{-5} to 40.0×10^{-3} mol L^{-1}; in Tris buffer 0.25 mol L^{-1}, pH 7.5) in the solution reaction of 4 mL of 4-POBN (3.2×10^{-3} mol L^{-1}; in water containing 5.8% (v/v) of ethanol) and 20 μL of $FeSO_4 \cdot 7H_2O$ (22.0×10^{-3} mol L^{-1}; in water). The Fenton reaction was initiated by adding 80 μL of H_2O_2 (24.0×10^{-3} mol L^{-1}; in Milli-Q water) at 25.0 ± 0.2 °C. After 2 min, the measurement was carried out by EPR monitoring the formation of the spin adduct 1-hydroxyethyl/4-POBN as a function of the TCEP concentration.

Effect of TCEP on Beer EPR Lag-Phase Measurements. The lag-phase measurements were carried out using the method previously described by Uchida et al.[18] Briefly, 100 mL of beer (sample 4; strong pilsner type, 7.2% of alcohol by volume, pure malt) was degassed by adding 10 μL of 1-octanol and vigorously stirred for 5 min. After that, 24 mL of the degassed sample was transferred to a reaction flask, and different concentrations of TCEP (ranging from 0.0 to 0.5×10^{-3} mol L^{-1}) were added. After 5 min of TCEP addition, 100 μL of POBN (3.60×10^{-3} mol L^{-1}, in water) and 300 μL of ethanol were added. The resulting solution was then incubated at 60.0 ± 0.2 °C, and aliquots were transferred to the EPR flow cell at given time intervals. The EPR spectra were collected at 25.0 ± 0.2 °C, and the relative intensities of the spin adduct signals were determined.

Ferric Reducing Assay. The ability of TCEP to reduce Fe(III) to Fe(II) was evaluated using the ferrozine colorimetric essay.[19] Briefly, 100 μL of Milli-Q was added to 25 μL of aqueous solutions with various concentrations of TCEP (0.0, 0.9×10^{-5}, 1.8×10^{-5}, 3.7×10^{-5}, and 7.4×10^{-5} mol L^{-1}) and $FeCl_3$ (1.8×10^{-5}, 3.7×10^{-5}, and 7.4×10^{-5} mol L^{-1}). Then a 100 μL aliquot of aqueous ferrozine solution (5.0×10^{-4} L mol^{-1}) was added, and after 1 min of incubation, the absorbance was recorded at 560 nm.

■ RESULTS AND DISCUSSION

Competitive Kinetic Studies. The reactivity of thiol-containing compounds toward the 1-hydroxyethyl radical was studied following the methodology established by Almeida et al.[6] The decrease in the spin adduct 1-hydroxyethyl/4-POBN signal intensity as a function of the increasing concentration of the investigated compounds was monitored by EPR spectroscopy, as illustrated in Figure 1 for the presence of reduced LTP1. The EPR spectra of the 1-hydroxyethyl/4-POBN spin adduct is well characterized by a triplet of doublets presenting a nitrogen hyperfine coupling constant of A_N = 15.6 G and a hydrogen hyperfine coupling constant of A_H = 2.6 G, in agreement with the literature.[4] In this way, the expression $(F/1 - F)k_1 \times [\text{4-POBN}]$, where F refers to the percentage of inhibition for the formation of

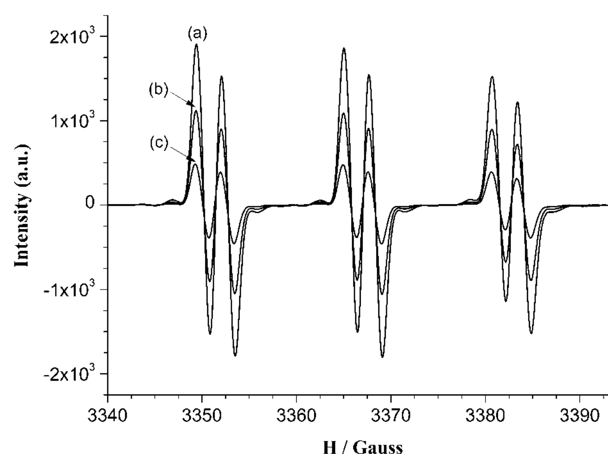


Figure 1. EPR spectra of 1-hydroxyethyl/4-POBN spin adduct generated in the presence of different levels of the protein LTP1. Reactions were carried out in nitrogen-saturated aqueous solution at 25.0 ± 0.2 °C containing ethanol (6% v/v), H_2O_2 (15.0×10^{-3} mol L^{-1}), $FeCl_2 \cdot 4H_2O$ (10.0×10^{-3} mol L^{-1}), 4-POBN (3.2×10^{-3} mol L^{-1}), and various concentrations of reduced LTP1 protein: (a) 2.7×10^{-4} mol L^{-1}; (b) 3.9×10^{-4} mol L^{-1}; and (c) 6.2×10^{-4} mol L^{-1}.

1-hydroxyethyl/4-POBN spin adduct, is expected to be linearly correlated to the concentration of the thiol added to the reaction solution (Figure 2). k_2 has the value of 3.1×10^7 L mol^{-1} s^{-1} and

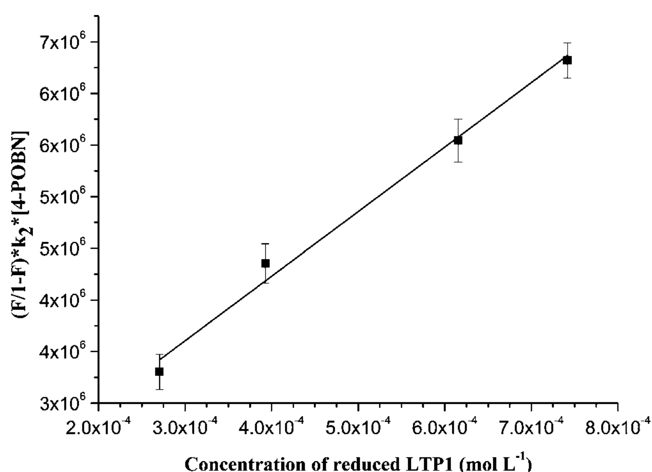


Figure 2. Plot of $(F/1 - F) \times k_2 \times [\text{4-POBN}]$ versus the concentrations of reduced LTP1 protein as obtained by spin-trapping EPR.

is the second-order rate constant for the reaction between the spin trap 4-POBN and the 1-hydroxyethyl radical.[20] The apparent second-order rate constants, k_2, for the reaction between 1-hydroxyethyl radicals and substrates (in this case thiols) are calculated from the slope of the linear dependence as established by eq 1.

$$\left(\frac{F}{1 - F}\right) \times [\text{4-POBN}] \times (3.1 \times 10^7) = k_2 \times [\text{substrate}] \quad (1)$$

The apparent second-order rate constants for the scavenging of 1-hydroxyethyl radicals were determined for a selection of thiols and oxidizable amino acids (Table 1). Thiol-containing compounds proved to be very reactive toward the 1-hydroxyethyl radical with rate constants approaching the diffusion limit in aqueous solution. The apparent second-order rate constants, k_2,

Table 1. Apparent Second-Order Rate Constants for the Reaction of Selected Substrates with the 1-Hydroxyethyl Radical[a]

substrate	k_2 (L mol^{-1} s^{-1})
cysteine	1.0×10^9
glutathione	7.1×10^8
Cys-Gly	9.0×10^8
His-Cys-Lys-Phe-Trp-Trp	4.9×10^8
Asn-Arg-Cys-Ser-Gln-Gly-GlySerCysTrpAsn	1.7×10^9
N-acetylcysteine	8.7×10^8
homocysteine	1.4×10^9
cysteine methyl ester	1.3×10^9
DTT	2.7×10^9
cystine	not reactive
oxidized glutathione	not reactive
methionine	not reactive
S-methyl-L-cysteine	not reactive
isolated LTP1 (isolated)	not reactive
reduced LTP1 (TCEP addition)	6.1×10^9
histidine	7.5×10^4
serine	8.8×10^4
tryptophan	6.7×10^5
lysine	4.4×10^5

[a]Reactions were conducted in aqueous/ethanol solution (94:6% v/v), at 25.0 ± 0.2 °C, under nitrogen.

ranged from 4.9×10^8 to 2.7×10^9 L mol^{-1} s^{-1} for the His-Cys-Lys-Phe-Trp-Trp peptide and DTT, respectively. The non-sulfur-containing oxidizable amino acids histidine, serine, tryptophan, and lysine showed moderate reactivity with apparent second-order rate constants ranging from 7.5×10^4 to 6.7×10^5 L mol^{-1} s^{-1} for histidine and tryptophan, respectively. However, oxidized thiol compounds, such as cystine and the oxidized glutathione (GSSG) and the compounds with a blocked thiol group, *S*-methyl-L-cysteine and methionine, did not show any reactivity toward the 1-hydroxyethyl radical. This proves that the free thiol groups are the reactive sites toward the 1-hydroxyethyl radicals.

The thiol-containing LTP1 protein isolated from beer did not react with the 1-hydroxyethyl radicals, suggesting that the thiol groups in the protein side chain are oxidized to disulfide bonds (RSSR), as is the case in native LTP1 from barley.[21] The thiol groups in LTP1 have been shown to be reduced by the yeast in fresh beer,[22] but may be in an oxidized form in stored beers.[23] The oxidation of the thiols in LTP1 may therefore already have taken place before the isolation from the stored beers used in the present study or during the isolation and purification processes. The isolated LTP1 was therefore subjected to a selective reduction of the four disulfide bonds by the addition of 4.5 equiv of TCEP. The reduced protein, which is expected to have 8 equiv of thiol groups, proved to be reactive toward 1-hydroxyethyl radicals with an apparent second-order rate constant $k_2 = 6.1 \times 10^9$ L mol^{-1} s^{-1}, approaching the diffusion limit in aqueous medium.

The kinetic isotope effect for the reduction of 1-hydroxyethyl radical by thiols was investigated using cysteine and glutathione as the thiol-containing substances in deuterated medium (D_2O/CH_3CH_2OD). A clear decrease in activity for the reduction of the 1-hydroxyethyl radical in deteurated media was observed, resulting in lower apparent second-order rate constants (Figure 3 and Table 2). The reaction showed a moderate normal kinetic

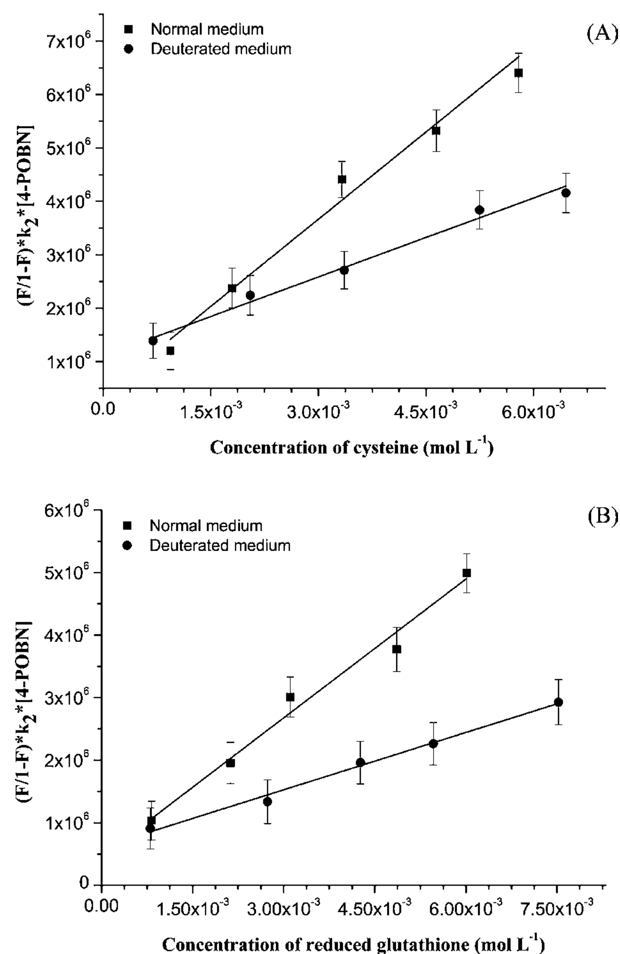


Figure 3. Plot of $(F/1 - F) \times k_2 \times$ [4-POBN] versus the concentration of (A) cysteine and (B) reduced glutathione as obtained by spin-trapping EPR.

Table 2. Kinetic Isotopic Effect As Determined by the Apparent Second-Order Rate Constants for the Reaction of Deuterated Cysteine and Glutathione (RSD) with 1-Hydroxyethyl Radical[a]

deuterated substrate	k_{app} (L mol^{-1} s^{-1})	$k_H{}^b/k_D$
cysteine	4.6×10^8	2.2
glutathione	3.1×10^8	2.3

[a]Reactions were conducted in deuterated medium (D_2O/CH_3CH_2OD 94:6% v/v)), at 25.0 ± 0.2 °C, under nitrogen. [b]k_H values displayed in Table 1.

isotope effect, k_H/k_D ratio of 2.2 and 2.3 for cysteine and glutathione, respectively. The magnitudes around 2 suggest a primary kinetic isotope effect, which is in agreement with a mechanism where the rate-determining step of the reduction of the 1-hydroxyethyl radical by thiols is a hydrogen atom abstraction from the RSH group rather than an electron-transfer reaction. This is in agreement with our recent observations that compounds with low X−H (C, N, O, or S) bond dissociation energies have a high reactivity toward 1-hydroxyethyl radicals,

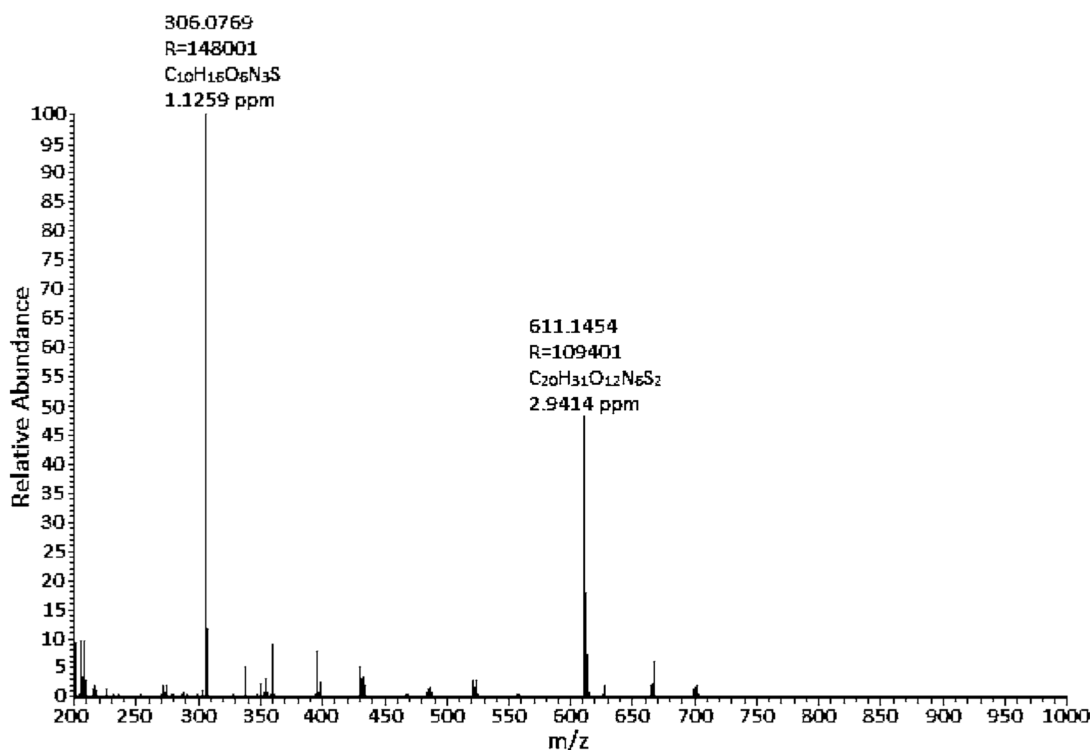
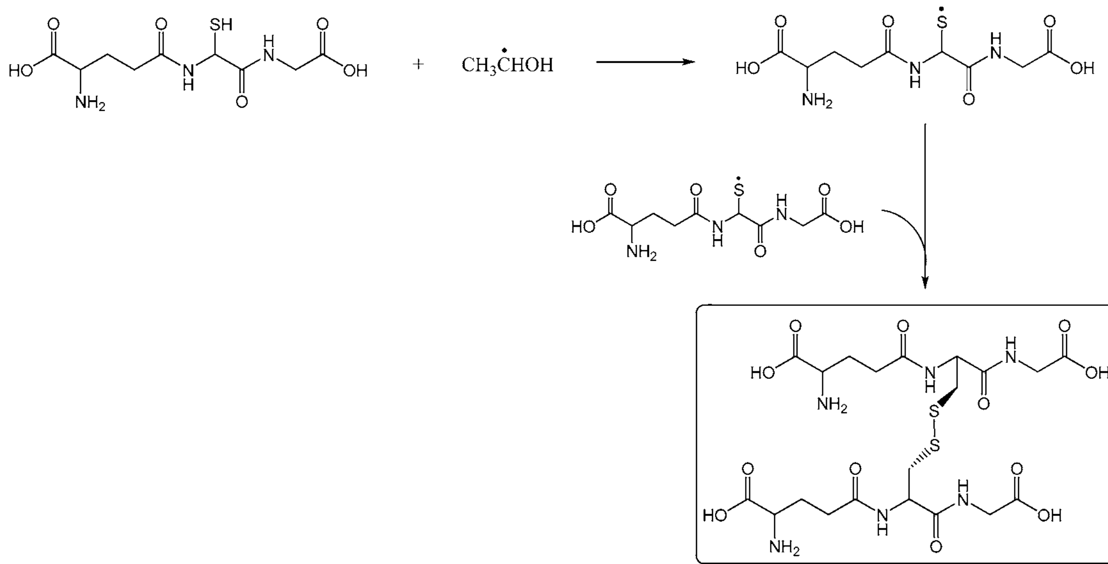


Figure 4. Orbitrap ESI-FT-MS of the products of the reaction between reduced glutathione and the 1-hydroxyethyl radical, after 1 min, at 25.0 ± 0.2 °C, operating in negative-ion detection mode. MW = 306.0769 refers to reduced glutathione; MW = 611.1454 refers to the respective disulfide.

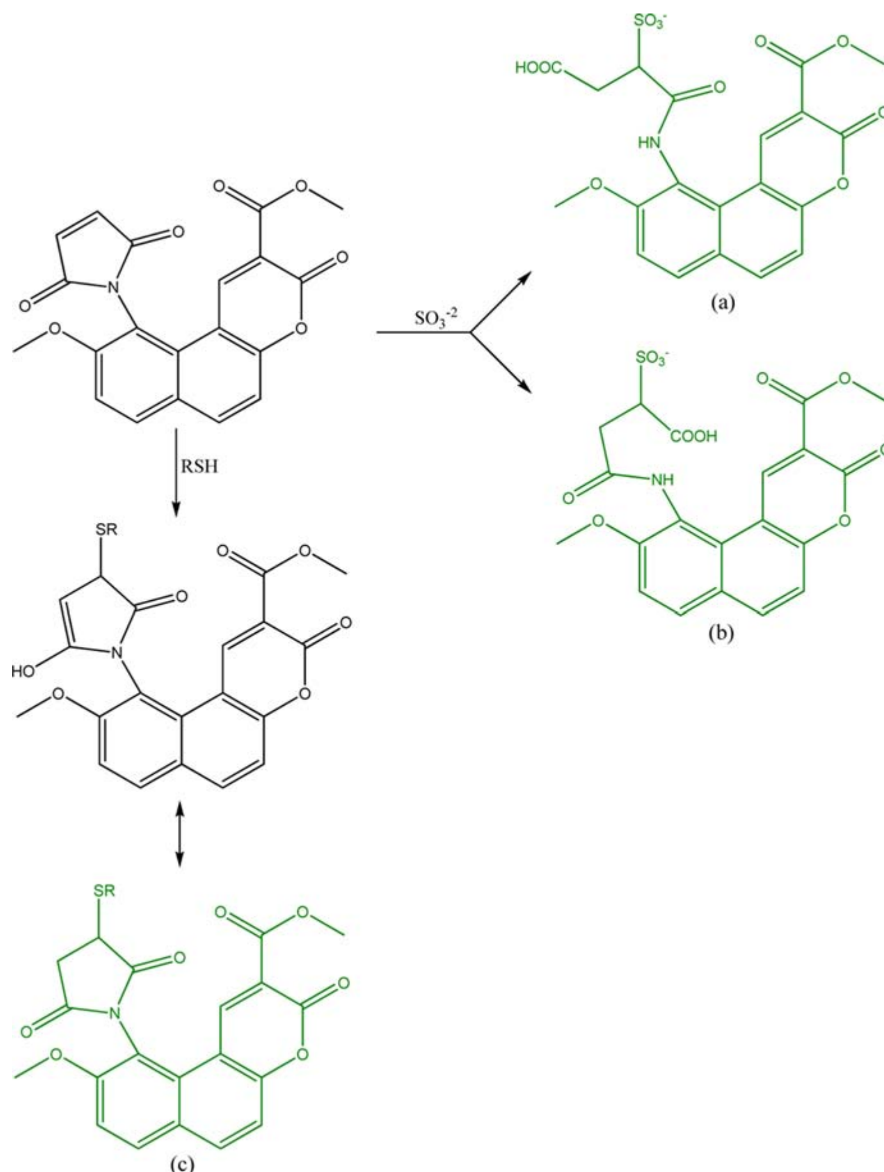
Scheme 1. Proposed Reaction Mechanism of 1-Hydroxyethyl Radical with Thiol-Containing Compounds Represented by Glutathione



whereas the kinetics is less dependent on the substrates one-electron oxidation potential.[6,7]

The thiol-containing compounds and the beer hop-derived bitter acids have very similar rate constants for the reaction with 1-hydroxyethyl radical. The apparent second-order rate constants reported for beer bitter acids are 1.8×10^8 L mol $^{-1}$ s^{-1} for *cis*-isohumulones,[6] 9.2×10^9 L mol $^{-1}$ s^{-1} for *trans*-isohumulones,[6] 1.5×10^9 L mol $^{-1}$ s^{-1} for dihydroisohumulones,[6] and 2.7×10^8 L mol $^{-1}$ s^{-1} for lupulones.[7] The content of bitter acids in beer is around 4 times higher than the content of thiols.[24] This suggests both types of compounds will trap comparable amounts of 1-hydroxyethyl radicals. Phenolic compounds are present in concentrations 40-fold higher than the concentration of thiol-containing compounds in beer;[8,25,26,11] however, the second-order rate constants are around 10^4 L mol $^{-1}$ s^{-1} for their reactions with 1-hydroxyethyl radicals,[8] and they are therefore expected to trap only a minor amount of the 1-hydroxyethyl radicals. Polyphenols have previously been shown to have negligible antioxidant effects on radical formation in lager beers.[27] Therefore, the overall higher reactivity of the thiols and bitter acids suggest that they will be oxidized in beer at a faster rate than the polyphenolic compounds.

Scheme 2. Fluorescent Labeling of Thiol Compounds with ThioGlo-1



Oxidation Products of Glutathione. To identify the major oxidation products arising from the reaction of the thiol-containing compounds toward the 1-hydroxyethyl radical, the reaction mixture containing glutathione and the radical was analyzed by ultrahigh-resolution accurate mass spectrometry (Figure 4). The ESI-MS spectrum revealed the presence of two major compounds with quasi-molecular ions $[M - H]^-$ at m/z 306.0769 and 611.1454 corresponding to glutathione (calculated for $[C_{10}H_{17}O_6N_3S - H]^-$ = 306.0759, error of 1.1 ppm) and oxidized glutathione (calculated for $[C_{20}H_{32}O_{12}N_6S_2 - H]^-$ = 611.1441, error of 2.9 ppm). The formation of sulfenic acid was checked by the addition of dimedone in the reaction mixture, however, in our experimental conditions, it was not possible to detect any trace of the dimedone/RSOH adduct, indicating that this reactive intermediate was not formed in the reaction.

Thus, according to the mechanism previously suggested in the literature,[13] the reaction is initiated by the hydrogen atom transfer from the thiol group (RSH) to the 1-hydroxyethyl radical, giving rise to the formation of a thiyl radical ($RS^{\bullet}$), which yields the respective disulfide compound (RSSR) (Scheme 1).

Quantification of Total and Reduced Thiol Levels in Beer. Quantification of the level of reduced thiols in different beers was done by employing the methodology established by Abrahamson et al.[14] and Hoff et al.[15] ThioGlo-1 was used as derivatization reagent, yielding fluorescent adducts (Scheme 2). Quantification of the total level of thiols in beer was achieved by prior reduction of the disulfide bonds in beer by the addition of TCEP as the reductant and following ThioGlo-1 derivatization and HPLC analysis.

The HPLC separation of the derivatized thiols from the interfering sulfite was performed using elution gradients 1 and 2 for the quantification of reduced thiols and total level of thiols in beer, respectively (Figure 5). The chromatogram displays four eluting peaks at t_r = 5.4 (peak A), 7.2 (peak B), 14.9 (peak C), and 18.4 min (peak D). The first two peaks (A and B) are assigned to the sulfite derivatives (Scheme 2a,b),[14] whereas peak C corresponds to a byproduct formed by the presence of TCEP in excess. Peak D eluting at 18.4 min refers to the beer thiol derivatives (Scheme 2c),[15] which clearly increases with TCEP addition until all disulfides are reduced to RSH. The chromato-

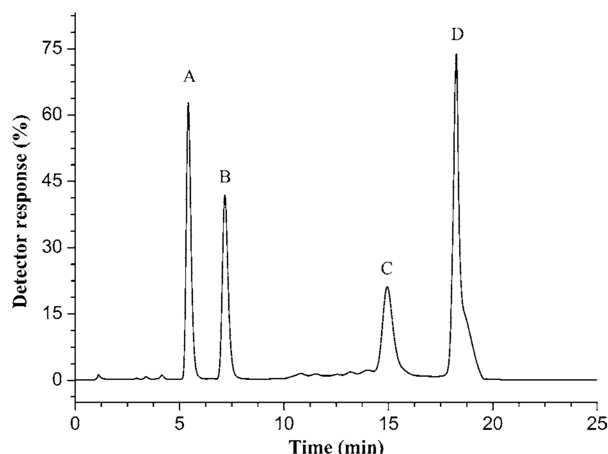


Figure 5. HPLC-FD chromatogram of beer sample 4 containing TCEP after derivatization with ThioGlo-1. Excitation was at 242 nm, and emission was detected at 492 nm. Peaks are assigned to derivatized sulfite (A, B), TCEP (C), and thiol-containing compounds (D).

grams used for quantification of reduced thiols are similar, but without the presence of peak C.

The total concentrations of reduced thiol compounds in different beers were studied after reduction with various amounts of TCEP (Figure 6). Nearly linear correlations were observed for

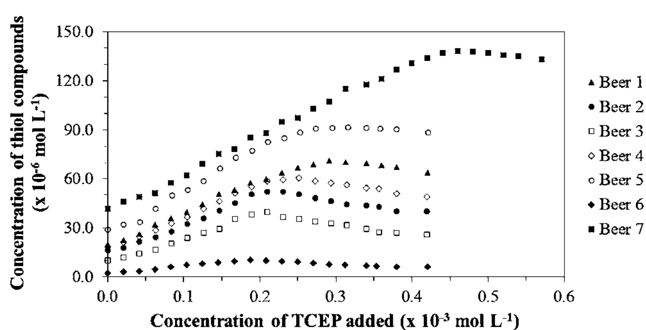


Figure 6. Total thiol concentration determined in different beers after TCEP addition and derivatization with ThioGlo-1 as determined by HPLC-FD. HPLC conditions were similar to those in Figure 4 and described under Materials and Methods.

the total level of thiol compounds as a function of added TCEP. In all beers a maximum level of thiols was achieved, indicating that all disulfide bonds were reduced to thiols. It was found that the necessary concentrations of TCEP to promote the reduction of the maximal amount of disulfide compounds were different for each beer, but it appeared to be proportional to the initial amount of thiol compounds in the beer. Interestingly, a decrease in the total thiol content was observed by the addition of TCEP in large excess. This behavior was further examined by the addition of two different concentrations of TCEP (2.5 and 4.5 × 10^{-4} mol L^{-1}; in Tris buffer, 0.25 mol L^{-1}, pH 7.5) into solutions containing different concentrations of glutathione or oxidized glutathione. The concentration of free thiols was subsequently analyzed with the ThioGlo-based method (Figure S1, Supporting Information). The linear correlation with a slope close to 1 between added thiol and measured thiol demonstrates that TCEP does not interfere with the derivatization and quantification of the thiol-containing compounds. Furthermore, in the experiment with oxidized glutathione the *y*-intercept is close to 0 and the slope is twice as high as compared to the experiment with reduced glutathione (Figure S1(A),(B), Supporting Information). This result demonstrates the efficacy of the reduction of disulfides with TCEP for the quantification of total thiol (RSH) groups.

The observed decrease in the total thiol content in beer after the addition of TCEP in large excess is therefore most likely not caused by a decrease in TCEP's ability to reduce disulfides. Therefore, a possible interference of TCEP on the redox reactions of transition metals in beer was investigated using a model Fenton reaction. Model solutions containing the spin-trap 4-POBN, H_2O_2, Fe(II), and ethanol were prepared with different concentrations of TCEP, and the EPR spectra corresponding to the formation of the spin adduct 1-hydroxyethyl/4-POBN were monitored. Indeed, a gradual increase in intensity of the EPR signal was verified starting from 1.0 × 10^{-4} mol L^{-1} of TCEP in the reaction (Figure 7). This increase in the formation of the spin

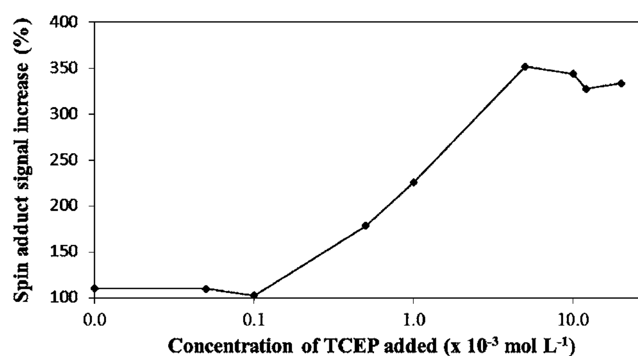


Figure 7. 1-Hydroxyethyl/4-POBN spin adduct generation in a model aqueous solution containing ethanol (5.8% v/v), 4 mL of 4-POBN (3.2 × 10^{-3} mol L^{-1}), 80 μL of H_2O_2 (24.0 × 10^{-3} mol L^{-1}), and 20 μL of $FeSO_4 \cdot 7H_2O$ (22.0 × 10^{-3} mol L^{-1}; in water) as influenced by TCEP addition (range at 1.0 × 10^{-5} to 40.0 × 10^{-3} mol L^{-1}).

adduct indicates a larger extent of radical formation by the Fenton reaction. The ability of TCEP to catalyze the Fenton reaction may be explained by the reduction of Fe(III) to Fe(II) ($E^{0\prime}$ = +0.77 V vs NHE)[28] by TCEP (E = −0.045 V vs NHE),[29] leading to increased concentrations of Fe(II). Ferrous ions are known to decompose H_2O_2, generating OH radical more efficiently than Fe(III).[30] The ability of TCEP to reduce Fe(III) to Fe(II) was checked by adding ferrozine to solutions containing various concentrations of Fe(III) and TCEP. The reduction of the metal center was confirmed by the spectrophotometric detection of the [Fe^{II}(ferrozine)] complex (Supporting Information).

Additional experiments were carried out by adding TCEP to commercial beer samples and evaluating the effect on the oxidative stability of beer as probed by the lag phase for the formation of the spin adduct 1-hydroxyethyl/4-POBN in beer with added POBN and submitted to a thermal oxidative stress. As shown in Figure 8, the increasing concentration of TCEP added to a commercial beer sample (4) clearly reduced the lag phase for radical formation under accelerated aging conditions (60.0 ± 0.2 °C). Accordingly, it may be concluded that TCEP addition to beer samples intensifies the generation of radicals, which readily react with free thiols to produce disulfides and, therefore, cause the decrease in the thiol content observed for some beers by the addition of TCEP at high concentrations (Figure 6).

The reduced and total thiol concentrations in different beers were found to be highly correlated with the total protein contents

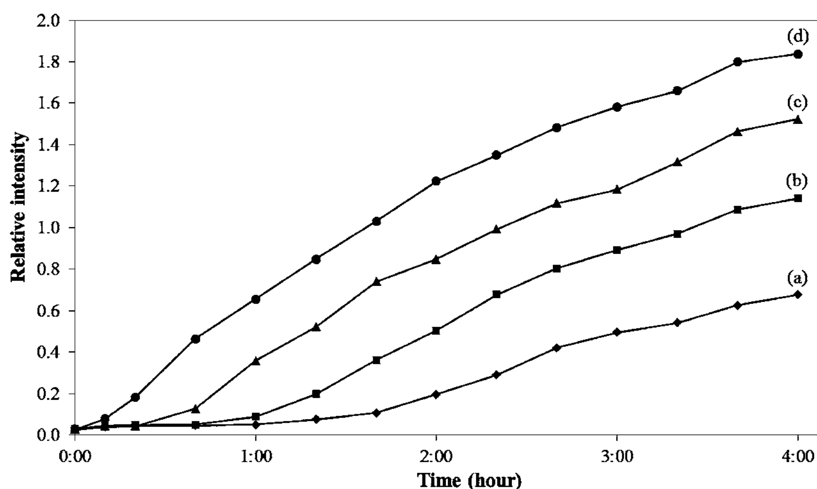


Figure 8. Generation of 1-hydroxyethyl/4-POBN spin adduct in beer (4) as influenced by various concentrations of the reductant TCEP at forced aerobic aging at 60.0 ± 0.2 °C: (a) control, 0.0 mol L^{-1} TCEP; (b) 1.0×10^{-4} mol L^{-1}; (c) 2.5×10^{-4} mol L^{-1}; (d) 5.0×10^{-4} mol L^{-1}. The spin adduct was quantified by EPR.

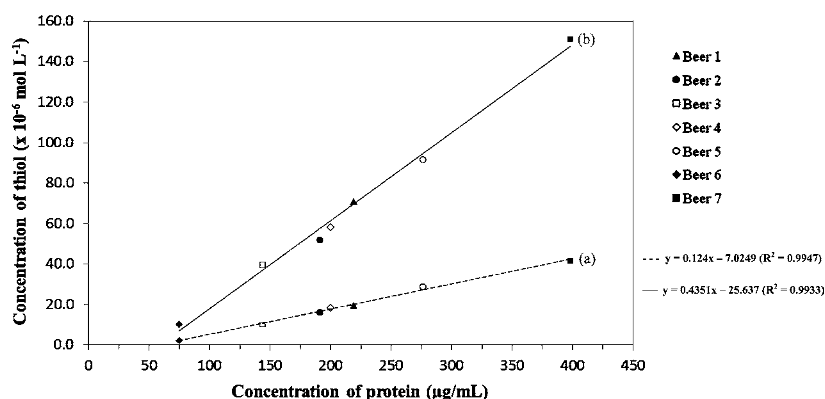


Figure 9. (A) Reduced and (B) total thiol concentration in different beers as determined by HPLC-FD versus total protein concentration as determined by using the Bradford method.

as determined by using the Bradford method (Figure 9). This relationship corroborates the fact that the majority of beer proteins contain thiol groups (cysteine) in the protein backbone. However, a small part of the beer proteins does not contribute very much to the thiol content because a certain protein concentration (approximately 75 μg/mL) is required before thiols are detected. Protein Z is the most abundant protein in beer, and contains only two cysteine residues, whereas LTP1 (with eight cysteine residues) is typically present as the second-most abundant protein in beer.[31−34] Therefore, the higher concentration of protein Z over LTP1 may explain the nonzero y-intercept. Furthermore, the protein concentration correlated linearly with both the reduced and total thiol concentrations. From the slopes of the two linear correlations, it can be calculated that the ratio between the concentrations of thiols and disulfides, [RSH]/[RSSR], is 0.79 in all of the studied beers, which shows the thiol redox status of different stored beers is surprisingly similar. This is possible if other redox-active compounds present in considerable amounts in the beer are able to maintain a constant redox balance of the thiols.

In conclusion, the apparent second-order rate constants for the reaction between thiol-containing compounds and the 1-hydroxyethyl radical are close to the diffusion limit in water and show the importance of thiol-containing peptides and proteins on the redox stability of beer. The observed primary kinetic isotopic effect suggests that the reduction of the 1-hydroxyethyl radical by thiols is governed by a hydrogen abstraction mechanism, yielding thiyl radicals that further generate disulfide compounds and protein cross-links in beer. A good correlation is seen between both total and reduced thiol contents and protein concentration in beer, suggesting that the majority of beer proteins are rich in cysteines. Due to the fact that proteins in collaboration with polyphenols contribute to haze formation in beer,[32,33] brewers often aim for lower protein contents in beer. However, the concentration of thiol-containing proteins is correlated to the oxidative stability of the product,[10,11,34] suggesting that inducing a higher protein content in beer may result in a product with enhanced oxidative stability.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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