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Effect of Pasteurization on the Protein Composition and Oxidative Stability of Beer during Storage

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ABSTRACT: The impacts of pasteurization of a lager beer on protein composition and the oxidative stability were studied during storage at 22 °C for 426 days in the dark. Pasteurization clearly improved the oxidative stability of beer determined by ESR spectroscopy, whereas it had a minor negative effect on the volatile profile by increasing volatile compounds that is generally associated with heat treatment and a loss of fruity ester aroma. A faster rate of radical formation in unpasteurized beer was consistent with a faster consumption of sulfite. Beer proteins in the unpasteurized beer were more degraded, most likely due to proteolytic enzyme activity of yeast remnants and more precipitation of proteins was also observed. The differences in soluble protein content and composition are suggested to result in differences in the contents of prooxidative metals as a consequence of the proteins ability to bind metals. This also contributes to the differences in oxidative stabilities of the beers.

KEYWORDS: *pasteurization, beer, oxidative stability, electron spin resonance spectroscopy, protein composition, LTP1, metals*

■ INTRODUCTION

Flavor stability of beer is one of the most important concerns for the brewing industry, because flavor is considered the primary quality parameter. During beer aging, fresh flavor notes decrease while aged flavor compounds are increasingly formed. Especially, *trans*-2-nonenal that is considered to be responsible for the development of cardboard-like flavors formed through oxidative reactions, has received much attention but a number of other aged flavor notes such as winery and solvent-like flavors are also formed.[1] Due to the various chemical reactions taking place in beer during storage it remains difficult to understand and control flavor stability.

Pasteurization is often employed in order to obtain beer that is stable in terms of microbiological growth and spoilage. Pasteurization influences the oxidative stability of beer as well as the protein solubility and composition, although the reported effects on oxidative stability depends on the type of analysis.[2−5] Kaneda et al.[3] showed by measuring chemiluminescence intensity that pasteurization increases the level of oxidation in beer. Furthermore, the radical concentration was lower in pasteurized beer, which was suggested to be caused by uptake of residual oxygen leading to accelerated radical reactions during the pasteurization followed by a subsequent reduction in the radical concentration after pasteurization. However, Pascoe et al.[2] used antioxidant assays (radical scavenging ability of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS, and ferric-reducing antioxidant power, FRAP) to demonstrate an increasing antioxidant capacity for fresh pasteurized beer compared to fresh unpasteurized beer. It was suggested that the increased antioxidant capacity was created by Maillard reaction products (MRPs) formed by the pasteurization treatment. Furthermore, a significant increase in the phenolic compound, catechin was also determined, which would also give an antioxidant response in the assays, although the authors did not have any explanation for the observed increased content of catechin during pasteurization. In contrast, a recent study showed that increasing pasteurization intensity decreased the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability of beer together with a decreased level of total polyphenols.[5] These contradictory reports show the need for further studies on the effect of pasteurization on the oxidative stability of beer.

Sulfite is believed to be a primary antioxidant in beer due to its ability to remove trace levels of H_2O_2 in a direct nonradical reaction. It has been suggested that protein thiols can be involved in the antioxidant mechanism by acting as catalysts.[6,7] Briefly, protein thiols are suggested to react with H_2O_2 forming mixed disulfides either directly or via the formation of a protein sulfenic acid. This disulfide may be reduced by either sulfite or other smaller reducing compounds like enzymes and regenerate the original protein thiol, which may then again react with another H_2O_2 molecule. It has been shown that the content of thiols in beer correlate with the sulfite content and the oxidative stability in beer as evaluated by forced aging combined with electron spin resonance (ESR) detection of radicals.[8] Furthermore, lipid transfer protein 1 (LTP1), which is believed to act as a foam stabilizer in beer, has been shown to be important for the flavor stability of aged beer determined by sensory analysis and possesses antioxidative activity determined by DPPH radical scavenging ability and a *Saccharomyces cerevisia*-based antioxidant screening assay.[9] LTP1 is stabilized by four disulfide bonds in its native form (in unmalted barley) giving a potential of eight thiol groups if the protein is fully reduced in the beer.[10]

Apart from influencing the oxidative stability of beer, pasteurization has also been shown to influence foam stability through a modified protein content and composition.[4]

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Pasteurization improved foam stability and increased the protein content in beer, and a protein, putatively identified as LTP1 was found to disappear in unpasteurized beer over three months of storage. The loss of LTP1 in unpasteurized beer was explained by proteinase A activity derived from yeast, which were inactivated in the pasteurized beer.

The primary objective of the present study was to investigate the relationship between the oxidative stability and protein composition in pasteurized and unpasteurized beer during storage for over one year at room temperature. Although it is known that protein composition is important in relation to colloidal stability of beer (i.e., haze and foam stability) it is unknown to which extent the protein composition affects oxidative stability of beer. A typical lager beer was produced and bottled, and half of the bottles were pasteurized to 20 pasteurization units (PU). The beers were stored for 426 days at 22 °C in the dark and characterized by pH, color, oxidative stability by ESR spectroscopy, volatile profile by GC-MS, protein content by the Bradford method, protein composition by SDS-page and MS analysis, sulfite and thiol quantification by derivatization with ThioGlo 1 fluorescent reagent followed by separation by RP-HPLC and fluorescent detection, color, metal analysis (Fe and Cu) by ICP-MS, and total phenol content by the Folin-Ciocalteu method.

■ MATERIALS AND METHODS

Chemicals. Acetonitrile, N-tert-butyl-α-nitrone (PBN), glutathione (GSH), 1-octanol, 4-methyl-1-pentanol, gallic acid, HCl, chlortetracycline, chloramphenicol, trichloroacetic acid, and 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO) were purchased from Sigma-Aldrich (St. Louis, MO). Tris-(hydroxymethyl)amino methane (tris), ThioGlo 1 fluorescent thiol reagent, trifluoroacetic acid (TFA, >99.8%), Folin-Ciocalteu phenol reagent, sodium carbonate, sodium sulfite, sorbic acid, cycloheximide, malt extract agar and MRS (de Man, Rogosa and Sharpe) agar were obtained from Merck (Darmstadt, Germany). NuPAGE Novex 12% bis-tris gels, LDS sample buffer, MES running buffer, Mark 12TM unstained standard, and Molecular Probes SYPRO ruby protein gel stain were obtained from Invitrogen, CA. Dithiothreitol (DTT) and acetic acid were obtained from Applichem GmbH, Darmstadt, Germany. Iodoacetamide was from Acros Organics, Geel, Belgium. Bradford Bio-Rad Protein Assay Reagent was obtained from Bio-Rad Laboratories, Hercules, CA. Bovine Serum Albumin (BSA) standard of 2.0 mg/mL was obtained from Thermo Fisher Scientific Inc. (Rockford, IL). Ethanol (96%) was obtained from Kemetyl (Køge, Denmark). Trypsin was from Promega, (Madison, WI), and NH_4HCO_3 was from ICN (Aurora, Ohio). All chemicals were of analytical grade or highest possible purity. Water was purified through a Milli-Q water purification system (Millipore, Billerica, MA).

Brewing of Beer. A typical all-malt lager beer (alc. 5.7%) was produced at a local microbrewery. After fermentation the beer was filtered by sheet filtration. The beer was bottled into 0.5L brown bottles and closed with a crown cork. After packaging, half of the bottles were heat treated in a tunnel pasteurizer to 20 PU, while the other half remained unpasteurized.

Storage of Beer. Beers were stored at 22 ± 2 °C in the dark, and the temperature was monitored using a temperature logger. The beers were analyzed for oxidative stability by ESR spectroscopy and volatile profile by GC-MS approximately every 2 months during the storage period. After storage for 426 days, beer samples for protein and phenol analysis were frozen and stored at −20 °C. The unpasteurized beer contained a small amount of precipitate, and this precipitate was not transferred to samples that were freeze-stored. Beer samples for sulfite, thiol, metal, and microbial growth analyses were kept at 5 °C until analysis.

Oxidative Stability of Beer by ESR Lag Phase Measurements during Storage. ESR lag phase measurements were performed according to Uchida et al.[11] Beer was degassed by stirring on a magnetic stirrer for 5 min. Degassed beer containing 30 mM PBN was heated at 60 °C in closed Blue Cap bottles with a headspace of atmospheric air. Samples were analyzed at given time intervals. ESR spectra were recorded at room temperature with a JES-FR30 ESR spectrometer (Jeol, Tachikawa, Japan) using a quartz capillary (ID 0.75 mm) sample cell (Wilmad Glass, Buena, NJ). The settings were as follows: microwave power, 4 mW; sweep width, 50.0 G; sweep time, 2 min; modulation width 1.25 G; amplitude 1000; time constant 0.3 s. All spectra consisted of single scans. Intensities of the ESR signals were calculated relative to an internal Mn(II) standard (set to 650) attached to the ESR cavity to compensate for day-to-day variation. All ESR measurements were performed in duplicate as a minimum.

Volatile Profile by GC-MS during Storage. Head space analysis was carried out in triplicate using 5 mL beer and 1.00 mL 4-methyl-1-pentanol (50 mg L^{-1}) as internal standard. The volatile compounds were collected on a Tenax-TA trap (Buchem bv, Apeldoorn, The Netherlands). Samples were equilibrated to 30 ± 1 °C in a circulating water bath and then purged with nitrogen (75 mL·min^{-1}) for 15 min. The trapped volatiles were desorbed using an automatic thermal desorption unit (ATD 400, Perkin-Elmer, Norwalk, CT) and transferred to a gas chromatograph-mass spectrometer (GC-MS, 7890A GC-system interfaced with a 5975C VL MSD with Triple-Axis detector from Agilent Technologies, Palo Alto, CA). Separation of volatiles was carried out on a DB-Wax capillary column 30 m long × 0.25 mm internal diameter, 0.25 μm film thickness. The temperature program is detailed in Deza-Durand and Petersen.[12] Volatile compounds were identified by probability based matching of their mass spectra with those of a commercial database (Wiley275.L, HP product no. G1035A). The software program, MSDChemstation (Version E.02.00, Agilent Technologies, Palo Alto, CA), was used for data analysis. Concentrations are presented as relative areas calculated as peak area of the volatile compound divided by the peak area of internal standard.

Detection of Yeast and Lactic Acid Bacteria after Storage. At the end of the storage experiment beers were analyzed for growth of yeast or lactic acid bacteria (LAB).[13] Together with beers stored at 22 °C, beers stored at 5 °C were included in the analysis in order to determine the initial level of yeast and lactic acid bacteria in the beer since microbial growth at 5 °C is reduced. The beers were opened under sterile conditions. Briefly, 100 mL of pasteurized beer were filtered through either 0.22 or 0.45 μm filter to detect acetic acid bacteria and yeast, respectively, while unpasteurized beer were serially 10-fold diluted before detection of microorganisms. Yeast were grown on malt extract agar containing 100 mg/L chloramphenicol and 50 mg/L chlortetracycline, to inhibit bacterial growth, for three days at 25 °C. LAB were grown anaerobically for three-seven days at 30 °C on MRS agar containing 0.2% sorbic acid and 0.1% cycloheximide to

suppress yeast growth. Following incubation the number of colony forming units (CFU) was recorded.

Color Determination. Absorbance of the wort samples was measured at 430 nm using a Cintra 40 spectrophotometer (GBC, Melbourne, Australia), and EBC color determined according to Analytica EBC.[14]

Determination of Fe and Cu. The samples were acid digested in a microwave oven using the solvents and temperature program detailed in Wyrzykowska et al.[15] The multielemental composition of sample digests were subsequently analyzed using inductively coupled plasma-mass spectrometry (ICP-MS) equipped with an octopole reaction cell for interference removal (Agilent 7500ce, Manchester, UK) following the instrumental settings listed in Hansen et al.[16] Beer samples were determined in duplicate.

Total Phenol Concentration by Folin-Ciocalteu. The phenolic concentration was determined by Folin Ciocalteu's method as described by Singleton and Rossi 1965.[17] The thawed beer samples were diluted 10 times in Milli-Q water and let to react with Folin-Ciocalteu phenol reagent for maximum 8 min. Subsequently, 20% sodium carbonate was added and the reaction mixture was incubated at room temperature for 2 h. The phenol concentration was determined spectrophotometrically at 765 nm on a Cintra 40 spectrophotometer against a standard curve prepared from gallic acid. Triplicate measurements were performed on each sample. The concentrations are given in mg gallic acid equivalents/L.

Protein Concentration by Bradford. Protein concentration of the beer samples was determined according to the manufacturer's procedure with a few modifications. Samples were prepared in triplicate by mixing 20 μL thawed beer sample, 800 μL 0.25 M tris buffer (pH 7.5), and 200 μL Bradford Quick Start Reagent. The samples were incubated at room temperature and absorbance at 595 nm was read after exactly 15 min using microcuvettes and a Cintra 40 spectrophotometer. Protein concentration was determined from a standard curve prepared with 0−5 μg/mL BSA (final concentration) where BSA standard solutions were added to the samples instead of degassed beer sample.

SDS-Page Analysis of Beer Proteins. Samples were analyzed by gel-electrophoresis using NuPAGE Novex 12% Bis-tris Gels according to the manufacturer's instructions. Loading samples were prepared with the same volume of each beer sample, and 0.1 M DTT (final concentration) was added to reduced samples. All loading samples were heated at min. 70 °C for 10 min before loading to the gel. Aliquots of 10 μL loading sample containing 5 μL thawed beer were loaded to the gel, and aliquots of 3 μL Mark 12 unstained standard were loaded to each gel. Electrophoresis was run at 200 V for 35 min in cassettes containing ice-cold MES running buffer. Following electrophoresis the gels were fixed in a solution containing 50% ethanol and 7% acetic acid for 30 min on a rocking table, where after the fix solution was exchanged and left overnight at room temperature. The gels were stained by the fluorescent SYPRO Ruby Protein Gel Stain overnight, washed with a solution of 10% ethanol and 7% acetic acid for 30 min and subsequently washed twice with Milli-Q water for 5 min, and photographed by a charge-coupled device (CCD) camera (Raytest, Camilla II, Straubenhardt, Germany).

Identification of Proteins from SDS-Page Analysis. The protein bands were visualized with UV light and selected protein bands were cut out of the gel and digested with trypsin. The resulting peptides were analyzed with matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). In-gel digestion was performed as described by Jensen et al.[18] Custom-made chromatographic columns were used for desalting and concentration of the peptide mixture prior to mass spectrometric analysis.[19] The proteins were identified with the use of a MALDI-TOF-TOF instrument (4800 Proteomics analyzer, Applied Biosystems, Foster City, CA). Both MS and MS/MS spectra were obtained and the proteins were identified using the Mascot database search program (Matrix Science, http://www.matrixscience.com) using the NCBInr database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). The searches were not restricted regarding taxonomy. The mass tolerance was limited to 70 ppm for peptide mass fingerprinting and to 0.6 Da for peptide sequence data.

Sulfite and Thiol Analysis. Quantification of sulfite and thiol groups was performed according to Abrahamsson et al.[20] and Hoff et al.[21] based on derivatization of sulfite and thiol groups with ThioGlo 1 fluorescent reagent followed by separation with reversed phase high performance liquid chromatography (RP-HPLC) and fluorescence detection. A standard addition protocol was used to compensate for beer matrix effects. A stock solution of ThioGlo 1 (2.60 mM) in anhydrous acetonitrile was prepared in the dark, and stored at 4 °C protected from light as described by Hawkins et al.[22] The solution was kept anhydrous by adding dried molecular sieves (0.3 nm, Metrohm Ltd., Herisau, Switzerland) directly to the stock solution. Stock solutions of sodium sulfite and glutathione (GSH) were freshly prepared every day in Milli-Q water, kept cold, and subsequently diluted to 0.5 mg/L and 4.0 μM, respectively, in 0.25 M tris buffer (pH 7.50). The dilution was performed within 30 min of using the standards to avoid air oxidation at the elevated pH values. Beer was opened immediately before analysis, degassed by magnetic stirring for exactly 5 min with addition of 0.01% 1-octanol to avoid foaming, and diluted 10 times. Samples with 0−0.125 mg/L SO_2 and 0−1.00 μM GSH each containing 20 μL degassed, diluted beer were prepared to a total volume of 100 μL made up with 0.25 M tris buffer (pH 7.50). Each sample was added 100 μL of 26 μM ThioGlo 1 (diluted just before use in 0.25 M tris buffer (pH 7.50) to avoid hydrolysis) and incubated for exactly 5 min at room temperature. The reaction was quenched by adding 10 μL concentrated HCl, and the samples were transferred to brown HPLC vials with 200 μL inserts and closed. HPLC analysis was performed as previously described.[20] All samples were run in duplicate as a minimum. Linear standard addition curves from SO_2 and GSH addition were prepared from the area of the corresponding peaks for each beer sample and used for quantification of sulfite and thiols. A blank sample of only ThioGlo 1 and buffer was run in parallel on each day and subtracted from the standard addition curves to compensate for background fluorescence from ThioGlo 1.

Data Analysis. Statistical analysis was performed using SAS 9.1 package, SAS Institute, Inc., Cary. NC. Data were analyzed by analysis of variance using proc glm. Means were used to compare differences and LSD was applied to compare the mean values. The significance level was $p < 0.05$.

RESULTS AND DISCUSSIONS

Microbial Stability of the Beers during Storage. Pasteurized beer stored at either 5 or 22 °C contained no growth of either yeast or lactic acid bacteria (LAB) proving that the pasteurization employed was sufficient to reduce microbial

growth in the beer initially. Unpasteurized beer contained very small amounts of yeast (0.5 CFU/ml) and LAB (5.4 CFU/ml) after storage at 5 °C for 426 days (Table 1). A small amount of microbes in the unpasteurized beer is expected; even though the beer has been sheet filtered before bottling, small amounts of yeast and LAB can pass through the filter. During storage of unpasteurized beer at 22 °C the amount of yeast (3×10^3 CFU/ml) and LAB (3×10^4 CFU/ml) increased in the beer. The amount of microbes found in the unpasteurized beer was in the same range as what has previously been reported.[23] However, the high amount of LAB present in the beer can cause a sour taste due to secreted lactic acid,[24] and possibly also the observed decrease in pH from 4.6 to 4.2 as shown in Table 2.

Table 1. Colony Forming Units (CFU) for Yeast and Lactic Acid Bacteria (LAB) of Pasteurized and Unpasteurized Beer Stored for 426 Days ($n = 2$)[a]

	colony forming units (CFU/ml)			
	5 °C		22 °C	
beer	yeast	LAB	yeast	LAB
pasteurized	0	0	0	0
unpasteurized	0.5	5.4	3×10^3	3×10^4

[a]For determination of microbial growth beers stored at both 5°C and 22°C were examined.

Table 2. Analytical Data for Pasteurized and Unpasteurized Beer Stored for 426 Days at Room Temperature in the Dark[a]

beer	pasteurized	unpasteurized
pH	4.62 ± 0.03^a	4.19 ± 0.08^b
EBC color	8.1 ± 0.3^{NS}	7.6 ± 0.2^{NS}
Fe (ppb)	46 ± 5^a	30 ± 2^b
Cu (ppb)	70 ± 1^a	79.0 ± 0.3^b
phenolic compounds (mg gallic acid equivalents/L)	55 ± 1^a	59.7 ± 0.8^b
protein (mg/mL)	0.2582 ± 0.0003^a	0.12 ± 0.01^b
thiol (μM)	8.66 ± 0.02^{NS}	9 ± 2^{NS}

[a]Values are given as mean values ± standard deviations of minimum two independent determinations. Numbers within the same row bearing different letters are significantly different ($p < 0.05$), NS=non-significant.

Effect of Pasteurization on the Oxidative Stability of Beer. Oxidative stability of the beers was evaluated by ESR lag phase measurements, where the lag phase is defined as the time before radical formation is initiated during aerobic incubation at 60 °C with the spin trap, PBN. After production of the beers (at storage day 0), there was no significant difference between pasteurized and nonpasteurized beers ($P = 0.31$). Both set of beers had initial lag phases around 220 min, which indicate beers with a good storage stability. During the storage at 22 °C the lag phases of the unpasteurized beers decreased, and at the end of the storage almost no lag phases were observed (Figure 1A). In contrast the pasteurized beers at day 426 had lag phases around 140 min. Pasteurization therefore seemed to have a significantly positive effect on the beer stability as evaluated from the changes in ESR lag phases during the storage. According to Kunz et al.[25] the use of the PBN spin trap increases pH in beer during ESR measurements, and the elevation in pH results in increased formation of hydroxyl radicals and hereby a shortened lag phase. In this study, however, pH is 4.6 in the pasteurized beer with the longest lag phase and the unpasteurized beer has pH 4.2, so assuming the observed effects of pasteurization were only dependent on pH, then they should have been reversed.

The rate of radical formation after the end of the lag phase is a measure of the radical forming potential of the beer, that is, the effect of prooxidants without the inhibiting effects of antioxidants. It is determined as the slope of the spin adduct formation curve after the end of the lag phase. Throughout the entire storage period the unpasteurized beers were found to have a significantly higher rate of radical formation than the pasteurized beers (Figure 1B). The higher radical formation rate in the unpasteurized beers may explain the observed faster change of their lag phases, due to a faster exhaustion of the initial antioxidants present in the beer. This was confirmed by measuring the levels of sulfite, which is believed to be the dominating antioxidant compound in beer. The pasteurized and unpasteurized beers originated from the same brew and they had undergone the same treatments until the pasteurization step, and therefore the two beers also had the same sulfite content before pasteurization. Although there was no significant difference between ESR lag phases at day 0, the content of sulfite in the unpasteurized beer was found to be slightly, but significantly, lower than in the pasteurized beer at day 0 ($P = 0.0448$). The concentrations of sulfite were found to change in parallel with the ESR lag phases during the storage (Figure 2). The rapid loss of sulfite in the unpasteurized beer during the first 63 days of storage is consistent with a fast reduction in the ESR lag phase by approximately 50%. Furthermore, the complete loss of sulfite is consistent with an almost complete disappearance of the ESR lag phase. The correlation between sulfite levels and ESR lag phases is in accordance with previous studies,[7,10,26] however in this case the observed differences in oxidative stability of the two set of beers appear to be caused mainly by differences in the effects of the prooxidants since the beers started out with nearly similar levels of sulfite.

Metal ions such as Fe and Cu are usually present in the ppb range in beer and known to be prooxidants due to their involvement in the Fenton reaction resulting in the formation of hydroxyl radicals ($^\bullet$OH) and other reactive oxygen species.[27,28] Unexpectedly, the contents of Fe and Cu was found to be significantly different in the two set of beers (Table 2). The original metal content before pasteurization was identical in the two beers. Different metal contents in the two beers indicate that metals are bound differently to the beer matrix dependent on pasteurization. The rate of radical formation (Figure 1B) has previously been found to increase with increasing concentration of Fe in beer,[27,29] but in the present study the pasteurized beer had a higher Fe content, lower Cu, and a lower rate of radical formation than the unpasteurized beer. Apparently there is no simple correlation between the metal levels and the radical formation rate, but the observed differences could be a result of different degrees of metal-binding to the beer matrix and a resulting variation in the metals prooxidative activity.

The importance of polyphenols on the flavor stability of beer is widely discussed and the results published on the subject are ambiguous.[30] While Pascoe et al.[2] found that pasteurization increases antioxidant capacity and polyphenol levels in beer; it has been shown that removal of protein-bound polyphenols decreases reducing capacity but without any impact on flavor

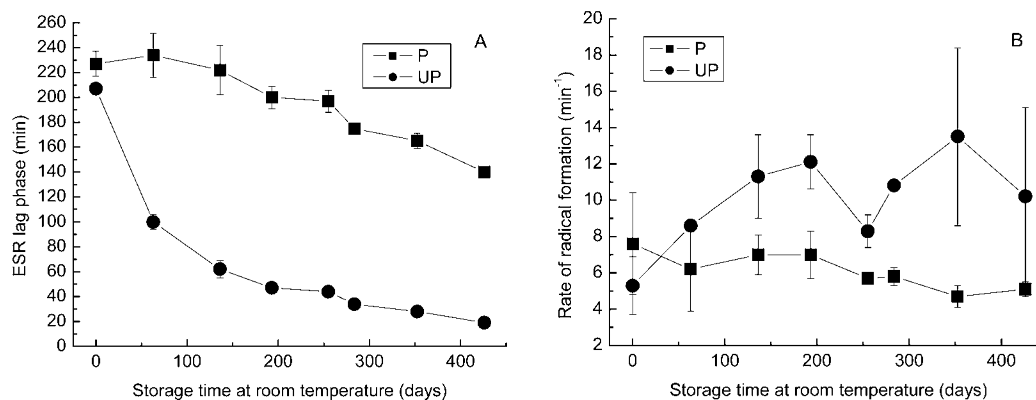


Figure 1. Oxidative stability of pasteurized (P) and unpasteurized beer (UP) during storage at 22 °C for 426 days determined by incubation of beer with PBN spin trap at 60 °C and measurement of radical intensity by ESR spectroscopy. (A) ESR lag phases are determined as the time until radical formation accelerates, and (B) ESR rate of radical formation is determined as the slope of the curve obtained after radical formation has accelerated.

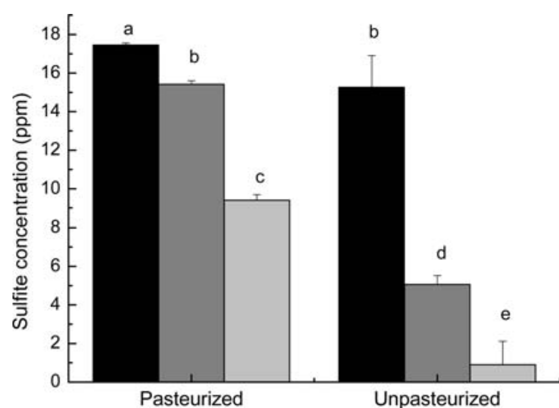


Figure 2. Sulfite concentration in beer after 0 (black bars), 63 (dark gray bars), and 426 (light gray) days of storage at 22 °C in the dark. All beers were kept at 5 °C after the given days of storage at 22 °C and analyzed after the storage period. Values are mean values of two independent samples and standard deviations are shown as error bars. Bars bearing different letters are significantly different ($P < 0.05$).

stability.[31] Furthermore, other studies indicate that polyphenols may not significantly affect the formation of radicals in beer during storage or in wort during brewing.[32] In the present study, determination of the total phenol content in the beers at the end of the storage period showed a slightly smaller, but significant, level of phenols in the pasteurized beer (Table 2) in agreement with previous findings.[5] Whether the loss of phenolic compounds is simply caused by the heat treatment, which would possibly result in phenol polymerization and subsequent precipitation or by a sacrificial oxidation of phenols into quinones through an oxidative mechanism cannot be concluded. However, the overall level of oxidation is expected to be highest in the unpasteurized beer since it showed a fast decrease in lag phase and a high potential for radical formation, suggesting the antioxidative effect of the phenols are limited since they were found in the highest concentration in this beer.

Volatile Compounds. The volatile profiles of the beers were determined during storage from day 63 to day 426 with identification of 60 volatile components among which Maillard Reaction Products (MRPs) and staling compounds were detected. The volatile profile varied between pasteurized and unpasteurized beer, but only to a small extent. Four volatile compounds associated with staling were found to change in intensity during storage: 3-methyl butanal, 3-methyl-2-butanone, 4-methyl-2-pentanone, and furfural (Figure 3). Pasteurization caused significantly increased intensities of all four volatile compounds compared to unpasteurized beer and this difference increased with increasing storage time. Increase in concentration of these four compounds in beer during storage has previously been reported.[1,33] Furfural and 3-methyl butanal are known to be derived from the Maillard reaction. Furfural has previously been identified as an indicator of heat-induced flavor damages, but the formation of furfural is most likely unaffected by oxygen and the concentrations typically found in beer are not thought to be significant in terms of overall beer flavor.[1,33,34] 3-methyl butanal, may also be considered a suitable marker for beer oxidation but is most likely not important for stale flavor formation.[1] In conclusion, these four compounds are likely to be heat induced with their generation initiated by the pasteurization. Their generation is therefore likely to occur independently from other oxidative reactions taking place in the beer. Furthermore, a slight tendency of a smaller loss of volatile ester compounds, such as ethyl 2-methylpropanoate, ethyl pentanoate, ethyl hexanoate, ethyl octanoate, ethyl nonanoate and ethyl decanoate was observed in the unpasteurized beers during storage compared to the pasteurized beers. These compounds are often associated with fruity flavors and beer freshness. Considering the overall volatile profile of the beers, the differences between the two beers were small, but unpasteurized beer contained slightly more volatile ester compounds associated with a fruity character and less compounds associated with staling suggesting that the unpasteurized beer actually have a better sensory quality than the pasteurized beer. These contradictious observations may be explained by the fact that the generation of these particular staling compounds as well as the loss in volatile ester compounds is likely to occur independently of the radical generation. The higher concentration of the ester compounds in the unpasteurized beer could be due to a loss in the pasteurized beer due to the heat treatment or increased formation of these compounds from active yeast remnants in the unpasteurized beer.

MRPs are formed during roasting of malt and heat treatments during the brewing process, and have been reported to act both as antioxidants[35,36] and prooxidants[37−39] in wort and beer. The pasteurization of beer is expected to induce a slight production of MRPs. Measuring the color of beer gives an indication of the level of MRPs, but although a slight increase in color of the pasteurized beer was observed, then no

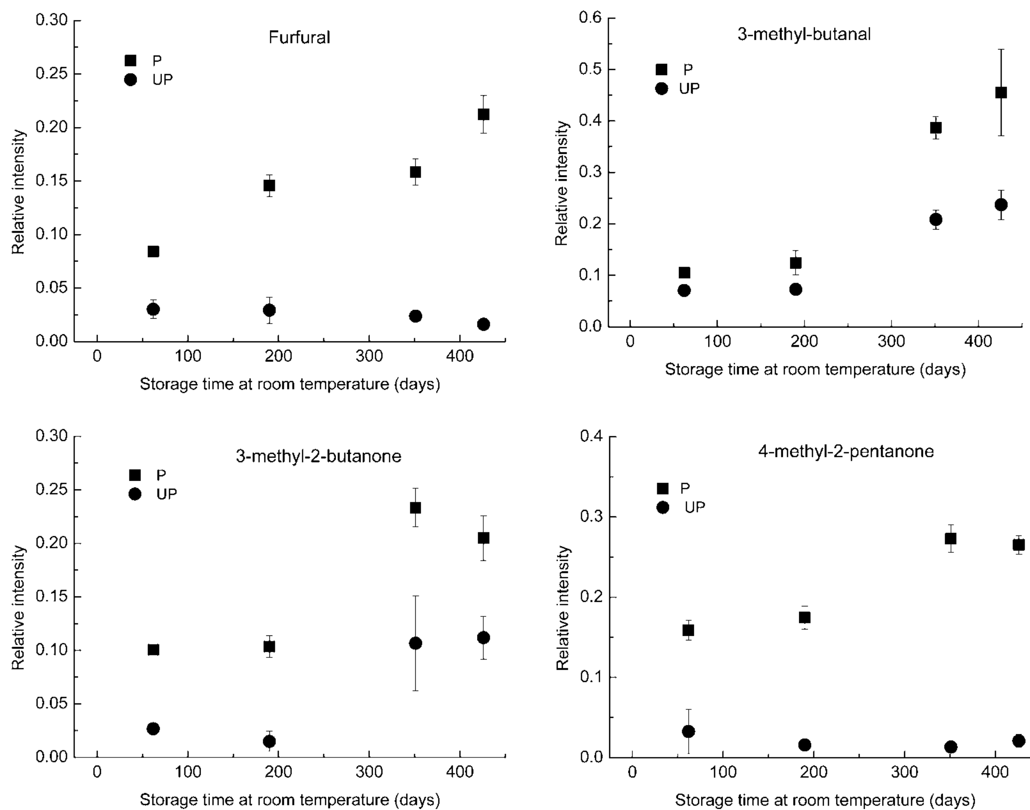
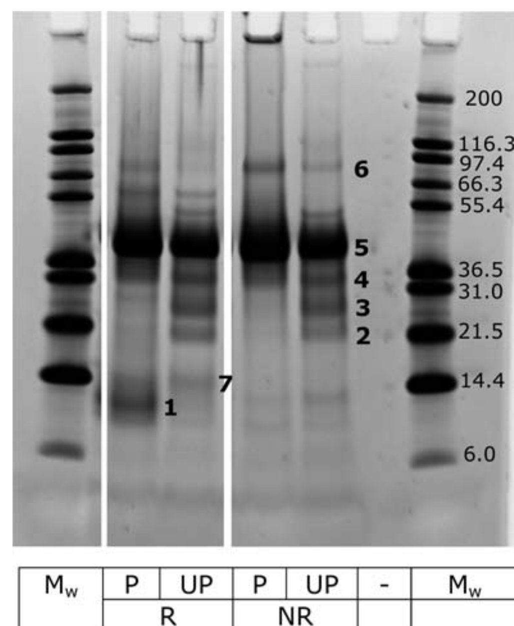


Figure 3. Relative intensities of the four volatile compounds (furfural, 3-methyl-butanal, 3-methyl-2-butanone, and 4-methyl-2-pentanone) that differed in intensity in pasteurized (P) and unpasteurized beer (UP) during storage at 22 °C for 426 days.

statistical significant difference was found between the color of the pasteurized and the unpasteurized beer (Table 2).

Thiols and Proteins. Thiols have been suggested to act as antioxidants in beer and the concentration of thiols has been found to correlate with the oxidative stability in beer as previously described.[6,8] However, in the present study no significant difference in thiol concentrations was observed after storage (Table 2). In fact, the thiol concentration was relatively low compared to the concentrations determined by Lund and Andersen,[8] which indicates that the thiols were oxidized during storage, but whether this is due to any antioxidative mechanism of the thiols during storage cannot be concluded based on these results.

The content of soluble protein was considerably higher in the pasteurized beer than in the unpasteurized beer after storage for 426 days (Table 2). This difference is probably due to (i) protein precipitation as a small precipitate was observed after storage at 22 °C in the unpasteurized beer, and (ii) protein degradation due to proteolytic activity of yeast and bacteria. The Bradford method used for protein determination only detects peptides or proteins of at least 3 kDa so if proteins are degraded below this limit they are not detected.[40] SDS-page analysis revealed major differences in the composition of the soluble proteins between the pasteurized and unpasteurized beers after storage (Figure 4), and the major differences in protein composition were studied by MS analysis (Table 3). In band no. 1 two proteins were identified as trypsin/amylase inhibitor and LTP1. For reduced samples, these proteins were found to be present in pasteurized beer but not in the unpasteurized beer. Furthermore, several breakdown products of protein Z (bands nos. 2, 3, and 4) were observed in the unpasteurized beer which was consistent with a smaller



M_w	P	UP	P	UP	-	M_w
	R		NR			

Figure 4. A representative SDS-page gel of pasteurized (P) and unpasteurized (UP) beer samples after 426 days of storage at 22 °C in the dark that was either reduced (R) by DTT or nonreduced (NR) prior to electrophoresis. The numbers in bold inserted on the gel refer to the protein band to the left of the number that was identified by MS analysis as shown in Table 3. M_w is the molecular weight marker and numbers (not bold) refer to the molecular weight in kDa. All lanes are from the same SDS-page gel, but lanes with irrelevant samples have been cut out of the figure for clarity.

Table 3. Identification of Protein Bands from the SDS-Page Gel[a]

band no.	identified protein	accesion number	score	mass (Da)	sequence coverage (%)
1	trypsin/amylase inhibitor	gi|225102	263	15 307	47
	lipid transfer protein 1	gi|47168353	101	10 145	38
2	protein Z-type serpin	gi|1310677	308	43 307	30
3	protein Z-type serpin	gi|1310677	542	43 307	27
4	protein Z-type serpin	gi|1310677	667	43 307	30
5	protein Z-type serpin	gi|1310677	794	43 307	38
6	protein Z-type serpin	gi|1310677	257	43 307	19
7	trypsin inhibitor cme precursor	gi|1405736	137	16 341	31

[a]Protein band numbers refer to the number on the SDS-page gel in Figure 3. All proteins were derived from barley (*Hordeum vulgare*).

intensity of the original band of protein Z (band no. 5). This effect of heat treatment on LTP1 and protein Z is likely to be caused by activity of proteinase A from yeast in unpasteurized beer while proteinase A is inactivated by the pasteurization process as previously shown by He et al.,[4] and is likely to result in degradation of trypsin/amylase inhibitor as well. The optimum for proteinase A activity on hemoglobin has been reported to be pH 2−4.5 and varies with the protein substrate,[41] so proteinase A activity is to be expected within the range of the beers from the present study (Table 2).

For nonreduced samples, an additional protein band above protein Z (band no. 6) was observed in both beers and was identified as protein Z. Since this band could not be observed in reduced samples, it is an oxidized form of protein Z cross-linked through disulfide bonding. The content of disulfide cross-linked protein Z was mainly present in the pasteurized beer which could be a result of either (i) disulfide bonding in protein Z is induced by pasteurization or (ii) that disulfide bonding of protein Z took place in both beers but is degraded by proteinase A in unpasteurized beer during storage.

The major differences in protein composition observed for pasteurized and unpasteurized beer could explain the observed differences in oxidative stability of the beers. The presence of LTP1 in the pasteurized beers with greater oxidative stability is in agreement with the study of Wu et al.[9] showing a stabilizing effect of LTP1 on flavor in beer and a radical scavenging ability of the protein. However, in the beers after storage LTP1 seems to be oxidized as observed both in the SDS-page results and since the thiol contents are the same in the two beers. Since there is still sulfite present in the pasteurized beer, LTP1 should be able to be reduced according to Rogers and Clarke,[6] and since this is not the case pasteurization may have destroyed the functionality of LTP1. However, this is speculative since the reducing ability of sulfite on the disulfide bonds in LTP1 is unknown. The identified trypsin/amylase inhibitor present in the same band as LTP1 contains 10 cysteine residues and may therefore also potentially work as an antioxidative protein in beer. Furthermore, proteins are known to bind metals, and since the Fe content is higher in the pasteurized beer with higher oxidative stability, the proteins that have not been degraded by proteinase A in the pasteurized beer may bind Fe and perhaps make it less effective in the Fenton reaction. The determination of metals by ICP-MS provides a total content of metals irrespective of protein-binding, but if proteins are precipitated the protein-bound metals will also be precipitated and therefore not included in the quantification. Barley LTP have been shown to bind Co(II) and Pb(II) but has no affinity toward Cu(II) and it is unknown to which extent it binds Fe.[42] The protein identified as trypsin inhibitor cme precursor (band no. 7) did not seem to be affected by pasteurization.

Protein Composition and the Correlation to Oxidative Stability in Beer during Storage. Pasteurization of beer was found to improve the oxidative stability during storage at 22 °C for over one year as determined by measuring the radical formation by ESR spectroscopy. A faster rate of radical formation was observed in unpasteurized beer, which is in agreement with a faster consumption of sulfite. The pasteurized beer was found to have a higher content of heat induced volatile staling compounds as well as a slightly lower content of volatile ester compounds. So although the pasteurized beer clearly shows a better oxidative stability determined by ESR spectroscopy, the pasteurization induces a slightly negative effect on the volatile profile. The level of oxidative stability of the two set of beers is suggested to be determined mainly by differences in the prooxidative activity of the metals. The different metal contents in the beers indicate that metals are bound differently to the beer matrix dependent on pasteurization since the original metal content before pasteurization were identical in the two beers. The metal-binding beer matrix components are suggested to be protein-derived since a large difference in protein content and composition was observed in the two beers. The unpasteurized beer contained more degraded protein but also more precipitated protein, which could explain the observed differences in metal contents. If metals are bound to beer proteins, protein precipitation would result in a reduction in metal content due to removal of metal from the liquid phase as observed for Fe. Protein degradation could on the other hand result in the release of metals due to a decreased metal-binding capacity as observed for Cu. Hence, proteins are suggested to contribute positively to the oxidative stability either (i) by binding metals and hereby making them less reactive or available as prooxidants during the Fenton reaction or (ii) by reacting as a catalyst in the removal of H_2O_2 formed during oxidative reactions in beer as previously described for LTP1.[9] The mechanism of proteins during oxidation in beer is currently being exploited further in our lab as well as the redox status of thiols during storage and the oxidation potential of protein-bound metals.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BSA, bovine serum albumin; CFU, colony forming units; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DTT, dithiothreitol; ESR, Electron Spin Resonance; GC-MS, gas chromatography−mass spectrometry; GSH, glutathione; HPLC, high performance liquid chromatography; ICP-MS, Inductively Coupled Plasma-Mass Spectrometry; LAB, lactic acid bacteria; LTP1, lipid transfer protein 1; MALDI-TOF MS, matrix assisted laser desorption ionization-time-of-flight mass spectrometry; MRPs, Maillard reaction products; PBN, N-*tert*-butyl-α-nitrone; SDS-page, Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxy; TFA, trifluoroacetic acid; tris, tris(hydroxymethyl)amino methane

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