FACULTY OF SCIENCE
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PhD thesis

Signe Hoff

From Barley to Beer

Oxidation throughout the brewing process

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PhD Thesis · 2013

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Oxidation throughout the brewing process

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Preface

This PhD project was carried out at the Institute of Food Science in corporation between the groups Quality and Technology and Food Chemistry. The work has been funded by the Danish Ministry of Food, Agriculture and Fisheries and University of Copenhagen. The research was carried out during the period October 2009 to October 2013. From September 2011 to September 2012 I was on a leave working on the FØLOX research project at Food Chemistry.

This PhD project has been supervised by Associate professor Birthe Møller Jespersen, Associate professor Marianne Lund Lametsch, Associate professor Mikael A. Petersen and Professor Mogens L. Andersen.

I am grateful to Birthe for inviting me to do this PhD and thereby introducing me to the exciting world of beer research. I would also like to thank you for loyal support and good colaboration. Thank you Marianne, for sharing your knowledge and enthusiasm and for encouraging me to aim high. I would like to thank Mikael for valuable discussions and advice regarding volatile analysis. Thank you Mogens, for always taking the time to talk and for sharing your knowledge.

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Signe Hoff, October 2013

Abstract

The factors determining the flavor stability of beer are extremely complex and remain the largest challenge in brewing today. The overall purpose of this PhD thesis was to gain more knowledge about flavor stability and oxidation throughout the brewing process and in the final beer including investigation of raw materials as well as the possible antioxidative effect of protein thiols.

Evaluation of the oxidative stability of malt, wort and beer was performed by electron spin resonance spectroscopy. Thiols were quantified by a newly developed method based on HPLC separation and fluorescence detection and volatiles were detected through headspace sampling and GC-MS analysis.

The storage of malt resulted in oxidative reactions, formation of lipid oxidation products and a large change of the volatile profile of the resulting worts. Roasted malt is much more unstable than pilsner malt illustrated by a higher initial radical intensity, larger radical decay during storage, a larger change in volatile profile of the wort with increased amounts of lipid oxidation products. The results suggest that storage time of malt and handling of sweet wort in general should be minimized to prevent initiation of oxidative reactions and to limit the loss of volatiles. The Maillard reaction products, melanoidins, are suggested to contribute to the instability of dark malt and wort through a prooxidative effect in combination with Fe by acceleration of the Fenton reaction. The research suggests that barley variety can influence the volatile profile of wort and thereby possibly also the beer flavor.

The research supports the hypothesis about protein thiols having an antioxidative effect in wort and beer. The amounts of protein thiols in sweet wort varied between barley varieties. Thiols are very sensitive to oxidation, and exclusion of oxygen is crucial for keeping the thiols on their reduced form. Increased thiol oxidation was linked to a lowering of wort filtration rates due to formation of gel proteins. Sweet wort was found to contain compounds able to oxidize protein thiols, referred to as “thiol-removing capacity”, which is suggested to be mainly caused by an enzymatic effect. The levels of thiols were found to decrease during beer storage, suggesting that thiols are intermediates in redox reactions during beer staling.

Pasteurization has a positive influence on the oxidative stability and radical intensity of both filtered and non-filtered beer, which was consistent with a decreased consumption of sulfite. Pasteurization has a minor negative effect on the volatile profile by increasing the level of volatile compounds that is generally associated with heat treatment and by decreasing the levels of ester volatiles generally associated with fruity aroma.

Sammendrag

Smagsstabilitet af øl under lagring er meget komplekst og forbliver den største udfordring i ølfremstilling i dag. Det overordnede formål med denne PhD var at undersøge smagsstabilitet og oxidation igennem brygprocessen, såvel som i den færdige øl, inklusiv analyse af råmaterialer og den mulige antioxidative effekt af protein-thioler.

Den oxidative stabilitet af malt, urt og øl blev undersøgt ved hjælp af elektron spin resonansspektroskopi. Thioler blev kvantificeret med den nyligt udviklede metode baseret på HPLC separation og fluorescens detektion. Flygtige stoffer blev detekteret ved hjælp af headspace opsamling og GC-MS analyse.

Maltlagring resulterer i en generel stigning i oxidation, dannelse af lipidoxidationsprodukter samt en stor ændring i sammensætningen af flygtige komponenter i den søde urt. Ristet malt er væsentligt mere ustabilt end pilsner malt illustreret ved en højere radikalintensitet, større radikalhenfald under lagring, større ændring i sammensætningen af flygtige stoffer samt øget mængde af lipidoxidationsprodukter. Resultaterne viser, at maltlagringstid og håndteringen af sød urt bør minimeres for at forebygge oxidation og begrænse tabet af flygtige stoffer. Maillardprodukterne, melanoidiner, bidrager til nedsat oxidativ stabilitet af mørk malt og urt ved hjælp af en prooxidativ effekt i kombination med Fe og acceleration af Fentonreaktionen. Bygsorten har indflydelse på sammensætningen af flygtige stoffer i urt og dermed muligvis også på smagen af øllet.

Forskningen understøtter hypotesen om, at protein-thioler har en antioxidativ effekt i urt og øl. Mængden af protein-thioler i sød urt varierer mellem bygsorter. Thioler er meget følsomme overfor oxidation, og begrænsning af ilttilgængeligheden er afgørende for at holde thiolerne på reduceret form. Øget thioloxidation er korreleret med en nedsat filtreringshastighed på grund af dannelsen af gelproteiner. Sød urt indeholder et stof, der er i stand til at oxidere protein-thioler, kaldet "thiol-removing kapacitet", og denne kapacitet menes at være primært enzymatisk. Thiolindholdet falder under lagring af øl, hvilket tyder på, at thioler indgår i redoxreaktioner.

Pasteurisering har en positiv effekt på den oxidative stabilitet og radikalintensitet af både filtreret og ufiltreret øl, hvilket var konsistent med nedsat forbrug af sulfit. Pasteurisering havde en mindre negativ effekt på sammensætningen af flygtige stoffer udtrykt ved en øget mængde af de flygtige stoffer, som ofte associeres med varmebehandling samt ved tab af flygtige esterforbindelser, som normalt associeres med frugtagtig aroma.

List of Publications

Paper I

Hoff, S.; Lund, M. N.; Petersen, M. A.; Jespersen, B. M.; Andersen, M. L. Quality of Pilsner Malt and Roasted Malt during Storage. 2013, *Journal of the institute of brewing*, submitted 19/09-13.

Paper II

Hoff, S.; Lund, M. N.; Petersen, M. A.; Jespersen, B. M.; Andersen, M. L. Influence of Malt Roasting on the Oxidative Stability of Sweet Wort. 2012, *Journal of Agricultural and Food Chemistry*, 60, 5652-5659.

Paper III

Hoff, S.; Damgaard, J.; Petersen, M. A.; Jespersen, B. M.; Andersen, M. L.; Lund, M. N. Influence of Barley Varieties on Wort Quality and Performance. 2013, *Journal of Agricultural and Food Chemistry*, 61, 1968-1976.

Paper IV

Lund, M. N.; **Hoff, S.**; Berner, T. S.; Lametsch, R.; Andersen, M. L. Effect of Pasteurization on the Protein Composition and Oxidative Stability of Beer during Storage. 2012, *Journal of Agricultural and Food Chemistry*, 60, 12362-12370.

Paper V

Hoff, S.;Lund, M.L; Petersen, M. A.; Frank, W.; Andersen, M. L. Storage Stability of Pasteurized non-filtered Beer. 2013, *Journal of the institute of brewing*, 119, 172-181.

Paper VI

Abrahamsson, V.; **Hoff, S.**; Nielsen, N. J.; Lund, M.L; Andersen, M. L. Determination of sulfite in beer based on fluorescent derivatives and liquid chromatographic separation. 2012, *Journal of American Society of Brewing Chemists*, 4, 296-302.

Paper VII

Hoff, S.; Larsen, F. H.; Andersen, M. L.; Lund, M. N. Quantification of protein thiols using ThioGlo 1 fluorescent derivatives and HPLC separation. 2013, *Analyst*, 138, 2096-2103.

Popular Science Publication in Danish

Hoff, S.; Lund, M. N.; Jespersen, B. M.; Andersen, M. L. Protein-thioler og stabilitet af øl. 2013, *Dansk Kemi*, Nr. 9.

Abbreviations

Cu	Copper ion
DMS	Dimethylsulfide
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
EBC	European Brewery Convention
ESR	Electron spin resonance
Fe	Iron ion
GSH	Glutathione
HPLC	High performance liquid chromatography
LTP1	Lipid transfer protein 1
NADPH	Nikotinamid-adenin-dinukleotid-fosfat
PBN	N-*tert*-butyl-α-nitrone
PCA	Principal component analysis
PLS	Partial least squares
POBN	α-(4-pyridyl-1-oxide)-N-t-butylnitrone
SA	Spin adduct
ST	Spin trap
TCEP	Tris(2-carboxyethyl)phosphine

Table of Content

1 Introduction

In the past, appearance of haze and the growth of beer spoilage micro-organisms were considered the main challenge in beer production. These problems are now under control, due to extensive research and improved brewing technology, and the interest has therefore shifted to factors affecting the changes in beer flavor. The factors determining the flavor stability of beer is extremely complex and remains the largest challenge in brewing today.

Flavor deterioration, or beer staling, is a result of both formation and degradation of volatile compounds and their precursors. These flavor changes are highly influenced by the beer type, storage temperature and oxygen concentration in the can or bottle as well as oxygen concentration throughout the brewing process. The level of oxidation has a large impact on oxidative reactions leading to beer staling. Beer staling however, has also been found to occur under minimal oxygen concentrations suggesting that parts of staling is caused by non-oxidative processes (Vanderhaegen et al., 2006; Bamforth et al., 2009).

Figure 1 shows a simplified schematic drawing of flavor changes during beer aging according to Dalgliesh (1977). The staling pattern vary highly between beers and the drawing is brought to illustrate the complexity of beer staling.

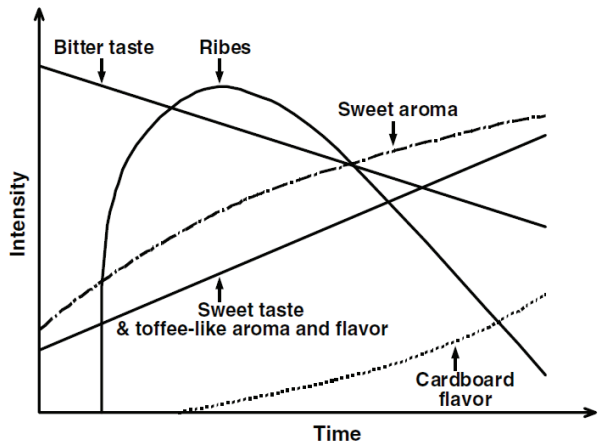


Figure 1: Simplified schematic drawing of sensory changes during beer aging according to Dalgliesh (1977).

A large part of the flavor deterioration has been assigned to the formation of carbonyl compounds (Malfliet et al., 2008; Baert et al., 2012). Especially trans-2-nonenal, with its cardboard-like flavor, has for many years been referred to as the main staling compound in beer and therefore this compound has received a great deal of attention (Jamieson and Van Gheluwe, 1970; Noël et al., 1999; Liegeois et al., 2002). Lately however, researchers tend to agree that the impact of trans-2-nonenal has been over emphasized and that trans-2-nonenal is only a part of the bigger picture of beer deterioration. Beer deterioration is now believed to be caused by formation of a large number of staling compounds as well as loss of positive flavor compounds (Vanderhaegen et al., 2003; Baert et al., 2012).

Beer quality is highly influenced by the quality of the raw materials which vary with the growing and weather conditions. Therefore production of an equally stable beer year after year may not always be possible. It is generally accepted that malt has a storage stability of one year. However, in practice, many brewers notice changes in malt aroma during storage, but only very few scientific studies have been carried out on flavor stability and oxidative stability of malt during storage. Therefore this important issue has been dealt with in the research of the current thesis.

Many of the unwanted reactions are initiated by reactive oxygen species leading to chain reactions resulting in formation of especially carbonyl compounds (Bamforth and Parson, 1985). The balance between pro- and antioxidants in the beer is to a great extent determining the rate of deterioration (Kaneda et al., 1992; Andersen and Skibsted, 1998; Nøddekær and Andersen, 2007; Frederiksen et al., 2008). Two important prooxidants are the transition metals Fe and Cu which can act as catalysts in oxidative reactions by donating electrons and participating in the Fenton and Haber-Weiss reactions (Figure 2). In the presence of ferrous or copper ions (Fe^{2+}/Cu^{+}) oxygen can capture an electron and form the superoxide anion (O_2^{-}). At the pH values of most beers the superoxide anion is protonated and forms the perhydroxyl radical (OOH·) with a much higher reactivity. The super oxide anion can also be reduced by Fe^{2+}/Cu^{+} to the peroxide anion (O_2^{2-}) which in beer is protonated to H_2O_2. Hydroxyl radicals (OH·) can then be produced from H_2O_2 through metal induced reactions (Kaneda et al., 1999).

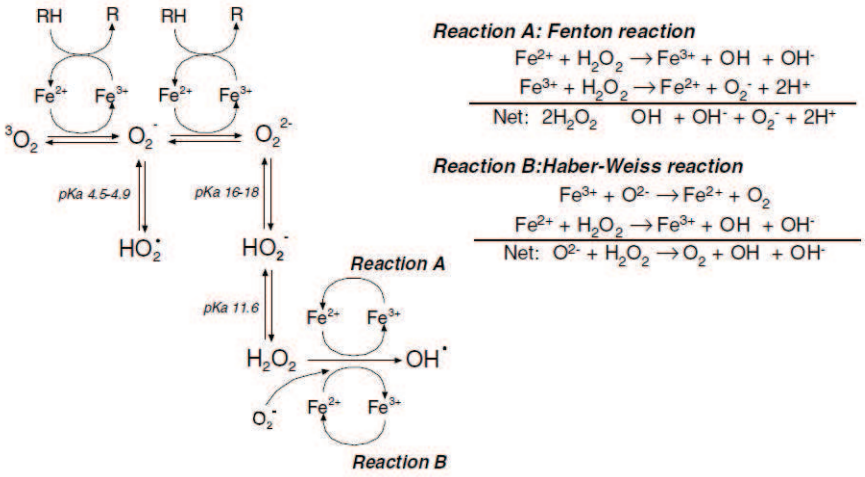


Figure 2: Reactions producing reactive oxygen species (ROS) in beer (Kaneda et al., 1999; Vanderhaegen et al., 2006).

It has been widely discussed whether the Maillard reaction products such as melanoidins, formed during malt roasting, act as prooxidants (Nøddekær and Andersen, 2007; Cortes et al., 2010; Kunz et al., 2011; Kunz et al., 2013b) or antioxidants (Bright et al., 1999; Coghe et al., 2003; Samaras et al., 2005; Vandecan et al., 2011) and this issue has been dealt with during this PhD study. Also enzymatic reactions are taking place during beer staling and lipoxygenase, originating from the barley, has been found to have a large impact on the formation of carbonyl staling compounds (Baxter, 1982; Walker et al., 1996; Sovrano et al., 2006) however, this has not been dealt with in the current thesis.

Sulfite has been identified as the main antioxidant in beer, which has been ascribed to its ability to remove H_2O_2 and its presence is crucial for the beers oxidative stability (Kaneda et al., 1996; Uchida and Ono, 1999; Andersen et al., 2000). Up until now protein research in beer has mainly been focused on the negative influence of beer proteins such as their connection to colloidal stability and generation of protein derived carbonyl staling compounds (Vanderhaegen et al., 2006). Recently, thiols originating from proteins and peptides have received attention due to their hypothesized antioxidative activity in beer and throughout the brewing process (Rogers and Clarke, 2007; Lund and Andersen, 2011; Wu et al., 2012; Chen et al., 2012). Thiols are known to have strong antioxidant capacity in mammalian cells (Murphy, 2012) however, it is a new approach to study thiols in relation to the oxidative stability of beer. Some promising research studies have been carried out indicating that thiols may play a fundamental role as antioxidants in beer in combination with sulfite (Rogers and

Clarke, 2007). Thiols as antioxidants throughout the brewing process and in beer have been further studied during this thesis.

Pasteurization and filtration are standard procedures in many larger brew houses. However, many microbrewers choose not to filter and not to pasteurize their beer either by choice or simply due to lack of equipment. Despite the wide application of pasteurization only few scientific studies have dealt with the influence of pasteurization on the chemical flavor stability of beer (Kaneda et al., 1994; Cao et al., 2011). Therefore the influence of pasteurization has been investigated during the research of this thesis.

There is a vast complexity of reaction and interactions that can occur in the complex systems of wort and beer. Furthermore, the perceived stale flavor is highly dependent of the beer type. Some staling compounds may easily be detected in light colored beer whereas the oxidation products in a darker beer may not be detected at all because of the strong and complex taste of the beer. To add to the complexity some oxidation products may even be preferred by the consumer. The volatile intensities as well as the changes in volatile profile over time are usually rather small in pilsner beer compared to darker beer. However, the changes occurring in pilsner beer are usually more important as its aroma in general is more pure and delicate. Therefore a big challenge in determination of flavor stability of beer is whether a significant change in oxidation results in a significant change in flavor identified by the consumer.

There are great differences in the research needs for microbrewers compared to larger breweries. Today especially microbrewers are challenged in producing an oxidative stable beer due to the use of more basic equipment. The larger breweries are able to control and adjust this process to a greater extent. The focus of this thesis has been on the challenges faced by a small scale brewer. Selection of yeast and fermentation itself may also influence the oxidative stability of beer. However, the study of yeast was not within the scope of this thesis.

1.1 The Brewing Process

The steps in the brewing process, important for the studies in the current PhD thesis, are shown in Figure 3. The figure includes the names of the different processes and intermediate products and the brewing process is briefly described in the following.

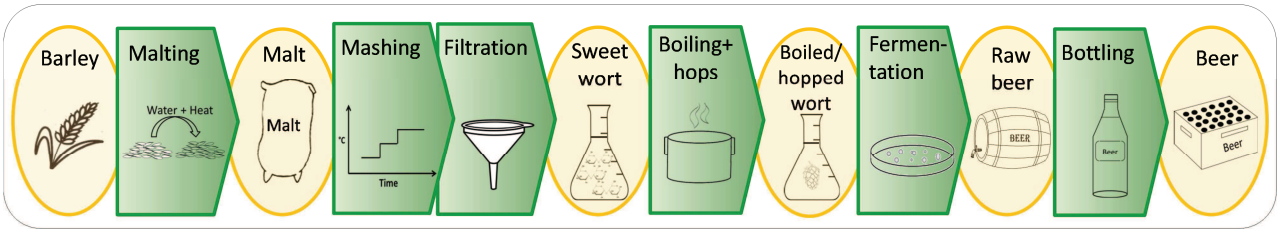


Figure 3: Main steps and intermediate products in the brewing process important for the current PhD thesis.

Malt is produced from barley grains following the three phased process of steeping, germination and kilning. During steeping the grains are soaked in water and germination is initiated. After steeping the germination continues on a germination floor causing breakdown of the cell walls and formation of enzymes which initiate the degradation of starch molecules as well as protein and lipids. The germination is stopped through the kilning process (drying process) which also preserves the enzymes enabling the malt to be stored. Mashing is the first step in the brewing process. At this step the grinded malt is mixed with water and heated in steps to reach the optimal temperatures of the different enzymes. This results in efficient degradation of starch as well as of the small content of proteins and lipids making the nutrients available for the yeast to ferment and further degrade. After mashing the remains of the malt are removed through filtration, leaving the sweet wort. The sweet wort is boiled under the addition of hops. When the boiled and hopped wort is cooled down yeast is added which starts to ferment the sugars and produce alcohol. After fermentation the beer is bottled, filtered or non-filtered, and in some cases pasteurized (Kunze, 2004).

1.2 Overview of the Thesis

This PhD thesis is based on seven papers covering the oxidative stability of malt, wort, and beer including the influence of the brewing process on the oxidative stability of beer. The focus has been on the raw materials, the brewing process, and beer storage (Figure 4).

This thesis includes a discussion of; choice of methods (section 2), influence of raw materials and the brewing process on oxidative stability of wort and beer (Section 3) a detailed discussion of thiol chemistry during beer production and storage (Section 4) and a short discussion concerning beer pasteurization (Section 0). The influence of filtration and pasteurization on the oxidative stability of beer is discussed in detail in Paper IV and V and only a brief summary of the results are presented in the thesis.

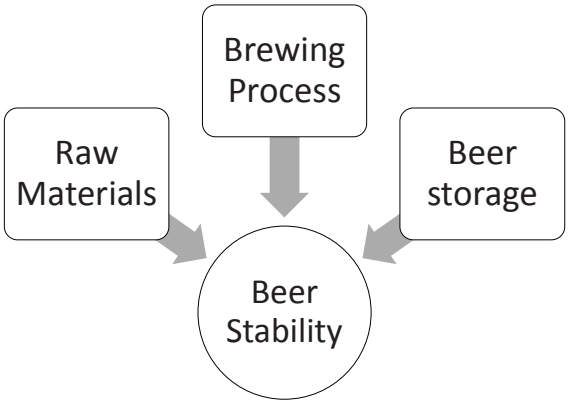


Figure 4: The three factors influencing beer stability during storage mainly investigated during this PhD study.

Figure 5 shows how the seven papers relate to the brewing process. Paper I, Paper II and Paper III are based on research carried out in the initial stages of brewing either directly on the malt or in the wort. The term "wort" refers to sweet, boiled and hopped wort. Paper I describes a study of the quality of pilsner malt and roasted malt during storage. Paper II describes the influence of malt roasting on the sweet wort and Paper III describes the influence of barley varieties on wort quality and volatile profile. Paper IV and V each describe a study concerning the influence of pasteurization and beer filtration on the oxidative stability and volatile profile of the beers. Paper VI and Paper VII describe a method developed for sulfite quantification and thiol quantification, respectively.

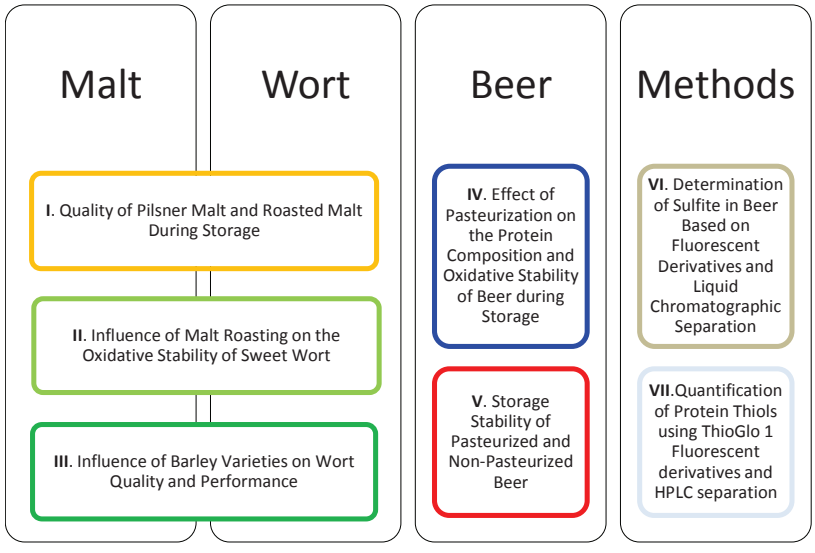


Figure 5: Overview of papers and where in the brewing process the studies were carried out.

Overall Purpose

The overall purpose was to gain more knowledge about oxidation throughout the brewing process and in the final beer.

2 Methods and Experimental Design

A number of methods have been applied in the research related to this thesis. The four main methodologies used to evaluate the quality of malt, wort and beer are a) radical detection by electron spin resonance (ESR) spectroscopy, b) thiol quantification based on a newly developed method; HPLC separation and fluorescence detection c) analysis of volatiles through headspace sampling and GC-MS analysis and d) characterization by European Brewery convention (EBC) methods. Figure 6 shows where in the brewing process these methods have been applied. The sweet wort was to a large extent used to evaluate malt quality and therefore thiol quantification, volatile analysis and characterization through EBC methods were not performed directly on the malt. Generally, it is possible to study the malt quality in the sweet, boiled and hopped wort but not in the final beer as fermentation has a large influence on both volatile profile and oxidative stability. Fermentation changes the matrix completely when turning the wort into beer.

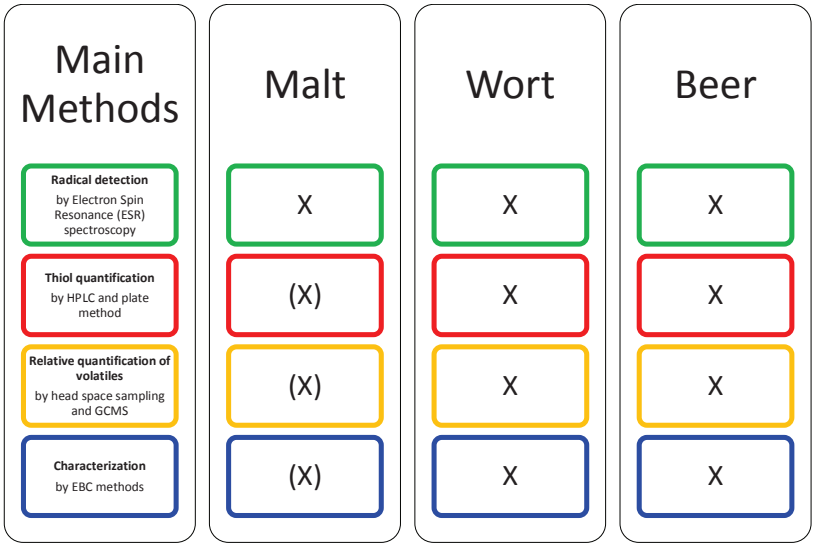


Figure 6: Main methods used for analysis of malt, wort and beer. The thiol content, volatile profile and characterization of malt were investigated in wort which explains the parenthesis.

Issues concerning ESR analysis and thiol quantification will be addressed in the following. Analysis of volatile compounds was carried out according to the procedure developed in our

lab and has been carefully described in the relevant Papers (Paper I-V). Therefore, only a few perspectives concerning volatile analysis are mentioned in the following. EBC methods were applied using the protocols (Analytica-EBC, 2008) and are not described or discussed in the following.

2.1 Electron Spin Resonance (ESR) Spectroscopy

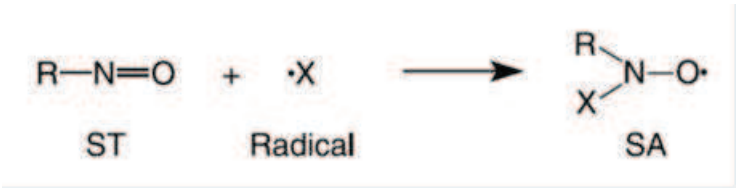
Radicals are intermediates in oxidative reactions in malt, wort and beer potentially leading to flavor deterioration. Electron spin resonance (ESR) spectroscopy enables detection of radicals making ESR a strong tool to study the early stages of these oxidative reactions. How these oxidative processes actually influence the aroma profile is less clear but correlation between radical formation determined by ESR analysis and flavor stability has been found in light colored beer (Uchida et al., 1996; Uchida and Ono, 1996). These results indicate that radical intensity measured through ESR spectroscopy positively correlates to the oxidative stability. In darker beer the correlation between ESR spectroscopy and flavor stability is more complicated as a large number of both oxidative and non-oxidative reactions take place. The evaluation of the oxidative stability of dark beers is furthermore complicated by the great loss of volatile compounds found during storage of dark malt and dark wort (Paper I and Paper II) and naturally this evaporation of volatile compounds cannot be detected by ESR spectroscopy. ESR spectroscopy has the advantage of being non-invasive which makes it possible to perform analysis on the complete system, and thereby show the competition between pro- and antioxidants; being in contradiction to assays providing information about single reactions in a complex reaction mixture.

Basic theory

Very briefly, ESR spectroscopy is based on the fact that radicals are a paramagnetic substance, which have an odd number of electrons and thereby exhibit characteristic magnetic properties when exposed to an externally applied magnetic field. Radicals are detected based on the absorption of microwaves from energy state changes of the unpaired electron. The unpaired electrons are characterized by an intrinsic mechanical angular momentum called spin, meaning that they behave like spinning tops. The electron spins are excited during absorption of microwaves in the magnetic field resulting in the radical signal. Absorption of the electromagnetic radiation is observed when the energy levels of the spin system match the energy of the irradiation. The ESR spectrum is a diagram where the absorption of microwave

frequency radiation is plotted against the magnetic field intensity (Andersen and Skibsted, 2002; Corvaja, 2009).

Most organic radicals in fluid products are very reactive and therefore very short-lived. Their lifetime is therefore too short to be detected by ESR spectroscopy. This challenge can be overcome by use of spin traps (ST) being molecules reacting with highly reactive radicals while forming relatively stable and detectable spin adducts (SA):



The radicals in wort and beer are very short lived and throughout the research carried out during this thesis, spin traps were added to stabilize and accumulate the radicals in spin adducts enabling them to reach a detectible concentration (Janzen, 1971; Andersen and Skibsted, 1998).

Malt, wort and beer analysis

Radicals in dry products such as malt are stabilized by the reduced mobility in the matrix and the radical intensity can be measured directly in the malt kernels (Cortes et al., 2010), as performed in Paper I. Analysis of malt results in a powder spectrum as illustrated in Figure 7, A. A powder spectrum enables detection of the sum of radicals but not identification of specific radicals due to line broadening.

For wort analysis the spin trap α-(4-pyridyl-1-oxide)-N-t-butylnitrone (POBN) was used according to the method described in Frederiksen et al. (2008) and for beer analysis the spin trap N-*tert*-butyl-α-nitrone (PBN) spin trap was used according to Uchida et al. (1996). Figure 7, B shows an ESR spectrum from a forced aging experiment of wort being incubated with POBN (40 mM) and ethanol (5 %) for 90 min at 60 °C. It is necessary to add ethanol during spin trapping in wort to accumulate spin adducts to concentrations that give ESR signals with sufficient intensities. The hydroxyl and alkoxyl radicals present in wort react with ethanol and generate the 1-hydroxyethyl radicals (CH_3·$CHOH$), which are trapped by POBN resulting in a stable spin adduct (·POBN/$CH(CH_3)OH$) (Frederiksen et al., 2008).

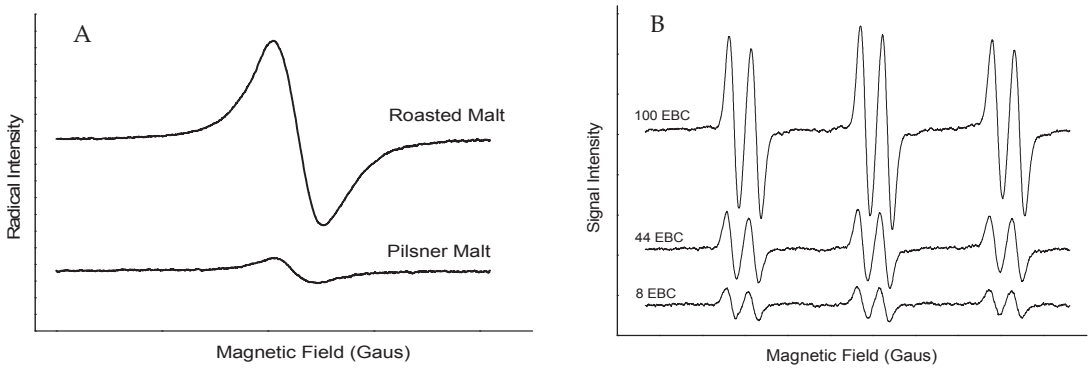


Figure 7: A, ESR spectra of pilsner malt and roasted malt without addition of spin trap (Paper I). B, ESR spectra of wort made from malt of 8, 44 and 100 EBC color with POBN spin trap (40 mM) and ethanol (5%) (Paper II).

Oxidative stability of beers was evaluated by ESR lag phase measurements. The lag phase is defined as the time before radical formation is initiated during aerobic incubation at 60 °C with the spin trap, PBN (Figure 8). The ESR lag phase has been found to be a very good measure for oxidative stability of beer as the lag phase decrease during storage with a strong correlation to the endogenous antioxidative activity in the beer, with sulfite being the most important antioxidant (Uchida and Ono, 1996; Nøddekær and Andersen, 2007). 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.

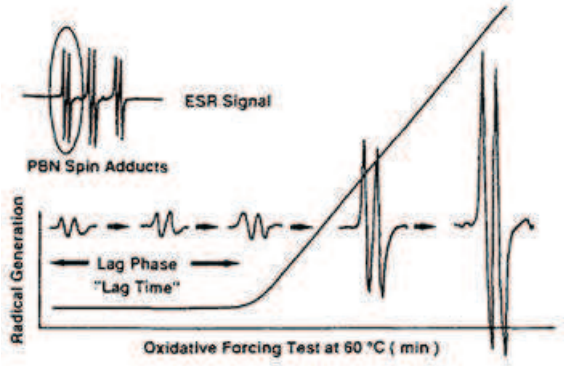


Figure 8: Behavior of the radicals detected in beer during the forced aging test (Uchida and Ono, 1996).

Spin traps

There is no complete control of possible unintended effects the spin trap can have on the system. It was recently found that the PBN spin trap caused an increase in pH during ESR measurements in beer (Methner et al., 2007; Kunz et al., 2008). Figure 9 shows how different

concentrations of PBN and POBN spin traps influence beer pH over time. Increased pH is known to cause increased formation of hydroxyl radicals, influencing the radical content detected during the forced aging test. However, using POBN, a much lower amount of spin trap can be used while still providing a strong radical signal and this is almost without changing pH (Figure 9). As the sensitivity of PBN is much lower than the sensitivity of POBN (Pou et al., 1994) the signal to noise ratio was too large to use small concentrations of PBN for the evaluation of lag phase (Methner et al., 2007; Kunz et al., 2008).

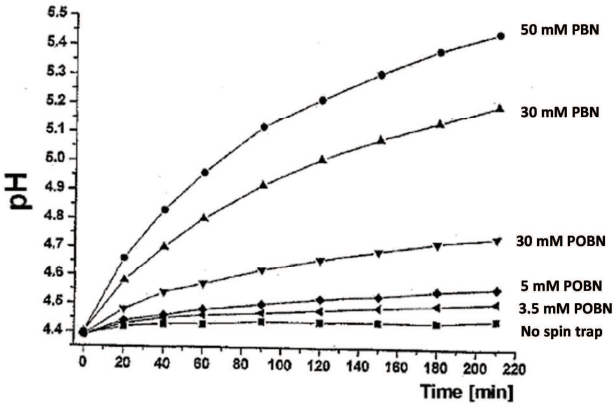


Figure 9: pH change during lag-time determinations using PBN and POBN spin traps (modified from Kunz et al. (2008).

The method applied for beer analysis in the current thesis (Paper IV and V) was based on Uchida et al. (1996) using 30 mM PBN. The influence of PBN on pH is not likely to have influenced the results of these experiments as they were carried out using the same concentrations of PBN and studied over the same period of time. Hence, the comparison made between the samples are not likely to be influenced by a possible pH change. Furthermore, the pH of the least stable beer was lowest, so if the observed effects were only dependent on pH, the pH of the least stable beer should be highest (Paper IV and Paper V) emphasizing that pH is not influencing the obtained results. The spin trap POBN was successfully used for beer analysis (Methner et al., 2007; Kunz et al., 2008) but this spin trap also increased the analysis time significantly (Figure 10). Based on the influence of PBN on pH, POBN should be considered as a substitute for PBN in future beer studies.

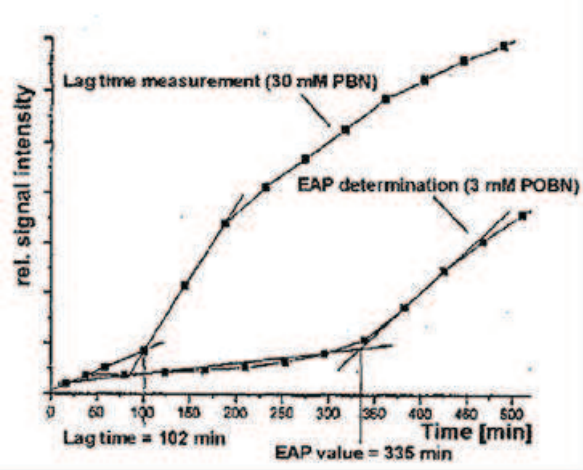


Figure 10: Comparison between PBN and POBN as spin trap for determination of lag time in beer (Kunz et al., 2008).

Lag time and endogenous antioxidative potential (EAP) value illustrated in Figure 10 is in principal the same phenomenon. However, the phrase, lag time, is used in studies with PBN as spin trap and the phrase, EAP value, is used in studies with POBN as spin trap. As illustrated in Figure 8 lag phase and lag time refers to the same phenomenon.

2.2 Thiol Quantification throughout the Brewing Process

During the research of this thesis a number of methods for thiol quantification at different conditions were developed as well as a method for sulfite quantification.

Thiol quantification in beer

The most commonly applied method for thiol determination is based on a spectrophotometric detection of the generated 5-thio-2-benzoic acid at 412 nm after reaction with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). Another derivative for thiol detection is the fluorescent maleimide derived probe ThioGlo 1, but like Ellman's reagent, this reagent is greatly challenged by the presence of sulfite in beer. Figure 11 shows the two fluorescent adducts formed through the reaction between ThioGlo 1 and sulfite (HSO_3^-) or thiol (R-SH). The discovery of interference from sulfite in the detection of thiols, led to the development of the two published methods for sulfite quantification (Paper VI) and thiol quantification (Paper VII) in beer based on fluorescent detection of ThioGlo 1 adducts through HPLC separation.

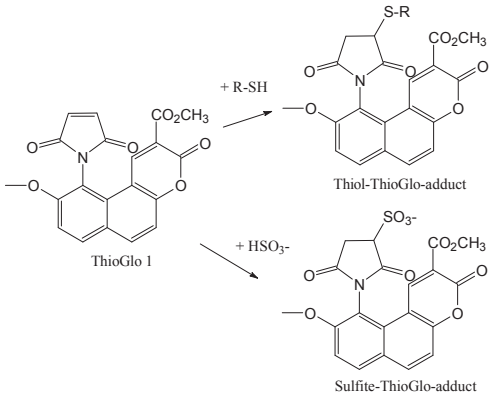


Figure 11: Reaction between ThioGlo 1, thiol (R-SH) and sulfite (HSO3-) and formation of the fluorescent adducts (λex 242 and λem 492).

Lund and Andersen (2011) presented the first ThioGlo 1 based method, where the interference from sulfite was overcome by external sulfite determination and preparation of standard addition curves for both sulfite and thiol. This enabled the contribution from the sulfite-ThioGlo 1 adducts to be subtracted from the total contribution. The drawbacks of this method are that external sulfite determination is necessary. More importantly, the sulfite content often is several folds higher than the thiol content which makes the fluorescent signal from sulfite much larger than the signal from the thiols and thereby the thiol determinations less accurate. The Danish all malt bock type lager beer (7.2 %) had for example a sulfite content which was 7.5 times higher than the thiol content (28.2 μM thiol vs. 210.6 μM sulfite) (Paper VII).

The methods developed in Paper VI and Paper VII are based on the reaction of sulfite and thiols with ThioGlo 1 where HPLC separation enables separate determination of sulfite and thiol in the same system. Figure 12 shows the baseline separation of two sulfite derivatives and elution of thiol-containing peptide- and protein-derivatives within one single peak enabling quantification of the total amount of thiols as well as sulfite.

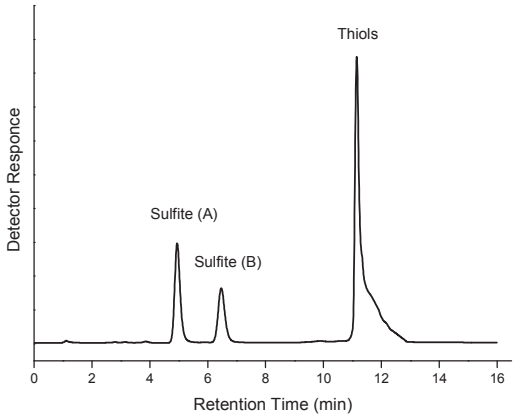


Figure 12: HPLC separation of sulfite and thiols in beer and the chemical reaction between ThioGlo 1, thiol (R-SH) and sulfite (HSO_3^-) (Paper VI).

The standard addition procedure was applied In the method presented by Lund and Andersen (2011) as well as in the methods presented in Paper VI and Paper VII. This procedure results in improved precision compared to an external standard curve and overcomes the inner filter effect and thereby enable thiol and sulfite quantification in dark beers. Standard addition procedure means adding standard (in this case thiol or sulfite) directly to aliquots of the sample (wort or beer). By this an internal standard curve is created. The curve is vertically parallel displaced on the Y-axis depending on the thiol content. The curve is extrapolated to intercept the X-axis and based on a mathematical calculation and the thiol or sulfite concentrations can be read on the curves intercept with the x-axis (Figure 13)(see Paper VII for details).

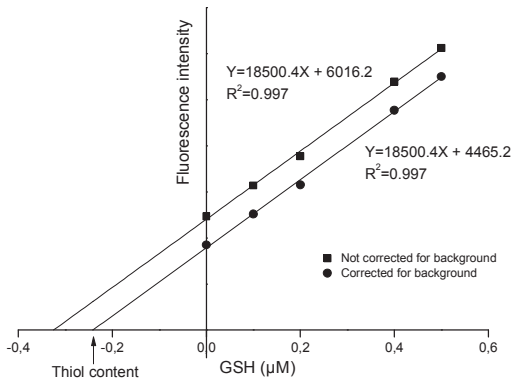


Figure 13: Standard addition curve of beer A spiked with 4 different concentrations of GSH, showing the standard addition curve with and without correction for background from ThioGlo 1 (Paper VII).

The method described in Paper VII is more than 100 times more sensitive than the commonly used DTNB based method for thiol quantification, and our results also show that in samples

with sulfite interference, the DTNB based method is inapplicable due to lack of sensitivity. Application of the standard addition procedure makes it possible to determine and compare the thiol content in both light and dark samples, which is highly relevant when working with different beer types.

In Paper VII it is described that decreased fluorescence intensity is detected from free cysteine-ThioGlo 1 adducts compared to other thiols. This problem results in underestimation of the thiol concentration in any biological sample containing reduced free cysteine, when using ThioGlo 1 as derivatizing agent, and this has not been reported previously. ^{1}H nuclear magnetic resonance (NMR) studies showed that the reaction pathway between cysteine and ThioGlo 1 is different from that of glutathione (GSH) and ThioGlo 1, explaining the different fluorescence intensities. Matsui et al. (1984) found the amount of free cysteine in beer to be on average 47.7 μM and approximately 4.5 μM was on the reduced form suggesting that decreased fluorescence from the cysteine-ThioGlo 1 adduct may be a small problem in beer. Recently free cysteine was successfully separated from the thiol bulk peak on HPLC (Almeida et al. 2012 data not published). This separation makes it possible to compensate for the decreased fluorescence intensity observed for the cysteine-ThioGlo 1 adduct. However, using this method no cysteine was detected in the analyzed beers. This can be caused by the fact that a) there is no free cysteine in the beers or b) the method is not sensitive enough to detect the free cysteine. The detection limit for thiols was determined to 0.028 μM. However, as the fluorescence response from the cysteine-ThioGlo 1 adduct is only approximately half of the response from other thiols, the detection limit increase but remain below 0.1 μM. This suggests that the decreased fluorescence intensity observed from the cysteine-ThioGlo 1 adduct does not result in an underestimation of thiols in beer.

Most methods for thiol quantification are designed to measure the content of thiols relative to the amount of protein (Hawkins et al., 2009). The method described in Paper VII is designed to quantify the total thiol content in the beer from both proteins and peptides.

Thiol quantification in wort

During the development of methods for thiol quantification in wort, several issues were assessed; i), interference from "thiol-removing capacity", ii) effect of boiling, iii) inner-filter effect, and iv) absent sulfite interactions.

Ad i) Sweet wort was found to contain a "thiol-removing capacity" able to oxidize small thiols present in the sweet wort (Paper II and Paper III) (details on the "thiol-removing capacity" see 4.2.1). The presence of the thiol-removing capacity caused a great challenge for the thiol quantification in the sweet wort as the standard addition procedure could not be applied due to oxidation of the added thiol standard.

Ad ii) The "thiol-removing capacity" seems to be inactivated through extensive boiling. Thiol levels of boiled and hopped wort therefore can be determined using the standard addition procedure and colored worts can be included.

Ad iii) For thiol quantification of light sweet worts, being similar in color, an external standard curve can be prepared in water. Dark worts however, may quench the out coming fluorescence through an inner filter effect and as the standard addition procedure is inapplicable it so far remains impossible to analyze the thiol content of dark sweet worts.

Ad iv) No sulfite is present in wort and HPLC separation for quantification of thiols is therefore not necessary. Thiol quantification of wort can therefore successfully be carried out in microtiter plates following the principals described in Lund and Andersen (2011). Carrying out the analysis in microtiter plates is both much faster and cheaper than HPLC analysis. The inner filter effect can be overcome using both the microtiter plate method and the HPLC method.

Table 1 sums up the methods suited for thiol quantifications in wort and beer.

Table 1: Methods suited for thiol quantification in wort and beer.

Method	Light Sweet Wort	Dark Sweet Wort	Boiled/Hopped Wort	Light Beer	Dark Beer
Plate, external standard curve	X		X		
Plate, standard addition method			X	X[a]	X[a]
HPLC, external standard curve	X[b]		X[b]	X	
HPLC, standard addition method			X[b]	X	X

[a] if no sulfite is present [b]plate method is preferred

As a help in the process of deciding on a method for thiol quantification a decision tree is presented in Figure 14.

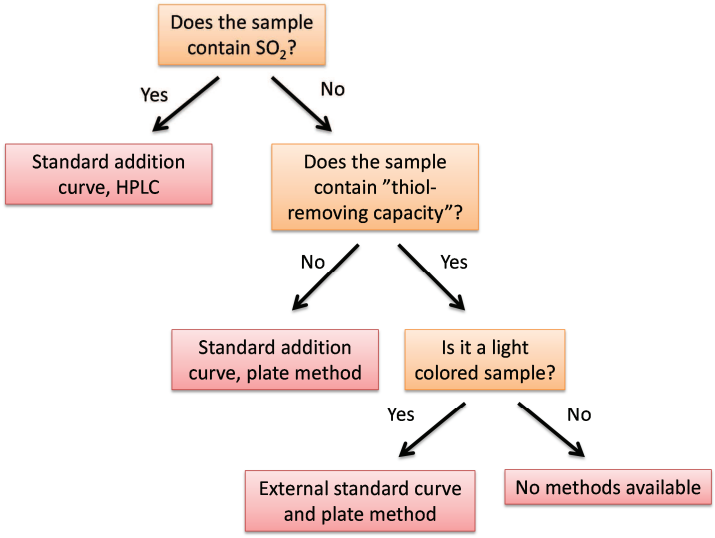


Figure 14: Decision tree for choosing a method for thiol quantification.

2.3 Analysis of Volatiles

In Paper I-V dynamic head space sampling and GC-MS analysis were used to evaluate changes in the volatile profiles of wort and beer samples, but also to complement the other methods in evaluating the oxidative stability through identification of oxidation products. Analysis of volatiles made it possible to study volatile formation and degradation under different conditions as well as comparing the volatile profiles between samples. In this PhD thesis a volatile compound defines a compound extracted from headspace of wort or beer. An aroma compound is defined as a compound that is likely to be sensed when the wort or beer is consumed. The different volatiles have different threshold values and it is important to note that the relative quantification of volatiles, performed in the current PhD thesis, does not provide information about the actual aroma profile. Whether a volatile is an aroma compound or not can only be determined through quantification and comparison to its threshold value, which has not been done in the research work of this thesis.

Principal Component Analysis

The large amount of data generated through volatile analysis was handled using Principal Component Analysis (PCA). PCA was formulated by Hotelling (1933) and is a multivariate projection method designed to extract and display the systematic variation in a data matrix X. It is a common method within multivariate data analysis and can be used together with a well selected set of objects and variables to build a model of how a physical or chemical system behaves, and this model can be used for prediction when new data are measured for the

same system. PCA makes it possible to decompose the large amount of data in order to detect and model the possible hidden phenomena and describe trends. PCA has throughout this PhD thesis been an essential tool for evaluation of the volatile profile in wort and beer.

PCA decomposes the data into scores (T) and loadings (P) according to the equation:

$$X = TP + E$$

T is the score matrix, P is the transposed loadings matrix and E is the error matrix (residuals). The scores are related to the samples (wort or beer) and the loadings to the variables (volatile compounds). The plane is described by two orthogonal lines (Principal Components) and the deviation between the projections and the original coordinates are termed, the residuals. The first Principal Component (PC) is fitted in order to explain most variance using the least square process. The second PC is placed orthogonal on the first PC to explain second most variance. Each new PC accounts for as much of the remaining variability as possible (Wold et al., 1987; Eriksson et al., 2003; Næs et al., 2004).

Partial Least Squares (PLS) Regression

PLS is a regression extension of PCA. It uses factors determined by employing both X and y directly in the estimation. Each component is obtained by maximizing the covariance between y and all possible linear functions of X. The idea is to find a couple of linear combinations of the original X-values and to use only these linear combinations in the regression equation. In this way irrelevant and unstable information is discarded and only the most relevant part of the x-variation is used for regression. The model structure is given by the two following equations, where the first equation is data compression in x-space and the other is regression based on the compressed components:

$$X = TP + E$$

$$Y = Tq + f$$

The matrix P and the vector q are called loadings and they describe how the variables in T relate to the original data matrices X and y. The matrix E and the vector f are residuals and represent the noise or irrelevant variability in X and y, respectively (Eriksson et al., 2003; Næs et al., 2004). From the validated PLS model the parameters Coefficient of Determination (R^2) and Root Mean Square Error (RMSE) can be extracted as performed in Paper II.

3 Malt as Raw Material

Much focus has been drawn to the starch degradation and sugar extract of the malt whereas less attention has been drawn to malt quality and its influence on the oxidative stability of wort and beer. This section summarizes and compares the main results concerning barley and malt as raw materials in brewing obtained from Paper I, II and III.

3.1 Background

It has been widely accepted that the barley variety has a large influence on the malting process and thereby malt quality (Bravi et al., 2012). However, to which extent the barley variety influences beer and wort quality is less well understood. Some studies have been carried out on the subject and barley varieties were for example found to vary in protein composition, which was also identified in the corresponding malt (Østergaard et al., 2002). Similarly Silva et al. (2008) found that the different protein profiles of the malting barley varieties *Scarlett* and *Prestige* were reflected in both wort and beer showing that the variations in barley variety is not eliminated during the brewing process.

The influence of malt quality on wort and beer quality has been studied and Suarez et al. (2011) found that the radical content of the malt was closely connected to the sulfite content of the final beer and thereby that the oxidative stability of malt was directly correlated to the oxidative stability of beer. Also the oxidative stability of wort was found to be directly correlated to the oxidative stability of beer (Cortes et al., 2010). A good correlation was also found between malt, wort and beer flavor based on sensory analysis (Voigt et al., 2013). Research within the quality of raw materials remains very important as their influence on the final product is not yet fully understood.

Malt types

Malt can be divided into regular brewer's malt and specialty malt. Regular brewer's malt covers what is usually called pilsner malt, lager malt or normal malt. Specialty malts cover a large spectrum of different malt types, which differ from regular brewer's malt by varying in

raw material (wheat, rye, oats, rice or other) or varying in malting parameters applied during steeping, germination or kilning (Coghe et al., 2003). Furthermore, regular brewer's malt possesses significant enzymatic activity in contrast to roasted malt, which is characterized by a lack of enzymatic activity. According to Coghe et al. (2003) dark specialty malt can be classified into four groups being color malt, roasted malt, caramel malt and roasted barley. A flowchart of their production is shown in Figure 15.

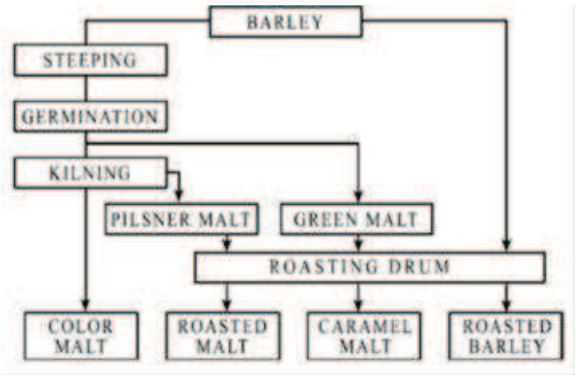


Figure 15: Flowchart for the production of different specialty malt groups (Coghe et al., 2003).

Regular brewer's malt is mainly produced with the purpose of providing a good extract yield. The specialty malt on the other hand is produced with the purpose of providing specific aroma character. Therefore, specialty malt is only added in small concentrations as it can be very intense in both color and flavor and often with a lower content of degraded starch. The regular brewer's malt is usually the main content of the brew.

3.2 Maillard Reaction and Oxidative Stability

Maillard reactions are taking place throughout the brewing process and Maillard reaction compounds are important in relation to both flavor formation and oxidative stability of the beer.

Maillard reactions are initiated by the condensation of an amino group and a reducing sugar followed by a large number of complex reactions. The Maillard reactions are heat induced and non-enzymatic and result in formation of volatiles and colored compounds (Maillard, 1912). Reductones are intermediates in the Maillard reactions and of interest in relation to the oxidative stability of beer due to their reactive enediol structure (Figure 16).

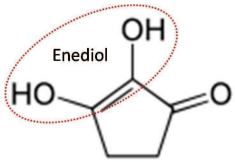


Figure 16: Reductone.

Reductones can polymerize to the brown colored melanoidins while preserving their enediol structure (Wang et al., 2011). The Maillard reaction products are mainly introduced to the beer through kilned and roasted malt though some Maillard reaction products may be formed during wort boiling (Paper III) or in some cases beer pasteurization (Paper IV and V). Whether the reductones act as prooxidants (Nøddekær and Andersen, 2007; Cortes et al., 2010; Kunz et al., 2011) or antioxidants (Bright et al., 1999; Coghe et al., 2003; Samaras et al., 2005; Vandecan et al., 2011) in beer have been widely discussed. In Paper II the radical intensity was found to increase with increasing wort color. Furthermore, a correlation between Fe content, radical intensity and color was found. This suggested that the reductones act as prooxidants in beer by driving the Fenton reaction (H.J.H. Fenton, 1894). The findings from Paper II was confirmed in the recent and more detailed study by Kunz et al. (2013b) where the reductones were suggested to be the main cause of rapid reduction of metal ions causing acceleration of oxidative reactions and increased consumption of antioxidants such as sulfite. Figure 17 describes the mechanisms of the suggested prooxidative effect of reductones in beer (Kunz et al., 2013b).

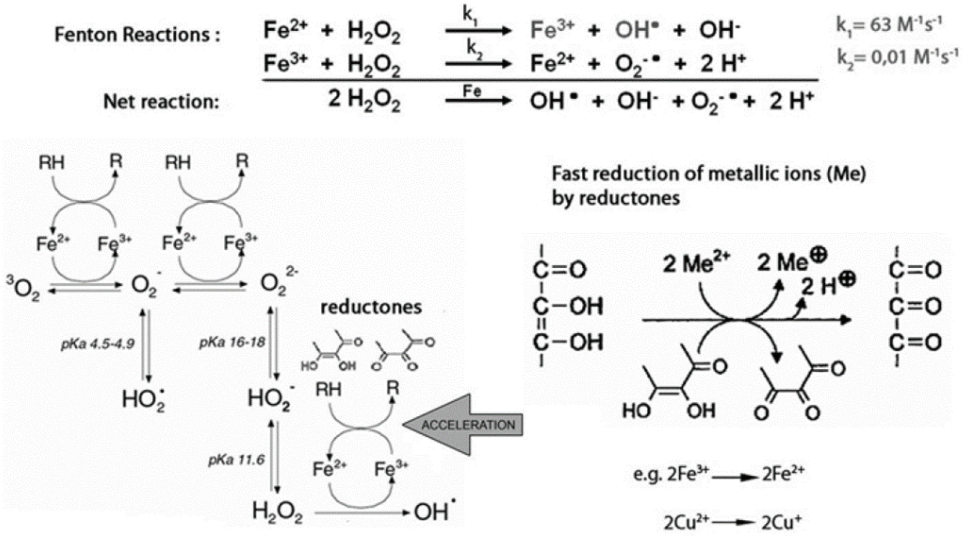


Figure 17: Proposed mechanism for the accelerated oxygen activation and generation of reactive hydroxylradicals by Fenton reaction system based on the strong reducing potential of reductones toward oxidized metal ions (Kunz et al., 2013b).

Reductones may mistakenly be categorized as antioxidants in beer when the methods for detection of antioxidative potential are based on radical scavenging activity using a semistable radical with high reactivity towards many types of compounds. Therefore, studies based on radical scavenging activity assays often result in antioxidative activity, whereas prooxidative activities are neglected. The contradictious results concerning the pro- or antioxidative effect of melanoidins may simply be caused by the difference in methods applied. Application of ESR spectroscopy makes it possible to evaluate the complete wort and beer system and study the competition between pro- and antioxidants making ESR spectroscopy more suitable for evaluation of pro- and antioxidant activity.

Melanoidins have been suggested to form stable radicals during malt roasting (Baltes, 1982; Cortes et al., 2010; Jehle et al., 2011). Interestingly, a very stable melanoidin derived radical, originating from roasted malt, and with a molecular weight of approximately 10^6-10^8 g/mol was recently identified in dark wort and beer (Jehle et al., 2011). How, or if, this stable radical influences the oxidative stability of wort and beer remain uncertain.

The prooxidative effect of melanoidins was confirmed during storage of roasted malt, where higher radical content correlated with increased lipid oxidation compared to pilsner malt (Paper I). As a way to handle the prooxidative activity of the colored Maillard reaction compounds, Suarez et al. (2011) found that larger beer being color adjusted with melanoidin malt (being higher in its content of melanoidin) induces the smallest increase in flavor-active aging compounds during storage compared to the colored malt Carafa Special type III and Carahell. In a recent study it was furthermore found that both fermentable and non-fermentable sugars can have reducing potentials at pH values typical in beer showing that carbohydrates, in similar ways as melanoidins, may have prooxidative activity in beer through reduction of metal ions (Kunz et al., 2013a).

The Maillard reaction has a large influence on the volatile profile as more than 3,500 volatile compounds have been identified from this reaction and many of them have a low odor threshold value (van Boekel, 2006; Reineccus, 2006). Figure 18 shows an outline of the pathways leading to the various groups of flavor compounds.

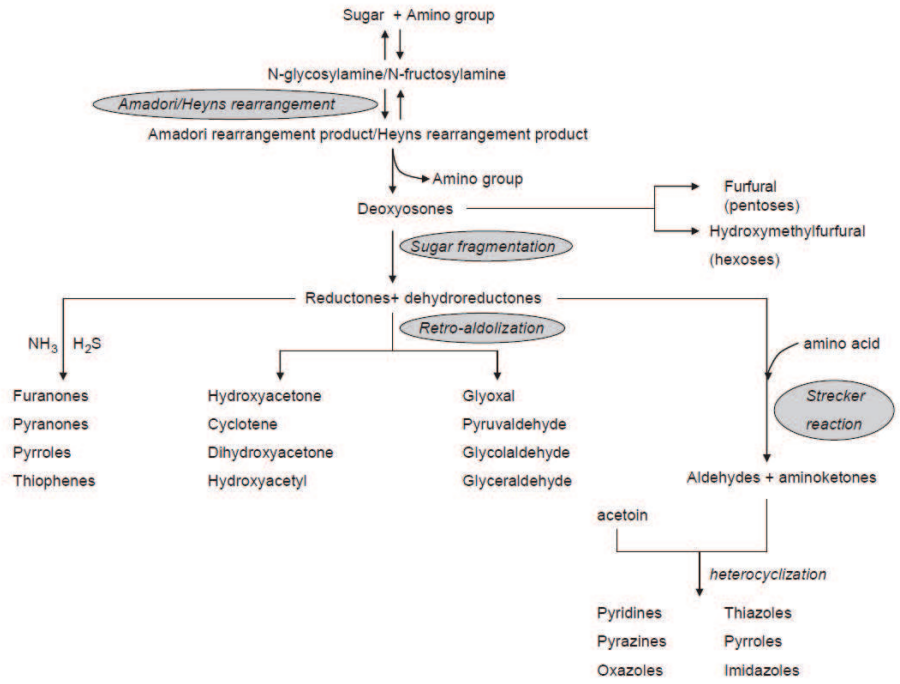


Figure 18: Volatiles produced during the Maillard reaction (van Boekel, 2006).

The impact of the Maillard reactions on the flavor profile can be difficult to interpret. In dark beer many of the volatiles released through Maillard reaction are key compounds essential for the beer aroma such as furfural, furans, pyrazines and Strecker aldehydes such as 2- and 3-methylbutanal. However, in light colored beer most of these compounds are most often perceived as staling compounds.

3.3 Malt Roasting and Storage Stability

It is generally accepted that malt has a storage stability of one year independently of malt type. However, many brewers observe changes in the smell of the malt during malt storage. There seem to be a lack of scientific knowledge on the changes occurring during malt storage. The research described in Paper I was designed to help clarify the changes in oxidative stability and volatile profile during one year of storage.

Figure 19 shows a PLS score plot based on the volatile profile of sweet wort made from fresh pilsner malt and roasted malt as well as after storage for six and twelve months (Paper I). Naturally, there is a big difference between the volatile profile of the wort made from pilsner malt and the wort made from roasted malt illustrated by the fact that the roasted malts are located in the top and pilsner malt in the bottom of the score plot. The volatile profile of roasted malt changed much more during one year of storage than the volatile profile of pils-

ner malt illustrated by the larger differentiation between the volatile profiles during storage. These changes were caused by a large loss of volatiles as well as by formation of compounds through oxidative reactions (data not shown).

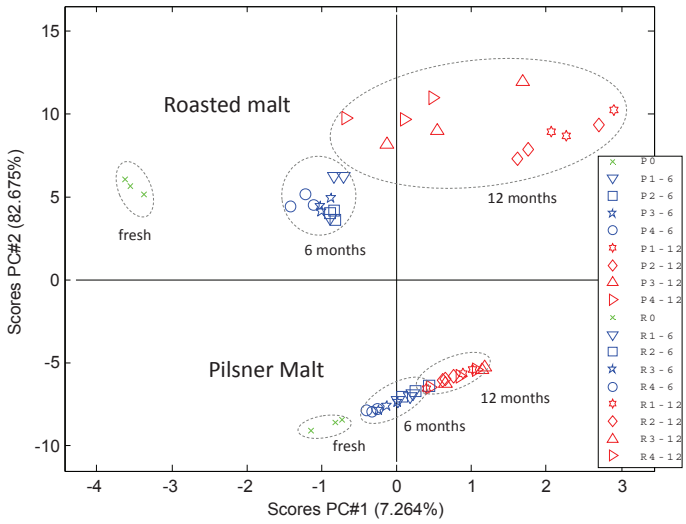


Figure 19: PLS score plot based on the volatile profile of sweet wort made from pilsner malt and roasted malt stored for 0, 6 and 12 months at 10 °C or 20 °C at water activity (a_w) of 0.231 or 0.432 (n=3). P=pilsner malt and R=roasted malt. (P1/R1=0.231, 20 °C; P2/R2 =0.432, 20°C; P3/R3=0.231, 10°C; P4/R4 =0.432, 10°C). (Paper I).

Many compounds were lost due to evaporation during malt storage (Paper I). Interestingly, this was closely correlated to the fact that sweet wort made from malt roasted to the color of 44 EBC and above showed a large loss of volatiles during mild heat treatment, with some compounds loosing more than 50 % (Paper II). Worts made from less colored malts did not loose any volatiles during heat treatment. These results (Paper I and Paper II) indicate that the heat induced changes in the malt matrix, caused by malt roasting, have a large impact on the ability of both malt and wort to retain the volatiles. Evaporation takes place in malt and sweet wort and it therefore seems likely that evaporation also takes place during wort boiling, fermentation and beer storage, and attempts should be made to minimize the loss.

Roasted malt, and the corresponding sweet wort, had a significantly decreased oxidative stability compared to pilsner malt and the corresponding sweet wort (Paper I and Paper II). For both roasted malt and pilsner malt, a good correlation was found between radical decay and changes in volatile profile where storage at high temperature (20 °C) and high water activity (0.432) resulted in the most unstable malt compared to malt stored at 10 °C with a water activity of 0.231 (Paper I). The results showed that the change in volatile profile correlated with the fact that roasted malt had a large radical content, larger radical decay during

storage, larger change in volatile profile and increased lipid oxidation. The extract yield remained constant throughout one year of storage showing that the physical malt parameters are intact. The malt storage experiment (Paper I) shows that malt quality is significant decreased in terms of a volatile loss and formation of oxidation products. Based on this, the claimed shelf life should be much shorter than one year. However, the shelf life has no problem matching the quality of the extract yield.

3.4 Barley, Malt and Beer Quality

Barley selected for use in the brewing industry must meet special quality requirements to be approved for malting and beer production. These requirements are based on the varieties genetic background, physical conditions during growth, harvest and storage and in the end an optimal extract yield (Analytica-EBC, 2008; Silva et al., 2008). However, the idea of using barley to influence beer flavor has in some ways been overlooked. A recent study has shown that different wheat varieties were significantly different from each other in both odor and flavor profiles based on sensory analysis (Starr et al., 2013). This leads to the assumption that a critical selection of barley based on its flavor profile may influence the beer flavor. With this perspective in mind the possible influence of three barley varieties on the volatile profile of wort was investigated in Paper III.

Influence of Barley Variety on Beer Flavor

The organic barley varieties Pallas, Fero and Archer were grown on the same field and malted simultaneously. Figure 20, A, shows a PCA score plot based on the volatile profile of sweet, boiled and hopped wort from the three barley varieties Pallas, Fero and Archer. In sweet wort the volatile profile of Archer differentiated from Pallas and Fero whereas in boiled wort the volatile profile of Fero differentiated from Pallas and Archer. The addition of hops to some extent masked the barley varieties influence on the volatile profile when the results from both sweet and boiled worts were included in the model. However, the volatile profile of Fero remains different from Pallas and Archer when sweet and boiled wort are excluded from the PCA analysis (Figure 20, B). These results support that a barley variety can influence the volatile profile of wort and thereby possibly also the beer flavor though this was not investigated. The impact of five different barley varieties on the final beer was investigated by Guido et al. (2007) who showed that barley varieties had an influence on flavor of aged beers but interestingly, there was no difference between the flavor profiles of the fresh

beers. It was further suggested that variations in weather and soil conditions had a larger influence on malting quality than on beer flavor stability found by comparing Spanish and French grown *Scarlet* barley (Guido et al. 2007). This suggests that the environmental factors have larger influence on the technological malting quality whereas the beer flavor stability is more influenced by barley variety.

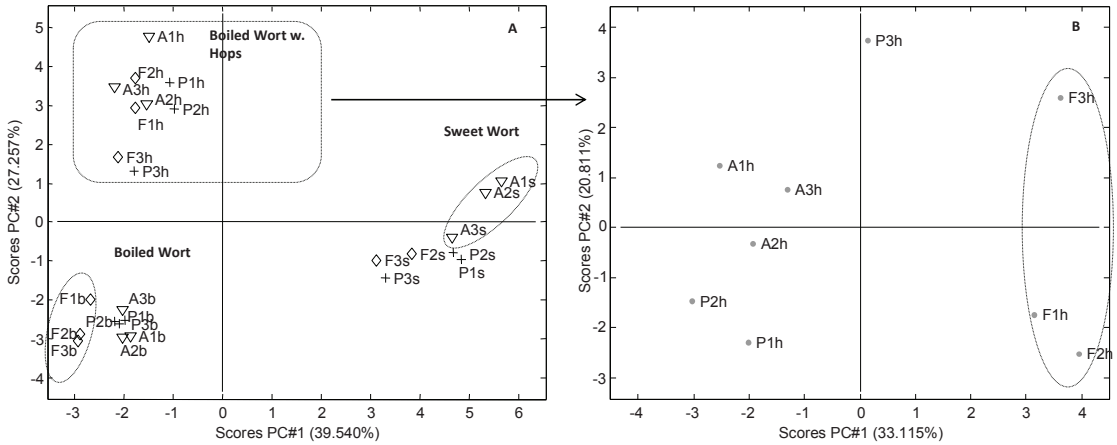


Figure 20: PCA score plot based on the volatile compounds found in sweet, boiled and hopped Pallas, Fero and Archer wort. Only the volatiles also detected in sweet and boiled wort has been included for the hopped wort. The oval circles enclose the wort samples that differentiate from the others. (A) Sweet Archer wort (A1s, A2s, A3s), boiled Archer wort (A1b, A2b, A3b) hopped Archer wort (A1h, A2h, A3h). Sweet Fero (F2s, F3s), boiled Fero (F1b, F2b, F3b) hopped Fero (F1h, F2h, F3h). Sweet Pallas wort (P1s, P2s, P3s), boiled Pallas wort (P1b, P2b, P3b) hopped Pallas wort (P1h, P2h, P3h). (B) PCA score plot of hopped Pallas, Fero and Archer wort (Paper III).

Few research studies have been carried out on the barley varieties influence on wort and beer flavor however, barley varieties have to a great extent been investigated for levels of lipoxygenase 1 which was found to contribute to the formation of trans-2-nonenal in beer (Kaukovirta-Norja et al., 1998; Kuroda et al., 2003; Guido et al., 2005). Carlsberg laboratories have had great success with removal of this enzyme from malting barley through selective breeding resulting in a more oxidative stable beer with very low contents of trans-2-nonenal (Hirota et al., 2006; Breddam et al., 2008; Douma et al., 2011).

Challenges in Investigation of Barley Variety

The malting process has a large impact on malt quality. Therefore, a big challenge in performing experiments including different barley varieties is carrying out the malting process in a way that makes the barley varieties comparable. The malting process can either be optimized according to the specific barley variety or carried out by following a standardized method. Either with or without optimization, according to the specific variety, the malting

process may introduce some variations. Especially the kilning process has a large influence on malt quality. Barley variety and the choice of malting process can both have a significant influence on the malt quality which is important to consider when designing experiments including a larger number of barley varieties.

Wort processing

Just as well as the malting process has a large influence on the malt quality the wort processing (mashing, boiling, hopping) also has a large influence on wort quality. In Paper III and Paper V it was found that wort boiling increased the radical content significantly compared to sweet wort. Interestingly, the addition of hops was able to compensate for the increased radical content formed during boiling (Paper III) caused by the antioxidative activity of hops (Wietstock et al., 2010). In Paper V it was furthermore found that boiling with hops remove a large part of the Fe and Cu content being positive for the oxidative stability. Therefore both malt and wort processing has a large influence on volatile profile and oxidative stability.

3.5 Summary

The research work of this PhD thesis shows that roasted malt and the corresponding dark colored wort is much more unstable than light colored malt and wort. Roasted malt had a larger change in the volatile profile during storage correlating with a larger initial radical content, larger radical decay, larger change in volatile profile and increased lipid oxidation compared to pilsner malt. The heat induced changes of the malt matrix through roasting caused both malt and the corresponding wort to loose many volatiles through evaporation. The Maillard reaction products melanoidins were suggested to work as prooxidants in combination with Fe through acceleration of the Fenton reaction. Based on the current research the storage time of malt and wort should be kept at a minimum to prevent initiation of oxidative reactions and to limit the loss of volatiles.

Both raw materials and the brewing process influence the quality of the beer. It is difficult to separate the impact from the two parameters as they many times interact. Boiling increase radical formation, whereas addition of hops limits the oxidative reactions through its content of antioxidative compounds and ability to bind Fe and Cu. The current research suggests that the barley variety can influence the volatile profile of wort and thereby possibly also the beer flavor.

4 Thiols in Beer and throughout the Brewing Process

Protein research in beer has mainly been focused on colloidal stability and generation of protein derived carbonyl compounds correlated with staling (Siebert et al., 1996; Miedl et al., 2005; Vanderhaegen et al., 2006). A large part of the research carried out during this thesis has been focused on the possible antioxidative effect of protein thiols and how preservation of these thiols throughout the brewing process can be used to produce a beer of naturally high quality for a long time after filling. The following section reviews the knowledge generated throughout this PhD project in relation to the current literature.

4.1 Background

Glutathione and other thiol containing molecules have been found to play significant antioxidative roles in living organisms (Jacob et al., 2003; Gungor et al., 2011; Wu et al., 2012). The thiol group of cysteine is easily oxidized and the rate of thiol oxidation in some systems reaches diffusion controlled rates (Pattison and Davies, 2001). The thiolate anion (RS^-) is one of the strongest biological nucleophiles, and consequently, the thiol group of cysteine is one of the most reactive functional groups in proteins (Jacob et al., 2003). Based on the knowledge about the strong antioxidative capacity of thiols in living organisms, there is reason to think that thiols can have a similar positive effect on the oxidative stability of beer as well as other foods. The antioxidative activity of thiols in beer was first described by Rogers and Clarke (2007) who found that beer with high protein content tend to be more oxidative stable and suggested that the protein thiols were responsible. This hypothesis was recently supported by three studies indicating that thiols act as antioxidants in beer (Lund and Andersen, 2011; Wu et al., 2011; Chen et al., 2012). The 1-hydroxyethyl radical is most likely the most abundant radical in beer (Section 2.1) and in a recent study the reaction between thiol-containing compounds and the 1-hydroxyethyl radical was found to be close to the diffusion limit in water which underlines the importance of thiol-containing peptides and proteins on the redox stability of beer (de Almeida et al., 2013). Thiols are mainly introduced to the beer through the barley in the form of lipid transfer protein 1 (LTP1) consisting of 91

amino acids and with a mass of ca. 10 kDa. In the mature barley grain LTP1 is present in its native curled structure with 4 intramolecular disulfide bridges and has no antioxidative capacity. However, the protein is denatured during malting and wort boiling, where the disulfide bonds become disrupted resulting in 8 free thiol groups (Perrocheau et al., 2006; Jin et al., 2009; Wu et al., 2011). LTP1 was also found to have foam stabilizing properties, which become active as the molecule gradually unfold and glycosylate through Maillard reactions during wort boiling (Jegou et al., 2001; Perrocheau et al., 2006).

Sulfite is usually referred to as the main antioxidant in beer (Kaneda et al., 1996; Uchida and Ono, 1999; Andersen et al., 2000) and sulfites (HSO_3^-) reaction with hydrogen peroxide is shown in the following equation.

$$HSO_3^- + H_2O_2 \rightarrow HSO_4^- + H_2O$$

The antioxidative reaction mechanism including protein thiols in beer suggested by Rogers and Clarke (2007) is illustrated in Figure 21. This is a simplified scheme of thiol oxidation reactions as several other reactions may take place.

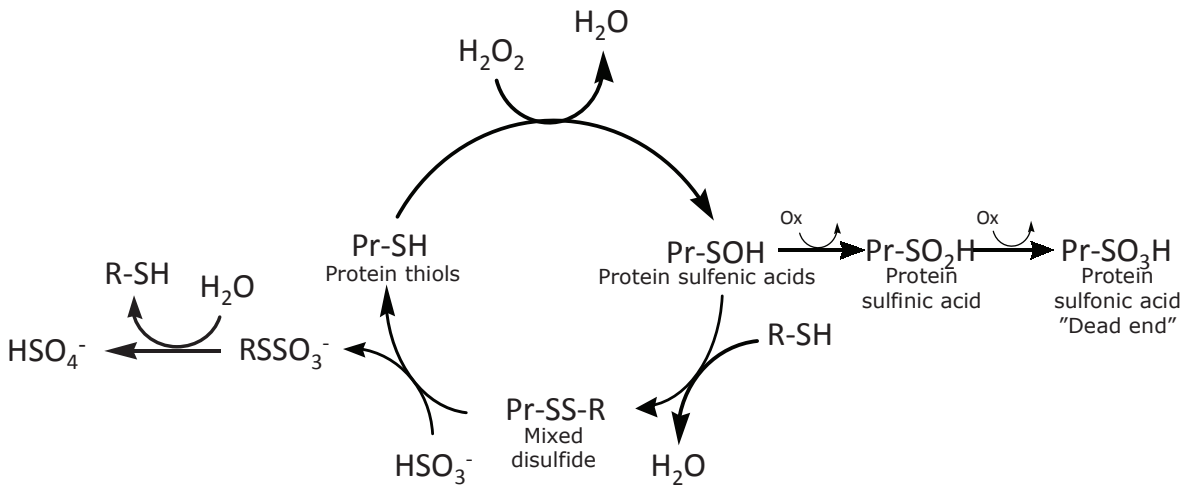


Figure 21: Proposed antioxidant mechanism of protein thiol groups in beer.

Initially, the protein thiol group reacts with the hydrogen peroxide and form protein sulfenic acid. The hypothesis suggests; that the original thiol can be recovered by two sequential reactions when sulfite is present. This is taking place, as the sulfenic acid is highly reactive and will generate a disulfide through reaction with another thiol. In the following sequence sul-

fite is used to regenerate the thiol from the disulfide. A bunte salt ($RSSO_3^-$) is formed and then broken down into the original thiol compound and sulphuric acid by reaction with water.

In this illustration (Figure 21) sulfite is suggested to act as an electron donor however, it is still uncertain whether the reducing potential of sulfite is large enough to reduce disulfide bonds, in the concentrations relevant in beer. It is likely that other antioxidative compounds also would be able to reduce the disulfide bond and complete the reaction chain. An example of such a compound is the small thiol containing protein thioredoxin (11 kDa), which is secreted by the yeast during fermentation (Swan et al., 2003; Takeuchi et al., 2007; Berner and Arneborg, 2012). Thioredoxin has two cysteine residues in its reactive site and is able to reduce other proteins by formation of a disulfide bond. Yeast strains vary in secretion of thioredoxin and beer produced with a yeast strain secreting a high concentration of thioredoxin was recently found to produce a beer with increased oxidative stability (Berner and Arneborg, 2013). This introduces the possibility of selecting a yeast that can produce a more stable beer. Thioredoxin is activated by thioredoxin reductase on the expense of NADPH and can be regenerated when thioredoxin reductase and NADPH are present (Figure 22) (Holmgren, 1989). However, whether NADPH is present in the beer remains uncertain.

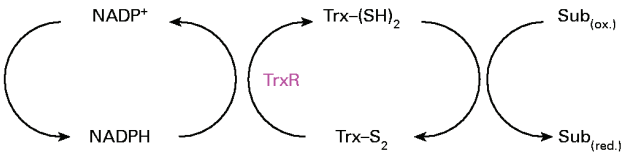


Figure 22: Reaction catalyzed by thioredoxin reductase. Trx (thioredoxin) and TrxR (thioredoxin reductase) (Mustacich and Powis, 2000).

Another important issue concerning the proposed antioxidative method is the possibility of protein sulfenic acid reacting and forming sulfinic and sulfonic acid (Figure 21) which thereby disrupts the regenerative chain reaction. The reaction pathways leading to formation of sulfinic and sulfonic acid, instead of the proposed disulfide, depend on the beer pH, pKa of the sulfenic acid as well as the surrounding thiols, their concentrations and possibly a large number of other factors (Nagy and Winterbourn, 2010). How the conditions for these reactions are in beer, remain unknown. Neither sulfinic nor sulfonic acid can be reduced by thiols and sulfonic acid is considered a non-reversible, "dead end" oxidation product. It is however possible, that sulfenic or sulfinic acids can be reduced by enzymes or other reducing

compounds in the beer. Sulfenic acid may also react with the amines present in the beer with further reaction to sulfinamide and sulfonamide (Nagy and Winterbourn, 2010). Whether these reactions occur may be influenced by the oxygen content in the beer and throughout the brewing process. It would be expected that sulfenic acid in a beer with high oxygen content would be more likely to form sulfonic acid and sulfonamide.

Finally, radical induced thiol oxidation by 1-hydroxyethyl radicals was recently described by de Almeida et al. (2013) to directly resulting in formation of a disulfide bond without forming protein sulfenic acid as an intermediate.

Thiol chemistry in beer is complex. However, supporting the hypothesis, about the thiols ability to become regenerated (Figure 21), it was recently found that thiols in beer in fact were reducible by the chemical reductant tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (de Almeida et al., 2013). This was also demonstrated in wort (Paper III). The unique about thiols in beer is that they are likely to be regenerated and therefore not become exhausted as it is the case with sulfite.

The risk of off-flavor formation

Thiols have been known to be contained in some off-flavor compounds. Two famous thiol-derived off flavors in brewing are dimethyl sulfide (DMS), with a cabbage like odor and the sun struck flavor caused by 3-methyl-2-butene-1–thiol. DMS originate from the malt but knowledge about its formation and how to limit its formation during the brewing process has made it possible to keep it under its odor threshold value (Anness and Bamforth, 1982). The sun struck flavor is caused by photo oxidation of iso-α-acids originating from the hops which in the presence of sun light and a thiol donor can form 3-methyl-2-butene-1–thiol (Gunst and Verzele, 1978). Yeast was found to excrete the small thiol glutathione (GSH) during fermentation, which had a positive effect on beer stability (Erfran, 1992; Gijs et al., 2004; Chen et al., 2012). However, Gijs et al (2004) also identified a negative consequence of increased GSH levels, as severe sulfurous off flavors were detected in beer being added 50 ppm GSH and then stored for five days at 40 °C. It should be noted that 50 ppm GSH corresponds to approximately 163 μM thiols which is larger than the total thiol content previously detected in beers (Lund and Andersen, 2011). Off-flavors originating from thiols in beer are something to consider, when doing research in the preservation of protein thiols in beer. However, compounds with volatile nature originate from small molecules and a rule of

thumb says that compounds larger than 300 g/mol are not volatile. The thiol compounds of interest in the current study consist of protein and peptides, most likely originating from LTP1, being much larger than GSH. These compounds are therefore not expected to influence on the volatile profile. However, extensive breakdown of the protein thiols may cause a problem in relation to off flavors.

4.2 Thiols throughout the Brewing Process

Protein thiols are introduced to the beer through the barley. Their fate through the brewing process and in the final beer has been investigated in Paper II, III, IV and V. This section summarizes the main findings from this PhD study while comparing to findings in other research studies.

4.2.1 Thiols in Wort

Thiol oxidation in wort

In the current PhD, mashing's were carried out using the EBC congress mash procedure, which takes place at atmospheric conditions with access to oxygen. Stephenson et al. (2003) showed that beer thiols are very sensitive to oxygen and found that anaerobic mashing resulted in increased thiol levels in worts made from regular malt as well as for wort made with low proanthocyanidin malt (being low in polyphenols). Figure 23 shows that the thiol content in the sweet wort did not decrease during 40 min of analysis, and it was suggested that most of the thiol oxidation had already taken place during mashing and filtration.

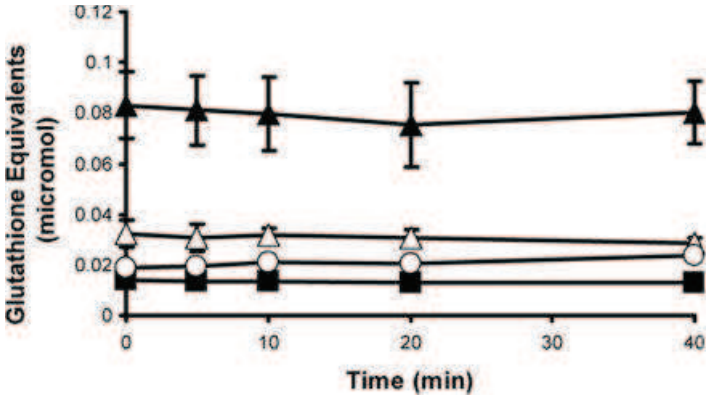


Figure 23: Impact of aerobic and anaerobic mashing on thiol levels in sweet worts determined in micromole using the DTNB method. Standard malt, ■ aerobic, △ anaerobic. Low proanthocyanidin malt, ○ aerobic, ▲ anaerobic. (Stephenson et al. (2003)).

The thiol content is presented in micromol (μmol) glutathione equivalents (Figure 23) however, based on the thiol quantification method, described in Stephenson et al. (2002), 0.08

μmol must be equivalent to 105 μM and 0.01 μmol equivalent to 13.4 μM being similar to the amounts detected in the current research (detailed in the following).

In the current studies almost all thiols were oxidized during the EBC congress mashing process. However, de Almeida et al. (2013) showed that the thiols could be reduced using the chemical reductant TCEP. In Paper III wort was prepared from the three barley varieties Pallas, Fero and Archer. After the reduction with TCEP it was clear that sweet Archer wort had almost twice the amount of reducible thiols compared to sweet Fero and Pallas wort (Figure 24). During mashing the thiols were oxidized and had formed disulfide bonds causing the formation of gel-proteins. These proteins are known to result in a viscous layer leading to increased filtration times (Baxter et al., 1980; Moonen and Graveland, 1986; Poyri et al., 2002). The increased amount of oxidized thiols correlated with the fact that the filtration time of Archer wort (42 min) was more than twice as long as for Fero (21 min) and Pallas wort (16 min).

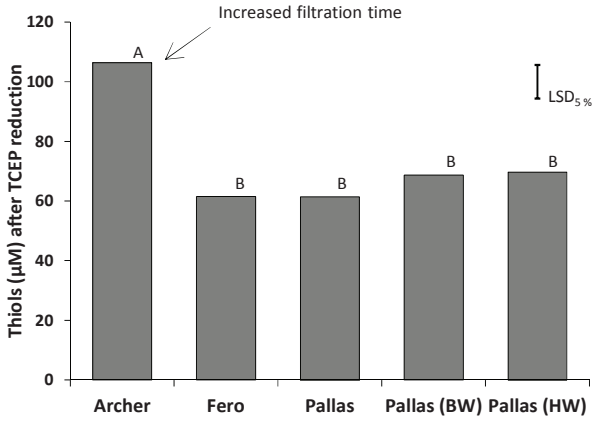


Figure 24: Thiol concentration in wort samples treated with the thiol reducing agent TCEP. (n=3). BW= boiled wort and HW=hopped wort. (Paper III).

These results underline the importance of keeping the thiols on their reduced form both in order to preserve their antioxidative capacity as well as to keep a short filtration time. It furthermore stresses the importance of carrying out the mashing under oxygen controlled conditions. The thiol levels correlate with levels determined by Stephenson et al. (2003). The fact that more thiols were actually detected in wort produced under aerobic conditions may be explained as the wort was kept anaerobically directly after filtration. Research in our lab has indicated that a lot of thiol oxidation takes place during filtration. The protein composition of Pallas, Fero and Archer barley was not determined. However, Silva et al (2008) found quantitative differences between the protein profiles of wort made from two different barley varie-

ties. This further supports the possibility of the barley variety having an impact on wort and beer quality.

Jin et al (2009) found that the amount of reduced thiols increased during wort boiling and suggested that it was caused by heat induced protein unfolding. As illustrated in Figure 4 there is a weak tendency for the total reducible thiol content to increase after boiling. In Paper V the thiol content in boiled and hopped wort was also slightly higher than the thiol content in sweet wort. However, as also the boiling was carried out under atmospheric conditions at least some of the possibly reduced thiols are likely to become oxidized again. The current experiments have not been designed to provide a clear answer to whether more thiols become reducible due to heat induced protein unfolding or not.

Thiol removing capacity

Bamforth et al. (2009) identified an enzyme responsible for oxidation of the thiols; cysteine, dithiothreitol, glutathione and mercaptoethanol added to sweet wort (Figure 25). This enzyme, named thiol oxidaze, was found to be very active using cysteine and dithiothreitol as a substrate but much less active when using reduced glutathione and merchaptoethanol. Interestingly, a small amount of thiol oxidizing activity remained in the malt extract after autoclaving (Figure 25, B). This either indicates a very heat stable enzyme or more likely some remaining non-enzymatic thiol oxidation (Bamforth et al., 2009).

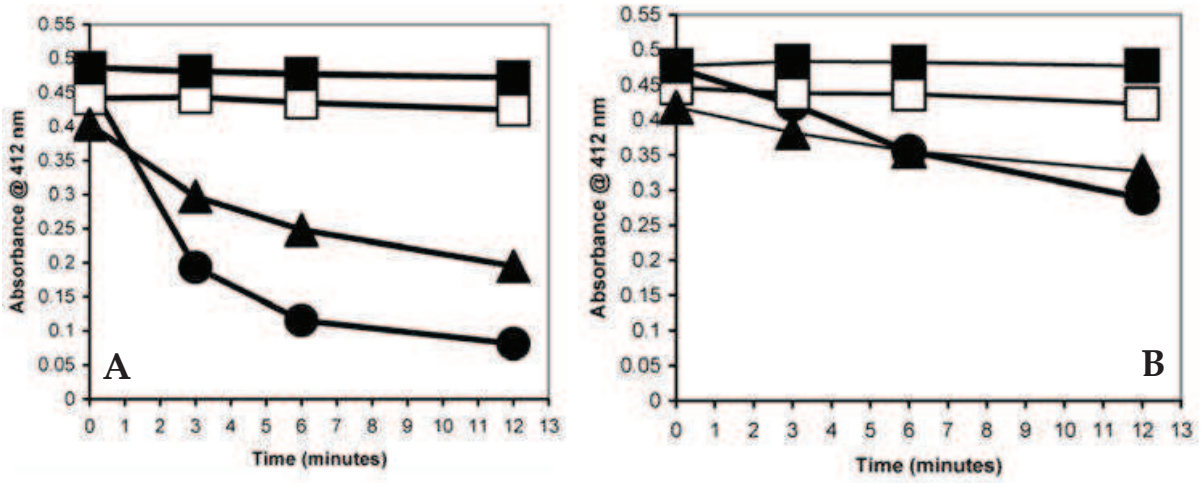


Figure 25: A, loss of thiols in reaction mixture of malt extract. B, loss of thiol in autoclaved reaction mixture of malt extract. ● Cysteine, ▲ dithiothreitol, □ glutathione and ■ mercaptoethanol. From Bamforth et al (2009).

In line with the results from Bamforth et al. (2009) a "thiol-removing capacity" was detected in sweet wort (Paper II and Paper III) being able to oxidize small thiols added to the sweet wort. Similar to the enzyme described by Bamforth et al. (2009) the "thiol-removing capacity" was more reactive towards cysteine than towards glutathione (Figure 26). This makes it likely that what is described as "thiol-removing capacity" in Paper II and III is what is described as the enzyme thiol oxidase by Bamforth et al. (2009).

The "thiol removing capacity" was inactivated during wort boiling (Figure 26, A) as well as during malt roasting, where increasing EBC wort color resulted in decreased "thiol-removing capacity" (Figure 26, B).

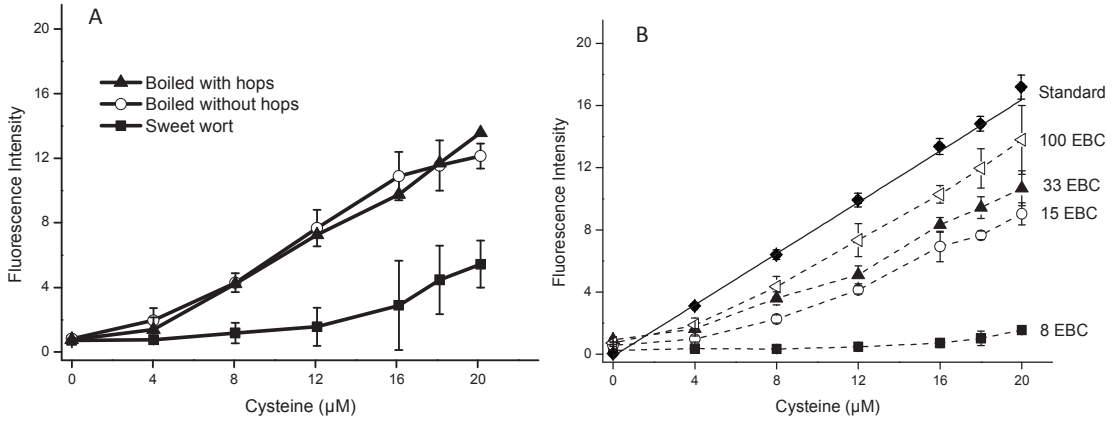


Figure 26: Standard addition curves of cysteine in sweet wort based on derivatization with the fluorescent reagent ThioGlo 1 and wort diluted 40 times (n=3). (A) Fluorescent response from Pallas wort (sweet, boiled and hopped) with added cysteine in concentrations between 0 and 20 µM (Paper III). (B) Fluorescent response from sweet wort made from pale ale malt roasted to colors varying from 8 to 100 EBC. Cysteine is added in concentrations between 0 and 20 µM. The standard curve of cysteine in buffer is illustrated with a regression line (standard). (Paper II).

These results indicate that the "thiol-removing capacity" is mainly enzymatic. Thiol oxidation in sweet wort however, was also suggested to be non-enzymatic (Stephenson et al., 2003; Bamforth et al., 2009). Based on the current and previous results it seems most likely that the "thiol removing capacity" consists of both enzymatic and non-enzymatic oxidation. The current results and Bamforth et al. (2009) suggest that the enzyme mainly acts on smaller thiols. It therefore seems likely that the thiol groups of the peptides remain reduced when the wort is produced under anaerobic conditions. This also correlates with the fact that reduced thiols have been detected in sweet wort in previous studies (Muller, 1995; Stephenson et al., 2003;

Jin et al., 2009) along with increased amounts of thiols detected when the sweet wort was produced under anaerobic conditions (Stephenson et al., 2003).

It is important to note that the "thiol-removing capacity" was eliminated during wort boiling (Paper III) and is therefore not an active thiol oxidizing agent in the final beer (Paper VII). The presence of the "thiol removing capacity" made it impossible to use the standard addition procedure for thiol detection in sweet wort (section 2.2).

Reduced thiols in wort

Despite the "thiol-removing capacity" detected in sweet wort and despite the fact that this sweet wort was made under aerobic EBC congress mashing conditions; thiols were detected in sweet wort using external standard curve of glutathione prepared in water (Figure 27) (data not published). The levels were low and the sweet wort made from Munich malt had a thiol content of 4.8 μM and the sweet wort made from Pilsner malt had a thiol content of 3.4 μM and decreased to a level of 1.9 μM and 1.1 μM respectively after being stirred in an open beaker for 6 to 8 hours at room temperature. Between 8 and 10 hours the thiol content did not decrease further.

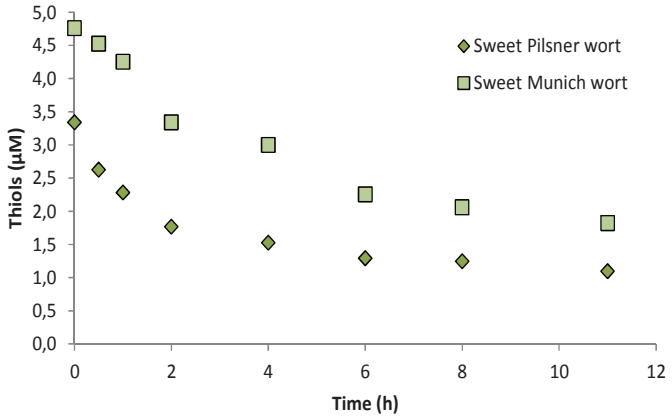


Figure 27: Thiol levels in sweet pilsner wort and sweet Munich wort during magnetic stirring in an open beaker.

Interestingly, the thiol levels had increased to approximately 17 μM after overnight infection clearly indicating that the infected microorganisms had secreted thiols, or possibly thiol reducing enzymes, during growth. In Figure 27 sweet Munich wort shows a larger thiol content than the sweet pilsner wort, which may be explained by higher kilning temperatures and a greater extent of inactivation of thiol oxidizing enzymes. However, due to the use of external standard curve we cannot exclude the fact that the difference detected in thiol con-

tent may be caused by inner filter effect due to the slightly darker color of the sweet Munich wort (see method section 2.2).

4.2.2 Thiols in Beer

In Paper IV and V thiols were studied in the final beer. Pasteurization had a minor influence on the thiol levels in fresh beer but all thiols were found to decrease in concentration during storage however, the thiol content was low compared to other studies (Lund and Andersen, 2011) (Figure 28).

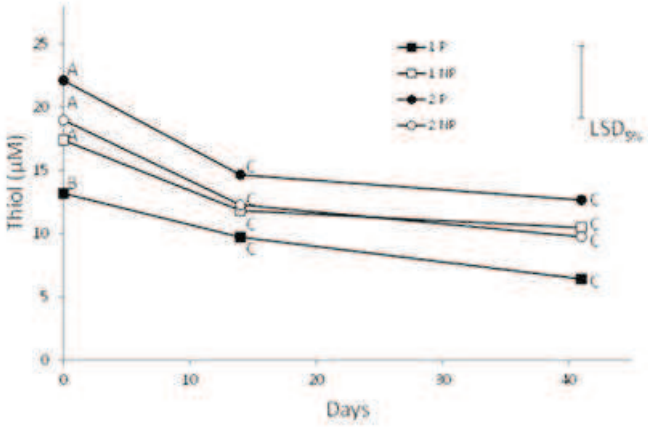


Figure 28: Thiol levels in Beer 1 pasteurized (1P) and non-pasteurized (1 NP) and Beer 2 pasteurized (2 P) and non-pasteurized (2 NP) during storage for 41 days at 40 °C (n=4). Due to a day to day variation between the measurements, LSD (Least Significant Difference) value is presented. Letters indicate the samples statistical differences and the levels bearing different letters are significantly different ($p < 0.05$).

The decrease in thiol content during storage, suggests that thiols are intermediates in redox reactions during beer staling supported by other recent findings (Lund and Andersen, 2011; Wu et al., 2011; Chen et al., 2012). The thiol levels detected in pasteurized and non-pasteurized beer, described in Paper IV, was 8.66±0.02 µM and 9±2 µM after 426 days of storage and the thiol levels detected in pasteurized and non-pasteurized beer after 41 days at 40 °C, described Paper V, are similarly low (Figure 28). We may speculate that the thiols are not likely to become completely exhausted and that their reaction below 8 µM is very slow. This result along with the similar findings in wort (Figure 27) indicates that some thiols are not able to react or will end up in a steady-state or redox balance by some other reducing components. Kunz et al. (2013a) recently showed that carbohydrates are likely to have reducing properties, and these may be worth investigating in relation to beer thiols. It seems as if

thiols in wort are more sensitive towards oxidation than thiols in beer. This suggests that something in the beer stabilizes or regenerate the thiols being either the sulfite or other compounds from the yeast. The fact that the thiols are more stable in the beer may be an indicator of their regeneration. An unpublished experiment showed that the thiol content in a sulfite containing beer, left in a beaker with magnetic stirring at room temperature had a very stable thiol content only showing a minor decrease after 3 hours of stirring.

A large number of beers were analyzed during the optimization of the method described in Paper VII. During the large number of pre-experiments the thiol content was found to vary between brands as well as between batches of the same beer. This was supported by Lund and Andersen (2011) who performed a screening of 11 commercial beers. The PCA scores and loadings plot (Figure 29) visually illustrates that thiol content, sulfite content, and the beers oxidative stability determined by ESR lag phase were closely correlated. The ESR rate, beer color and ethanol, Fe and Cu content were also correlated. This suggests that PC1 mainly describes antioxidative properties and that thiols have antioxidant capacity in beer. PC2 mainly describes prooxidative properties supporting previous findings about the fact that the rate of radical formation (ESR rate) correlate with beer color, Fe and Cu content and ethanol content (Uchida and Ono, 1996; Andersen and Skibsted, 1998; Nøddekær and Andersen, 2007).

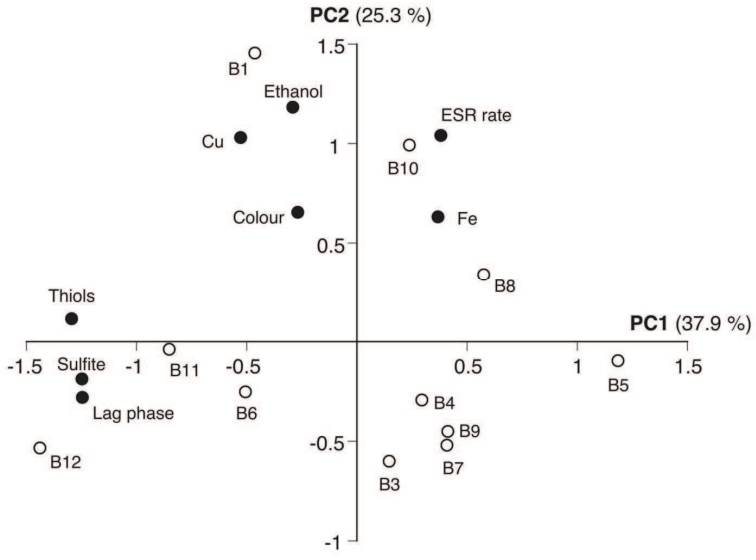


Figure 29: Principal component analysis (PCA) score plot of 11 stored beers (B1, B3-B12), illustrating the correlation between thiol content, sulfite content and ESR lag phase measured by electron spin resonance (ESR) spectroscopy. From Lund and Andersen (2011).

4.3 Summary

Figure 30 sums up the results about the fate of the thiols discovered on the work from the current PhD thesis.

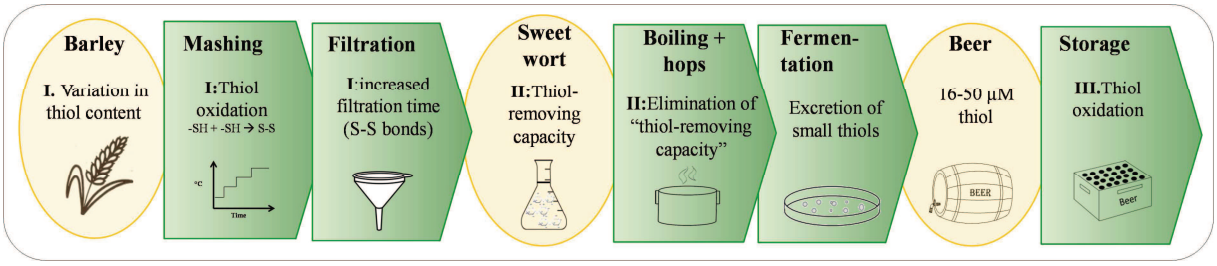


Figure 30: Thiol relevant reactions at different stages of beer brewing

Barley derived thiols are present during all stages of brewing and in the final beer. Thiols are very sensitive to oxidation, and exclusion of oxygen is crucial for keeping the thiols in the reduced form. The amounts of protein thiols varied between barley varieties. Increased thiol oxidation is linked to a lowering of wort filtration rates. Sweet wort was found to contain compounds able to oxidize protein-thiols referred to as a "thiol-removing capacity". The activity of these thiol-oxidizing compounds decreased through increased malt roasting; and wort boiling eliminated their activity, suggesting an enzymatic origin of the effect. The levels of thiols were found to decrease during storage, suggesting that thiols are intermediates in redox reactions during beer staling.

5 Pasteurization and Oxidative Stability of Beer

In spite of the wide application of the pasteurization technique for microbiological reasons, the influence of pasteurization on the chemical storage stability of beer is not very well described. It is the opinion among many microbrewers that pasteurization has a negative influence on beer flavor and for this reason pasteurization is avoided. This assumption was tested in Paper IV and V along with the influence of pasteurization on protein content and composition. This section summarizes the main results and presents some thoughts and speculations, which were not included in the papers.

Scientific Approach and Main Results

Paper IV describes a study designed to evaluate the storage stability and protein composition in filtered, pasteurized or non-pasteurized beer during storage for 426 days at room temperature. Paper V describes a study designed to evaluate the storage stability of two different non-filtered pasteurized or non-pasteurized beers, produced from the same wort during accelerated storage (40 °C, 41 days).

In both studies the oxidative stability was evaluated by ESR spectroscopy and sulfite measurements and the volatile profile was evaluated by headspace sampling and GC-MS analysis. Results from Paper IV, illustrated in Figure 31 A, shows that the ESR lag phases of the non-pasteurized beers decreased during storage at 22 °C, and at the end of the storage almost no lag phases were observed. In contrast the pasteurized beers at day 426 had lag phases around 140 min. The concentrations of sulfite were found to change in parallel with the ESR lag phase, and the complete loss of sulfite is consistent with the complete loss of ESR lag phase for the non-pasteurized beer. (Figure 31, B).

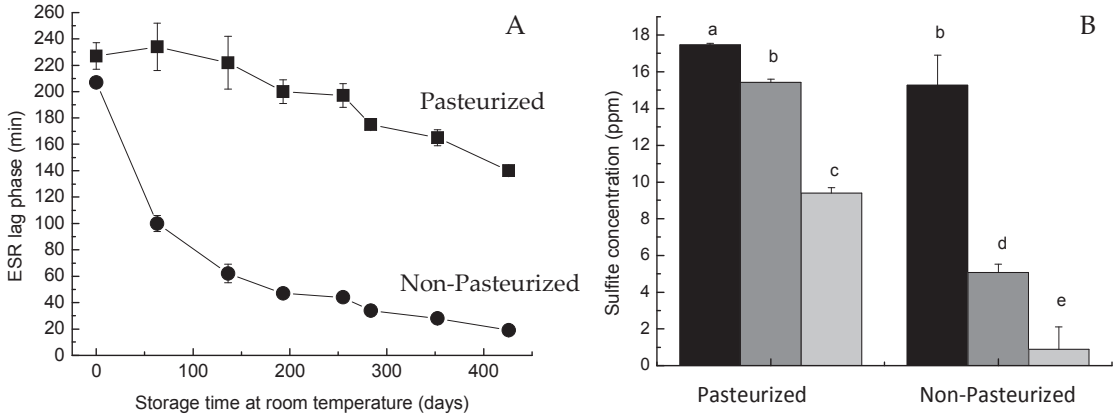


Figure 31: A) Oxidative stability of pasteurized and non-pasteurized beer during storage at 22 °C for 426 days determined by ESR lag phase measurements. B) Sulfite concentrations in beer after 0 (black bars), 63 days (dark grey bars), and 426 days (light gray bars) of storage at 22 °C. (Paper IV).

This result was confirmed in Paper V. However, the beers were already oxidized and no sulfite and no lag phases were present and the differences were evaluated based on only the rate of radical formation. It was therefore concluded in Paper IV and Paper V that pasteurization has a significantly positive effect on the oxidative stability of beer, which is in contradiction to common understanding among microbrewers.

However, despite the increased oxidative stability found in pasteurized beer, pasteurization was found to induce formation of Maillard reaction compounds as well as to cause a minor decrease in volatile ester compounds. Maillard reaction compounds are associated with beer staling in light colored beer, and ester compounds are associated with more fruity aroma and beer freshness (Vanderhaegen et al., 2003; Verstrepen et al., 2003). The formation of Maillard reaction products is likely to occur independently from other oxidative reactions. Interestingly, the Maillard reaction products developed during storage in Paper IV, whereas in Paper V they were developed already during pasteurization and was present in the fresh beers. In Paper V the differences between pasteurized and non-pasteurized beer was leveled out during accelerated storage (40 °C 41 days), which is not surprising considering the high storage temperature. The Maillard reactions are complex, and minor variations in pasteurization method may also have an influence on both loss and formation of volatiles. Finally, as the volatiles were not quantified, but only given as relative concentration, it remains uncertain how the changes in concentration of these volatiles actually influence the aroma of the beer.

These results show that ESR spectroscopy, in this case, does not reflect the changes in volatile profile. This is most likely explained by the fact that the change in volatile profile is not initiated by oxidative reactions. In this study the ESR analysis does therefore not provide a complete picture of the beer quality. In comparison the ESR method and the analysis of volatiles each has different qualities. The ESR method is able to predict shelf life based on radical reactions leading to oxidative changes in the beer (Uchida et al., 1996), whereas the volatile profile can provide a picture of the actual volatile status of the beer detecting all volatiles, from both oxidative and non-oxidative reactions. However, it remains unknown to which extent the volatile profile represents beer aroma and taste (See section 2.3). In Paper V pasteurization initially caused a change in volatile profile compared to non-pasteurized beer but the volatile profile did not change further during storage. Maybe this is an expression of the fact that pasteurization decreases the quality of the fresh beer but result in an improved stability during storage.

The results from Paper IV and Paper V clearly shows that pasteurization limits radical generation, but the mechanisms behind it are unknown. It is usually agreed that heating cause an increase in oxidative reactions, and it therefore remains some kind of a paradox that pasteurization has a significant, positive influence on the oxidative stability. In Paper IV it was found that the difference between pasteurized and non-pasteurized beer was mainly caused by a difference in effect of prooxidants. It can therefore be speculated that the decreased radical generation is caused by inactivation of prooxidants however, may also be influenced by the fact that the pasteurized beer is sterilized and contains no active yeast, bacteria or enzymes, which may promote oxidation during storage. If the decreased radical generation is caused by heat inactivation of prooxidants, these prooxidants must be generated during the fermentation process and therefore, be present only post fermentation. If the prooxidants had been present in the wort they would have been heat inactivated earlier, as the wort undergoes extensive boiling.

Maillard reaction products were suggested to have prooxidative effect (Paper I and II, Kunz et al. 2013b). However, in pasteurized beer Maillard reaction products are developed parallel to the fact that beer becomes more oxidative stable (Paper IV and V). The amount of Maillard reaction products formed during pasteurization is most likely relatively small as they only

has a minor influence on beer color. Therefore, despite of the prooxidative effect of Maillard reaction products the protective effect of pasteurization is much larger.

The protein composition is known to influence the colloidal stability of beer in the form of haze and foam stability. It is unclear to which extent the protein composition affects the oxidative stability of beer. Therefore, also the protein composition was investigated in the two studies however, the results were not as straight forward. Fe and Cu are known for their prooxidative effect while acting as catalysts in the radical generation and oxidation reactions during beer aging (Nøddekær and Andersen, 2007; Frederiksen et al., 2008). In Paper IV filtered, pasteurized beer had a soluble protein content of approximately twice the amount of non-pasteurized beer, which was likely to have influenced the amount of soluble Fe and Cu levels. In Paper V pasteurization did not influence the soluble protein content or soluble levels of Fe and Cu.

6 Conclusion

Malt and Wort

The oxidative stability of pilsner malt and roasted malt decreases during storage and is sensitive to temperature and water activity. Roasted malt and the corresponding sweet wort are much more unstable than pilsner malt as well as the corresponding sweet wort in terms of increased radical content, more lipid oxidation, and a larger change in volatile profile during storage. The results suggest, that storage time of malt and handling of sweet wort in general should be minimized to prevent initiation of oxidative reactions and to limit the loss of volatiles. The Maillard reaction products, melanoidins, are suggested to contribute to the instability of dark malt and wort through a prooxidative effect in combination with Fe by acceleration of the Fenton reaction.

Beer quality is a result of a balance between the raw materials and the brewing process. Transition metals such as Fe and Cu, originating from the barley, has a large influence on the oxidative stability of beer. Boiling with hops is found to limit the amount of Fe and Cu through binding by hop compounds. Also proteins in the final beer are likely to have a positive effect on the oxidative stability through binding of metals. Finally, the current research suggests that barley variety can influence the volatile profile of wort and thereby possibly also the beer flavor.

Thiols throughout the Brewing Process

The research supports the hypothesis about protein thiols having an antioxidative effect. The thiols are derived from barley proteins and the amounts of protein thiols varied between barley varieties. Thiols are very sensitive to oxidation, and exclusion of oxygen is crucial for keeping the thiols on their reduced form. Increased thiol oxidation is linked to a lowering of wort filtration rates due to formation of gel proteins. Sweet wort is found to contain compounds able to oxidize protein thiols and referred to as a "thiol-removing capacity". The activity of these thiol-oxidizing compounds decrease through increased malt roasting; and wort boiling eliminate their activity, suggesting that it is an enzymatic effect. The levels of

thiols are found to decease during storage, suggesting that thiols are intermediates in redox reactions during beer staling.

Pasteurization

Pasteurization has a positive influence on the oxidative stability and radical intensity of both filtered and non-filtered beer, which is consistent with a decreased consumption of sulfite. Pasteurization has a minor negative effect on the volatile profile by increasing volatile compounds that is generally associated with heat treatment and by decreasing the levels of ester volatiles generally associated with fruity aroma.

7 Perspectives

The present thesis forms the basis for further research within oxidative stability of malt, wort and beer as well as for research to obtain more basic mechanistic understanding of thiol chemistry in wort and beer. Further studies should reveal to which extent oxygen levels influence the oxidative stability of malt, wort and beer. Very important for future studies is the inclusion of sensory analysis, using a trained sensory panel, to evaluate how the changes in radical intensity, thiol content and volatile profile actually are perceived.

Malt

How the volatile profile of different barley varieties influences the beer could be verified through a larger screening experiment including many barley varieties and their corresponding worts and beers.

Thiols

It is important to confirm, if thiols have a positive influence on beer staling and do not contribute with off-flavors themselves. The regenerative mechanism of thiols in beer should be further investigated, including a clarification of the possible contribution from both sulfite and reducing enzymes such as thioredoxin. Selection of barley variety could be investigated with the purpose of increasing thiol content in wort and beer. Other raw materials used in brewing such as wheat, maize and rice should be investigated for their influence on thiol levels as well as on oxidative stability.

Investigation of thiols as antioxidants in other food products should also be considered. Dairy products could be interesting for this type of research due to its naturally high content of proteins.

Pasteurization

The mechanisms behind the stabilizing effect of pasteurization on the oxidative stability of beer should be investigated with the purpose of enabling a more targeted use of pasteuriza-

tion to improve the shelf life of beer. Improved understanding of these mechanisms in beer brings up perspectives for other food products.

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Paper I

Quality of Pilsner Malt and Roasted Malt during Storage

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Quality of Pilsner Malt and Roasted Malt during Storage

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Abstract

Malt is usually expected to be stable during 12 months of storage. However, in practice many brewers notice changes in malt aroma during storage. The oxidative stabilities of pilsner malt and roasted malt were evaluated during a 12 month storage at different temperatures (10 °C and 20 C°) and water activities (0.231 and 0.432) as the radical content in malt kernels measured by electron spin resonance spectroscopy and the volatile profile of the resulting sweet worts measured by head space analysis followed by GC-MS analysis. The storage of malt resulted in oxidative reactions and a large change of the volatile profile of the resulting worts. Roasted malt was much more unstable than pilsner malt illustrated by a higher initial radical intensity, larger radical decay during storage, a larger change in volatile profile of the wort with increased amounts of lipid oxidation products. For both roasted malt and pilsner malt, good correlations were found between radical decay and change in volatile profile of the wort, where high temperature and high water activity resulted in the largest changes. During the 12 months of storage the sugar extract of the wort made from the malts remained constant and were not affected by the chemical changes. This study suggests that chemical changes occurring in malts during less than 12 months of storage may potentially affect the aroma of beer, and that water activity and storage temperature both should be kept low in order to maintain a high malt quality.

Key worts: Malt, volatiles, storage stability, radicals, oxidation, electron spin resonance

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Introduction

Beer quality is highly influenced by malt quality (1,2). It is generally accepted that malt has good storage stability when kept in a dry environment and most malts are accepted to have a storage stability of 12 months. In practice however, many brewers notice changes in malt aroma during storage, but only very few scientific studies have examined the flavour and oxidative stabilities of malt during storage. The effect of storage on malts has been investigated in relation to wort filtration rates (3) where longer storage times were found to have a positive effect, most likely explained by the decay of a thiol oxidizing enzyme *(4-6)*. Lipid oxidation is a known cause of off flavours in beer (1) and the levels of the lipid oxidizing enzyme lipoxygenase (LOX), has been studied during malt storage (7). Selected volatiles were studied in relation to storage and it was observed that Strecker aldehydes and N-heterocyclic compounds increased slightly in concentration within the first few months and then decreased to concentrations below the initial content, and interestingly similar variations in malt and beer aromas were identified (2). In a recent study it was found that sweet worts made from roasted malts with colours above 33 EBC units were very susceptible to a loss of volatiles when exposed to mild heating, whereas the profile of volatile compounds of wort made from pilsner malt was not affected by the heating (6).

Water activity (a_w) is a major factor influencing storage stability of food products. A high water activity results in a higher mobility of reactive compounds within the product and thereby the possibility of more damaging reactions taking place. Water activity is directly related to the equilibrium relative humidity (ERH) at a certain temperature following the equation [1]:

$$a_w = P/P_0 = ERH/100 \qquad [1]$$

Where P is the partial vapour pressure of moisture and P_0 is saturation vapour pressure of pure water. Water activity has been found to have a significant influence on radical content in dry products (8,9) and water activity is therefore likely also to influence radical content in malt. The levels of radicals in malt kernels have previously been determined by direct measurement using electron spin resonance (ESR) spectroscopy *(10-12)*.

The purpose of the current study was to gain an understanding of chemical changes and oxidative stability of pilsner malt and roasted malt during storage. Also the influence of temperature (10 °C and 20 C°) and water activity (0.231 and 0.432 being equal to 23.1 and 43.2 % ERH) has been tested. The observations were compared to the volatile profile of wort produced from malt after 0, 6 and 12 months of storage. The study has been based on pilsner and roasted malts derived from the same batch of malt, and all variations between pilsner malt and roasted malt can be ascribed to the differences in roasting. A detailed study of

the influence of water activity on radical formation was also carried out on pilsner malt at 20 °C using a range of water activities between 0.113 and 0.910.

Materials & Methods

Malt

Fresh pale ale malt (two-row spring barley (*Hordeum vulgare*) harvest 2010) was kindly provided by Danish Malting Group, Vordingborg, Denmark.

Roasting

Pilsner malt was roasted by distributing it in a single layer on a cloth on a baking plate and heating it in an oven at 190 ˚C for 50 minutes.

Storage of pilsner malt at 10 different water activities

Approximately 50 g of fresh pilsner malt was placed in 10 containers where the water activities were adjusted with the presence of saturated aqueous salt solutions: Lithium chloride (a_w 0.113), potassium acetate (a_w=0.231), magnesium chloride (a_w=0.331), potassium carbonate (a_w=0.432), magnesium nitrate (a_w=0.529), sodium nitrate (a_w=0.693), sodium chloride (a_w=0.756), ammonium sulfate (a_w=0.817), potassium chloride (a_w=0.859) and barium chloride (a_w=0.910) *(13)*. The malt was stored in a thermostatted cabinet at 20 °C.

Storage of pilsner malt and roasted malt

Pilsner malt and roasted malt were stored in the dark at 10 °C or 20 °C at a water activity (a_w) of 0.231 or 0.432 (Table 1). The water activity of the malt was adjusted with saturated salt solutions of potassium acetate (a_w=0.231) and potassium carbonate (a_w=0.432). Water activity may be influenced by temperature, however, no significant difference was found between 10 °C and 20 °C for these two salts *(13)*.

Electron Spin Resonance (ESR) Spectroscopy

Samples were taken from both experiments for ESR spectroscopy analysis throughout the 12 months storage. Radical intensities were measured directly on the malt kernels and the method was developed with inspiration from Cortes et al (10), Kaneda et al. (11) and Tokai et al (12). The malt kernel was placed in the center of the cavity using a cylindrical thin-walled 702-PQ-7 clear-fused quartz (CFQ) tube (Wilmad Glass Company Inc,. Nuena, NJ). The weight of each kernel was measured before ESR analysis and the peak to peak height of the resulting ESR signal was divided by the weight of the kernel. Each result consists of an average of 10 measurements. ESR spectra were recorded at room temperature with a Miniscope MS 200 X-band spectrometer (Magnettech, Berlin, Germany) using the settings: Microwave power, 10 mW; sweep width, 73.8 G, sweep time 30 s; number of passes, 2; modulation, 2000 mG; and microwave attenuation, 10 mW. Pilsner malt was recorded at gain 700 and roasted malt was recorded at gain 100.

Mashing

Mashings of the malts were carried out after 0, 6 and 12 months of storage according to Analytica EBC 4.5.1 "Extract of Malt: Congress Mash" *(14)*, with the modifications described by Frederiksen *et al. (15)*. The colour of the fresh worts was measured spectrophotometrically according to Analytica EBC 8.3 "Colour" *(14)* and sugar content was determined in ˚Brix values, using a refractometer (Analytic Jena, Jena, Germany). Wort filtration time was noted as the time where no more liquid appeared on the top of the filter cake, and the extract yield was measured 45 min after filtration start. The worts were stored at -20 ˚C and thawed before volatile analysis.

Volatiles

Dynamic headspace analysis was carried out in triplicate using 5 ml wort and 0.25 mL 4-methyl-1-pentanol (5 mg/L) added as internal standard. The volatile compounds were collected on a Tenax-TA trap (Buchem bv, Apeldoorn, The Netherlands). The trap contained 250 mg of Tenax-TA with mesh size 60/80 and a density of 0.37 g mL^{-1} (Buchem bv, Apeldoorn, The Netherlands). The samples were equilibrated to 30±1°C for 5 min in a circulating water bath and then purged with nitrogen (200 mL min^{-1}) for 30 min. Analysis was carried out in triplicate.

The trapped volatiles were desorbed using an automatic thermal desorption unit (ATD 400, Perkin Elmer, Norwalk, USA). Primary desorption was carried out by heating the trap to 250°C with a flow (60 mL min^{-1}) of carrier gas (H_2) for 15.0 min. The stripped volatiles were trapped on a Tenax TA cold trap (30 mg held at 5°C), which was subsequently heated at 300°C for 4 min (secondary desorption, outlet split 1:10). This

allowed for rapid transfer of volatiles 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, California) through a heated (225°C) transfer line. Separation of volatiles was carried out on a DB-Wax capillary column 30 m long x 0.25 mm internal diameter, 0.5 µm film thickness. The column pressure was held constant at 2.4 psi resulting in an initial flow rate of approximately 1.2 mL min^{-1} using hydrogen as carrier gas. The column temperature programme was: 10 min at 40°C, from 40°C to 240°C at 8°C min^{-1}, and finally 5 min at 240°C. The mass spectrometer was operating in the electron ionisation mode at 70 eV. Mass-to-charge ratios between 15 and 300 were scanned. 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, MSD Chemstation (Version E.02.00, Agilent Technologies, Palo Alto, California), was used for data analysis. Relative concentrations are presented as areas based on single ions divided by the peak area of internal standard.

Multivariate data analysis

Multivariate data analysis was applied to the relative areas of the identified volatiles analyzed by GC-MS to visualize the influence from storage, temperature and water activity using Principal Component Analysis (PCA) and Partial Least Squares-Descriminant Analysis (PLS-DA) regression analysis. Multivariate data analysis was performed using Latentix software (LatentiXTM 2.0, Latent5, Copenhagen, Denmark, www.latentix.com). Analyses were carried out on the relative peak areas and data was auto scaled and fully cross validated.

Statistical data analysis

Statistical analysis was carried out as one-way ANOVA using the software SASjmp 9, SAS Institute, Inc., USA. Storage Day and Sample were included as fixed effects. The differences in radical intensities between Treatments (temperature and water activity) were evaluated by including the Average radical intensity of the malt and Treatment as fixed effects and Storage Day as random effect.

Results

Storage of pilsner malt at different water activities

Temperature and water activity have a large influence on the storage stability of foods and the effects of these factors were investigated during malt storage. An initial study was set up where pilsner malt was stored at 20 °C in closed boxes with atmospheres where the water activities were kept fixed at 10 different levels from 0.113 to 0.910.

Radical intensity was measured directly on the malt kernels using electron spin resonance (ESR) spectroscopy during the storage. Each result is based on an average of the peak to peak height of the ESR spectrum of 10 malt kernels divided by the weight of each kernel (Figure 1, pilsner malt).

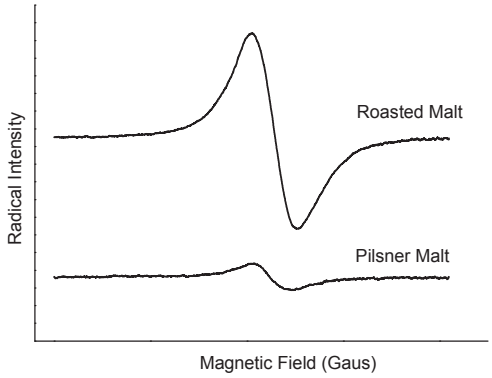

Figure 1: ESR spectrum of roasted malt and pilsner malt. The peak to peak height is used as a measure of radical content.

After one month of storage visual mold appeared on malt stored at the high water activities, 0.817 and 0.910. After two months of storage mold appeared also on malt stored at a water activity of 0.756, and after three months on malt stored at a water activity of 0.693 as well. Malt samples were excluded from the study after visible mold formation appeared. Malt with a water activity at 0.529 and below remained uninfected by mold throughout the 12 months of storage in agreement with previous findings *(16,17)*. The initial water activity of the malt kernels was low (on average 0.15). The radical content decreased in the malt samples and eventually stabilized at different levels during the storage (Figure 2, A). The decrease in radical contents during the first months of storage is most likely caused by increased mobility of the kernel components thus allowing chemical reactions to take place. Radicals observed in the malt kernels are very likely highly reactive, but the entrapment in the physically locked matrix of the dry kernels prevent their decay. The increased mobility in the malt kernels due to the higher water activity enables the radicals to react and their increased activity can be detected as a radical decay as observed by ESR spectroscopy. A

drop in radical content in malt kernels is therefore a sign of higher component mobility and therefore also the possibility of decreased oxidative stability. After 4-6 months of storage the radical intensities of the malt kernels had stabilized at lower levels, which reflect a steady state level between formation and decay (Figure 2, A). After 4 months of storage strong linear correlations were found between the stabilized radical intensities and the water activities where a high water activity resulted in a low radical content (Figure 2, B).

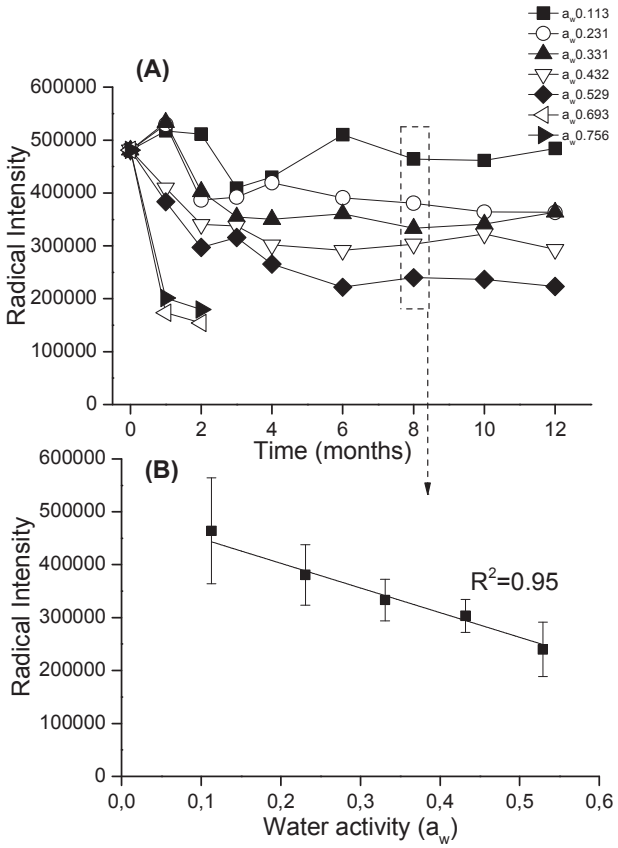

Figure 2: (A) radical intensity of pilsner malt stored for 12 months at 20 °C at different water activities (aw). (B) Correlation between radical intensity and water activity of pilsner malt stored for 8 months at different water activities.

Storage of pilsner and roasted malts

In a separate storage experiment a pilsner malt and a roasted malt were stored for 12 months at 10 °C or 20 °C and at water activities of 0.231 or 0.432 resulting in four different storage conditions. The roasted malt was produced from the pilsner malt by heating 190 °C for 50 min resulting in a color of 114 EBC. The two malts were therefore identical except for the roasting. In this way differences due to different growth, harvesting, and malting conditions were eliminated. The pilsner malt was freshly kilned and the roasting was carried out the day before initiation of the storage experiment. Pilsner malt had initially a radical intensity of 3.9 x 10^5 and roasted malt a radical intensity of 1.9 x 10^6. The radical signal from roasted malt

was approximately 7 times larger than the signal from pilsner malt, demonstrating the high level of radicals generated and trapped during the roasting in the roasted malt *(18)* (Figure 1). After 1 month of storage the radical content of pilsner malt and roasted malt was lower due to the adjustment of water activity causing increased mobility and radical decay (Figure 3). The radical contents in both malts were found to be highly influenced by the water activity, and malts stored at the low water activity (0.231) had a significantly larger radical content and a smaller decay compared to malts stored at high water activity (0.432) (Figure 3). When the storage water activity of the malt was low (0.231), the radical intensity was not influenced by the storage temperature (10 and 20 °C). However, when the water activity of the malt was high (0.432), the storage temperature had a significant influence on the radical formation, and pilsner malt and roasted malt stored with a high water activity (0.432) at 20 °C had a significantly larger radical decay compared to storage at 10 °C. The radical content in the roasted malt changed the most during the 12 month storage, and the high storage temperature and water activity (20 °C, 0.432) conditions resulted in a significant decay in radical content to 62 % of the initial level.

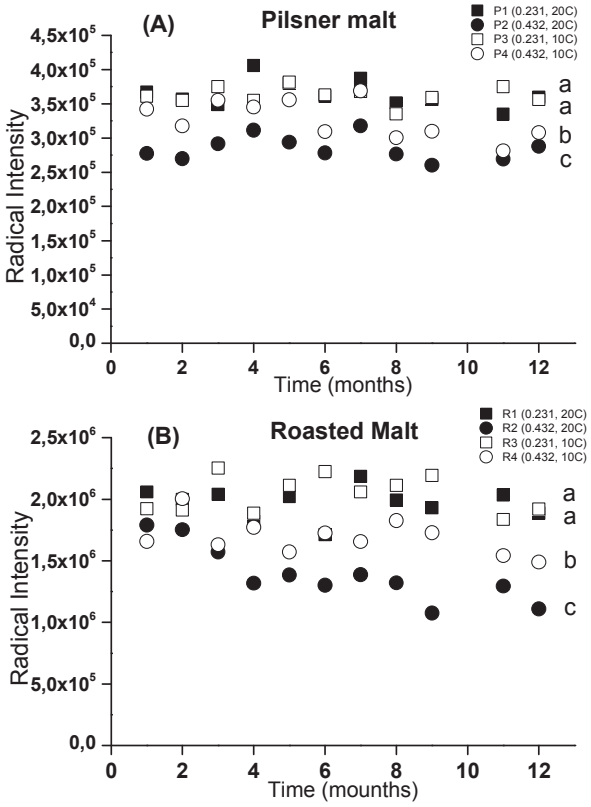

Figure 3: (A) radical content of pilsner malt stored for 12 months at 10 °C and 20 °C and at a water activity (aw) of 0.231 or 0.423). (B) Radical intensity of roasted malt stored for 12 months at 10 °C and 20 °C and at a water activity (aw) of 0.231 or 0.423). Each value is a result of an average of 10 malt kernels each corrected for their weight. Letters (a,b,c,d) indicate the statistical difference of the four treatments using "month" as a random effect ($p < 0.05$).

Sweet wort made from stored malt

Sweet worts were made from fresh (0 months) pilsner malt and roasted malt as well as malts stored for 6 and 12 months at the different storage conditions. The sweet worts were characterized by color, pH, filtration time, wort extract yield after 45 min of filtration, and sugar content (°Brix) (Table 1).

Table 1: Color, filtration time, extract yield after 45 min of filtration, pH and sugar content (°Brix) determined on wort produced from pilsner malt (P1, P2, P3, P4) and roasted malt (R1, R2, R3, R4) after 0, 6 and 12 months of storage at 10 °C or 20 °C and at water activities (aw) of 0.231 or 0.423. Values are given as means ± standard deviation (n=2). Letters indicate the statistical difference of samples within the same square, and the levels bearing different letters are significantly different (p < 0.05).

			Color (EBC)			pH			Filtration Time (min)			Yield (mL)			°Brix		
Name	a_w	Temp °C	0 m	6 m	12 m	0 m	6 m	12 m	0 m	6 m	12 m	0 m	6 m	12 m	0 m	6 m	12 m
P1	0.231	20		5.6±0.2^{a}	5.6±0.5^{a}		6.00±0.01^{c}	6.03±0.08^{bc}		34±1^{b}	49±13^{ab}		335±12^{a}	310±31^{a}		13.46±0.00^{ab}	13.46±0.00^{abcd}
P2	0.432	20	5.6± 0.1^{a}	5.4±0.2^{a}	5.6±0.0^{a}	6.01± 0.01^{abc}	5.99±0.01^{c}	6.06±0.07^{bc}	34.5± 6.4^{ab}	39±10^{ab}	42±5^{ab}	325.0± 8.5^{a}	332±8^{a}	322±3^{a}	13.46± 0.00^{a}	13.46±0.00^{abcd}	13.45±0.00^{d}
P3	0.231	10		5.4±0.1^{a}	5.3±0.2^{a}		6.02±0.01^{bc}	6.09±0.03^{ab}		46±1^{ab}	48±9^{ab}		328±0^{a}	315±7^{a}		13.46±0.00^{ab}	13.46±0.00^{abc}
P4	0.432	10		5.3±0.2^{a}	5.8±0.3^{a}		6.10±0.01^{ab}	6.15±0.00^{a}		37±2^{ab}	57±16^{a}		336±4^{a}	304±33^{a}		13.45±0.01^{bcd}	13.45±0.00^{cd}
R1	0.231	20		104.5±5.1^{b}	103.7±3.1^{b}		4.97±0.01^{d}	5.06±0.02^{a}		31±13^{b}	*69^{a}		215±4^{a}	124±86^{b}		13.38±0.00^{a}	13.39±0.00^{a}
R2	0.432	20	114.7± 6.9^{a}	104.0±0.0^{b}	103.9±1.5^{b}	5.03± 0.01^{ab}	4.96±0.03^{d}	5.00±0.03^{bcd}	33.5± 16.2^{b}	20±1^{b}	26±16^{b}	194.5± 21.9^{a}	228±1^{a}	219±9^{a}	13.38± 0.01^{a}	13.37±0.00^{ab}	13.38±0.00^{ab}
R3	0.231	10		104.4±1.2^{b}	103.9±6.8^{b}		4.99±0.01^{bcd}	5.02±0.01^{abc}		16±2^{b}	19±1^{b}		223±4^{a}	226±2^{a}		13.36±0.02^{b}	13.38±0.00^{ab}
R4	0.432	10		100.6±2.2^{b}	104.4±1.0^{b}		4.99±0.01^{cd}	5.02±0.02^{abc}		15±1^{b}	17±3^{b}		223±1^{a}	226±2^{a}		13.37±0.00^{ab}	13.37±0.01^{ab}

*single determination as filtration of the second determination came to a complete stop.

For the roasted malt the wort color was significantly darker for wort produced from the fresh malt as compared to the color of wort produced from malt stored for 6 months independent of the storage temperature and water activity. The color of the wort did not significantly change upon further storage of the malt (12 months). The color of wort made from pilsner malt was not affected by storage time and conditions. The pH of worts was constant throughout the experiment, and generally the pH of pilsner worts was approximately one unit higher than of worts made from roasted malt in line with previous findings *(6,19)*. The sugar content was also constant throughout the 12 months of storage for both types of worts. Sugar content was generally lower for the dark worts than for the pilsner worts, most likely explained by increased enzyme inactivation and starch denaturation during roasting. Filtration time of wort made from pilsner malt had a tendency to increase during malt storage whereas the filtration time of worts made from roasted malt had a tendency to decrease however, this was not significant. Only wort made from roasted malt stored at a water activity of 0.231 and at 20 °C had a significantly higher filtration time after 12 months of storage than all other worts. All results were based on a double determination, however the one filtration of the latter worts came to a complete stop during filtration, and its filtration time is therefore not included in the table. The variations of the filtration times were reflected in the extract yields, which slightly decreased for pilsner wort and increased for roasted wort during storage with the only exception of the

roasted malt stored at low water activity (0.231) and 20 °C where also the extract yield decreased due to the slow filtration.

Volatiles

The volatile profile was determined on worts produced from pilsner malts and roasted malts stored for 0, 6 and 12 months at all storage conditions. Sixty-nine volatile compounds were identified in the wort volatile profiles (Table 2). As expected the volatile profile of wort made from pilsner malt was very different from the volatile profile of wort made from roasted malt. Furthermore, the volatile profile of roasted malt changed much more during malt storage than that of pilsner malt (Figure 4).

Table 2: Volatile compounds identified in the sweet worts. The target ion (Tgt) is used for identification and the number of each compound corresponds to the location in the loadings plots (Figure 4, Figure 5).

No	Name	Tgt Ion	No	Name	Tgt Ion	No	Name	Tgt ion
1	2-methylpropanal	72	**24**	1-pentanol	42	**47**	1-(2-Furyl)-2-propanone	81
2	2-Propanone	58	**25**	dihydro-2-methyl-3(2H)-furanone	43	**48**	benzaldehyde	106
3	2-methylfuran	82	**26**	methyl-pyrazine,	94	**49**	2-Acetoxymethylfuran	81
4	2-butanone	43	**27**	2-hexanone	43	**50**	5-methyl-2-furancarboxaldehyde	53
5	2-methylbutanal	57	**28**	1-hydroxy-2-propanone	43	**51**	2-butylpyridine	94
6	3-methylbutanal	58	**29**	2,5-dimethylpyrazine	108	**52**	1-ethyl-1H-pyrrole-2-carboxaldehyde	123
7	2,5-dimethylfuran	96	**30**	2,6-dimethylpyrazine	108	**53**	5-methyl-1H-pyrrole-2-carboxaldehyde	109
8	pentanal	44	**31**	ethyl-pyrazine	107	**54**	2-methylbenzaldehyde	91
9	hexanal	56	**32**	2,3-dimethylpyrazine,	108	**55**	2-isoamyl-6-methylpyrazine	108
10	2-methyl-2-butenal	84	**33**	2-ethyl-6-methylpyrazine	121	**56**	2-isopentyl-3-methylpyrazine	108
11	2-methyl-1-propanol	43	**34**	nonanal	57	**57**	phenylacetaldehyde	91
12	5-Hydroxy-2-hexanone	43	**35**	2-ethyl-5-methylpyrazine	121	**58**	2-furanmethanol	98
13	4,5-dimethyloxazole	97	**36**	2-ethyl-3-methylpyrazine	122	**59**	2,5-dimethyl-3-(3-methylbutyl)pyrazine	122
14	3-heptanone	57	**37**	Propylpyrazine	94	**60**	N-(4-hydroxyphenyl)-acetamide	109
15	2-penten-1-ol	57	**38**	3-furfural	95	**61**	1-phenyl-2-propanone	43
16	cyclopentanone	55	**39**	5-methyl-2(3H)-Furanone	55	**62**	2(5H)-furanone	55
17	pyridine	79	**40**	ethenylpyrazine	106	**63**	4-methyl-2-propylfuran	95
18	limonene	68	**41**	2,5-dimethyl-3-ethylpyrazine	135	**64**	3-phenyl-furan	115
19	trimethyloxazole	101	**42**	furfural	38	**65**	2-methyl-3-(furyl)propenal	79
20	pyrazine	80	**43**	2-acetyl-5-methylfuran	109	**66**	methylphenylpentenal	174
21	3-methylpyridine	93	**44**	5-methylfurfural	110	**67**	phenol	94
22	2-pentylfuran	81	**45**	2-methyl-6-propylpyrazine	108	**68**	5-methyl-2-phenyl-2-hexenal	117
23	Thiazole	85	**46**	3,5-diethyl-2-methylpyrazine	149	**69**	1-furfuryl-2-formylpyrrole	81

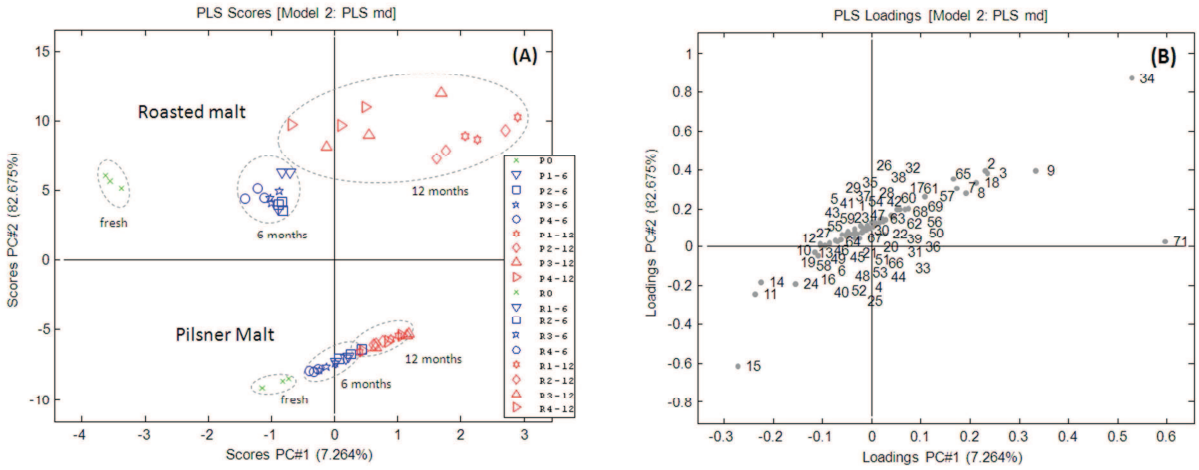

Figure 4: (A) PLS score plot based on the volatile profile of wort made from pilsner malt and roasted malt stored for 0, 6 and 12 months at 10 °C or 20 °C at a water activity (a_w) of 0.231 or 0.423 and (B) the corresponding loadings plot. Each volatile compound is represented by a number corresponding to that in Table 2. (n=3).

Analysis of the wort volatile profiles with a PCA provided detailed information on how the profiles changed during storage (Figure 5). The worts made from both pilsner malt and roasted malt was mainly influenced by a decrease of volatile compounds the first 6 months of malt storage and by formation of compounds from 6 to 12 months (data not shown). Furthermore the wort analysis showed that the malt remained uninfluenced by storage temperature and water activity within the first 6 months of storage. After 12 months of storage, the volatile profile of wort made from both pilsner malt and roasted malt was highly influenced by storage temperature. The volatile profile of wort from pilsner malt stored at 20 °C was also significantly influenced by the variation in water activity (Figure 5, 1A, 1B). However, the volatile profile of worts made from roasted malt was significantly influenced by the water activity when the malt had been stored at both 10 °C and 20 °C (Figure 5, 2A, 2B).

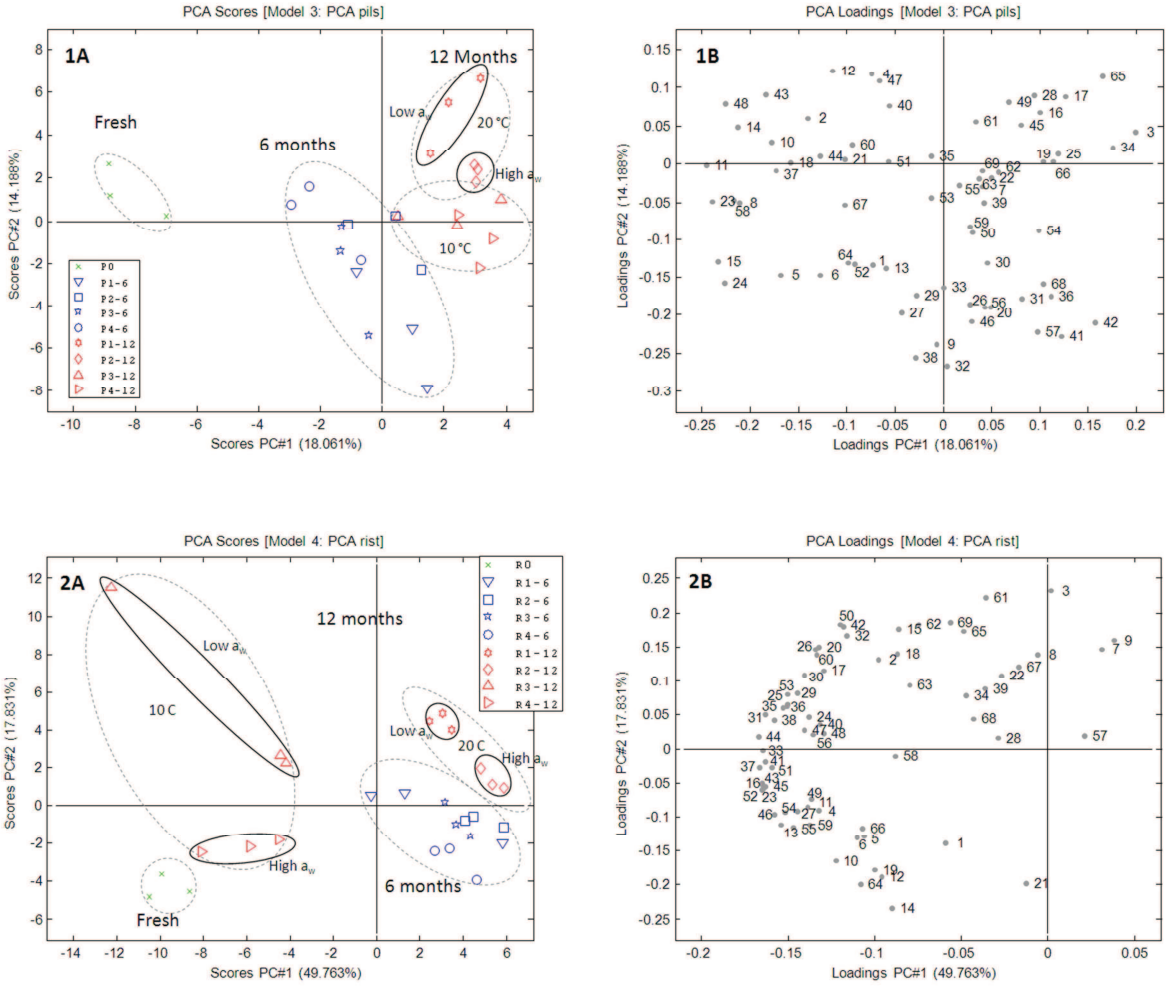

Figure 5: (1A) PCA score plot based on the total volatile profile of wort produced from pilsner malt stored for 0, 6 and 12 months at 10 °C or 20 °C at a water activity (a_w) of 0.231 or 0.423 and (1B) the corresponding loadings plot. (2A) PCA score plot based on the total volatile profile of wort produced from roasted malt stored for 0, 6 and 12 months at 10 °C or 20 °C at a water activity (a_w) of 0.231 or 0.423 and (2B) the corresponding loadings plot. Each volatile compound is represented by a number corresponding to that in Table 2. (n=3).

Storage temperature had a larger impact on the volatile profile during malt storage than the water activity. To illustrate how temperature influenced the volatile profile during storage the impact of water activity was neglected and the results averaged (Figure 6, Figure 7, Figure 8). As the roasted malt had a much more intense volatile profile compared to pilsner malt, the secondary axes are made proportional to the volatile intensity and different axes may appear for the same compound originating from pilsner malt and roasted malt (Figure 7, Figure 8). A major part of the change in volatile profile during storage is explained by a loss in volatile compounds exemplified for furanmethanol in wort from pilsner malt (Figure 6, A) and 3-heptanone in wort from roasted malt (Figure 6, B). The volatile profile of pilsner malt is furthermore characterized by a loss in alcohols and the volatile profile of roasted malt was characterized by a loss of pyrazines and ketones. Quantitatively more volatiles were lost for roasted malt compared to pilsner malt and generally more compounds are lost during storage at 20 °C compared to storage at 10 °C (Figure 6).

This loss illustrates what is likely to be the case for all volatile compounds in the malt during storage. Therefore, the compounds that are formed during storage are most likely also subject to evaporation. The degree of evaporation of each volatile is further complicated by its affinity to the malt matrix which may also be affected by the water activity.

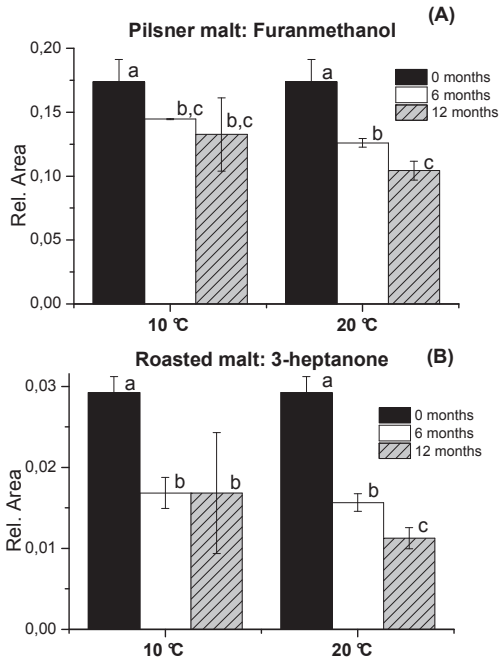

Figure 6: (A) Furanmethanol content in wort made from pilsner malt and (B) 3-heptanone content in wort made from roasted malt after 0, 6 and 12 months of storage. The relative area of the volatiles identified after storage at water activities of 0.231 and 0.423 has been averaged. 0 months (n=3), 6 and 12 months (n=6). Letters (a,b,c,d) indicate the statistical difference ($p < 0.05$).

In spite of the relatively large evaporation, compounds were also found to increase in concentration during storage. Lipid oxidation occurred, particularly in the roasted malt, expressed by the formation of nonanal, hexanal and pentanal (Figure 7). Hexanal and pentanal levels were found to increase only in roasted malt and not in pilsner malt. High temperature is known to increase lipid oxidation and despite the increased evaporation, also found in roasted malt, the concentration of hexanal was more than four times higher than in pilsner malt after 12 months storage at 20 °C. Interestingly, a slightly higher quantity of hexanal and pentanal was detected in roasted malt stored at 20 °C and at the low water activity of 0.231 compared to 0.432. The relative concentrations for pentanal was 0.0432±0.0030 at low water activity and 0.0265±0.0003 at high water activity and the relative concentration for hexanal was 0.2444±0.0078 at low water activity and 0.1307±0.0063 at high water activity. Nonanal concentration was found to increase significantly in both pilsner malt and roasted malt but was highest in the roasted malt. Also, methylfuran was formed in both pilsner and roasted malt continuously during storage and in pilsner malt also 2-methyl-3-furylpropenal and

pyridine were formed and in roasted malt also 2,5-dimethylfuran, propanone and limonene were formed (data not shown).

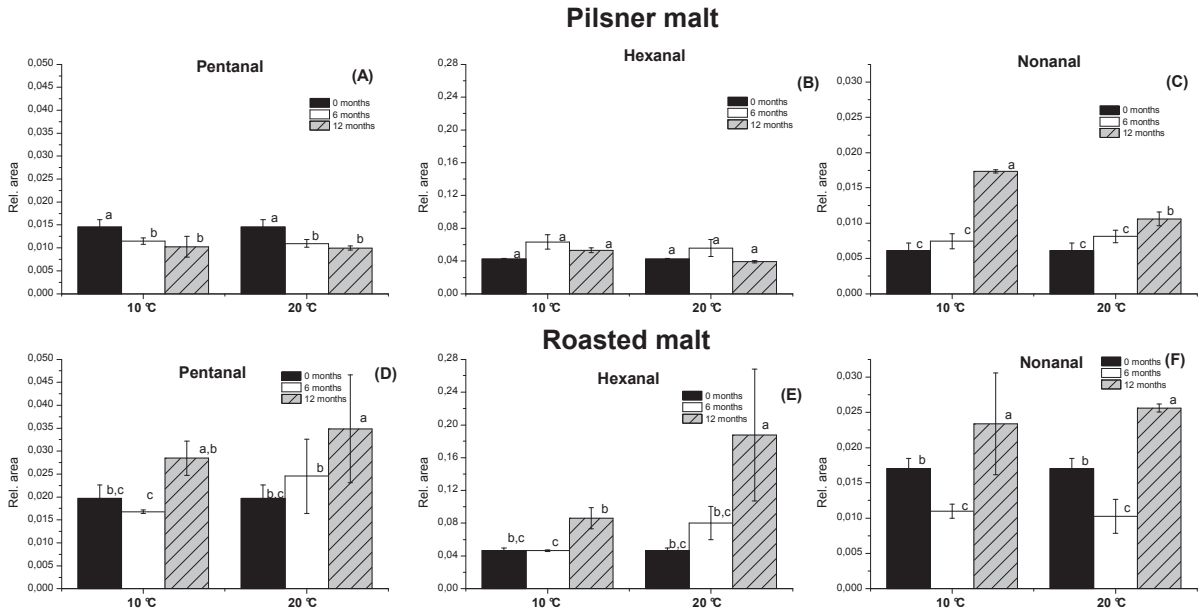

Figure 7: Lipid oxidation products in sweet wort made from pilsner malt and roasted malt after 0, 6 and 12 months of storage. Pentanal (A,D), hexanal (B,E) and Nonanal (C,F). The relative area of the volatiles identified after storage at water activities of 0.231 and 0.423 has been averaged. 0 months (n=3), 6 and 12 months (n=6). Letters (a,b,c) indicate the statistical difference ($p < 0.05$).

The Strecker aldehydes 2-methylbutanal, 3-methylbutanal and phenylacetaldehyde are all compounds characteristic for malt flavour *(2,4)* and they were present in roasted malt in much larger quantities than in pilsner malt (Figure 8), which is not surprising due to the Maillard reactions taking place during roasting. 2-methylbutanal and 3-methylbutanal tend to decrease in concentration during storage though this was only significant for storage at 20 °C, where the concentrations in roasted malt had decreased to approximately half of its original content after 12 months. Phenylacetaldehyde was formed during storage and its presence in pilsner malt was highly influenced by temperature where both evaporation and formation takes place. In roasted malt, stored for 12 months at 10 °C, there was a tendency for all three Strecker aldehydes to be present in slightly larger concentrations when stored at a water activity of 0.432 compared to 0.231. It was recently found that water treatment of malt and other dry products resulted in increased formation of phenylacetaldehyde and 2- and 3-methylbutanal *(20)* which may explain the observations in the current study.

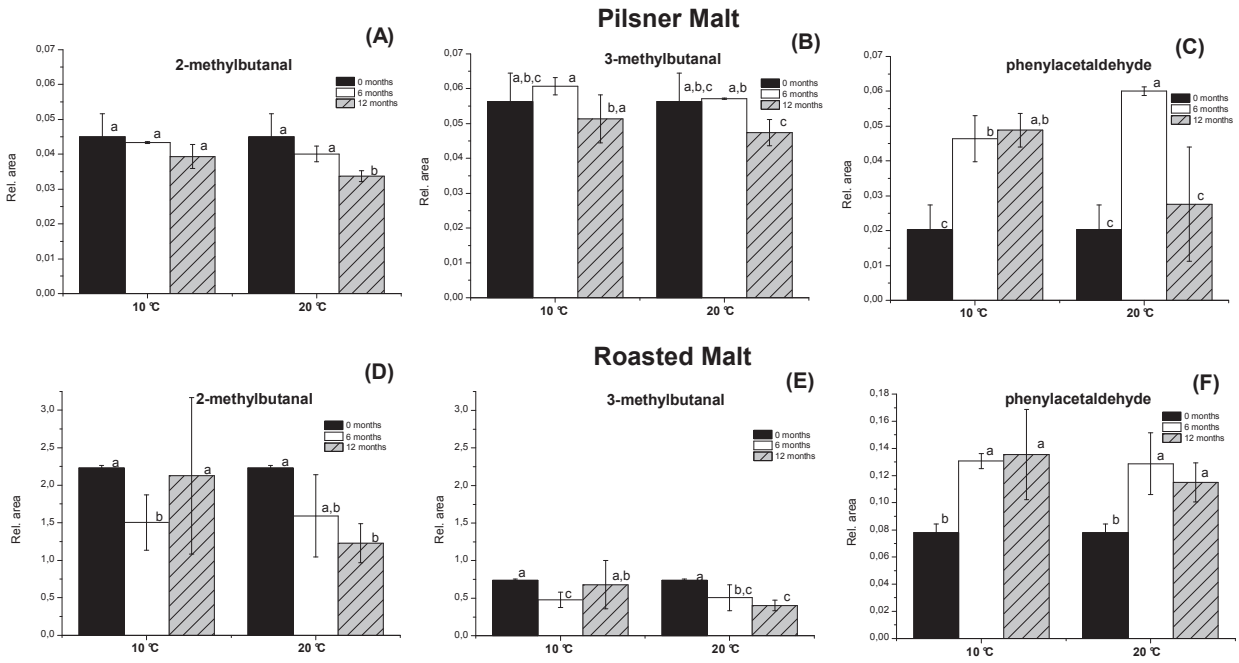

Figure 8: Strecker aldehydes in sweet wort made from pilsner malt and roasted malt after 0, 6 and 12 months of storage. 2-methylbutanal (A,D), 3-methyl butanal (B,E) and phenylacetaldehyde (C,F). The relative area of the volatiles identified after storage at water activities of 0.231 and 0.423 has been averaged. 0 months (n=3), 6 and 12 months (n=6). Letters (a,b,c) indicate the statistical difference ($p < 0.05$).

Discussion

The sugar content in the sweet wort was constant throughout malt storage showing that the major components in the malt and the starch degrading enzymatic activity were intact. However, the mobility of components in the malt depended on the water activity and temperature during the storage resulting in wort samples, where the volatile profiles and filtration rates changed during the storage of the malt.

The radical content in pilsner malt was highly correlated with water activity (R^2=0.95) indicating that water activity is an important factor for radical decay and radical reactivity in malt. The radical content of other dried products have also been found to be influenced by water activity although with less linear correlations *(9,21)*. The radical contents observed during the storage experiment with both pilsner malt and roasted malt was also influenced by water activity. At the high water activity (0.432) the storage at high temperature (20 °C) further affected the radical decay, whereas at low the water activity (0.231) the variation in storage temperature did not influence the radical content significantly. Radical content in roasted malt was approximately 7 times larger than the radical content in pilsner malt, and also the radical decay was larger for roasted malt. These results were reflected in the volatile analysis, as the volatile profile of wort made from roasted malt changed more during storage than the volatile profile of wort made from pilsner malt. Furthermore, the volatile profiles of both types of wort were highly influenced by

temperature, and thus correlating with the larger radical decay detected at 20 °C and high water activity (0.432). For the roasted malt the storage water activity had a significant influence on the volatile profile of the wort at both high (20 °C) and low (10 °C) storage temperatures after 12 months of storage. For pilsner malt the influence of storage water activity was only significant for storage at high temperature (20 °C). Some of the changes in the volatile profiles of the malts were caused by evaporation, which are independent of oxidative reactions. However, the oxidative changes detected by ESR were large enough to significantly impact the formation of volatiles detected. These results show that temperature is very important for both the rate of oxidative reactions and evaporation of volatiles and that low water activity (0.231) is a limiting factor for oxidative reactions.

Detailed studies of the volatile compounds showed that the radical decay was highly correlated to the oxidative stability of the malt in terms of development of more lipid oxidation-derived compounds such as nonanal, hexanal, and pentanal. Lipid oxidation is known to be one of the main contributors to off-flavours in beer (1). In the current study, formation of secondary oxidation products caused by lipid oxidation was not significant until after 12 months of storage (Figure 7). Hexanal and pentanal are both formed from oxidation of linoleic acid and nonanal is formed from oxidation of oleic acid *(22)*. Linoleic acid is the most abundant fatty acid in malt making up for approximately 60 % of the total lipid content followed by oleic acid making up for approximately 11 % depending on the barley variety *(23)*. The current study shows that pentanal, nonanal and hexanal in the wort is linked to the roasting of the malt and increases in concentration during the storage of the malt (Figure 7). Lipid oxidation may be caused by a combination of radical induced oxidation and lipoxygenase (LOX) induced oxidation, and the natural substrate for LOX is linoleic acid (7). The content of LOX has been found to decrease in activity during malt storage (7), however in the current study lipid oxidation products continued to increase in the wort indicating that the main part of the lipid oxidation is radical induced. Lipid oxidation became more pronounced in the worts during storage of the malts at 20 °C compared to 10 °C in accordance with existing knowledge *(24)*. An interesting observation was that a slightly higher quantity of hexanal and pentanal was detected in wort from roasted malt stored at the low water activity of 0.231 compared to 0.432 at 20 °C. As mentioned previously this could be caused by increased affinity towards the malt matrix at the lower water activity resulting in decreased evaporation, however, the reaction rate may also be influenced by the water activity. In a recent study oat meal, peanuts and pork scratchings were found to have significantly increased hexanal formation at a water activity of 0.231 compared to 0.432 (9). A similar tendency was found for oxidation of flaxseed oil where high oxidation rates were found at both low and high humidity conditions *(25)*.

The fact that a large number of compounds, including coloured Maillard reaction compounds, were lost during the first 6 months of storage correlated with the decrease in wort color (Table 1). Similar to this, Forster et al. (2) also found a correlation between colour and formation of Strecker aldehydes and N-heterocycles which were found to initially increase where after they decreased to concentrations below the initial content. In a recent study of sweet wort made from malt with colours varying from 8 to 100 EBC it was found that malt roasted to a level above 33 EBC had a large volatile loss when heated to 40 °C for 10 hours (6). This and the current study underlines the fact that malt roasting has a negative effect on the oxidative stability of beer caused by increased radical formation, increased lipid oxidation and a greater loss of volatiles during malt storage as well as in the initial stages of brewing. This is in agreement with other studies having identified a decrease in oxidative stability with increasing beer colour based on ESR lag phase measurements *(10,26)*. This suggests the oxidative stability of roasted malts can be correlated to the oxidative stability of the resulting beers.

The changes occurring during malt storage were found to be caused by a combination of 1) compound formation through oxidative processes and 2) loss of volatiles through evaporation. The volatile intensities as well as the changes in volatile profile of worts were relatively small during storage of pilsner malt compared to roasted malt. However, the changes occurring in pilsner malt may nevertheless be more important, as this malt aroma in general is more precise and delicate. The aroma of roasted malt is stronger and more complex and variations in volatile profile may to some extent be masked by its complexity.

Conclusion

The mobility of components in the malt depended on the water activity and temperature during the storage resulting in wort samples, where the volatile profiles and filtration rates changes during the storage of the malt. However, the sugar content in the sweet wort was constant throughout malt storage showing that the major components in the malt and starch degrading enzymatic activities were intact.

Malt roasting has a negative effect on the oxidative stability of beer caused by increased radical formation, increased lipid oxidation and a greater loss of volatiles during malt storage as well as in the initial stages of brewing. For both roasted malt and pilsner malt, good correlations were found between radical decay and change in volatile profile of the sweet wort, where high temperature and high water activity resulted in the largest changes. This study suggests that chemical changes occurring in malts during less than 12 months of storage may potentially affect the aroma of beer and that water activity and storage temperature both should be kept low in order to maintain a high malt quality.

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Paper II

Influence of Malt Roasting on the Oxidative Stability of Sweet Wort

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Influence of Malt Roasting on the Oxidative Stability of Sweet Wort

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ABSTRACT: Influence of malt roasting on the oxidative stability of sweet wort was evaluated based on radical intensity, volatile profile, content of transition metals (Fe and Cu) and thiols. Malt roasting had a large influence on the oxidative stability of sweet wort. Light sweet worts were more stable with low radical intensity, low Fe content, and ability to retain volatile compounds when heated. At mild roasting, the Fe content in the wort increased but remained close to constant with further roasting. Dark sweet worts were less stable with high radical intensities, high Fe content, and a decreased ability to retain volatiles. Results suggested that the Maillard reaction compounds formed during the roasting of malt are prooxidants in sweet wort. A thiol-removing capacity was observed in sweet wort, and it was gradually inhibited by malt roasting. It is possibly caused by thiol oxidizing enzymes present in the fresh malt.

KEYWORDS: *malt, sweet wort, roasting, oxidative stability, thiols, electron spin resonance spectroscopy, volatiles*

INTRODUCTION

Raw materials as well as the brewing process have a large influence on the quality and storage stability of the resulting beer. It has become evident that the extent of oxidation during the initial stages of beer brewing has a major influence on the flavor stability of the final beer.[1] Cortes et al.[2] found correlation between oxidative stabilities of boiled wort and the oxidative stabilities of the corresponding beers using electron spin resonance (ESR) spectroscopy. In both wort[3] and beer,[4] transitional metals were found to have significant effects on the oxidative stability as trace levels of Fe and Cu act as catalysts in radical generation and oxidation reactions during beer aging.

Malt is the major constituent in beer, and malt roasting results in different types of beer by influencing aroma and color. Previous malt studies have been carried out on commercial malts introducing uncertain variations due to different barley varieties and production conditions.[2,5] A standardized study of the influence of roasting alone has not been carried out. The influence of malt roasting on beer stability is preferably studied in wort as sulfite produced during fermentation has an impact on beer stability that may mask the influence of the roasting.

Thiols have recently been suggested to have an important role in oxidative processes in beer and in beer stability[6] and were recently quantified in beer with a correlation to the oxidative stability measured by ESR spectroscopy.[7] The significance of thiols in wort has not been investigated, and it remains uncertain where in the brewing process the thiols are introduced and whether they have an influence on the oxidative stability of sweet wort.

A correlation between radical intensity measured by ESR spectroscopy and stale flavor in beer has been found;[8,9] however, the possible correlation between radical intensity and the volatile profile of wort or the oxidative changes in wort over time have not been investigated. Some studies suggest that Maillard reaction products produced during malt roasting have antioxidative capacity and a positive influence on the oxidative stability of wort and beer.[5,10] However, in other studies these compounds were found to have a negative influence on wort and beer stability.[2,4] The purpose of this study was to test the effect of malt roasting on the oxidative stability of sweet wort using a standardized roasting process. The sweet worts were evaluated based on Fe and Cu content and thiol levels, whereas radical intensity and volatile profile were determined in both fresh and heat treated sweet worts.

MATERIALS AND METHODS

Chemicals. Anhydrous acetonitrile, glutathione, L-cysteine, 4-methyl-1-pentanol, 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO), and α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl) amino methane (tris) and ThioGlo 1 fluorescent thiol reagent were obtained from Merck (Darmstadt, Germany). Water was purified through a Milli-Q water purification system (Millipore, Billerica, USA). Ethanol (96%) was obtained from Kemetyl (Køge, Denmark). 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Fluka, Stenheim, Germany.

Roasting. Fresh pale ale malt (two-row spring barley (*Hordeum vulgare*), harvest 2009) was purchased from Weyermann, Bamberg, Germany through Maltbazaren, Copenhagen, Denmark. The malt was received less than five days after kilning and was roasted the following day. Malt was roasted by distributing it in a single layer on a cloth on a baking plate and heating it in an oven at 125 °C, 135 °C, 145 °C, 160 °C, or 190 °C for 50 min.

Mashing. Mashing was carried out on the 6 different malts (1 nonroasted and 5 roasted) on the first and second day after roasting, according to Analytica EBC 4.5.1 "Extract of Malt: Congress Mash",[11] with the modifications described by Frederiksen et al.[5] The 6 malts were mashed individually and named as illustrated in Table 1. Sweet wort was frozen at −80 °C.

Color. Sweet wort color was determined spectrophotometrically on a Cintra 40 Spectrofotometer (GBC, Melbourne, Australia) according to Analytica EBC 8.3 "Color":[11]

$$C = 25 \cdot f \cdot A430 \quad (1)$$

where C is the color in EBC units, f is the dilution factor, A430 is the absorbance at 430 nm, and 25 is a multiplication factor.

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Table 1. Characteristics of the 6 Sweet Worts Applied in This Study[a]

malt	roasting temp. (°C)	wort pH	wort °Bx	wort color (EBC)
1		5.82 ± 0.04	13.460 ± 0.000	8 ± 1
2	125	5.69 ± 0.05	13.462 ± 0.001	15 ± 1
3	135	5.58 ± 0.03	13.462 ± 0.002	33 ± 2
4	145	5.52 ± 0.02	13.460 ± 0.006	44 ± 4
5	160	5.38 ± 0.01	13.425 ± 0.000	68 ± 2
6	190	5.10 ± 0.10	13.380 ± 0.004	100 ± 10

[a]This included the roasting temperature, at which malt was roasted for 50 min, wort pH, wort sugar content measured in degree brix (°Bx), and wort color measured in European Brewery Convention (EBC) color units. Averages and standard deviations are based on two individual mashes.

Sugar Content (°Brix). °Brix values were determined using a refractometer (Analytic Jena, Jena, Germany).

Heat Treatment of Sweet Wort. Sweet wort was thawed in a 5 °C refrigerator overnight. An aliquot of 32 mL was transferred to a 250 mL blue cap flask and incubated in a 40 °C water bath. To avoid microbial growth, 100 μL of chloramphenicol (stock 30 mg/L ethanol) and 100 μL ampicilin (stock 100 mg/mL H_2O) were added to the sweet wort. The blue cap flask was closed with a cap to avoid evaporation, and a magnetic stirrer ensured constant stirring at 300 rpm.

Sampling for ESR measurements was carried out at 0, 1/2, 1, 2, 4, 6, 8, and 10 h. At each sampling, 6× 0.5 mL were distributed to safety-lock Eppendorf tubes, and all reactions were terminated by quick-freezing the tubes in liquid nitrogen. Samples were stored at −80 °C until analyzed.

Sampling for volatile analysis was carried out at 0 and 10 h. At regular intervals, the lid was led to match the ESR sampling process and to ensure that oxygen was present at all times.

Electron Spin Resonance (ESR). Ethanol and α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN) were added to sweet wort samples in final concentrations of 5% vol. and 40 mM, respectively, according to the method described by Frederiksen et al.[3] The sweet wort samples were subsequently incubated at 60 °C for 90 min. ESR spectra were recorded with a Miniscope MS 200 X-band spectrometer (Magnettech, Berlin, Germany) using 50 μL micropipets as sample cells (Brand GMBH, Wertheim, Germany). Spectra were recorded at room temperature using the following settings: microwave power, 10 mW; sweep width, 49,82 G; sweep time, 30 s; steps, 4096; number of passes, 6; modulation, 100 mG; and microwave attenuation, 5 mW. The amplitudes of the spectra were determined and are reported as the height of the first doublet. ESR spectra of an aqueous 2 μM 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) solution were recorded each day to compensate for day-to-day variation.

Transition Metals (Fe and Cu). Sweet wort samples were acid digested in a microwave oven using the solvents and temperature program detailed in Wyrzykowska et al. [12] The multielemental composition of sample digests were subsequently analyzed using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Perkin-Elmer 3300, United States) with the spectrometer equipped with an octopole reaction cell for interference removal (Agilent 7500ce, Manchester, UK) following the instrumental settings listed in Hansen et al.[13] Samples were determined in duplicate.

Thiol Levels Determined Using Fluorescent Reagent ThioGlo 1. Thiol levels were determined according to the standard addition procedure described by Lund and Andersen[7] based on the thiol reacting fluorescent reagent ThioGlo 1. Glutathione was replaced with cysteine for the standard addition curve, and analyses were carried out using microtiter plates where cysteine was added in concentrations between 0 and 20 μM to sweet wort diluted 40 times. Each standard addition curve was made in triplicate on each plate, and the average represents a single analysis. For each sweet wort sample, the single analysis was carried out in independent triplicates.

Thiol Levels Determined Using Ellman's Reagent, DTNB. The thiol level in one sweet wort sample (9.8 EBC) was determined spectrophotometrically after derivatization by Ellman's reagent, DTNB. The experiment was carried out based on the method described in Jongberg et al.,[14] and a standard curve of cysteine and a standard addition curve of cysteine and sweet wort diluted 40 times were constructed. Analysis was carried out by mixing 250 μL of sample, 1.00 mL of 0.25 M tris buffer (pH 7.5), and 250 μL of 10 mM DTNB dissolved in 0.25 M TRIS buffer (pH 7.5). The mixture was protected against light and allowed to react for exactly 30 min, and absorbance was measured at 412 nm. Blanks without DTNB and blanks without sample were prepared, and the blank values were subtracted from the sample values.

Volatiles. Headspace analysis was carried out in triplicate using 5 mL of sweet wort and 1.00 mL of 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 20 min. Separation and detection were carried out on 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, California) using a polar wax GC-column. Details on equipment and temperature program are found in Deza-Durand and Petersen.[15] Volatile compounds were tentatively 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, California), was used for data analysis. Concentrations were calculated as peak area of the volatile compound divided by the peak area of internal standard.

To increase the sensitivity for detection of (*E*)-2-nonenal, selected ion monitoring (SIM) was applied using ions 55 and 83, and a standard curve was created for quantification. Limit of quantification and detection were calculated from the average signal-to-noise ratio of two peaks from beer spiked with 0.8 μg·L^{-1} (*E*)-2-nonenal resulting in a quantification limit of 0.25 μg·L^{-1} and a detection limit of 0.07 μg·L^{-1} with a signal-to-noise ratio of 10 and 3, respectively.

Multivariate Data Analysis. Multivariate data analysis was applied to GC-MS data to describe the change in volatile profile of heat treated sweet worts over 10 h using principal component analysis (PCA) and to determine the correlation of the volatile profile to EBC color using partial least squares (PLS) regression analysis. PCA and PLS regression analyses were performed using Latentix software (LatentiXTM 2.0, Latent5, Copenhagen, Denmark, www.latentix.com). Analyses were carried out on the relative areas calculated as peak area of the volatile compound divided by the peak area of internal standard (4-methyl-1-pentanol). Data were auto scaled and cross-validated.

RESULTS AND DISCUSSION

Sweet Wort Characteristics. Differently roasted malts were prepared by heating a pale ale malt for 50 min at 5 different temperatures. Duplicate congress mashings were carried out for each of the six malts (including the pale ale malt), and the pH, sugar content, and color of the fresh sweet worts were determined (Table 1). Sweet wort color was highly correlated to malt roasting temperature (R^2 = 0.99) and was accordingly used as a marker of the roasting level. pH was negatively correlated to sweet wort color (R^2 = 0.99) in agreement with previous findings.[5] The sugar content in the sweet worts was lower for malt roasted at the two highest temperatures (sweet worts 5 and 6) than for the four malts roasted at lower temperatures (sweet worts 1 to 4). This is most likely due to increased enzyme inactivation as well as starch denaturation. All malts were made from the same batch of pale ale malt, and the observed differences can therefore be directly associated with their different levels of roasting.

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Oxidative Stability of Sweet Worts. The oxidative stability of the sweet worts was examined by incubating them at 40 °C with access to atmospheric oxygen for 10 h and then testing the radical forming ability of samples collected at different times. The ability of radical formation was examined by quantification of the amount of radicals measured by ESR spectroscopy generated after heating the samples at 60 °C for 90 min with added spin trap, POBN, and 5% ethanol.[4] The amplitude of the first doublet of each ESR spectrum recorded during the heat treatment was used as a measure of the amount of radicals generated (Figure 1A). The results showed that the

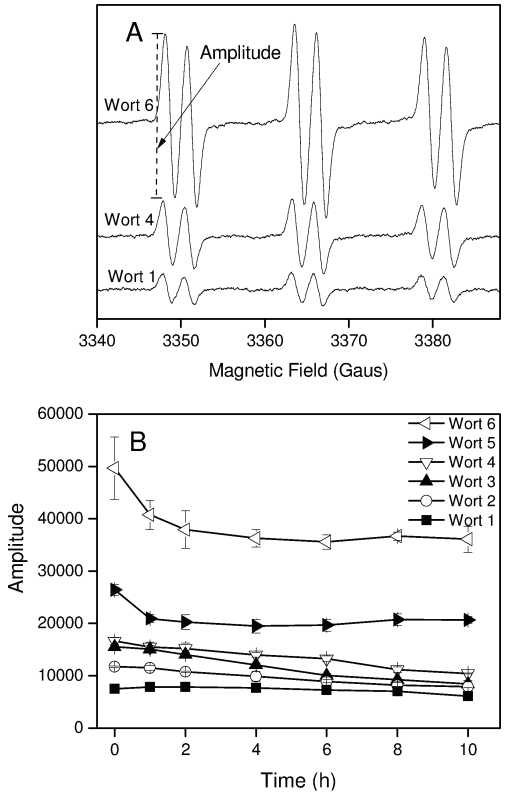

Figure 1. (A) ESR spectra of POBN spin adducts formed in fresh sweet worts 1, 4, and 6 after 90 min of incubation at 60 °C with 5% ethanol and 40 mM POBN spin trap. (B) The time dependence of the different radical formation capabilities of sweet worts during incubation at 40 °C. The capability of radical formation was evaluated by ESR spectroscopy by heating collected samples 90 min at 60 °C with added POBN and 5% ethanol. Results are shown as mean values, and standard deviations are given as error bars (n = 3).

formation of radicals in dark sweet worts was higher than that in lighter sweet worts (Figure 1B), which is consistent with previous observations.[2] The ability of radical formation decreased in the darkest sweet worts, 5 and 6, within the first hour of storage at 40 °C but still had a constant and high level during the following 9 h of incubation. The lighter sweet worts, 1, 2, 3, and 4, showed a lower but more constant decrease in radical formation over the 10 h; however, the difference in radical formation from 0 to 10 h of heat treatment decreased from sweet wort 4 to sweet wort 1 where the radical formation in sweet wort 1 was almost constant throughout the 10 h (Figure 1B). These results show that dark sweet worts, 5 and 6, are more oxidatively unstable than lighter sweet worts, and even though the storage at 40 °C had a larger influence on the ability of dark sweet worts to generate radicals compared to light sweet worts, they still had a high capacity for generation of radicals after 10 h at 40 °C.

The presence of Fe and Cu may promote metal-catalyzed oxidation and thereby affect the radical formation and the oxidative stability.[4] The transition metals, Fe and Cu, were therefore quantified in each of the six sweet worts. Malt roasting was found to have a large influence on Fe and Cu contents in the sweet worts but in different ways. The Cu concentration decreased from the lighter sweet wort 1 to the darker sweet worts 4, 5, and 6 (Figure 2). The concentrations

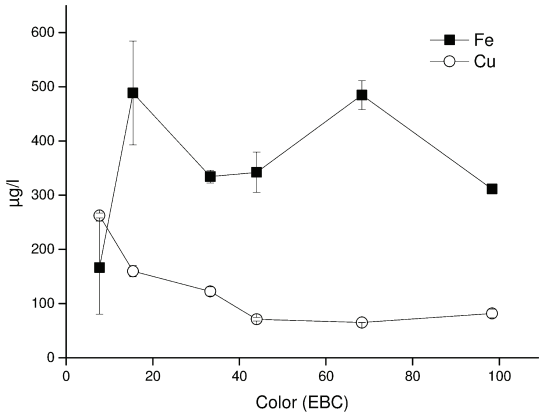

Figure 2. Fe and Cu content in sweet worts 1 to 6 plotted against color (EBC). Results are shown as mean values, and standard deviations are given as error bars (n = 2).

of Fe were generally higher, than the levels of Cu, except in sweet wort 1, which had a considerably lower level of Fe than the other sweet worts. Since the same pale ale malt was used for all malt roastings, the total metal content introduced into the mashings were the same in the six mashes. The variations observed in Fe and Cu contents in the sweet worts caused by the roastings must therefore be explained by different abilities of the malt solids to bind metals. Supporting this explanation, Frederiksen et al.[3] found that Fe and Cu added during mashing was trapped by insoluble malt solids resulting in an only slight increase in Fe and Cu content in the sweet wort after filtration. Roasting of the pale ale malt therefore reduces the ability of the malt solids to bind Fe but improves the ability to bind Cu. The fact that radical signal intensity in sweet wort increases with increasing malt roasting temperature where as the concentration of Cu decreases and Fe remains somewhat constant suggests that other components in the dark sweet worts also affect the oxidative stability.

Thiols in Sweet Wort. Protein-thiols have been suggested to have antioxidative activity in beer,[6] and their presence in sweet wort was investigated for their possible influence on the oxidative stability in the present study. Quantification of thiols in the sweet worts was attempted by using the standard addition procedure described by Lund and Andersen[7] and an external standard curve procedure, both of which are based on the thiol reacting fluorescent reagent ThioGlo 1. However, the external standard curve procedure was rejected due to the large variation in color of the sweet wort samples resulting in reduced excitation of ThioGlo 1 in dark samples compared to that of the uncolored standards and thereby resulting in inaccurate quantification of thiols. The standard addition

procedure was therefore tested in order to solve this problem; however, the sweet worts did not give rise to fluorescence emission typical for thiol-ThioGlo 1 derivatives. Upon addition of cysteine, the fluorescence emission was only observed when cysteine was added in concentrations higher than 8 μM (Figure 3). Dilution of the sweet wort resulted in fluorescence

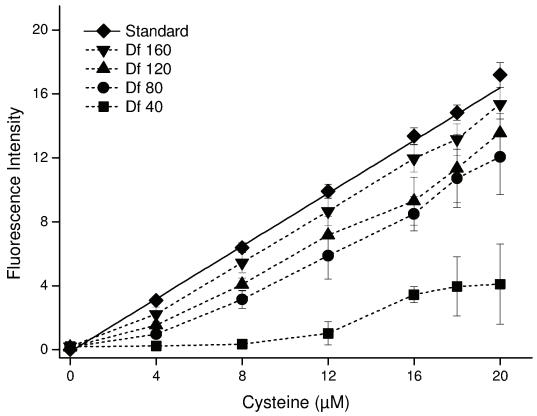

Figure 3. Thiol determination based on derivatization with the fluorescent reagent ThioGlo 1: standard addition curves of sweet pilsner wort (6.4 EBC) with cysteine added at concentrations between 0 and 20 μM. Wort was diluted with buffer between 40 and 160 times (Df 40−160). The standard curve of cysteine in buffer is illustrated with a regression line (standard). Results are shown as mean values, and standard deviations are given as error bars (n = 3).

responses, which approached the behavior of cysteine added to a pure buffer solution (Figure 3). The results demonstrate that the sweet wort contained compounds able to oxidize the cysteine added to the sweet wort. This effect will be referred to as thiol-removing capacity. The smallest dilution factor of 40 was chosen in order to minimize background fluorescence of the sweet wort sample as previously described for beer.[7] Glutathione was also oxidized when added to the sweet wort. However, it seemed to be less sensitive to oxidation than cysteine as a larger concentration of sweet wort was required to inhibit the emission of ThioGlo 1 derivatives (data not shown). Muller[16] found that addition of glutathione to a 65 °C mash under aerobic conditions resulted in an accelerated oxidation of glutathione compared to a simulated mash carried out on a solution of glutathione alone. The simulated mashing of glutathione carried out under anaerobic conditions inhibited all oxidation of glutathione. The results from the current study strongly support the theory about constituents of malt enhancing oxidation of not only glutathione but also cysteine. However, in previous studies, thiols have been detected in light-colored sweet worts.[16−18] In these studies, Ellman's reagent, DTNB, was applied for thiol determination. To verify that the current results were not caused by the different choice of thiol derivatizing agent, the standard addition procedure was tested on a sweet wort using the DTNB method. As shown in Figure 4 the same thiol-removing capacity was detected, which excludes that the difference in derivatization agent is the reason for the difference in results. Stephenson et al.[18] found that the presence of oxygen had a negative influence on the content of thiols in sweet wort based on aerobic and anaerobic mashing trials, and Jin et al.[17] detected thiols in sweet wort produced on an industrial scale, likely to be carried out under anaerobic conditions. The EBC congress mash method, applied

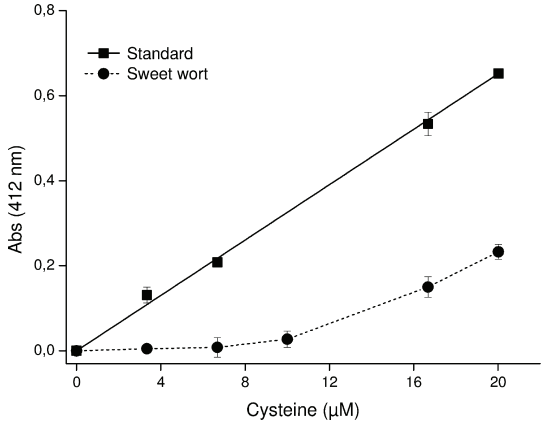

Figure 4. Thiol determination based on derivatization with DTNB: standard curve of cysteine and standard addition curve of sweet pilsner wort (9.8 EBC). Results are shown as mean values, and standard deviations are given as error bars (n = 2).

in the current study, included aerobic conditions during both mashing and filtration. Therefore, an analysis of thiol-removing capacity in sweet wort produced on an industrial scale was also carried out; however, the same thiol-removing capacity was detected (data not shown). This suggests that access to oxygen during mashing does not explain the presence of the thiol-removing capacity.

Malt roasting proved to influence the thiol-removing capacity in sweet wort, which decreased with an increasing level of roasting (Figure 5). Sweet wort 1 (not roasted) had thiol-

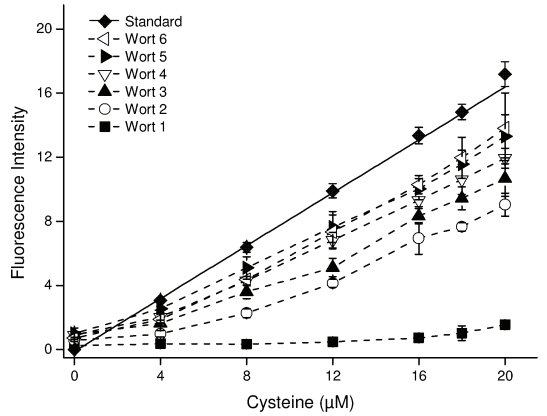

Figure 5. Thiol determination based on derivatization with the fluorescent reagent ThioGlo 1: standard addition curves of sweet wort 1 to 6 with cysteine added at concentrations between 0 and 20 μM. Worts were diluted 40 times, and the standard curve of cysteine in buffer is illustrated with a regression line (standard). Results are shown as mean values, and standard deviations are given as error bars (n = 3).

removing capacity enabling it to consume more than 15 μM of cysteine when diluted 40 times. A large part of the ability to oxidize cysteine was lost when malt was slightly roasted (sweet wort 2, malt roasted at 125 °C for 50 min). Further roasting of the malt lowered the thiol-removing capacity in the sweet wort until a roasting temperature of 160 °C (sweet wort 5). The difference between roasting at 160 and 190 °C was very small indicating that further roasting would not affect the thiol-removing capacity. Bamforth et al.[19] found a heat stable

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enzyme, present in fresh malt, capable of oxidizing thiol groups (cysteine, glutathione, and dithiothreitol) resulting in disulfide cross-linking. The enzyme is inactivated by autoclaving but retained approximately 70% of its activity after heating to 70 °C. Analyses in the current study were carried out on fresh malt with a mash out temperature of 70 °C, and according to Bamforth et al.,[19] the enzyme was therefore likely to be present in the sweet wort. Hence, the decrease in thiol-removing capacity from sweet wort 2 to 6 could be explained by increasing enzyme inactivation with increasing roasting temperature. If this is the case, the large difference in thiol-removing capacity between sweet wort 1 and 2 may reflect the lack of roasting of sweet wort 1 and thereby the lack of inactivation of the thiol oxidizing enzyme.

Also, nonenzymatic oxidation of thiols has been suggested to occur in sweet wort,[18,19] and the decrease in thiol-removing capacity in malt roasted at temperatures above 125 °C might also be influenced by nonenzymatic reactions. Samaras et al.[20] found that the total amount of phenolic compounds in wort decrease during the roasting of malt indicating that the phenols are oxidized and likely to produce more quinones. Thiol reactive quinones are typically generated through oxidation of phenols and have been found to react with thiols in both wine[21] and in myofibrillar proteins[22] forming thiol-quinone adducts. However, if the quinones were to be responsible for the removal of thiols in wort by forming thiol-quinone adducts, the thiol-removing capacity should increase with increasing roasting due to the formation of increased amounts of quinone; hence, this explanation is rejected. The Maillard reaction products formed during roasting may also be excluded as main contributors to the thiol-removing capacity as these compounds also increase in quantity with increasing roasting.

Volatile Compounds in Sweet Wort. Analysis of volatile compounds was carried out on fresh sweet wort and on sweet wort heat treated at 40 °C for 10 h. The results showed that many volatiles in the darker worts decreased in concentration after 10 h of heat treatment, whereas the concentration in the light sweet worts remained constant. A gas chromatogram and an example of the loss in volatiles are shown for sweet wort 6 in Figure 6. PCA was performed (Figure 7), based on the 49 volatile compounds listed in Table 2. Principal component (PC) 1 explains the variation caused by malt roasting with

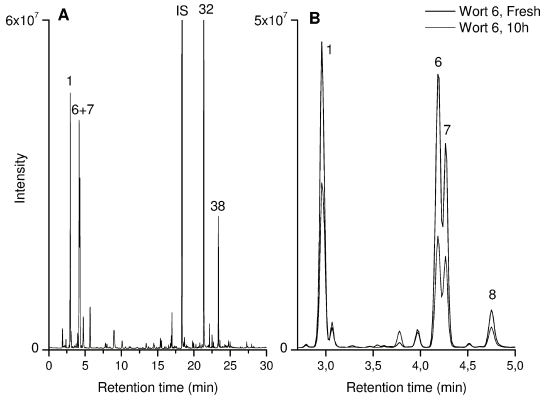

Figure 6. (A) Gas chromatogram of fresh sweet wort 6. (B) Gas chromatogram for fresh and heat treated (10 h) sweet wort 6 presented from retention time 2.5 to 5.0 min. Numbers refer to the compounds in Table 2. Internal standard (IS) is 4-methyl-1-pentanol.

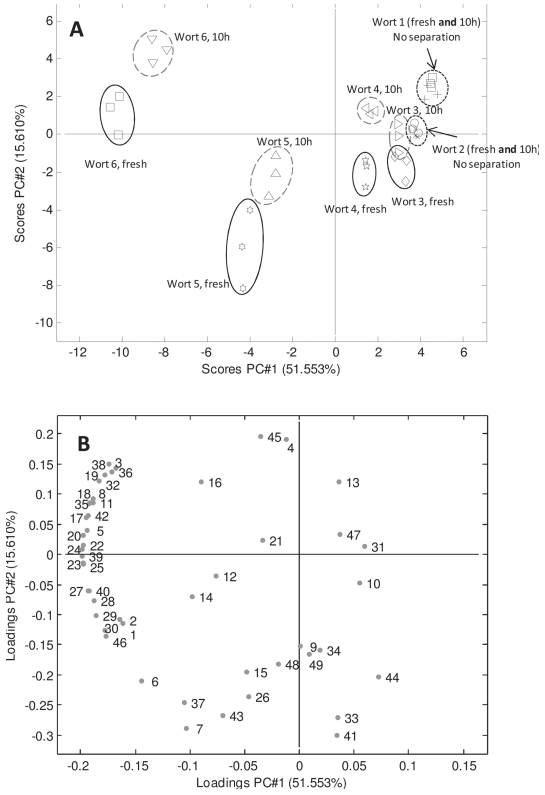

Figure 7. (A) Principal component analysis (PCA) score plot of volatile compounds in both fresh and heat treated sweet worts 1 to 6. The dotted oval encloses both fresh sweet worts and sweet worts heat treated at 40 °C for 10 h. The solid oval encloses fresh sweet worts. The dashed oval encloses sweet worts heat treated at 40 °C for 10 h. (B) Loadings plot. Each volatile compound is represented by a number corresponding to that in Table 2. Each volatile profile was determined in triplicate.

increasing concentrations of Maillard reaction products going from right to left. PC 2 explains the variation between heat treated and fresh sweet worts. The PCA score plot illustrates that sweet worts 1 and 2 were completely unaffected by 10 h of heat treatment (no separation between the fresh and heat treated sweet worts in Figure 7A). Sweet wort 3 showed a minor loss in volatile compounds, while heat treatment of sweet worts 4, 5, and 6 resulted in a larger loss of volatile compounds.

Approximately the same relative loss of volatile compounds was found for sweet worts 4, 5, and 6, and the average loss of the 10 volatile compounds showing the largest loss during heat treatment, compared to the values in fresh sweet wort, is listed in Table 3. This loss shows that the heat induced change of the malt matrix during the roasting process affects the ability of the sweet worts to retain the volatile compounds when exposed to heat and that malt roasting at temperatures between 145 and 190 °C result in the same relative loss of volatiles. Ethyl-dichloro acetate was added to the sweet worts before sampling and was released from sweet worts 4, 5, and 6 to the same degree as the volatiles from malt, showing that the malt matrix (and the degree of roasting) changes the retentivity of volatiles whether they come from the malt or not.

No volatile oxidation products were released after 10 h of heat treatment of sweet wort, uninfluenced by the degree of roasting. The staling compound (*E*)-2-nonenal, which with the

Table 2. Volatile Compounds in the 6 Sweet Worts (Heat Treated and Non-Heat Treated) Included in the PCA and PLS Analyses[a]

no.	name	Tgt ion	no.	name	Tgt ion	no.	name	Tgt ion
1	2-methylpropanal	43	18	thiazole	85	35	2-acetylfuran	95
2	2-propanone	43	19	2-methyltetrahydrofuran-3-one	43	36	furfuryl acetate	81
3	ethyl formate	31	20	methylpyrazine	94	37	2-methylpropanoic acid	43
4	ethyl acetate	43	21	octanal	41	38	5-methylfurfural	110
5	2-butanone	43	22	2,6-dimethylpyrazine	108	39	methylpyrazine	94
6	2-methylbutanal	41	23	ethylpyrazine	107	40	2-isoamyl-6-methylpyrazine	108
7	3-methylbutanal	41	24	2,3-dimethylpyrazine	67	41	benzeneacetaldehyde	91
8	3-methyl-2-butanone	43	25	2-ethyl-6-methylpyrazine	121	42	furfuryl alcohol	98
9	toluene	91	26	nonanal	41	43	3-methylbutanoic acid	60
10	3-methyl-pentanal	57	27	2-ethyl-3-methyl-pyrazine	122	44	2-methylbenzaldehyde	91
11	2,3-pentane-dione	43	28	dispirol-nonanone	94	45	ethylbenzaldehyde	133
12	dimethyldisulfide	94	29	ethyl pyrazine	106	46	furfuryl pyrrole	81
13	hexanal	56	30	3-ethyl-2,5-dimethylpyrazine	135	47	2-methyl-2,2-dimethyl-propanoic acid	71
14	2-methyl-1-propanol	43	31	acetic acid	60	48	benzothiazole	135
15	butanol	56	32	furfural	96	49	phenol	94
16	heptanal	70	33	2-ethyl-hexanol	57	-	ethyl-dichloro acetate[b]	29
17	pyrazine	80	34	decanal	57			

[a]The target ion (Tgt) is used for identification, and the number of each compound is based on the order of the retention time on the GC chromatogram. [b]Not included in the PCA model.

Table 3. Relative Loss of the 10 Volatile Compounds, for Sweet Worts 4, 5, and 6, Showing the Largest Loss after 10 h of Heat Treatment at 40 °C Compared to That of Fresh Sweet Wort[a]

no.	name	%	SD
6	2-methylbutanal	56.2	3.3
44	2-methylbenzaldehyde	54.2	38.7
9	toluene	53.2	31.2
10	3-methylbutanal	49.6	4.5
43	3-methylbutanoic acid	45.4	10.2
31	acetic acid	36.9	8.6
37	isobutyric acid	33.6	12.3
13	hexanal	33.2	19.9
36	furfuryl acetate	28.7	7.8
34	decanal	25.7	8.2
	ethyl-dichloro acetate	67.3	7.6

[a]Results are presented as an average loss in percent (%) with standard deviations (SD). Ethyl-dichloro acetate was added to all worts before heat treatment and was also lost during heat treatment.

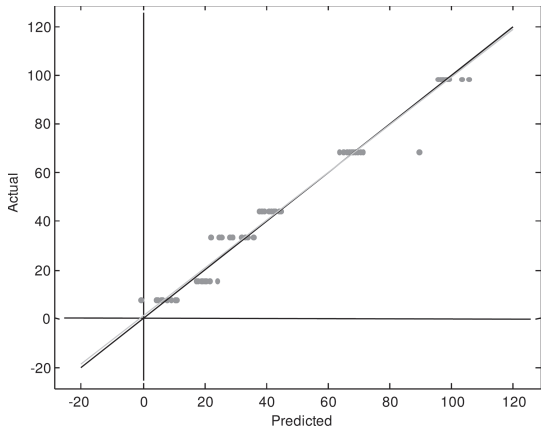

Figure 8. Partial least-squares (PLS) regression analysis of the volatile compounds in sweet worts 1 to 6 versus EBC color based on the first 4 principal components. The coefficient of correlation (r^2) is 0.969, and the root mean square error (RMSE) is 5.6 EBC units. The model is based on the same data set used in Figure 7.

current method could be detected down to 0.07 $\mu g \cdot L^{-1}$, was also not detected. Previous studies have shown that oxidation occurs during wort boiling[23,24] and that even though (*E*)-2-nonenal is not detected in either sweet wort or boiled wort, precursors may have been generated resulting in a release of the staling compound during beer aging,[25,26] as may also be the case for other secondary oxidation products.

The volatile profile of beer is highly influenced by the release of volatile fermentation products. However, the volatile profile of malt[27,28] is very similar to the volatile profile of sweet wort found in this study (Table 2) showing that the mashing process does not change the volatile profile of the sweet wort significantly from the volatile profile of malt. A high correlation between the sampled volatiles and EBC color was found (R^2 = 0.97) using PLS regression analysis (Figure 8). This suggests that the Maillard reaction products produced during malt roasting are the main ones responsible for both color change and the volatile profile of the sweet wort in this study. However, in a recent study color formation and flavor formation at higher EBC values were found to be nonlinear.[29]

Increased Roasting of Malt Results in Less Stable Sweet Worts. This study shows that malt roasting has a larger influence on the oxidative stability of sweet wort and that light and dark sweet worts behave very differently. Light sweet worts were less reactive toward oxidation with low radical intensity and low Fe content, and showed no loss in volatiles when stored at an elevated temperature over an extended period of time. The dark sweet worts were found to be less stable with high radical intensities, high Fe content, and a decreased ability to retain volatiles. It is well known that Maillard reaction compounds are produced during malt roasting; however, whether they act as antioxidants[5,10,20,29] or prooxidants[2,4,30] have been widely discussed. These contradicting results may be caused by the difference in methods applied. While the ESR-based experiments in the present study are based on a complete wort system providing information about the competition

between pro- and antioxidants, antioxidant assays have typically been based on simple model systems measuring the scavenging activity using a semistable radical that has high reactivity toward many types of compounds. Therefore, studies based on these radical scavenging activity assays often result in antioxidative activity, whereas prooxidative activities are neglected. The present study showed that malt roasting, radical intensity, and Fe content are closely linked suggesting that the Maillard reaction compounds act as prooxidants in sweet wort. Reductones are formed during the Maillard reaction and act prooxidatively by driving the Fenton reaction as shown in Figure 9, where Fe(III) is reduced to Fe(II) by the reductones

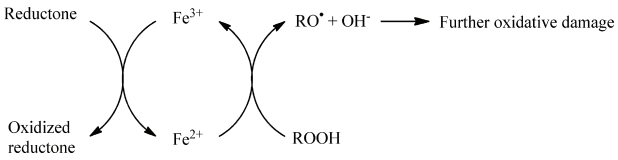

Figure 9. Reaction mechanism describing the prooxidative effect of reductones driving the Fenton reaction in sweet wort by reducing Fe(III) to Fe(II) leading to the oxidation of peroxides and the formation of reactive radical compounds.

leading to oxidation of peroxides and formation of reactive radical compounds which may induce further oxidative damage. This reaction could explain the decrease in oxidative stability of sweet worts produced by highly roasted malts. Contrary to these mechanisms, light sweet worts had a high Cu content and high thiol-removing capacity, whereas dark sweet worts had low Cu content and low thiol-removing capacity. Cu may also work as a catalyst in radical generation, but in the present study, Cu was found not to be correlated to roasting, which suggest that Cu is of minor importance in the oxidative stability of sweet worts. The thiol-removing capacity was gradually inhibited by malt roasting, and this capacity may be explained by the remaining activity of the thiol oxidizing enzyme in the light malts, which is being heat inactivated during roasting.[19] How or if the roasting influences the thiol content is uncertain. Despite the fact that the dark sweet worts had a high radical generation, no oxidation products were found from the analysis of volatiles. However, the dark sweet worts had another important characteristic as they showed a loss of volatiles over time in contrast to the light sweet worts. Therefore, to preserve the volatile profile of sweet wort produced from dark malts, the storage time should be held at a minimum to prevent initiation of oxidative reactions and to limit the loss of volatiles.

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Notes

The authors declare no competing financial interest.

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Paper III

Influence of Barley Varieties on Wort Quality and Performance

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Influence of Barley Varieties on Wort Quality and Performance

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ABSTRACT: Wort from the barley varieties (*Hordeum vulgare*) Pallas, Fero, and Archer grown on the same location were investigated for their influence on oxidative stability and volatile profile during wort processing. Barley varieties had a small influence on radical formation, thiol-removing capacity, and volatile profile. Wort boiling with and without hops had a large influence on these same parameters. Potentially antioxidative thiols were oxidized in sweet wort, but reduction of thiols using tris(2-carboxyethyl)phosphine hydrochloride revealed that Archer wort had a significantly larger content of total thiols than Pallas and Fero. Oxidized thiols resulted in gel proteins and longer filtration time for Archer wort. Our study shows that wort processing to a large extent will eliminate variations in volatile profile and thiol levels in wort which otherwise might arise from different barley varieties.

KEYWORDS: *barley variety, wort, thiols, oxidative stability, volatiles, boiling*

INTRODUCTION

Raw materials as well as oxidative reactions in the initial stages of beer brewing can have a large influence on quality and storage stability of the final beer.[1] Recently, a correlation was found between the oxidative stability of boiled wort and the oxidative stability of the corresponding beers measured by electron spin resonance (ESR) spectroscopy, [2] underlining the importance of wort quality in relation to beer quality. How the barley variety influences the oxidative stability of the wort remains uncertain; however, antioxidative potentials have been observed to vary between different barley varieties.[3] Detailed investigations of barley and malt in relation to beer stability is preferably studied using wort, as the fermentation process strongly influences the overall oxidative stability as well as the volatile profile, making it difficult to isolate the effects of barley and malt.

Protein thiols have been suggested to have antioxidative properties in beer,[4,5] and thiols have been quantified in beer exhibiting a high correlation to oxidative stability measured by ESR spectroscopy.[6] The thiols present in beer either come from the malt and are carried through to the beer as heat-stable proteins or smaller peptides or are produced by the yeast during fermentation. Upon oxidation the thiols form disulfide bonds which in the initial stages of brewing leads to protein complexes, also called gel proteins. High amounts of gel proteins in the mash have proved to result in longer filtration times,[7−9] which is unwanted by the brewers. In a recent study it was found that sweet wort contained compounds able to oxidize cysteine when it was added to sweet wort, and this capacity was named the thiol-removing capacity.[10] It was further found that increasing malt roasting resulted in a decrease in the thiol-removing capacity and that pilsner malt had a smaller thiol-removing capacity than pale ale malt. These observations were ascribed to the presence of a thiol-oxidising enzyme previously described by Bamforth et al.[21] How wort boiling and hopping influence on thiol content and thiol-removing capacity remains unknown but is important for understanding of the role of thiols and how they are preserved throughout the brewing process.

Often pilsner malt is chosen based on its malting and brewing performance with the purpose of increasing output. The idea of choosing a barley variety based on its contribution to the flavor profile or flavor stability of the final beer has received much less attention, and it is not clear to which extent the barley variety can be used to influence beer flavor and oxidative stability of wort and beer.

The aim of this study was to investigate how the barley varieties, wort boiling, and hopping influence the oxidative stability of wort as well as the volatile profile. It remains unclear how or if it is possible to select a certain malting barley variety that influences the oxidative stability of the final beer. Often such comparisons between different raw materials are difficult to make as the growing and malting conditions are difficult to standardize. In this study three two-row barley varieties (*Hordeum vulgare*) Pallas, Fero, and Archer were grown on the same location and harvested and malted simultaneously in order to minimize the effects of different handling. Furthermore, in an attempt to clarify reactions of thiols during the initial stages of the brewing process this investigation was also carried out with the purpose of investigating how the thiol-removing capacity responded to cysteine as well as glutathione.

METHODS

Chemicals. Acetonitrile, glutathione, 4-methyl-1-pentanol, tris (2-carboxyethyl)phosphine hydrochloride (TCEP), and 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl)amino methane (tris), ThioGlo 1 fluorescent thiol reagent, and trifluoroacetic acid (TFA,

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>99.8%) were obtained from Merck (Darmstadt, Germany). Bradford Bio-Rad Protein Assay Reagent was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Bovine Serum Albumin (BSA) standard of 2.0 mg/mL was obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Megazyme assay kit for determination of β-glucans was purchased from Megazyme International Ireland (Wicklow, Ireland). Ethanol (96%) was obtained from Kemetyl (Køge, Denmark). All chemicals were of analytical grade or highest possible purity. Water was purified through a Milli-Q water purification system (Millipore, Billerica, USA).

Malt. The three two-row barley varieties (*H. vulgare*) Pallas, Fero, and Archer were grown on the same location "Fuglebjerggaard" at Zealand, Denmark and harvested in 2009. The chosen varieties are former commercially grown varieties in Denmark and of renewed interest today for organic farming. To be sure to overcome dormancy, the barley was malted simultaneously in the spring of 2010 with steeping for 2 days and 4 h at 18 °C, germination for 4 days and 18 h at 17 °C, drying for 3 days at 15 °C, and kilning for 24 h at 85 °C. The freshly kilned malt was stored for more than 20 days before mashing.

Mashing. Three individual mashings were carried out according to Analytica EBC 4.5.1 "Extract of Malt: Congress Mash"[11] with a few modifications described by Frederiksen et al.[12] Wort produced from the third mashing was used for wort boiling. Filtration rate, pH, sugar content, and color were measured on the fresh sweet wort carried out in duplicate.

Boiling. Two 300 mL aliquots of each sweet wort were transferred to a 500 mL conical flask. To one flask of each wort was added 2 g of hops (*Humulus lupulus*) (First Gold, leafs, 6% AA (alpha)), and the content of each conical flask was kept boiling in a bath of rape seed oil (140 °C) for 1 h.

Color. EBC wort color was determined spectrophotometrically with a Cintra 40 Spectrophotometer (GBC, Melbourne, Australia) according to Analytica EBC 8.3 "Color"[11]

$$C = 25 \cdot f \cdot A_{430} \quad (1)$$

where C is the color in EBC units, f is the dilution factor, A_{430} is the absorbance at 430 nm, and 25 is a multiplication factor. Samples were filtered through a 0.45 μm filter prior to analysis.

Sugar Content (°Brix). Sugar content in °Brix was determined using a refractometer (Analytic Jena, Jena, Germany).

β-Glucan Determination in Malt. β-Glucan concentrations in malt in replicates were determined spectrophotometrically at 510 nm after digestion with the Megazyme assay kit according to AACC 32-23.[13]

Electron Spin Resonance (ESR) Spectroscopy. Wort samples were thawed and filtered (Mini Sart, 0.45 μm), and ethanol and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) spin trap were added to 5% v/v and 40 mM, respectively. Wort was incubated for 90 min at 60 °C before ESR spectra were recorded with a Miniscope MS 200 X-band spectrometer (Magnettech, Berlin, Germany) using 50 μL micropipets as sample cells. Spectra were recorded at room temperature using the following settings: microwave power, 10 mW; B_0 field, 3363 G; sweep width, 50 G; sweep time, 30 s; steps, 4096; number of passes, 6; modulation, 1 G; attenuation, 5 mW. Amplitudes of the spectra were measured and are reported as the height of the second doublet. The response of the ESR equipment was tested using an aqueous TEMPO solution (2 μM). Samples were analyzed in triplicate.

Determination of Thiol-Removing Capacity. Thiol levels were determined according to Lund and Andersen 2011[6] using both cysteine and glutathione as standards. Briefly, thiol levels in wort were determined using the thiol-selective reagent ThioGlo 1 which yields a fluorescent adduct after reaction with a thiol group (excitation wavelength, λ_{ex} = 384 nm, emission wavelength, λ_{em} = 513 nm). Analysis was carried out in microtiter plates after cysteine (wort diluted 40 times) or glutathione (wort diluted 10 times) were added to the wort at concentrations between 0 and 20 μM.

In our previous study[10] we found that sweet wort contains compounds able to oxidize cysteine and glutathione upon addition to the sweet wort. The amount of thiols that can be added to the wort before the thiol−ThioGlo 1 adducts appear is defined as the thiol-removing capacity.

Glutathione−ThioGlo 1 adducts exhibit a much stronger fluorescent response than cysteine−ThioGlo 1, which is seen in Figure 2. This feature was detailed by Hoff et al.[14] where it was found that thiols with a neighboring free amino group, which is the case for cysteine, result in ThioGlo 1 adducts with reduced fluorescence intensity. This difference in fluorescence intensity does not influence the interpretation of the thiol-removing capacity nor the fact that the thiol-removing capacity is more reactive toward cysteine than toward glutathione.

Thiol Determination Using External Standard Curve. The thiol-removing capacity makes it impossible to apply the standard addition procedure for thiol quantification in wort, so thiols can only be quantified using an external standard curve. Consequently, quantification and comparison can only be carried out on light worts of similar color and therefore with a similar matrix. A standard curve was prepared in buffer between 0 and 12 μM, and sweet, boiled, and hopped wort samples were quantified relative to this standard curve. Samples were undiluted.

Thiol Determination after Reduction with TCEP. The external standard curve was prepared in the range of 0−20 μM in 0.25 mM tris buffer (pH = 7.5). TCEP (tris(2-carboxyethyl)phosphine hydrochloride) was added to the wort to 1.92 mM (final concentration), and the mixture was incubated for 5 min. Thiol concentration was determined as described above. Any possible interaction between TCEP and the NEM (*N*-ethyl maleimide) group of ThioGlo 1[15] was controlled by background correction with a blank sample of ThioGlo 1 and TCEP at appropriate concentrations.

Volatile Profile. Head space analysis was carried out in triplicate using 5 mL of wort and 0.25 mL of 4-methyl-1-pentanol (5 mg L^{-1}) as the internal standard. Volatile compounds were collected on traps containing 250 mg of Tenax-TA with mesh size 60/80 and a density of 0.37 g mL^{-1} (Buchem bv, Apeldoorn, The Netherlands). Samples were equilibrated to 37 ± 1 °C in a circulating water bath and then purged with nitrogen (75 mL min^{-1}) for 30 min.

Trapped volatiles were desorbed using an automatic thermal desorption unit (ATD 400, Perkin-Elmer, Norwalk, USA). Primary desorption was carried out by heating the trap to 250 °C with a flow (60 mL min^{-1}) of carrier gas (H_2) for 15.0 min. The stripped volatiles were trapped in a Tenax TA cold trap (30 mg held at 5 °C), which was subsequently maintained at 300 °C for 4 min (secondary desorption, outlet split 1:10). This allowed for rapid transfer of volatiles 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) through a heated (225 °C) transfer line. 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. Column pressure was held constant at 2.4 psi, resulting in an initial flow rate of approximately 1.2 mL min^{-1} using hydrogen as carrier gas. The column temperature program was as follows: 10 min at 40 °C, from 40 to 240 °C at 8 °C min^{-1}, and finally 5 min at 240 °C. The mass spectrometer was operating in the electron ionization mode at 70 eV. Mass-to-charge ratios between 15 and 300 were scanned. 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. One chromatogram of sweet Fero wort (abbreviated, F1s) was removed from the data set due to excessive amounts of water on the trap.

Multivariate Data Analysis. Multivariate data analysis was applied to GC-MS data to evaluate the variation between the barley varieties as well as the influence of boiling with and without hops using principal component analysis (PCA). PCA is a multivariate projection method designed to extract and visually display the systematic variation in the data matrix of the volatile compounds, making it possible to include many statistical variables at the time. The aim of

Table 1. Characteristics of Wort Produced from Pallas, Fero, and Archer Malt[a]

	Archer	Fero	Pallas
β-glucan in malt (g/100g)	0.82 ± 0.00^{A}	1.73 ± 0.03^{B}	1.81 ± 0.00^{C}
filtr. time (SW) (min)	42 ± 5.7^{A}	21.5 ± 2.1^{B}	16 ± 2.8^{C}
°Brix (SW)	13.39 ± 0.09^{A}	13.44 ± 0.01^{A}	13.45 ± 0.00^{A}
°Brix (BW)	13.44 ± 0.02^{A}	13.44 ± 0.02^{A}	13.45 ± 0.00^{A}
°Brix (BHW)	13.44 ± 0.00^{A}	13.45 ± 0.00^{A}	13.45 ± 0.00^{A}
pH (SW)	5.94 ± 0.01^{D}	6.01 ± 0.00^{B}	5.98 ± 0.01^{C}
pH (BW)	5.97 ± 0.02^{C,D}	5.85 ± 0.01^{E}	6.08 ± 0.00^{A}
pH (BHW)	5.50 ± 0.00^{H}	5.57 ± 0.01^{G}	5.66 ± 0.01^{F}
EBC color (SW)	5.15 ± 0.08^{E}	4.35 ± 0.08^{F}	3.91 ± 0.05^{G}
EBC color (BW)	6.15 ± 0.10^{C}	6.15 ± 0.08^{C}	5.53 ± 0.05^{D}
EBC color (BHW)	9.30 ± 0.16^{B}	9.98 ± 0.03^{A}	9.25 ± 0.19^{B}
thiols (SW)	0.06 ± 0.01^{F}	−0.03 ± 0.01^{F}	0.01 ± 0.04^{F}
thiols (BW)	0.77 ± 0.06^{D}	1.24 ± 0.07^{A}	0.91 ± 0.02^{C}
thiols (BHW)	1.05 ± 0.01^{B}	0.61 ± 0.0^{E}	0.74 ± 0.07^{D}

[a]Sweet wort (SW), boiled wort (BW), and boiled hopped wort (BHW). Values are given as means ± standard deviations based on independent duplicates. A, B, C, D, E, F indicate the samples statistical difference, within each type of analysis, and the levels bearing different letters are significantly different ($p < 0.05$).

the PCA algorithm is to determine the latent factors or principal components (PCs) in the data set which describe most variation. On the basis of vector algebra the algorithm calculates and compresses the data material into scores (samples) and loadings (volatiles) for PCs which are plotted in the score plot and loadings plot, respectively. The position of a sample in the score plot expresses the pattern of the volatile profile, so those samples with similar scores reflect the same volatile profile.[16] PCA was performed using Latentix software (LatentiXTM 2.0, Latent5, Copenhagen, Denmark, www.latentix.com). Analyses were carried out on the relative peak areas, and data were autoscaled and cross validated.

Statistical Data Analyses. Statistical analysis was carried out as one-way ANOVA using the software JMP 9, SAS Institute, Inc., USA. Some day to day variance was observed from thiol quantification, day was included in the model as a random effect, and the LSD (least significant difference) value was determined

RESULTS

Sweet Wort Characteristics. Three barley varieties (*H. vulgare*) Pallas, Fero, and Archer were chosen for analysis in this study. Pallas is an old conventional barley variety used today in organic farming, while Fero and Archer are former grown varieties which also may have relevant properties in relation to organic farming. For the current study all three barley varieties were grown at the same location and harvested and malted simultaneously in order to obtain malts where the differences were primarily due to the selected variety. With this set up, it is possible to compare the effect of the variety on the influence on volatile profile and oxidative stability in the initial stages of brewing. The malts were mashed using the EBC congress mash procedure,[11] and the sweet worts were boiled simultaneously either with or without hops for 1 h. The extracts (°Brix) in the three worts were very similar and not influenced by boiling and hopping (Table 1). The barley varieties had a small but significant influence on sweet wort pH, and boiling resulted in a small increase in wort pH for all varieties being significant for Fero and Archer. Boiling with hops resulted in a significant decrease in pH for the three varieties, presumably due to introduction of alpha acids. The amount of hops applied in the current study was overdosed in order to detect increased effects compared to commercially produced wort. Sweet wort color was significantly influenced by the barley variety with Archer being the darkest at 5.15 ± 0.08 EBC and Pallas the lightest at 3.91 ± 0.05 EBC. The boiling process resulted in a significant increase in EBC color for all worts from all three barley varieties, and at the same time the boiling also eliminated some of the color differences between the barley varieties found in the sweet worts. However, boiled wort from Pallas remained significantly lighter than the other two. The increase in EBC color is most likely caused by heat-induced Maillard reactions, and boiling with hops resulted in a further increase in EBC color, eliminating the varietal differences. Filtration time varied between the three varieties and, in particular, gave Archer a long filtration time of 42 ± 5.7 min compared to Fero and Pallas with filtration times of 21.5 ± 2.1 and 16 ± 2.8 min, respectively. The content of β-glucan in Archer malt of 0.82 g/100 g ± 0.00 was significantly lower than in Fero and Pallas with 1.73 g/100 g ± 0.03 and 1.81 g/100 g ± 0.00, respectively.

Thiol Concentrations and Thiol Removing Capacity. *Thiol Concentrations.* In previous studies reduced thiols have been detected in wort.[17−19] In the current study the nonreduced thiol content was found to be close to zero (Table 1) and low compared to what has previously been reported in beer.[6] The fact that almost no thiols are present in the wort is most likely explained by application of the EBC congress mash procedure where the mash is exposed to atmospheric air throughout the mashing process as well as during filtration. The fact that thiols previously have been shown to be present in reduced form in wort underlines that the EBC congress mash method is not suitable for studies of thiols. Our observation is in agreement with the findings of Stephenson et al.,[19] who showed that aerobic EBC congress mashing reduced thiol levels considerably compared to EBC congress mashing performed in an anaerobic chamber. Even though the thiol concentrations in the present study are very low, it seems that boiling (with or without hops) actually increase the thiol concentrations. This could be due to unfolding of proteins during boiling, leaving the thiol groups more susceptible to reaction with the ThioGlo 1 reagent, or inactivation of the thiol-oxidizing enzyme.

To quantify the amount of oxidized thiols in the wort, the disulfide bonds were reduced to free thiols using the reducing agent TCEP (tris(2-carboxyethyl)phosphine). It was found that Archer had a larger amount of reducible thiols compared to Pallas and Fero and that boiling and hopping of the wort did not influence the amount of reducible thiols significantly

(Figure 1). These results show that the amount of reducible and potentially antioxidative thiols in wort is rather large (ca.

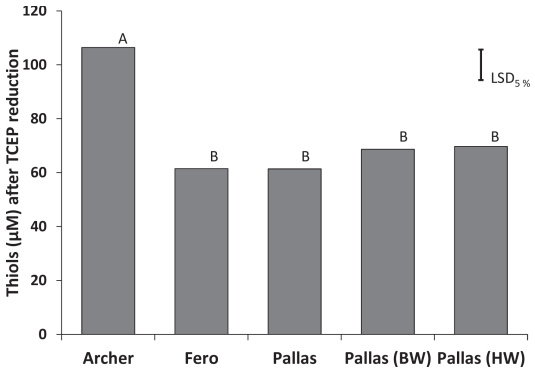

Figure 1. Thiol concentrations in wort samples treated with the thiol reducing agent TCEP. Due to a day to day variation between the measurements, LSD (least significant difference) value is presented. Values are given as means (n = 3), and the LSD value is presented by an error bar. Letters indicate the statistical difference of samples, and the levels bearing different letters are significantly different (p < 0.05).

60−110 μM), and as the thiols most likely became oxidized due to the aerobic mashing conditions, optimization of the mashing process is likely to have a positive influence on prevention of thiol oxidation. It is not certain that all thiols were on their reduced form before mashing; however, reduced thiols have previously been detected in wort where they were also found to be sensitive toward oxidation.[19] The fact that thiol oxidation occurs during the mashing process therefore seems to be the most reasonable explanation.

Sweet Archer wort had the longest filtration time (Table 1) along with the largest amount of reducible thiols (Figure 1). Formation of gel proteins, caused by thiol oxidation and generation of disulfide bonds between protein and peptide thiols during mashing, can result in a viscous layer leading to increased filtration times.[7−9] Basically all thiols were found to be oxidized in the current study. As sweet Archer wort was found to contain more reducible thiols, more disulfide bonds are likely to have formed, possibly explaining the longer filtration time. Also, the amount of β-glucans, present in the malt at the point of filtration, is known for its negative influence on filtration time.[20] However, as Archer malt had the lowest content of β-glucan (Table 1) compared to the other malts the possible influence of β-glucan can be neglected. Therefore, keeping thiols in their reduced form is important for preservation of their possible antioxidative potential as well as to keep a short filtration time, stressing the importance of carrying out the mashing production under oxygen-controlled conditions.

Thiol-Removing Capacity. In a recent study we found that compounds present in sweet wort were able to oxidize cysteine, and this ability to oxidize thiols was referred to as thiol-removing capacity.[10] In the current study it was investigated how barley varieties, wort boiling, and wort hopping influenced the thiol-removing capacity as well as how the thiol-removing capacity affected cysteine compared to glutathione. Wort boiling seemingly reduced the thiol-removing capacity completely when adding glutathione to the wort. However, a small amount of thiol-removing capacity remained after boiling when cysteine was added (Figure 2). All three barley varieties showed

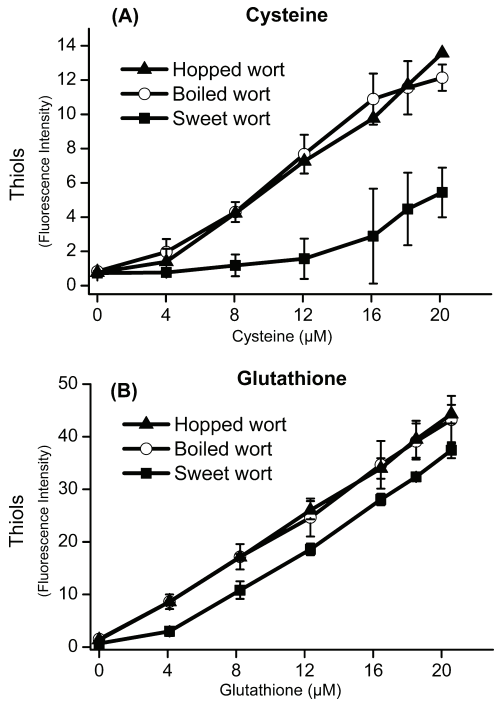

Figure 2. Addition of thiols to the worts. (A) Fluorescent response from Pallas wort (sweet, boiled, and boiled and hopped) with added cysteine in concentrations between 0 and 20 μM. Wort was diluted 40 times. (B) Fluorescent response from Pallas wort (sweet, boiled, and boiled and hopped) with glutathione added in concentrations between 0 and 20 μM. Wort was diluted 10 times. Values are given as means (n = 3), and standard deviations are shown by error bars.

the same tendency (Figure 3), and results are only presented for Pallas wort in Figure 2. Bamforth et al.[21] found a heat-stable

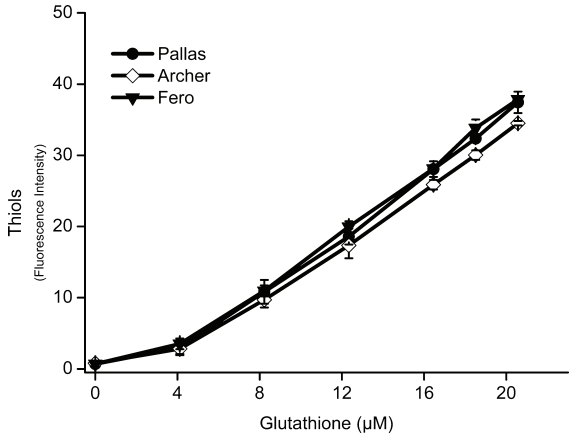

Figure 3. Fluorescent response from Pallas, Fero, and Archer sweet wort with glutathione added in concentrations between 0 and 20 μM. Wort was diluted 10 times. Values are given as means (n = 3), and standard deviations are shown by error bars.

enzyme, present in fresh malt, capable of oxidizing thiol groups (cysteine, glutathione, and dithiothreitol), resulting in disulfide cross-linking of proteins. This enzyme retained approximately 70% of its activity when heated to 70 °C, which is the mashing off temperature in the current study. After heating at 100 °C for 30 min, approximately 40% of the thiol-oxidizing activity

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remained and it was suggested that the remaining thiol-oxidizing activity was caused either by a very heat-stable enzyme or by nonenzymatic oxidation.[21] The results from the current study correlate with previous findings of the thiol-removing capacity being heat sensitive.[10,21] The occurrence of both enzymatic and nonenzymatic thiol oxidation in wort was also suggested in previous studies[10,19,21] though, whether the thiol oxidation occurring after wort boiling is explained by enzymatic or nonenzymatic oxidation remains uncertain.

The thiol-removing capacity caused a greater loss of cysteine than glutathione. In order to keep the results within the same concentration range, wort was diluted 40 times when using cysteine but only 10 times when using glutathione (Figure 2). As thiol adducts are detected by addition of 8 μM glutathione and addition of 16 μM cysteine a rough estimate makes the thiol-removing capacity at least 8 times more efficient toward cysteine than toward glutathione when including the dilution factor. In line with this, Bamforth et al.[21] found that even though glutathione displayed a much higher affinity for the enzyme than cysteine, cysteine was oxidized approximately 5 times more rapidly. The influence of barley variety was also investigated, but no effect was found on the thiol-removing capacity (Figure 3). The same result was found using cysteine (data not shown). The current study further showed that addition of hops did not systematically influence detection of thiols in the boiled wort (Table 1), and the potential antioxidative effect of the hops did not seem to influence the thiol-removing capacity when adding either cysteine or glutathione (Figure 2). In a previous study[10] a variation in thiol-removing capacity was found between two different malts (origin unknown), but in that study, barley, malting, and storage conditions were very different and not standardized as it was in the current study. This indicates that malting and storage conditions may have a larger influence on the thiol-removing capacity than the barley variety itself. It is known that freshly kilned malt should be stored for more than 20 days to improve filtration rates.[22] Bamforth et al.[21] explained this improvement of the malt quality during storage by inactivation of the thiol-oxidizing enzymes, supporting that storage time and conditions influence the thiol-removing capacity.

Radical Intensity Measured by ESR Spectroscopy. The radical-forming ability of sweet wort, boiled wort, and hopped wort made from Archer, Fero, and Pallas malt was determined by electron spin resonance (ESR) spectroscopy. Quantification was carried out by detection of radicals generated after heating samples at 60 °C for 90 min in the presence of the spin trap, POBN, and 5% ethanol. The amplitude of the second doublet of each ESR spectrum, recorded during heat treatment, was used as a measure of the amount of radicals generated (Figure 4A). Wort boiling without hops resulted in increased radical formation compared to sweet wort (Figure 4B), whereas radical formation of boiled/hopped wort and sweet wort was not statistically different. The increase in radical intensity during boiling and the antioxidative effect of hops supports the findings of Wietstock et al.[23] The barley varieties showed small effects on radical intensity though Fero was significantly more sensitive to boiling than Archer and Pallas.

Volatile Profiles. The volatile profile of sweet wort, boiled wort, and hopped wort was determined with the purpose of clarifying whether the barley variety or the boiling or hopping of the corresponding worts had an influence on the volatile profile. Hops contribute with a large amount of volatiles influencing wort aroma; however, the focus of this study

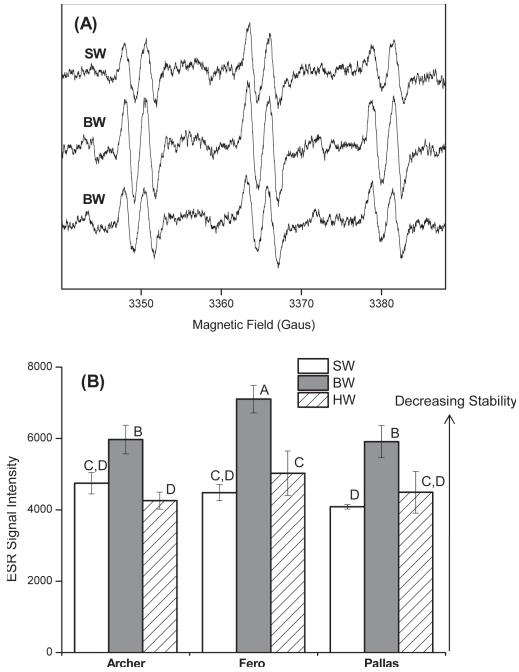

Figure 4. (A) ESR spectra of wort made from Pallas malt (sweet wort (SW), boiled wort (BW), and hopped wort (HW)) analyzed after 90 min of incubation at 60 °C with POBN (40 mM) and ethanol (5%). (B) Radical signal intensities measured by ESR spectroscopy of wort produced from Pallas, Fero, and Archer malt (sweet wort (SW), boiled wort (BW), and hopped wort (HW)). Values are given as means (n = 3). Letters indicate the statistical difference of samples, and levels bearing different letters are significantly different ($p < 0.05$).

concerns mainly the barley varieties, and only the fate of the compounds present in sweet and boiled wort were investigated and followed through to the hopped wort. Compounds coming exclusively from the hops were not included in analysis. Through headspace analysis 24 volatile compounds were identified in sweet and boiled wort (Table 2), and to investigate whether there was a difference between the volatile profiles of the barley varieties along with the boiling and hopping principal component analysis (PCA) was performed with scores and loadings presented in Figure 5. No volatile secondary oxidation products were detected; however, nondetectable precursors for oxidation may have been generated, resulting in release of off-flavors during storage of a final beer.[1] From the score plot it was found that sweet Archer wort differed from sweet Pallas wort and sweet Fero wort. After wort boiling, the differences between Archer and Pallas were leveled out while Fero was found to differ in volatile profile from the two others. Addition of hops masked the differences between the barley varieties; however, when excluding boiled wort from the score plot, Fero remained different from Pallas and Archer (data not shown), meaning that hops did not eliminate the differences completely as it appears in the present plot. When looking more specifically at the volatile compounds sweet Archer wort differentiated from sweet Fero wort and sweet Pallas wort by having a significantly higher concentration of 2-methyl-1-propanol, benzaldehyde, and limonene and a tendency for a higher concentration of phenylacetaldehyde and 2-pentanone. Furthermore, Archer had a significantly lower concentration of hexanal and a tendency for a lower concentration of 2-methyl-1-butanol and 2-ethyl-1-hexanol compared to sweet Fero and

Table 2. Volatile Compounds Included in the PCA Plot (Figure 5)[a]

no.	name	target ion	no.	name	target ion
1	propanal	29	13	3-methyl-1-butanol	55
2	2-methylpropanal	43	14	pentanol	42
3	butanal	44	15	1-hexanol	56
4	3-methylbutanal	44	16	3-ethyl-cyclobutanone	41
5	2-pentanone	43	17	furfural	96
6	2-methyl-butanal	57	18	2-ethyl-1-hexanol	57
7	2,4-dimethyl-3-pentanone	43	19	benzaldehyde	77
8	hexanal	56	20	2-methylpropanoic acid	43
9	2-methyl-1-propanol	43	21	phenylacetaldehyde	91
10	1-butanol	56	22	2,5-dimethyl-benzaldehyde	133
11	2-methyl-1-butanol	57	23	3-methyl-2-hexen-1-ol	71
12	limonene	68	24	phenol	94

[a]Compounds are identified in sweet, boiled, and boiled and hopped wort, though volatiles specific for the hops are not included. Target ion (Tgt) is used for identification, and the number of each compound corresponds to the location in the loadings plot (Figure 5B).

Pallas wort. After boiling the volatile profiles had changed and Fero differentiated from Pallas and Archer by having a significantly larger concentration of furfural and a tendency for a larger concentration of phenylacetaldehyde, 2-ethylhexanol, and 1-butanol. After boiling with hops Fero remained different from Archer and Pallas with a significantly larger content of furfural and a tendency for a larger content of phenylacetaldehyde and 1-butanol; however, again, this is not seen in Figure 5 as the contribution from the hops mask this tendency in the presented PCA plot. Furfural and phenylacetaldehyde are typical heat-induced compounds, and their formation correlates with the larger increase in color detected in boiled and hopped Fero wort compared to boiled and hopped Archer and Pallas wort. The results of volatile analysis show that the barley variety does influence the volatile profile, and barley may be selected with the purpose of influencing the volatile profile of the wort and possibly the final beer. Interestingly, boiling was found to eliminate some differences while introducing others unique to each barley variety, showing that also the processing influences and changes flavor composition.

It was previously found that no volatile compounds were evaporated from light sweet wort left at 40 °C for 10 h, whereas volatile compounds were found to evaporate from sweet worts made from malt roasted to more than 33 EBC.[10] In the current study boiling resulted in loss of many volatiles, even for light worts, which is exemplified in Figure 6 for the compounds showing the largest loss: hexanal, limonene, pentanol, and benzaldehyde. Few compounds increased in concentration during boiling, and some were present in approximately equal concentration before and after boiling, most likely due to formation during boiling. Furfural, phenol, propanal, and the Strecker aldehyde 2-methylpropanal were among the main compounds formed during boiling (Figure 7). Wort boiling also resulted in generation of Strecker aldehydes, and the contribution of Strecker aldehydes from hops is very limited. Development of 2-methylbutanal, 3-methylbutanal, and phenylacetaldehyde is shown in Figure 8. The antioxidative activities from hops did not seem to inhibit Strecker aldehyde production during wort boiling though; Wietstock et al.[23] found hops to have an inhibiting effect on generation of the Strecker aldehydes and staling compounds, 2-methylbutanal and 3-methylbutanal, during beer storage. Boiling with hops resulted in increased concentrations of most compounds present in sweet worts. Hops have a broad and complex volatile profile, and many compounds present in the wort are also present in hops. The compounds, which in this study are found to be unique for the malt, are isoamylalcohol, phenylacetaldehyde, 2,5-dimethylbenzaldehyde, propanal, hexanol, and 2- and 3- methylbutanal (data not shown).

DISCUSSION

Archer wort needed 42 ± 5.7 min of filtration compared to only 21.5 ± 2.1 and 16 ± 2.8 min for Fero and Pallas. This result could not be explained by the β-glucan determinations in malt where Archer had the lowest content of the three malt samples. Reduction of thiols using TCEP revealed a large difference between the thiol content of the worts, and Archer wort had a significantly larger content of total thiols. All thiols were found to be oxidized, causing generation of gel proteins[7−9] and longer

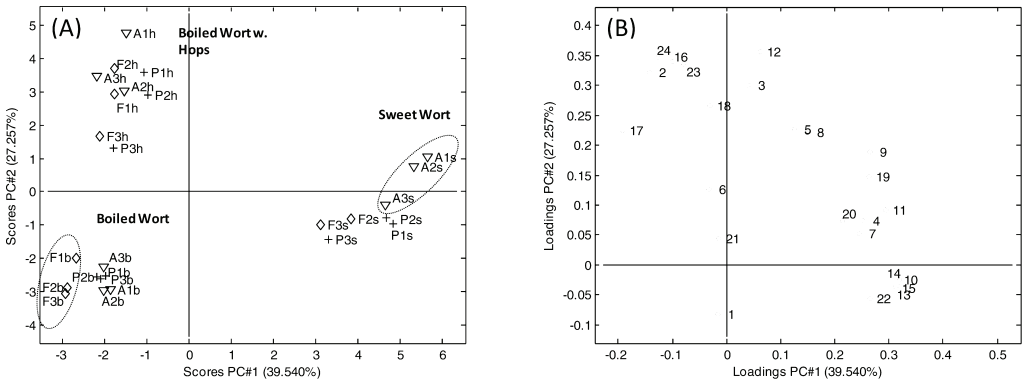

Figure 5. (A) PCA score plot based on the volatile compounds found in sweet, boiled, and hopped Pallas, Fero, and Archer wort. Only the volatiles also detected in sweet and boiled wort have been included for the hopped wort: sweet archer wort (A1s, A2s, A3s), boiled Archer wort (A1b, A2b, A3b), hopped Archer wort (A1h, A2h, A3h); sweet fero (F2s, F3s), boiled fero (F1b, F2b, F3b), hopped Fero (F1h, F2h, F3h); sweet Pallas wort (P1s, P2s, P3s), boiled Pallas wort (P1b, P2b, P3b), hopped Pallas wort (P1h, P2h, P3h). Ovals enclose the wort samples that differentiate from the others. (B) Loadings plot where each volatile compound is represented by a number and identified in Table 2.

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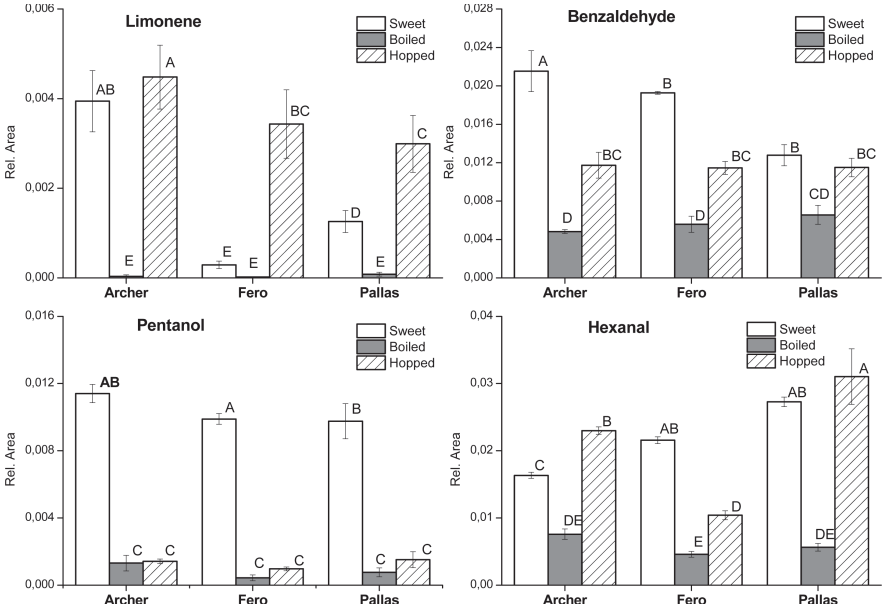

Figure 6. Relative concentrations of hexanal, limonene, benzaldehyde, and pentanol in sweet, boiled, and boiled and hopped Archer, Fero, and Pallas wort. These volatiles are chosen as examples of the loss happening during wort boiling. Values are given as means (n = 3), and standard deviations are shown by error bars. Letters indicate the statistical difference of samples, and levels bearing different letters are significantly different (p < 0.05).

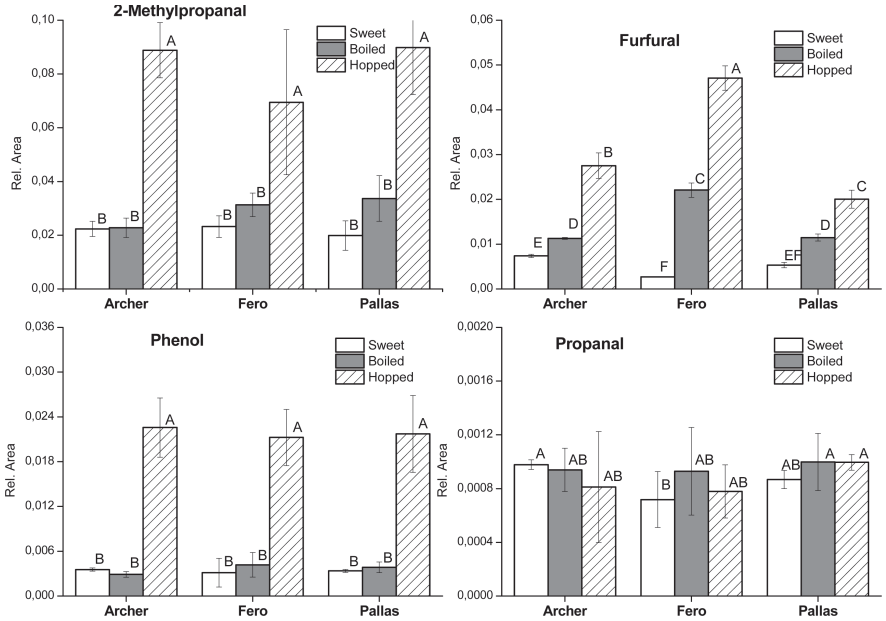

Figure 7. Relative concentration of furfural, 2-methyl propanal, phenol, and propanal in sweet, boiled, and boiled and hopped Archer, Fero, and Pallas wort. These compounds show the largest increase in concentration during boiling. Values are given as means (n = 3), and standard deviations are shown by error bars. Letters indicate the statistical difference of samples, and levels bearing different letters are significantly different (p < 0.05).

filtration time of Archer wort. Therefore, the challenge of keeping thiols in their reduced form during mashing and filtration is not only important to preserve the potential antioxidative capacity of the thiols but also to keep a short filtration time. These results underline the importance of oxygen-controlled processes. Due to the aerobic mashing and filtration all thiols were oxidized, and the differences found between the barley varieties were leveled out. Radical formation was also highly influenced by the processing. Boiling resulted in a large increase in radical formation, whereas boiling with hops prevented this increase in radical formation due presumably to its antioxidative activity as previously described.[23] Barley varieties had little effect on radical formation. Wort from the three barley varieties also had the same thiol-removing capacity, which was not completely eliminated after boiling. The thiol-removing capacity is not present in beer,[6] and as it is still present, to a small extent, in boiled and hopped wort, the remaining activity must somehow become eliminated during the fermentation process.

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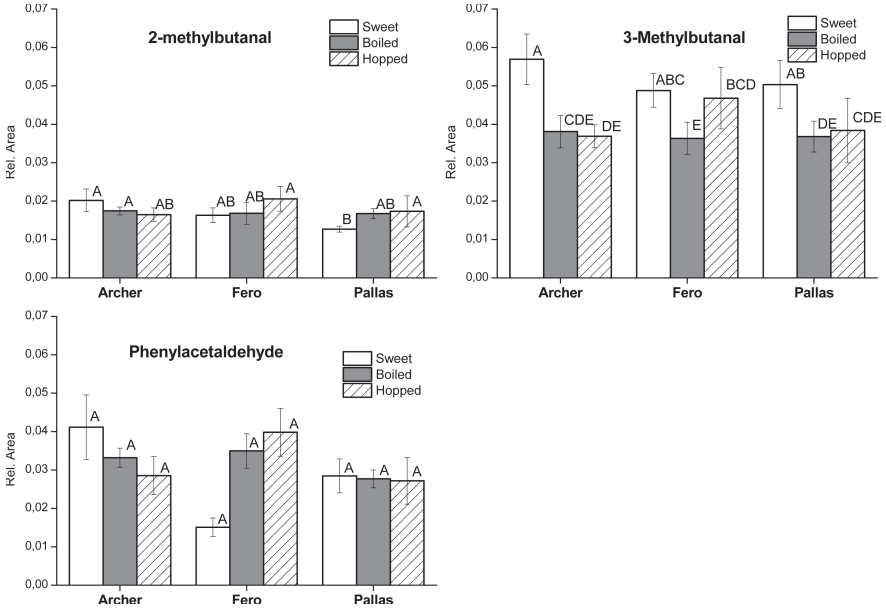

Figure 8. Relative concentration of the Strecker aldehydes 2-methyl-butanal, 3-methylbutanal, and phenylacetaldehyde in sweet, boiled, and boiled and hopped Archer, Fero, and Pallas wort. Values are given as means (n = 3), and standard deviations are shown by error bars. Letters indicate the statistical difference of samples, and levels bearing different letters are significantly different (p < 0.05).

Differences were detected in volatile profile caused by the barley variety however; the volatile profiles were highly influenced by the processing where boiling caused a decrease in many compounds due to evaporation but also an increase in other compounds. Sweet Archer wort differed from sweet Fero and Pallas wort by having a significantly larger concentration of 2-methyl-propanol, benzaldehyde, and limonene as well as a significantly lower concentration of hexanal. Fero wort differed in volatile profile from Archer and Pallas wort after boiling and was found to develop more heat-induced compounds such as furfural and phenylacetaldehyde. This correlated with increased radical intensity of boiled Fero wort measured by ESR spectroscopy. Not surprisingly, boiling caused mainly an increase in heat-induced compounds such as furfural, propanal, phenol, and 2-methylpropanal, and the compounds found most sensitive toward evaporation during boiling were hexanal, limonene, pentanol, and benzaldehyde. Many volatile compounds present in malt are also present in hops. However, the compounds which in this study were found to be unique for the malt are isoamylalcohol, phenylacetaldehyde, 2,5-dimethylbenzaldehyde, propanal, hexanol, and 2- and 3- methylbutanal. On the basis of this investigation of three barley varieties produced under the same conditions we found that when selecting a barley variety with the intension of influencing beer flavor it is important not only to evaluate the sensory properties of the malt and the sweet wort but also to evaluate how the volatile profile is influenced by processing.

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Notes

The authors declare no competing financial interest.

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Paper IV

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

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Article

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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, chlortretracycline, 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

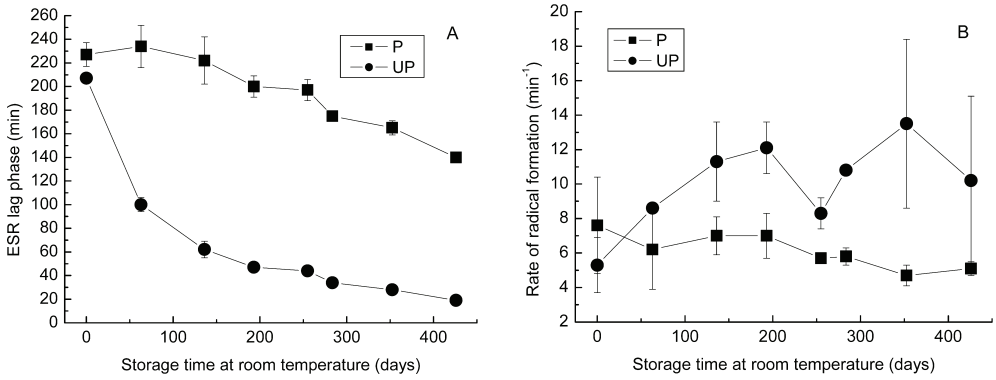

Figure 1. Oxidative stability of pasteurized (P) and unpasteurized beer (UP) during storage at 22 °C for 426 days determined by incubation of beer with PBN spin trap at 60 °C and measurement of radical intensity by ESR spectroscopy. (A) ESR lag phases are determined as the time until radical formation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

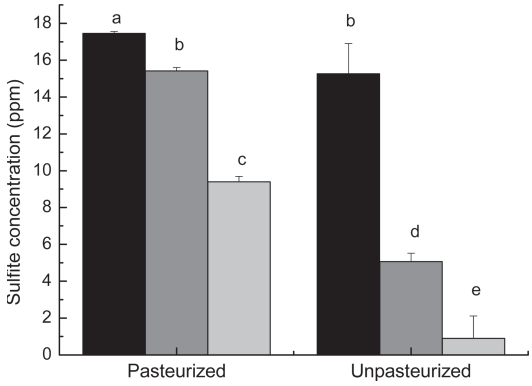

Figure 2. Sulfite concentration in beer after 0 (black bars), 63 (dark gray bars), and 426 (light gray) days of storage at 22 °C in the dark. All beers were kept at 5 °C after the given days of storage at 22 °C and analyzed after the storage period. Values are mean values of two independent samples and 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

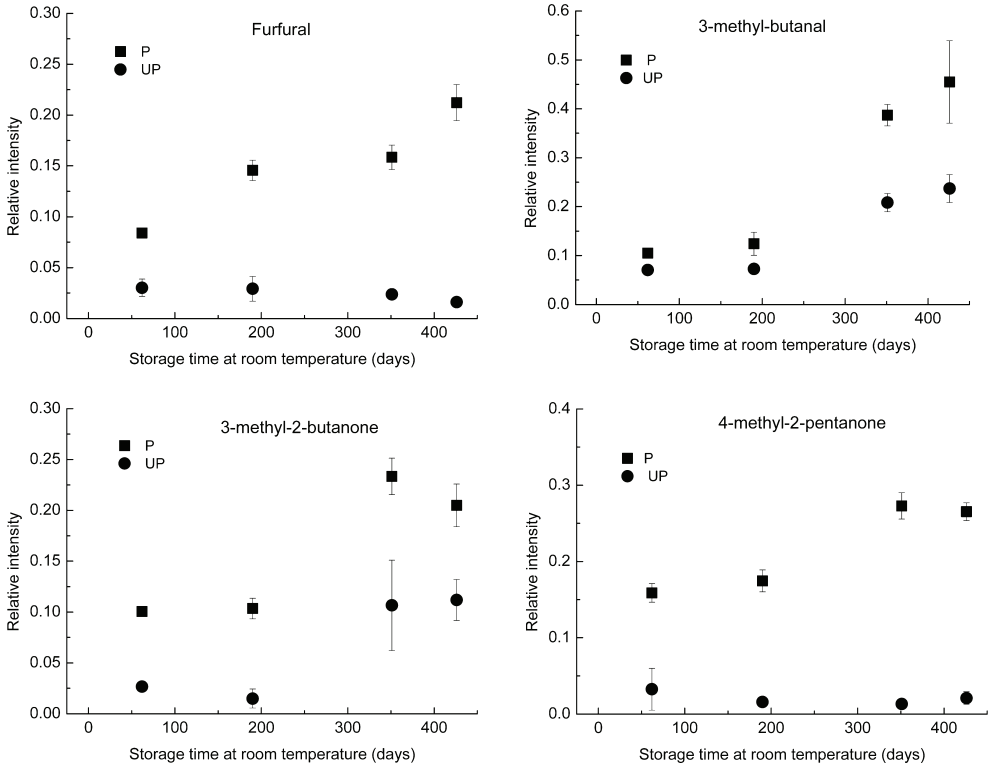

Figure 3. Relative intensities of the four volatile compounds (furfural, 3-methyl-butanal, 3-methyl-2-butanone, and 4-methyl-2-pentanone) that differed in intensity in pasteurized (P) and unpasteurized beer (UP) during storage at 22 °C for 426 days.

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

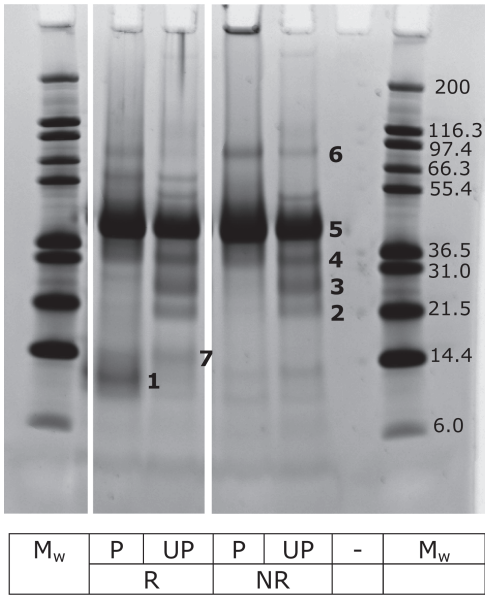

Figure 4. A representative SDS-page gel of pasteurized (P) and unpasteurized (UP) beer samples after 426 days of storage at 22 °C in the dark that was either reduced (R) by DTT or nonreduced (NR) prior to electrophoresis. The numbers in bold inserted on the gel refer to the protein band to the left of 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.

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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	gil225102	263	15 307	47
	lipid transfer protein 1	gil47168353	101	10 145	38
2	protein Z-type serpin	gil1310677	308	43 307	30
3	protein Z-type serpin	gil1310677	542	43 307	27
4	protein Z-type serpin	gil1310677	667	43 307	30
5	protein Z-type serpin	gil1310677	794	43 307	38
6	protein Z-type serpin	gil1310677	257	43 307	19
7	trypsin inhibitor cme precursor	gil1405736	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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Paper V

Storage Stability of Pasteurized Non-Filtered Beer

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Storage stability of pasteurized non-filtered beer

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The influence of pasteurization on non-filtered beers was investigated during accelerated storage (40°C, 41 days). Two beers were produced from the same hopped wort, which was fermented with two different yeast strains. Half of the bottled beers were tunnel pasteurized resulting in four different beer samples. The pasteurization influenced the volatile profile of both fresh beers, but during storage the differences between the volatile profiles of pasteurized and non-pasteurized beers disappeared. During the storage period, the pasteurized beers gave a lower rate of radical formation, as evaluated by electron spin resonance spectroscopy, indicating a better oxidative stability. The pasteurization had no effect on the levels of the pro-oxidative metals iron and copper. Pasteurization slightly increased the protein content of the beers. SDS–PAGE analysis showed that the two beers had different protein profiles, which changed during storage; however, pasteurization of both beers did not affect their protein profiles. The level of thiols were lowered in one beer and raised in the other beer by pasteurization, but during storage the levels of thiols decreased at the same rate in all of the beers. It was concluded that pasteurization had a positive influence on the oxidative stability of non-filtered beer.

Keywords: beer; pasteurization; oxidation; volatile profile; radicals; storage

Introduction

Beer is pasteurized in order to keep it microbiologically stable, and even though most breweries pasteurize their beer, a number of breweries prefer not to use pasteurization. Many microbrewers choose not to filter the beer, yet some prefer to pasteurize. Therefore, research including pasteurized, non-filtered beer remains important. In spite of the wide application of the pasteurization technique for microbiological reasons, the influence of pasteurization on the chemical storage stability is not very well described, and only a few studies have been carried out on the subject, and with contradicting results. Cao *et al*. *(1)* found that increasing levels of pasteurization had a negative influence on the chemical storage stability, as increasing levels of pasteurization resulted in an increase in colour, decrease in polyphenols and bitterness during storage, as well as an increase in staling aldehydes and a decrease in the concentration of volatile ester compounds associated with beer freshness *(1)*. Kaneda *et al*. *(2)* studied radical generation in both pasteurized and non-pasteurized beer using electron spin resonance (ESR) spectroscopy and chemiluminescence intensity, and suggested that pasteurization resulted in an initiation of radical reactions. In a recent study pasteurization of filtered beer was found to improve the oxidative stability during storage compared with non-pasteurized beer, based on ESR spectroscopy measurements and sulphite determination *(3)*.

The quality of wort is important in relation to the quality of the final beer and a correlation has been found between the oxidative stability of wort and that of beer *(4)*. Knowledge about the oxidative status of the original wort is therefore an important basis for understanding the complex mechanisms of beer stability. Transition metals such as Fe and Cu have been found to have significant effects in both wort *(5,6)* and beer *(7,8)* on the oxidative stability, as trace levels of Fe and Cu are known to act as catalysts in radical generation and oxidation reactions during beer aging. Beer proteins were recently found to contribute positively to the oxidative stability of beer, most likely caused by binding of metals, making them less reactive or available as pro-oxidants *(3)*. Furthermore, protein thiols have been correlated to the oxidative stability in beer measured by ESR *(9)*, and the thiol-containing lipid transfer protein 1 (LTP 1) has been found to play an important role in beer flavour stability *(10)*. The yeast enzyme proteinase A was found able to degrade LTP1, but was inactivated in pasteurized beer, and therefore remains important in relation to LTP1 preservation in non-pasteurized beer *(11)*.

The purpose of the current study was to evaluate the storage stability of two different non-filtered pasteurized and non-pasteurized beers, produced from the same wort. Both wort and beer were evaluated based on oxidative stability determined by ESR spectroscopy, protein content, protein composition and Fe and Cu levels. The beers were furthermore evaluated based on thiol content, sulphite content and their volatile profile during storage.

Methods

Chemicals

Acetonitrile, *N*-tert-butyl-α-nitrone (PBN), glutathione, 1-octanol, 4-methyl-1-pentanol, HCl, Perdrogen™ 30% H_2O_2, α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone and 2,2,6,6-tetramethylpiperidine-1-oxy

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were purchased from Sigma-Aldrich (St Louis, MO, USA). Plasma Pure 67–6% (HNO_3) was purchased from SCP Science (Courtaboeuf, France). Tris(hydroxymethyl)amino methane (tris), ThioGlo 1 fluorescent thiol reagent, trifluoroacetic acid (>99.8%) and sodium sulphite were obtained from Merck (Darmstadt, Germany). NuPAGE® Novex 12% bis-tris gels, LDS sample buffer, MES running buffer, Mark 12™ unstained standard and Molecular Probes SYPRO® Ruby Protein Gel Stain were obtained from Invitrogen (Carlsbad, CA, USA). Dithiothreitol (DTT) was obtained from Applichem GmbH, Darmstadt, Germany. Bradford Bio-Rad Protein Assay Reagent was obtained from Bio-Rad Laboratories, Hercules, CA. The bovine serum albumin (BSA) standard of 2.0 mg/mL was obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Ethanol (96%) was obtained from Kemetyl (Køge, Denmark). All chemicals were of analytical grade or highest possible purity. Water was purified through a Milli-Q water purification system (Millipore, Billerica, MA, USA).

The brewing process

Wort was produced from a 100% pilsner malt and mashing was carried out as follows: mash in was carried out at 53°C with an increase to 62°C held for 30 min followed by an increase to 65°C held for 40 min; thereafter the temperature was increased to 72°C and held for 15 min and mash out was carried out at 73°C. Wort was filtered and boiled for 30 min before 400 g Hallertauer Perle hop was added. After 60 min of total boiling time another 400 g of Hallertauer Perle hop was added and, after 75 min, 300 g of Centennial hop was added and the wort was left to boil another 15 min, resulting in a total boiling time of 90 min. Hops were added as type 90 pellets. The wort (12.7°P) was divided into two fermentation casks and cooled to 16°C. The two worts were fermented for 7 days with an ale brewer's yeast (yeasts 1 and 2) resulting in beers 1 and 2, respectively. Both beers were bottled, without filtration, in 330 mL flasks, and half of the bottles of each beer were pasteurized to 20 pasteurization units by tunnel pasteurization by holding the beers at 70°C for 30 min.

Radical intensity measured by ESR spectroscopy

Sweet, boiled and hopped wort. ESR wort measurements were carried out as a forced aging experiment according to Frederiksen *et al. (5)* with addition of ethanol (5% w/w) and α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone spin trap (40 mM). Wort, ethanol and spin trap were heated at 60°C in closed Blue Cap bottles with a headspace of atmospheric air and samples were analysed at given time intervals. ESR spectra were recorded at room temperature with a JES-FR30 ESR spectrometer (Jeol, Tachikawa, Japan) using a quartz capillary (i.d. 0.75 mm) sample cell (Wilmad Glass, Buena, NJ, USA). The settings were as follows: microwave power, 4 mW; sweep width, 5.0 mT; sweep time, 2 min; modulation width 0.125 mT; time constant 0.3 s. 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 wort ESR measurements were performed in duplicate.

Beer. ESR beer measurements were performed according to Uchida *et al. (12)* on the same equipment as described above. Beer was degassed by stirring with 1-octanol (0.01%) on a magnetic stirrer for 5 min and filtered through a funnel filter. Degassed beer containing PBN (30 mM) was heated at 60°C in closed Blue Cap bottles with headspace of atmospheric air. Samples were analysed with the same settings as listed for the wort samples at given time intervals, with only the amplitude changed to 1000. All ESR measurements on beer were performed in duplicate or triplicate.

Fe and Cu

A 10 mL aliquot of wort or beer sample was pre-concentrated to dryness and acid digested using 2.5 mL plasma–pure 67–69% nitric acid and 1.5 mL plasma–pure 15% hydrogen peroxide on a combined microwave and pressurizing system (UltraWave system, Milestone Srl, Sorisole, Italy). After 15 min, ramping samples were pre-pressurized to 40 bar and digested at 240°C for 10 min, using the pressurized microwave. After digestion, samples were diluted to an acid concentration of 3.5% using Milli-Q water. Multielemental composition of sample digests were subsequently analysed using inductively coupled plasma–mass spectrometry (Agilent 7500ce, Manchester, UK) using an octopole reaction cell for interference removal (Agilent 7500ce, Manchester, UK) following the instrumental settings listed in Hansen *et al. (13)* Samples were determined in duplicate.

Colour determination

Absorbance of the wort samples was measured at 430 nm using a Cintra 40 spectrophotometer (GBC, Melbourne, Australia), and EBC colour was determined according to Analytica EBC 8.3 *(14)*.

Sugar content (°P)

Original extract (wort °P) and apparent extract (beer °P) were determined using a hydrometer.

Alcohol content

Alcohol content was determined using a Beer Analyzer, Infratec 1256, ISW 3.20 (Foss, Denmark).

Protein concentration by Bradford

Protein concentrations of beer and wort samples were determined by the Bradford method, according to the manufacturer's procedure, with a few modifications. Samples were prepared in triplicate by mixing 20 μL thawed filtered beer sample or 5 mL wort sample with 1 mL Bio-Rad Protein Assay Dye Reagent Concentrate, diluted 4 times in Milli-Q-water. 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 (GBC, Melbourne, Australia). 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 or wort sample.

Sulphite and thiol quantification

The method for sulphite and thiol quantification was based on the fluorescent thiol reagent ThioGlo 1 and HPLC separation, making it possible to differentiate between thiol-ThioGlo 1 adducts and sulphite-ThioGlo 1 adducts. Quantification of sulphite was performed using the standard addition procedure described by Abrahamsson *et al. (15)*. Thiol quantification was carried out

based on the method described by Hoff *et al. (16)* using a matrix matched standard addition curve based on pasteurized beer 1.

SDS–PAGE analysis of beer proteins

Samples were analysed 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. Aliquots of a 10 μL loading sample, containing 5 μL beer, were loaded to the gel, and 0.1 M dithiothreitol (final concentration) was added to reduced samples. All loading samples were heated at minimum 70°C for 10 min before loading to the gel. Aliquots of 3 μL Mark 12TM 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, whereafter the fix solution was exchanged and left overnight. The gels were subsequently stained with 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 camera (Raytest, Camilla II, Straubenhardt, Germany).

Volatile profile

Headspace analysis was carried out in triplicate, using 5 mL wort and 0.25 mL 4-methyl-1-pentanol (5 mg/L) as internal standard. The volatile compounds were collected on a Tenax-TA trap (Buchem bv, Apeldoorn, The Netherlands). The trap contained 250 mg of Tenax-TA with mesh size 60/80 and a density of 0.37 g/mL (Buchem bv, Apeldoorn, The Netherlands). The samples were equilibrated to 30 ± 1°C for 5 min in a circulating water bath and then purged with nitrogen (75 mL/min) for 15 min.

The trapped volatiles were desorbed using an automatic thermal desorption unit (ATD 400, Perkin Elmer, Norwalk, CT, USA). Primary desorption was carried out by heating the trap to 250°C with a flow (60 mL/min) of carrier gas (H_2) for 15 min. The stripped volatiles were trapped in a Tenax TA cold trap (30 mg held at 5°C), which was subsequently heated at 300°C for 4 min (secondary desorption, outlet split 1:10). This allowed for rapid transfer of volatiles 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, USA) through a heated (225°C) transfer line. 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 column pressure was held constant at 2.4 psi resulting in an initial flow rate of approximately 1.2 mL/min using hydrogen as carrier gas. The column temperature programme was: 10 min at 40°C; from 40 to 240°C at 8°C/min; and finally 5 min at 240°C. The mass spectrometer was operating in the electron ionisation mode at 70 eV. Mass-to-charge ratios between 15 and 300 were scanned. 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, USA), was used for data analysis. Relative concentrations are presented as areas based on single ions divided by the peak area of the internal standard.

Statistical data analysis

Statistical analysis was carried out as one-way ANOVA using the software SAS Jump 9, SAS Institute, Inc., USA. Storage Day and Beer Sample were included in the models as fixed effects. Day to day variance between different analysis days was observed for thiol quantification and Analysis Day was included in the model as a random effect. Therefore the least significant difference value was determined and is presented in Fig. 6.

Multivariate data analysis

Multivariate data analysis was applied to GC-MS data to visualize the influence from pasteurization and storage, as well as the difference between the two beers using principal component analysis (PCA). PCA was performed using Latentix software (LatentiXTM 2.0, Latent5, Copenhagen, Denmark, www.latentix.com). Analyses were carried out on the relative peak areas and data was auto-scaled and cross-validated.

Results

The effect of pasteurization was evaluated by following the oxidative stability of non-filtered beer during accelerated storage at 40°C for 41 days. Two beers were produced from the same pilsner wort by fermentation with two yeast strains. Beers were bottled, and half of the bottles of each of the two beers were pasteurized and the other half remained non-pasteurized. The two yeast strains were chosen based on a preliminary unpublished study, where 20 different yeast strains were screened for their oxidative stress tolerance by exposing them to pro-oxidants. Yeast 1 was found to be a low oxidative stress resistant strain and yeast 2 to be a high stress resistant strain. The beers were brewed in a mid-sized Danish microbrewery, reflecting a small-scale production site.

Wort and beer

Beer quality is strongly linked to wort quality. In the sweet wort, boiled wort, and boiled/hopped wort, protein content and Fe and Cu levels were determined (Table 1) and the oxidative stability of the worts was evaluated using ESR spectroscopy (Fig. 1). The sample of boiled wort was collected after 30 min of boiling, immediately before hops were added, and the sample of hopped boiled wort was collected after 90 min of boiling,

Table 1. Protein, iron (Fe) and copper (Cu) content in sweet wort (SW), boiled wort (BW) and boiled/hopped wort (BHW). Values are given as means ± standard deviation (protein $n = 3$, Fe and Cu $n = 2$). Letters indicate the statistical difference of samples within the same column, and the levels bearing different letters are significantly different ($p < 0.05$)

Wort	Protein (μg/mL)	Fe (μg/L)	Cu (μg/L)
SW	252.5 ± 3.7^{A}	683.3 ± 9.7^{A}	265.3 ± 4.1^{A}
BW[a]	253.0 ± 6.0^{A}	725.9 ± 16.4^{A}	261.4 ± 2.3^{A}
BHW[b]	232.5 ± 1.9^{B}	314.1 ± 2.3^{B}	101.4 ± 5.6^{B}

[a]Boiled for 30 min
[b]Boiled for 30 min without hops and 60 min with hops

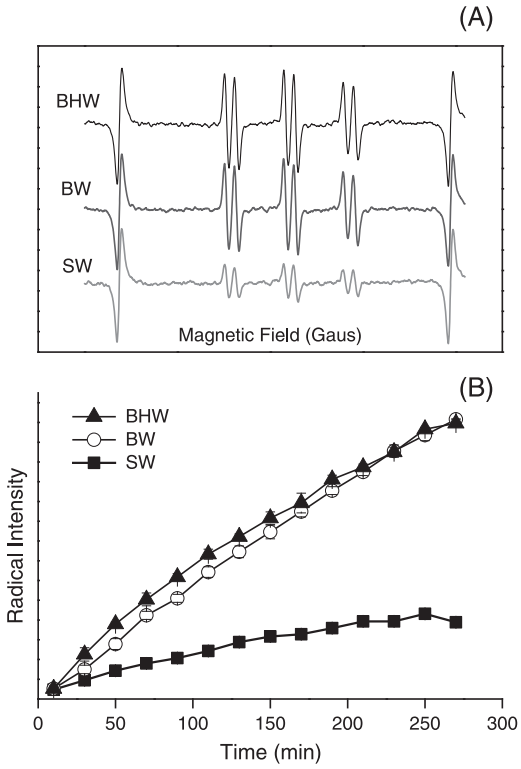

Figure 1. Oxidative stability of wort determined by ESR spectroscopy during forced aging by incubation at 60°C with ethanol (5%) and α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone spin trap (40 mM). (A) ESR spectra of α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone spin adducts formed in sweet wort (SW) boiled wort (BW) and boiled/hopped wort (BHW) after 90 min of incubation. (B) Radical intensity measured during incubation based on the amplitude of the spectra exemplified in (A) ($n = 2$).

immediately before cooling. Sweet wort was found to have the lowest radical forming intensity compared with boiled wort and hopped boiled wort in line with previous studies *(6,17)*. Boiling resulted in a large increase in radical intensity compared with sweet wort. Further boiling with hops resulted in a rate of radical formation similar to boiled wort. Previous studies have shown that hops have a radical scavenging effect *(17–20)*, which is most likely also the case in the current study, since wort boiling is known to increase radical formation. The additional 60 min of boiling should therefore result in an increase in radical formation *(17)*. Boiling with hops resulted in a small decrease in protein content, as well as in a rather large decrease in Fe and Cu levels (Table 1). This is in line with previous studies, where this phenomenon was explained by the effective binding of metals by proteins and polyphenolic compounds originating from the malt and the hops *(19,21,22)*.

The wort (12.7°P), which was fermented with yeast 1, gave a beer (beer 1) with an alcohol content of 5.9% (v/v) and an apparent extract of 1.9° P. Wort fermented with yeast 2 gave a beer (beer 2) with an alcohol content of 3.2% v/v and an apparent extract of 6.9°P (Table 2). The beers did not contain any sulphite immediately after bottling (Table 2). Sulphite is considered to be the main antioxidant in beer, and the two beers were therefore expected to be very susceptible to oxidation. Oxygen is important for radical generation *(23,24)* and beer staling is caused by oxidative as well as non-oxidative reactions *(24,25)*. Both beers were tested in a consumer survey and both scored high in 'likeability' (data not shown).

Volatiles

Analysis of volatile compounds was carried out on both fresh and stored beer (40°C for 41 days), resulting in the identification of 68 volatile compounds (Table 3). To visualize the influence from pasteurization and storage, as well as the difference between the two beers, a PCA was carried out on the volatiles from the fresh beers (Fig. 2, 1A/B) and stored beers (Fig. 2, 2A/B). Not surprisingly, beers 1 and 2 were very different in volatile profile, illustrated as beer 2 is located to the left and beer 1 to the right of both PCA score plots. Pasteurization had an influence on the volatile profile of the fresh beers as these were located at the upper part of the PCA score plot and the non-pasteurized beers located were at the lower part (Fig. 2 1A/B). Generally, the fresh pasteurized beers were characterized by having a larger content of compounds often associated with negative flavours *(24–26)* in lager beers: 2-methylbutanal, 3-methylbutanal, 4-methyl-2-hexanone, ethyl methanoate, 2-methylfuran, 2,5-dimethylfuran, 2-furancarboxaldehyde, benzaldehyde, 4,1-methylethyl benzoic acid and geranyl acetone. The fresh non-pasteurized beers had a larger content of alcohols and volatile ester compounds associated with beer freshness: ethyl heptanoate, hexyl formate, ethyl tridecanoate, ethyl octanoate, heptanol, ethyl nonanoate, 1-octanol, ethyl benzoate, ethyl decanoate, 2,3-dimethylpyrazine and phenylethyl acetate and ethyl phenylacetate. Esters are important for a beer's volatile profile and their degradation during storage has been found to be responsible for reduced fruit flavour *(24,27)*. Most esters are present in concentrations around the threshold value and minor changes in their concentration may have a large effect on beer flavour *(27)*. The loss of some volatiles, along with the generation of others, indicates that pasteurization had a negative influence on the volatile profile

Table 2. Beers 1 and 2 produced from the same pilsner wort with the yeast strains 1 and 2 were pasteurized (P) or remained non-pasteurized (NP). Alcohol content (Alc.%) and sugar content (°P) were determined for the fresh beers, and EBC colour and pH were determined on both fresh beer and stored beer (41 days at 40°C). Values are given as means ± standard deviation ($n = 2$). Letters indicate the statistical difference of samples within the same analysis, and the levels bearing different letters are significantly different ($p < 0.05$)

			Day 0			41 days 40°C	
	Alc.%	°P	SO_2 (mg/L)	EBC colour	pH	EBC colour	pH
Beer 1 P	5.9	1.9	0.3 ± 0.1	13.4 ± 0.0^{D}	4.4 ± 0.02^{AB}	16.1 ± 0.6^{C}	4.4 ± 0.00^{A}
Beer 1 NP	5.9	1.9	0.1 ± 0.1	13.5 ± 0.7^{D}	4.4 ± 0.00^{B}	15.8 ± 0.3^{C}	4.4 ± 0.00^{AB}
Beer 2 P	3.2	6.9	0.3 ± 0.1	16.5 ± 0.2^{C}	4.9 ± 0.00^{CD}	19.8 ± 1.5^{A}	4.9 ± 0.01^{C}
Beer 2 NP	3.2	6.9	0.3 ± 0.3	16.1 ± 0.0^{C}	4.8 ± 0.02^{D}	18.0 ± 0.3^{B}	4.9 ± 0.01^{C}

Table 3. Volatile compounds identified in the beers and included in the principal component analysis in Fig. 2. The target ion was used for quantification of the compounds

No.	Name	Target ion	No.	Name	Target ion	No.	Name	Target ion
1	2-Propanone	58	**24**	3-Methyl-1-butanol	31	**47**	Methoxyethane	45
2	2-Methylpropanal	72	**25**	2-Pentyl-furan	81	**48**	Benzaldehyde	106
3	Ethyl acetate	44	**26**	Ethyl hexanoate	88	**49**	Ethyl nonanoate	88
4	2-Methylbutanal	57	**27**	Thiazole	85	**50**	1-Octanol	56
5	3-Methylbutanal	58	**28**	1-Pentanol	42	**51**	Isobutyric acid	43
6	4-Methyl-2-hexanone	29	**29**	Hexyl acetate	43	**52**	Ethyl 9-decanoate	88
7	Ethyl methanoate	31	**30**	Difurfuryl ether	81	**53**	Phenylacetaldehyde	91
8	2-Methylfuran	82	**31**	3-Hexenyl butanoate	67	**54**	2-Furanmethanol	98
9	3-Methyl-2-butanone	43	**32**	Ethyl heptanoate	88	**55**	Ethyl decenoate	41
10	Ethanol	29	**33**	6-Methyl-5-hepten-2-one	43	**56**	Ethyl benzoate	105
11	2,5-Dimethylfuran	96	**34**	Hexyl formate	56	**57**	α-Humulene	93
12	Ethyl propionate	57	**35**	4-Hydroxypyridine	95	**58**	Ethyl decanoate	55
13	Ethyl isobutanoate	43	**36**	Heptyl acetate	43	**59**	Ethyl geranate	69
14	Propyl acetate	43	**37**	Ethyl tridecanoate	88	**60**	2,3-Dimethylpyrazine	71
15	4-Methyl-2-pentanone	43	**38**	Nonanal	57	**61**	4,1-Methylethyl benzoic acid	149
16	Isobutyl acetate	43	**39**	2,4-Hexadienal	81	**62**	Ethyl phenylacetate	91
17	Ethyl butanoate	71	**40**	Ethyl octanoate	101	**63**	Phenethyl acetate	104
18	1-Propanol	31	**41**	Acetic acid	43	**64**	β-Damascenone	69
19	Butyl acetate	43	**42**	Heptanol	70	**65**	Ethyl dodecanoate	88
20	Hexanal	56	**43**	Furfural	96	**66**	Geranyl acetone	43
21	2-Methyl-1-propanol	27	**44**	Octyl acetate	43	**67**	Phenylethyl alcohol	91
22	3-Methylbutyl acetate	55	**45**	Decanal	57	**68**	Phenol	94
23	Pentyl acetate	43	**46**	2-Furanylethanone	95			

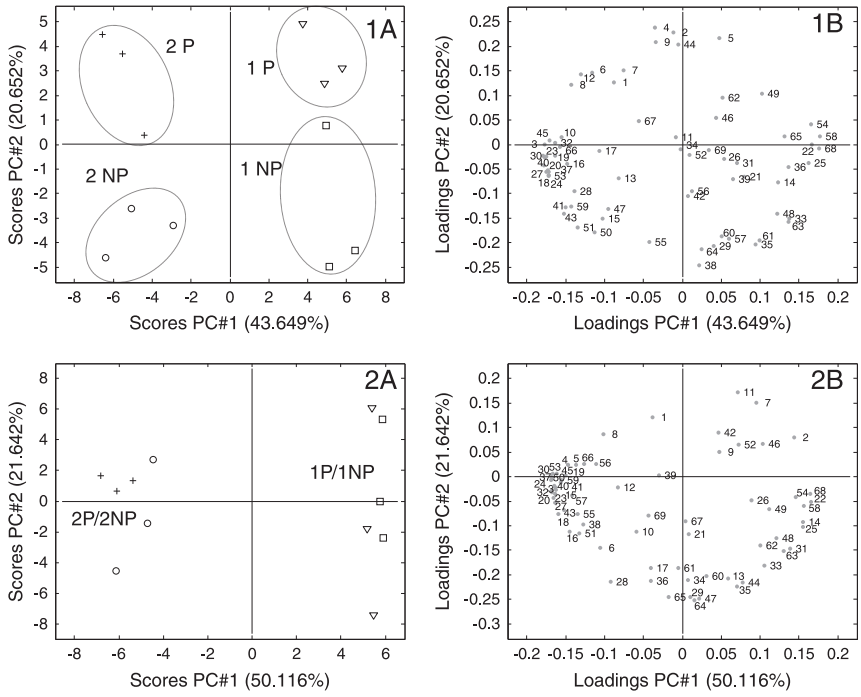

Figure 2. (1A) PCA score plot based on the total volatile profile in fresh beers and the corresponding loadings plot (1B). (2A) PCA score plot based on the total volatile profile of stored beers (41 days at 40°C) and the corresponding loadings plot (2B). Each volatile compound is represented by a number corresponding to that in Table 3.

of the fresh beers. Cao *et al. (1)* studied varying levels of pasteurization (2–14 pasteurization units) and found, similar to the current results, that an increase in pasteurization units resulted in increased loss of estery volatiles during storage, as well as an increase in aldehydes and sulphur-derived compounds *(1)*. However, in contradiction to the current study, these variations were not found in the fresh beer *(1)*, which could be explained by lower levels of pasteurization.

Storage at 40°C for 41 days had a large influence on the volatile profiles of all beers and resulted in elimination of the

differences caused by pasteurization in the fresh beers; in contrast to the fresh beers, it was not possible to differentiate between the volatile profiles of pasteurized and non-pasteurized stored beers, but only between the two beers, beers 1 and 2 (Fig. 2).

The overall volatile staling pattern was complex and caused by many different compounds. Selected staling markers from different origins *(25,26)* identified in the current study are shown in Fig. 3. Pasteurization resulted in increased concentrations of the Strecker degradation products 2-methylpropanal, 2-methylbutanal and 3-methylbutanal. During storage, these compounds increased even more in all beers and reached approximately the same level, which is likely to be caused by the elevated storage temperature. Interestingly, the staling compound and Strecker degradation product benzaldehyde decreased in concentration during storage and the staling compound and Strecker degradation product phenylacetaldehyde seemed to be uninfluenced by storage. The Maillard reaction product and marker of staling, furfural, was also generated during pasteurization, but increased dramatically in concentration during storage in all beers. The generation of furfural was previously found to be highly influenced by beer storage temperature and was found to be generated much faster with storage at 40°C compared with storage at 20°C *(24)*. In Lund *et al. (3)*, where filtered beer was stored at 22°C for over one year, furfural also developed in pasteurized beer, but almost no furfural developed in the non-pasteurized beer. The staling ketone β-damascenone was found to increase in

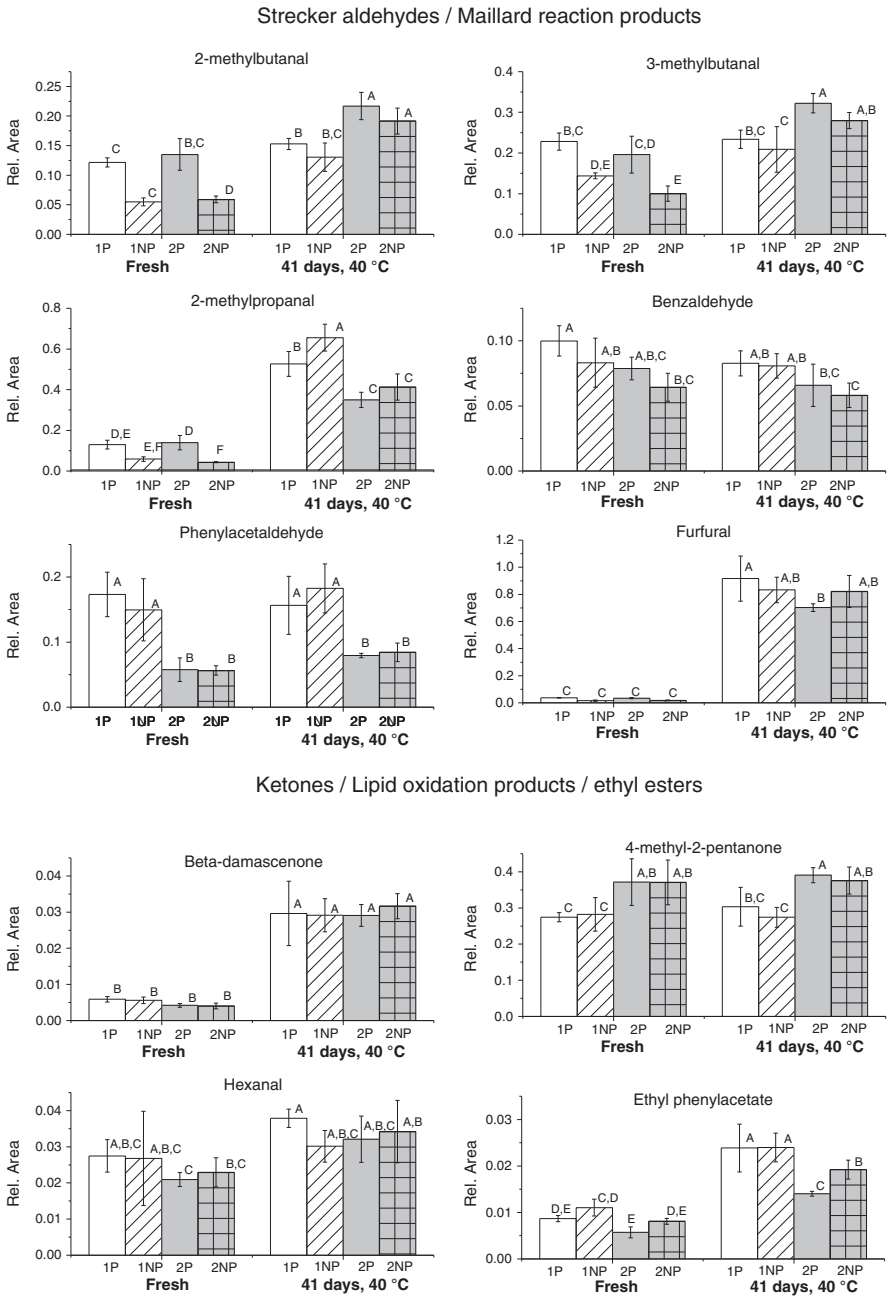

Figure 3. Relative concentration of some staling volatiles identified in the fresh and stored beer (41 days at 40°C; $n = 3$). Strecker degradation products: 2-methylbutanal, 3-methylbutanal, 2-methylpropanal, benzaldehyde and phenylacetaldehyde. Maillard reaction product: furfural. Ketones: β-damascenone and 4-methyl-2-pentanone. Lipid oxidation product: hexanal. Ehtyl ester: Ethyl phenylacetate. Letters indicate the samples statistical difference and the levels bearing different letters are significantly different ($p < 0.05$).

all beers, which was also the case for the ethyl ester and staling compound ethyl phenylacetate, which actually decreased in concentration during pasteurization. The staling ketone 4-methyl-2-pentanone was uninfluenced by storage in both pasteurized and non-pasteurized beer, but was previously found to increase in pasteurized filtered beer during storage at 22°C *(3)*. Only a slight increase in the fatty acid oxidation product hexanal during storage was detected and no other fatty acid oxidation products were detected, including (*E*)-2-nonenal and methional; however, the concentration of (*E*)-2-nonenal may have been below our detection limit. Generally, these results show an increase in many staling compounds during storage, indicating that significant oxidation has taken place in the beers.

Oxidative stability of beers during storage measured by ESR spectroscopy

The beers, stored for 41 days at 40°C, did not change in pH during the storage period. Initially the pasteurization had no significant effect on the colour of the beers; however, during the storage period both beers 1 and 2 became darker, which was probably caused by Maillard reactions owing to the high storage temperature. At the end of the storage, the pasteurized beers tended to have a darker colour compared with the non-pasteurized beer and this observation was significant for beer 2 (Table 2). The darker colour was in line with previous studies, where a linear correlation between storage time and colour was found *(1)* and explained by the generation of Maillard reaction compounds and degradation of polyphenols *(1,24)*. The oxidative stability of the beers was evaluated by measurement of the lag phase for radical formation during aerobic forced aging using ESR spectroscopy. Usually a lag phase is observed indicating the time before radical formation is accelerated. A lag phase was not observed for the two beers, owing to the lack of sulphite, which is the main antioxidant in beer *(8,28)*. Therefore the oxidative stability of the beers was evaluated by the rate of radical formation only. The rate of radical formation was determined as the slope of the spin adduct formation curve, and is a measure of the radical forming potential of the beer. The rate of radical formation evaluates the effect of pro-oxidants without the inhibiting effect of antioxidants. All beers showed a significant increase in radical generation from beginning of storage until 27 days of storage (Fig. 4). From day 27 to day 41 the radical formation levelled off and no further significant increase was observed. Furthermore, there was a tendency for the non-pasteurized beers to have a higher radical generation, as was also previously observed for filtered beer *(3)*, although this was not significant throughout the entire storage period. In a recent study, pasteurization was found to have a positive effect on the oxidative stability of beer as pasteurized beers gave a longer ESR lag phase than non-pasteurized beer during storage, as well as a slower decrease in sulphite content and decreased rate of radical formation *(3)*. Results based on ESR, very similar to the current study, where no lag phase was detected in pasteurized and non-pasteurized beer, were found by Kaneda *et al*. *(2)*, also showing that pasteurized beer had a lower content of radicals. However, in contrast to the current study, this result was suggested to be caused by the fact that some radicals in the pasteurized beer had already reacted during the pasteurization process, indicating that the radical-forming potential was exhausted.

Beer 2 had a lower alcohol content than beer 1. Ethanol radicals are the main radicals detected in beer by spin trapping in combination with ESR spectroscopy *(5)*. An experiment with addition of ethanol to Beer 2 was carried out confirming that the decreased ethanol content in beer 2 was not a limiting factor for the detection of radicals (data not shown).

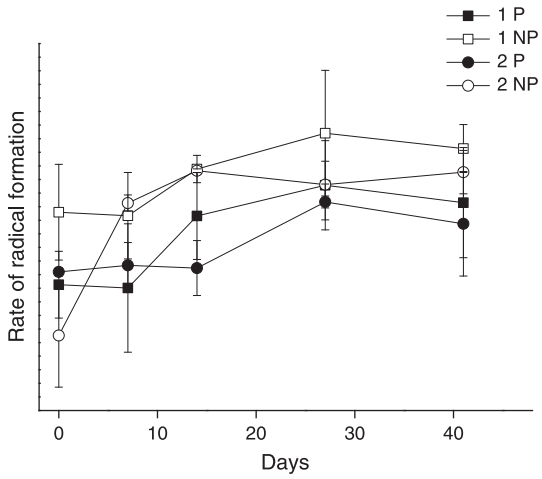

Figure 4. Oxidative stability of beers 1 and 2, pasteurized (P) and non-pasteurized (NP) measured by ESR spectroscopy during 41 days of storage at 40°C. The rate of radical formation on each particular day was determined as the slope of the curve obtained after radical formation had accelerated and measured during forced aging by incubation with *N*-tert-butyl-α-nitrone spin trap (30 mM) at 60°C.

Thiols, Fe and Cu levels and protein content

Thiols are in general sensitive to oxidation and the concentration of thiols in beer was therefore monitored during storage. Generally, the concentration of thiols was in the low range of what had previously been observed in other beers *(9)*, but this was consistent with a corresponding low protein concentration (Fig. 5). A significant loss of thiols was observed in all beers during the first 14 days of storage. However, storage from 14 to 41 days did not result in a further significant loss of thiols (Fig. 6). These results indicate that thiols are quickly lost at the beginning of storage, until a certain level where the loss of thiols is slowed down. Pasteurized beer 1 had a significantly lower content of thiols on day 0. However, no further significant differences were found between the beers.

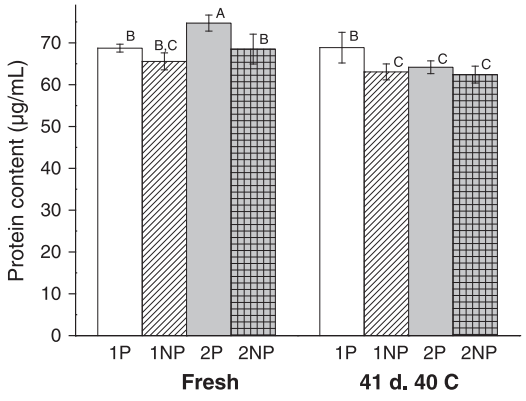

Figure 5. Protein content in fresh and stored beer (41 days at 40°C) ($n = 3$). Letters indicate that the sample's statistical difference and the levels bearing different letters are significantly different ($p < 0.05$).

The presence of the transition metals Fe and Cu promotes metal-catalysed oxidation and thereby affects radical formation and the oxidative stability of wort *(5)* and beer *(7,23,28)*, and these metals were therefore quantified in the wort (Table 1) as well as in the fresh and stored beer (Fig. 7). A large proportion of Fe and Cu was removed from the wort, by both yeast strains during fermentation, in line with previous studies *(21,29,30)*. Beer 1 had a significantly higher content of both Fe and Cu than beer 2. The variations could be caused by the yeast, as yeast with less vital cells is ineffective in concentrating and binding metals within the cell *(29,30)*. Also, the level of oxidative reactions can affect the content of Fe and Cu, since the exposure of yeast cells to exogenous reactive oxygen species was recently shown to affect their uptake and export of metal ions *(31)*. The variations in Fe and Cu levels between the two beers may therefore be a combination of differences in yeast vitality and exposure to oxidative conditions during the fermentation.

Pasteurization seemed to have only a minor influence on soluble Fe and Cu levels, with the exception of fresh beer 2, where the fresh pasteurized beer had a higher Fe content than the fresh non-pasteurized beer. This difference, however, was not observed after storage. Storage had no influence on Fe and Cu levels apart from beer 1, where a minor but significant decrease in Cu levels was found in both pasteurized and non-pasteurized beer.

After fermentation the protein content in all of the beers had decreased from 232.5 μg/mL in the hopped boiled wort to approximately 70 μg/mL in all beers (Fig. 5). This content of soluble protein was low for both wort and beer *(32)*. Non-pasteurized fresh beers tend to have a slightly lower content of soluble proteins, but there was only a significant difference for beer 2. In contrast to beer 1, beer 2 was also affected by storage, where the soluble protein content decreased significantly for both pasteurized and non-pasteurized beer.

SDS–PAGE analysis of both wort and beer samples was performed to investigate the protein composition. The sweet wort and boiled wort clearly showed a more diverse protein composition compared with hopped/boiled wort and beer in agreement with previous studies *(33)*, where it has been found that many of the trypsin/α-amylase inhibitors present in sweet wort were lost during wort boiling. The loss of a number of proteins during boiling, found by SDS–PAGE analysis correlates with the protein content observed in the current study (Table 1). SDS–PAGE analysis also revealed a difference in protein composition between the beers, as more protein bands were visible for beer 2 compared with beer 1 (Fig. 8). Storage influenced the protein composition of beer 2, resulting in the loss of a number of protein bands and formation of a few new protein bands, while storage only had a minor effect on protein composition of beer 1. In beer 2 protein Z (the strongest band just above the 36.5 kDa marker) is suggested to be degraded by protease activity from the yeast as the additional bands appearing below protein Z in the stored beers previously have been identified as protein Z *(3)*. The band appearing just below the 14.4 kDa marker has previously been identified to contain trypsin/α-amylase inhibitor and LTP1 *(3)*, and this band was also lost during storage, which was also likely to be due to protease activity of the yeast. Protein Z, trypsin/α-amylase inhibitor and LTP1 degradation were also observed in the pasteurized beer, which may be caused by non-enzymatic protein degradation *(11)*, or alternatively

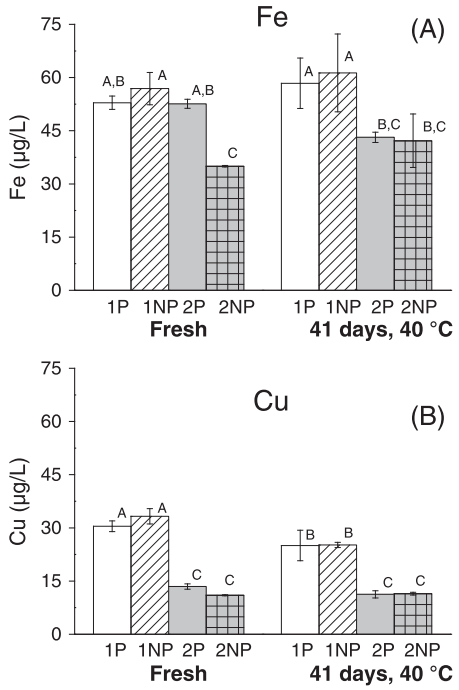

Figure 7. (A) Iron (Fe) content in fresh and stored beer (41 days at 40°C). (B) Copper (Cu) content in fresh beer and stored beer (41 days at 40°C). Letters indicate the sample's statistical difference and the levels bearing different letters are significantly different ($p < 0.05$).

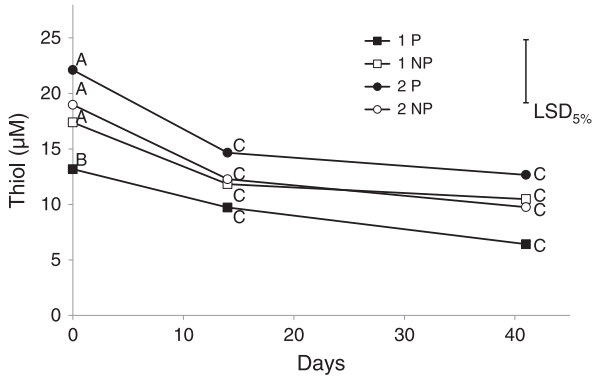

Figure 6. Thiol levels in beer 1 pasteurized (1 P) and non-pasteurized (1 NP) and beer 2 pasteurized (2 P) and non-pasteurized (2 NP) during storage for 41 days at 40°C ($n = 4$). Owing to a day to day variation between the measurements, the LSD (least significant difference) value is presented. Letters indicate the sample's statistical difference and the levels bearing different letters are significantly different ($p < 0.05$).

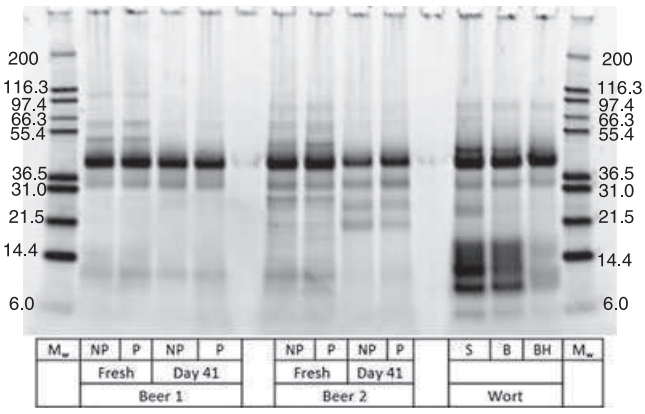

Figure 8. A representative SDS–PAGE gel of fresh and stored (41 days at 40°C) beers 1 and 2 pasteurized (P) and non-pasteurized (NP). All samples had been reduced. Mw is the molecular weight marker and numbers refer to the molecular weight in kDa.

because the pasteurization was not sufficient to inactivate the yeast protease. As opposed to our previous study *(3)*, pasteurization had only a small influence on the protein composition in the present study, which may be due to several reasons, such as a different brewhouse, different yeast strains and different storage conditions. Based on the SDS–PAGE analysis, it appears that the protein composition of beer 2 changed more during storage than the protein composition of beer 1. This correlates with a decrease in protein content (Fig. 5), as well as with a decrease in Cu content, during storage of beer 2. Pasteurization, however, did not influence the protein composition during storage as was previously found for a filtered beer *(3)*.

Discussion

Pasteurized non-filtered beer gave a lower level of radical formation than non-pasteurized beer. Storage eliminated the initial differences in volatile profiles induced by the pasteurization of the beers. A slightly higher protein content was detected in the pasteurized beers, but no effect on the protein composition was found and only minor effects of pasteurization were detected on Fe and Cu levels. This study shows that pasteurization has an overall positive influence on the oxidative stability of non-filtered beer, despite the formation of a number of Maillard-related aroma compounds. This is in agreement with a previous study of pasteurization of filtered beer *(3)*.

Pasteurization is likely to initiate a number of reactions in the beers, and in the current study this was the case for generation of both Maillard reaction products and Strecker aldehydes, which were found in increased concentrations in the fresh pasteurized beers. However, during storage, differences in volatile profiles were eliminated. This elimination of these differences may be influenced by the elevated storage temperature (40°C) as the small differences between filtered pasteurized and non-pasteurized beer previously were found to increase during storage at 22°C *(3)*. The difference in the results could be due to the different storage conditions and the fact that filtered beers and another yeast strain had been used. For foods, a temperature increase in approximately 10°C often results in at least a doubling of the rate of chemical reactions. Accelerated storage was applied in the current study to decrease storage time. However, different reactions have different activation energies, and therefore the aging pattern of the beers at 40°C might be slightly different than when carried out at room temperature *(26,34)*.

The current study and Lund *et al. (3)* show that pasteurization limits the level of radical generation. The mechanisms behind this effect are unknown, but it has been suggested that the difference between pasteurized and non-pasteurized beer was mainly caused by a difference in the levels and the effects of pro-oxidants *(3)*. Trace levels of iron and copper have an important catalytic role as pro-oxidants in beer. The binding of metals by proteins and polyphenolic compounds, originating from malt and hops, can affect the levels of iron and copper *(21,29,30)*. Not surprisingly, a significant difference in metal content and protein composition was found between beers 1 and 2, as they were produced with different yeast strains. Pasteurization did not influence the Fe and Cu levels, protein content and protein composition, in either the fresh or stored beer in this study. It was previously found that pasteurized beer after storage (22°C for 426 days) had a protein content of approximately twice the amount of non-pasteurized beer, along with a higher Fe content and lower Cu *(3)*. Generally only a minor change in protein content during storage was found in the present study, which correlates with the observed minor changes of Fe and Cu levels. However, the minor changes in protein content and composition observed in the present study, compared with our previous study *(3)*, could also be due to the low protein content in the present beers, hence making differences more difficult to detect.

Thiols were oxidized during storage in all beers, suggesting that they play an important role in the oxidative stability of beer by consuming oxidizing species generated during storage. Pasteurization did affect the initial levels of thiols; however, the rates of thiol oxidation were very similar in all of the beers and were not affected by the pasteurization. The beers did not contain sulphite, and the rates of oxidation of thiols were therefore not affected by the potential regeneration of thiols by sulphite.

Hops were found to improve the oxidative stability of the wort when the pro-oxidative effect of boiling was considered and this was correlated with a decrease in Fe and Cu levels. Hops are known for their antioxidative effect in wort and the current study suggests, in accordance with previous studies *(19,21,22)*, that malt proteins and hop constituents bind metals and cause their removal from the solution through precipitation during the brewing process. The ability to bind metals is likely to be an important part of the antioxidative effect of hops. Large amounts of the metals were also removed during fermentation by both yeast strains. Interestingly the high stress resistant strain (yeast 2) gave beers with the lowest levels of iron and copper.

Conclusion

This study showed that pasteurization had a positive influence on the oxidative stability of non-filtered pilsner beer. Pasteurized beer gave a lower radical formation than non-pasteurized beer and storage eliminated the differences in volatile profiles induced by pasteurization. Fresh pasteurized beer contained some Maillard-related volatile compounds and the fresh unpasteurized beer contained slightly more alcohols and volatile ester compounds. Slightly higher protein content was detected in the pasteurized beers, but no influence of pasteurization on the protein composition was found. Only minor effects of pasteurization were detected on thiol concentrations and on the concentrations of pro-oxidative metals. The decreased oxidative stability observed for both beers during storage correlated with the increased amount of staling compounds, decrease in protein content, decrease in thiol levels and lack of sulphite.

Acknowledgements

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Paper VI

Determination of Sulfite in Beer based on Fluorescent Derivatives and Liquid Chromatographic Separation

Victor Abrahamsson, Signe Hoff, Nikoline J. Nielsen, Marianne N. Lund and Mogens L. Andersen

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Determination of Sulfite in Beer Based on Fluorescent Derivatives and Liquid Chromatographic Separation

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ABSTRACT

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A method was developed for quantification of sulfite in beer based on derivatization with the maleimide-derived probe ThioGlo 1 followed by separation of fluorescent adducts by reversed-phase high-performance liquid chromatography and fluorescence detection. Sulfite gave two ThioGlo 1 derivatives and it was shown by mass spectrometry that both had identical mass spectra. Matrix effects were observed when constructing sulfite standard curves in different beers and, therefore, use of a matrix-matched calibration curve is proposed. ThioGlo 1 was found to generate fluorescent adducts with both bound and free sulfite, providing a quantification of the total sulfite content in beer. The limit of quantification of sulfite was 0.6 mg/L and the method can be used for quantification of sulfite in highly colored beers.

Keywords: Analysis, Beer, Matrix match, Sulfite, ThioGlo 1

RESUMEN

Se desarrolló un método para para la cuantificación de sulfito en cerveza sobre la base de derivatización con la sonda derivada de maleimida, ThioGlo 1, seguido por la separación de los aductos fluorescentes usando fase inversa cromatografía líquida de alto rendimiento y detección por fluorescencia. El sulfito dio dos derivados de ThioGlo 1 y se demostró por espectrometría de masas que ambos tenían espectros de masa idéntica. Efectos de matriz se observaron en la construcción de las curvas estándar de sulfito en diferentes cervezas, y por lo tanto el uso de una curva de calibración ajustada a la matriz se propone. ThioGlo 1 fue encontrado para generar aductos fluorescentes con tanto unido y libre de sulfito, proporcionando una cuantificación del total de sulfito contenido en la cerveza. El límite de cuantificación fue de 0.6 mg/L de sulfito y el método puede ser utilizado para la cuantificación de sulfito en cervezas muy coloreadas.

Palabras claves: Ajustada a la matriz, Análisis, Cerveza, Sulfito, ThioGlo 1

Sulfite (SO_3^{2-}), also often referred to as sulfur dioxide (SO_2), is an additive in foods, which functions as an antioxidant and as a preservative to reduce or prevent microbiological spoilage (27). In beer, sulfite is produced during the fermentation by the yeast as an intermediate in the amino acid synthesis, and it occurs naturally in concentrations of 0.5 to 10 ppm as the protonated species bisulfite (HSO_3^-) (11). The oxidative stability of beer has been linked to the presence of sulfite, and it has been suggested that sulfite inhibits oxidative reactions in beer by scavenging the reactive oxygen species hydrogen peroxide (4,17,25). Sulfite may also bind to compounds containing carbonyls and, thereby, render them nonperceptible to the overall beer staling (7,22). Thus, sulfite has two important functions in relation to beer stability: it works as both an antioxidant and a camouflage of off-flavors; for example, (E)-2-nonenal (7).

Sulfite is a well-known allergen and sensitive individuals who are exposed, particularly asthmatics, may suffer reactions ranging from dermatitis, urticaria, flushing, hypotension, abdominal pain, and diarrhea to life-threatening anaphylactic and asthmatic reactions (26). The Joint FAO/WHO Expert Committee on Food Additives (28) has specified an acceptable daily intake of up to 0.7 mg of sulfur dioxide per kilogram of body weight. Within the European Union, food and beverages containing sulfur dioxide and sulfites at more than 10 ppm must be labeled according to directive 2003/89/EC (24). Beer, including non- to low-alcoholic beer, may not contain sulfur dioxide at more than 20 ppm. However, sulfur dioxide at 50 ppm is permitted in beer with a second fermentation in the cask according to directive 95/2/EC (23).

The level of sulfites in beer can be quantified by numerous techniques, including titrimetry, electrochemistry, fluorometry, chemiluminescence spectrometry, colorimetry, gas chromatography, biosensors, and liquid chromatography, including ion chromatography and flow injection analysis, of which the latter two have been intensely explored in recent years (8,12,18,21,29). The most frequently used methods are those recommended by the American Society of Brewing Chemists (ASBC) and the European Brewery Convention (EBC). The Institute of Brewing (IOB) Analysis Committee has evaluated alternative approaches to the traditionally accepted Monier-Williams based method (6). In a comparative study including the Monier-Williams method, the IOB rapid method, the ASBC *p*-rosaniline method, and a method based on Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) [DTNB]), it was concluded that determination by means of *p*-rosaniline provided the better repeatability. Today, ASBC recommends the *p*-rosaniline method (5). EBC recommends the distillation method (Monier-Williams method) and the EBC enzymatic method for determining the total sulfur dioxide in beer (10). The Monier-William method is time consuming and labor intensive. Both the Monier-William and *p*-rosaniline methods use hazardous chemicals.

Lund and Andersen (19) have recently quantified thiol-containing compounds in beer using fluorescence detection after derivatization with 10-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-9-methoxy-3-oxo-3H-naphthol[2,1-b]pyran-2-carboxylic acid methyl ester (ThioGlo 1). Sulfite was found to interfere because it also forms fluorescent adducts with ThioGlo 1. ThioGlo 1 is a maleimide derivative which has high affinity for thiol groups. The probe is weakly fluorescent when un-reacted, but highly fluorescent as sulfite- or thiol-derived adducts (Fig. 1) (9). A related maleimide derivative, N-(9-acridinyl)maleimide (NAM), has been reported to form derivatives with sulfite and, combined with reversed-phase high-performance liquid chromatography (RP-HPLC), it has been used for determination of sulfite in alcoholic beverages and environmental samples (1–3,20). A drawback of the method was the formation of three NAM derivatives after

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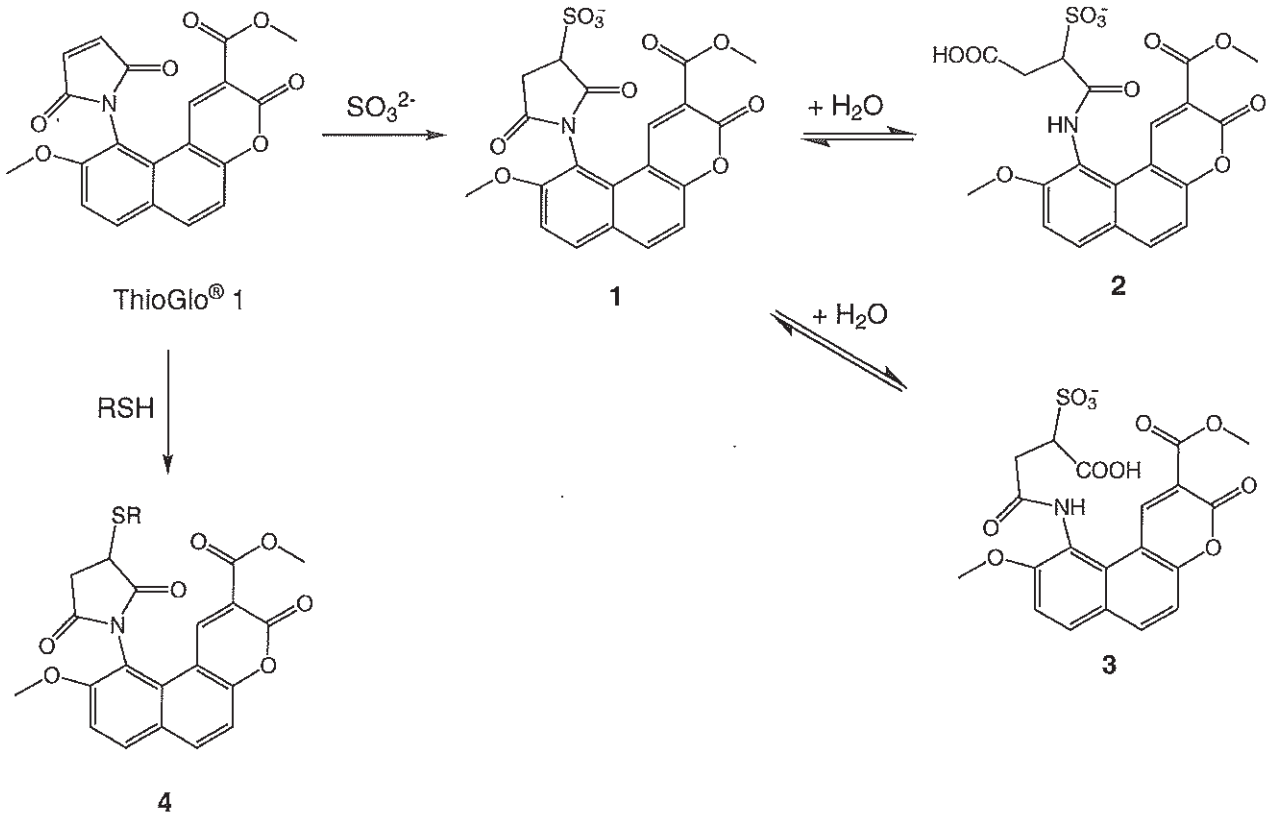

Fig. 1. Derivatization of sulfite and thiols with ThioGlo 1. Sulfite adds to the maleimide group by a Michael addition mechanism. The succinimide ring of the initial adduct 1 may undergo reversible hydrolysis to two isomeric compounds, 2 and 3. Thiols give fluorescent adducts 4.

reaction with sulfite, one initial adduct, and two isomeric hydrolyzed adducts. The method had to be performed at elevated pH (pH = 10) in order to accelerate the formation of the two hydrolyzed compounds, which were stable for more than 48 hr.

Here, we report the development of a method based on derivatization of sulfite in beer with ThioGlo 1 combined with RP-HPLC separation with fluorescence detection for determination of total sulfite content in beer. The use of RP-HPLC separation and fluorescence detection allows determination of sulfite in low concentrations, as well as in very dark beer types.

EXPERIMENTAL

Reagents and Chemicals

Stock solution of ThioGlo 1 (2.6 m*M*; Covalent Associates Inc., Woburn, MA) was prepared by dissolving 5.00 mg of ThioGlo 1 in 5.07 mL of anhydrous acetonitrile (Sigma-Aldrich, St. Louis) and storing it at 4°C, protected from light. A buffer solution (Tris-buffer) was made with 0.25 M tris(hydroxymethyl)aminomethane (Merck, Darmstadt, Germany) and the pH was adjusted to 7.5 with hydrochloric acid (HCl, 37%; Sigma-Aldrich, Steinheim, Germany). ThioGlo 1 solution was diluted 1:100 (v/v) with Tris-buffer to a concentration of 26 µ*M* prior to use. Sodium sulfite (J. T. Baker, Deventer, Holland) was used for making calibration curves and for synthesis of acetaldehyde-sulfite adduct using acetaldehyde (>99.9%; Sigma-Aldrich, St. Louis). 1-Octanol (>99.5%; Fluka, Buchs, Switzerland) was used as foam controller. Milli-Q water from a Q-plus purification system was used for standard solutions and mobile phases (Millipore Corp., Bedford, MA). Methanol (>99.9%; Merck) was of analysis grade and acetonitrile (>99.9%; Sigma-Aldrich, St. Louis) was of HPLC-gradient grade. Formic acid (>98%; Fluka), ammonium formate (>99.0%; Fluka), acetic acid (100%; Merck), and ammonium acetate (>98.0%; Merck) were of puriss grade. Trifluoroacetic acid (TFA) (>99.8%; Merck) was of spectroscopy grade. Triethanolamine (TEA) (>99.5%; Sigma-Aldrich, St. Louis) used in sample preparation was of analysis grade. For LC-MS mobile phases, water was glass distilled, methanol (>99.9%; Fisher Scientific, Loughborough, UK) was of LC-MS grade, and TFA (>99.5%; Fluka, St. Louis) was of protein sequence analysis grade.

Beers were bought from local shops. Beer A was a Danish lager made with barley and barley malt (can, 4.4% alcohol), beer B was a typical Danish all-malt lager beer (can, 4.6% alcohol), beer C was an all-malt bock-type of lager beer (bottle, 7.2% alcohol), beer D was a dark all-malt lager (can, 4.4% alcohol), beer E was a top-fermented all-malt ale (bottle, 5.5% alcohol), and beer F an all-malt porter (bottle, 7.8% alcohol).

Sample Derivatization

Beer samples were degassed by adding 10 µL of 1-octanol to 100 mL of beer and stirring for 5 min. Aliquots of 100 µL of degassed beer were diluted to 1 mL in Tris-buffer and 20 µL was transferred to a vial. Sulfite was added as a 1 mg/L solution in Tris-buffer and, finally, the total volume was adjusted to 100 µL with Tris-buffer. The added sulfite concentration ranged from 10 to 50 µg/L. Subsequently, 100 µL of ThioGlo 1 (26 µ*M* in 0.25 M Tris-buffer, pH 7.5) was added to the vial. The reaction was quenched after 5 min by addition of 10 µL of 12 *M* HCl. The final dilution factor of beer was 1:105 (v/v) and the concentration of

ThioGlo 1 was approximately 13 μ*M*. This corresponded to a sulfite standard addition calibration curve prepared in the range of SO_2 at 0 to 25 mg/L.

Free and Bound Sulfite: Addition of Acetaldehyde or Acetaldehyde-Sulfite Adduct

Acetaldehyde-sulfite adduct (sodium 1-hydroxyethanesulfonate) was synthesized according to the procedure described by Andersen et al (4). A beer of the same type as beer C was degassed as described above and divided into two portions. One portion was used as control and to the other portion was added either acetaldehyde (25 m*M*) followed by incubation for 30 min at room temperature or acetaldehyde-sulfite adduct (3 mg/L). Sulfite content was determined using the standard addition calibration procedure described above.

Instrumentation and Chromatographic Conditions

An Agilent 1100 Series liquid chromatographic system consisting of a model G1312A binary pump, a G1379A vacuum degasser, a G1313A autosampler, a G1321A fluorescence detector, and Agilent ChemStation data handling program (Agilent Technologies Inc., Palo Alto, CA) was used. Separation was performed on a Jupiter C18 (150 by 2.0 mm, 5-μm particle size, 300-Å pore size) column (Phenomenex, Torrance, CA). Water (mobile phase A) and methanol (mobile phase B) were both acidified with equal amounts of TFA (pH 2.0, 10 m*M*). The gradient was held at 25% B for 8 min (isocratic), instantly increased to 95% B and kept at 95% B for 6 min. The mobile-phase conditions were then returned to starting conditions and reequilibrated for 7 min, resulting in a total run time of 21 min. Injection volume was 20 μL. Flow rate was 0.5 mL/min, and detection was performed at excitation of 242 nm and emission of 492 nm. The sum of the areas of the two peaks corresponding to ThioGlo 1-derivatized sulfite was used for the quantification of sulfite.

Mass Spectrometry

The ThioGlo 1-sulfite adducts were characterized using an Acquity ultra-performance liquid chromatograph (Waters, Milford, MA) equipped with a photodiode array detector and a fluorescence detector, coupled to an Ultima Global quadrupole/orthogonal acceleration time-of-flight (TOF) mass spectrometer with electrospray ionization operated in negative ion mode, and MassLynx v4.1 was used for data acquisition and processing (Waters Micromass, Manchester, UK). The ThioGlo 1 derivatized compounds were separated on the Jupiter C18 column described above. Samples were prepared as previously stated with the following exceptions: TEA buffer (pH 7.5, 0.25 *M*) was used instead of Tris-buffer (pH 7.5, 0.25 *M*) and TFA was used instead of HCl to quench the ThioGlo 1 reaction. The gradient was held at 25% B for 12 min (isocratic), instantly increased to 95% B, and kept at 95% B for 5 min. Flow rate was decreased to 0.4 mL/min to increase performance of the mass spectrometer source. The mass spectrometer was operated at ion source temperature 120°C, desolvation gas temperature 420°C, cone gas flow 50 L/hr, desolvation gas flow 700 L/hr, capillary voltage 0.9 kV, cone voltage 20 V, scan time 1 sec, and interscan delay 0.1 sec. The mass spectrometer was operated in TOF scan mode (*m/z* 100 to 900). External calibration (*m/z* 180 to 793, 10-point calibration, fifth order of polynomial fit) was performed before analysis with 1 m*M* sodium hydroxide and 0.01% formic acid in methanol.

Color Determination

The absorbance of the different beers was measured at 430 nm according to EBC method 9.6 (10) using an HP-8453 spectrophotometer (Hewlett Packard, Portland, OR). Quantification of the beer color (EBC units), *C*, was then determined according to the equation $C = 25 \times f \times A_{430}$, where A_{430} is the absorbance at 430 nm and f is the dilution factor of the beer.

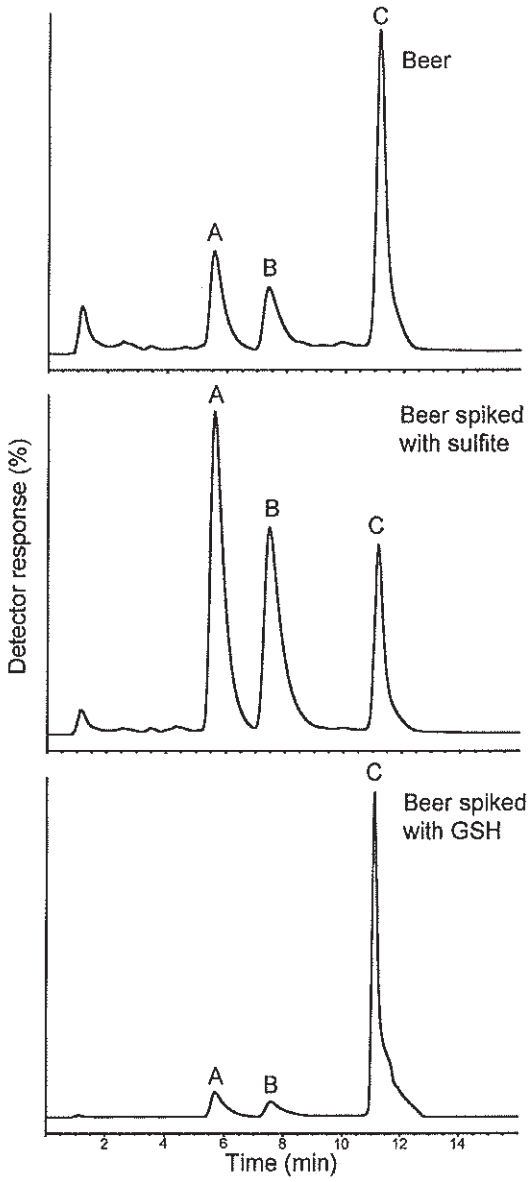

Fig. 2. Chromatogram of beer C analysis after method optimization. Peaks corresponding to derivatized sulfite (peaks A and B) and thiol-containing compounds (peak C) were identified through identification by spiking beer C with either sulfite or glutathione (GSH). Peaks are presented as percentage of the highest peak in the chromatogram.

RESULTS AND DISCUSSION

HPLC Separation and Detection of ThioGlo 1 Adducts

The HPLC separation of ThioGlo 1 adducts with sulfite and beer thiols on a C18 column was tested with various mixtures of water, methanol, and acetonitrile as eluents; eluent gradient profiles; and pH values of eluents between 2.0 and 5.0 either by adjusting with TFA or buffering with formic acid and ammonium formate or acetic acid and ammonium acetate. Optimization resulted in an eluent gradient based on only water and methanol

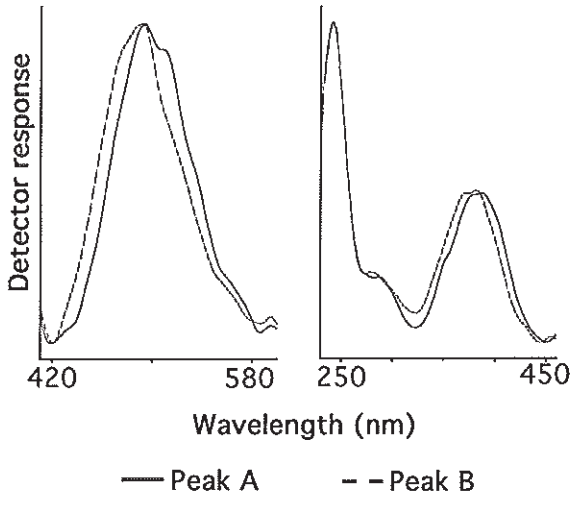

Fig. 3. Wavelength spectra at the peak apex of sulfite adducts (peaks A and B eluted first and second, respectively) in a reversed-phase HPLC chromatogram of a beer sample as shown in Figure 2. Emission spectra (left) were obtained with 384 nm excitation and excitation spectra (right) were obtained with 492 nm emission detection. The chromatographic separation was performed with a 25% methanol eluent for 8 min and then increased to 95% methanol for 6 min. The mobile phases were adjusted to pH 2.0 with trifluoroacetic acid. Flow rate was 0.5 mL/min.

with pH 2.0 adjusted with TFA, held at 25% B for 8 min (isocratic), instantly increased to 95% B, and kept at 95% B for 6 min. The mobile-phase conditions were then returned to starting conditions and the system reequilibrated for 7 min, resulting in a total run time of 21 min. These chromatographic conditions gave chromatograms where sulfite derivatives were baseline separated and the instant change of eluent composition after elution of the sulfite derivatives resulted in all the thiol-containing derivatives eluting within one single peak (Fig. 2, peak C). Sulfite derivatives were identified by injecting a derivatized standard of sulfite in buffer and always gave two peaks (Fig. 2, peaks A and B). Generally, eluents with pH higher than 2.0 or containing acetonitrile resulted in some thiol-containing derivatives eluting earlier than both the bulk thiol and sulfite derivatives.

Optimization of the fluorescence detection using ThioGlo 1-derivatized beer samples showed that optimal excitation wavelengths were 242 and 385 nm and that, in both cases, maximal emission occurred at 492 nm (Fig. 3). However, the excitation at 242 nm gave the highest emission response, and it was chosen for the further quantitative studies.

ThioGlo 1-sulfite Derivatives

Meguro et al (20) investigated the impact of pH and temperature on the derivatization of sulfite with the analogous maleimide compound NAM and found that NAM, over time, formed three products with sulfite, and that both the formation rate and relative amounts of the products were pH dependent. ThioGlo 1-derivatized sulfite gave rise to two peaks in the chromatograms, and the relative intensities of the two peaks were constant, provided the same buffer and pH were used during derivatization. The two sulfite derivatives were assessed with LC-MS in order to confirm that the peaks were derived from sulfite. The MS spectra of the two derivatized sulfite peaks were identical. With negative ion mode electrospray and full scan mode (*m/z* 100 to 900) with subtracted background, ions of *m/z* 460.0, 596.0, 612.0, 726.0, and 732.0 were found in the MS spectra of both peaks of derivatized

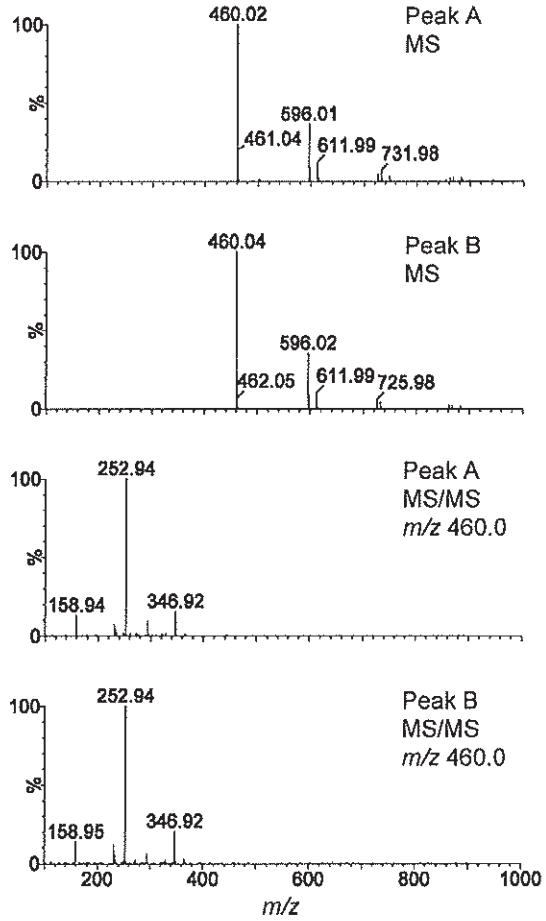

Fig. 4. Mass spectra of peaks (Fig. 2, peaks A and B) corresponding to derivatized sulfite in full scan between *m/z* 100 and 900 and daughter ions of *m/z* 460.0 (negative ion mode) of ThioGlo 1-sulfite derivatives in peaks A and B fragmented with collision energy of 30 V.

sulfite. The ion of *m/z* 460.0 was assigned to be a sulfite-ThioGlo 1 derivative, while the other ions were not identified but assumed to be adducts or cluster ions. A comparison of the theoretical isotope pattern of the ThioGlo 1-sulfite derivative with the elemental composition $C_{20}H_{14}NO_{10}S$ and the detected isotope pattern around *m/z* 460.0 showed indistinguishable compositions.

The two ThioGlo 1-sulfite derivatives (*m/z* 460.0) were further studied by fragmentation in MS/MS mode at collision energies of 10 and 30 V. In a scan mode of the daughter ions, the mother ion was intact at 10 V and only minor fragmentation was observed (*data not shown*). Only an ion of *m/z* 346.9 was observed in both spectra. At a collision energy of 30 V, the mother ion was not present. Fragments of *m/z* 346.9, 294.1, 252.9, and 158.9 were found in both spectra and with similar relative abundances (Fig. 4). The similar fragmentation patterns indicate that the ThioGlo 1 derivatives in the two HPLC peaks are converted to the same ion during the MS-analysis. Akasaka et al (2) suggested, based on IR and NMR data, that the succinimide ring structure of NAM-sulfite adducts could be hydrolyzed to two isomeric compounds, and a similar behavior has been reported for thiol-maleimide adducts (15). The HPLC separation of the ThioGlo 1-sulfite adducts into two peaks can be explained by a similar mechanism where two isomeric hydrolyzed adducts, 2 and 3, are formed (Fig. 1). However, the identical MS results for the two peaks and the lack of

TABLE I
Characteristics of the Six Standard Curves Made by Addition of Sulfite to Beer and the Beer Color (95% Confidence Interval)

	Beer					
	A	B	C	D	E	F
Response Factor	540.6 ± 28.0	527.3 ± 11.3	443.9 ± 32.1	449.6 ± 27.9	414.1 ± 12.6	407.2 ± 19.0
R^2	0.9986	0.9998	0.9973	0.9980	0.9995	0.9989
EBC Color[a]	8.62 ± 0.04	7.84 ± 0.05	13.8 ± 0.1	30.0 ± 0.4	128.5 ± 0.5	236.9 ± 0.5

[a] n = five replicates.

TABLE II
Quantitative and Statistical Results Derived from Analysis of Sulfite in Different Types of Beer

	Analyzed Beer, SO_2 Concentration (mg/L)						
Beer for Standard[a]	A	B	C	D	E	F	RMSE[b]
A	1.89[c]	5.32	6.81	3.42	0.46	1.86	1.01
B	1.57	5.44[c]	6.98	3.51	0.47	1.90	0.93
C	1.86	6.48	8.73[c]	4.17	0.56	2.26	0.49
D	1.84	6.40	8.19	4.51[c]	0.55	2.23	0.50
E	1.99	6.95	8.89	4.47	0.57[c]	2.42	0.68
F	2.03	7.07	9.04	4.54	0.61	2.32[c]	0.74
SD	0.16	0.71	0.97	0.52	0.05	0.24	...
Mean	1.83	6.12	7.92	4.01	0.52	2.14	...
RSD (%)	9	12	12	13	10	11	...

[a] Beer used for constructing standard curve. SD = standard deviation and RSD = relative standard deviation.
[b] Root mean squared error.
[c] Value attained from the standard addition method.

real mother ion of m/z 478.0 suggest that the hydrolysis is reversible, and that the conditions during the electrospray result in loss of water and a closure of the succinimide ring, regenerating the initial sulfite adduct. The MS results together with the HPLC spiking experiments prove that the two ThioGlo 1 adducts detected after the HPLC separation are derived from sulfite and, therefore, that they can be used for the direct quantification of sulfite.

Matrix-Matched Calibration Curve

Quantification of sulfite by means of a standard calibration curve made with water proved to be unsatisfactory due to the significantly different ($P < 0.01$) response factors of 101.4 ± 1.6 and 89.7 ± 2.6 (95% confidence interval) for calibration curves prepared in water and in beer C, respectively. The matrix effects of beer imply that correct measurements may be obtained through matrix-matched calibration or standard addition but not from aqueous calibration solutions. In order to overcome the matrix-related effects in beer, an attempt was made to establish a matrix-matched calibration curve. Different beers ranging from light to very dark types were tested as matrix match in order to determine whether the observed matrix effect was independent of beer type. Samples and spiked samples were prepared in triplicates and each injected twice for both types of calibration curves. The response factor appeared to be related to the darkness of the beer (Table I). A higher response factor was observed for the two brightest beers (A and B) while the darkest beer (F) gave the lowest response factor. These effects are likely due to inner-filter effects, where the intensities of either the light used for excitation or the light emitted by the fluorescent derivatives are affected by co-eluting nonfluorescent beer components.

To determine which beer was most suitable as a matrix match, the root mean squared error (RMSE) of the fit between matrix-match calibration curve results and results obtained by standard addition was calculated. The matrix-matched calibration curves were prepared in each of the six beers, keeping in mind the initial levels of sulfite. The sulfite level in each beer was then determined by each matrix-matched calibration curve. Levels below the calibration range were quantified by extrapolating the calibration curve. Using external standard calibration curves by extrapolating below the calibration range is not good practice. However, for a matrix-matched calibration curve, where the matrix match is not a true blank (such as beer, which has a background level of sulfite), extrapolations below the calibration range are often necessary. The value attained from the standard addition method was considered as the "true" value. Beers C and D had the best results, with RMSE values of 0.49 and 0.50, respectively (Table II). Beer E had the lowest initial level of sulfite (SO_2 at 0.6 mg/L, determined by the standard addition method), as well as acceptable RMSE. Consequently, beer E was chosen as matrix match. The standard addition experiment is always assumed to yield a more accurate measure of concentration and, indeed, the external standard calibration curve approach—with or without matrix match—should be considered an operational alternative, because it is less labor intensive and can be performed on smaller sample sizes but with lower accuracy as trade-off.

Method Validation

The limit of detection (LOD) and limit of quantification (LOQ) were determined by standard addition method using beer E, which contained the lowest level of sulfite. Determination of LOD and LOQ was done according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) 6.3 and 7.3 based on the standard deviation of the response (n = 9) and slope (13). The standard deviation of the response was based on the manually integrated analyte peaks in the nine replicate injections of beer E. Beer without added standard was injected nine times (injection volume = 20 µL). The LOD of SO_2 was determined to be 0.2 mg/L and the LOQ was 0.6 mg /L in beer.

The precision was based on sulfite determination of beers A and F utilizing the matrix-matched calibration curve of beer E (Table III). Three replicates of each sample were analyzed on three different days. The repeatability and intermediate precision were calculated according to ISO 5725-2 (13). Sulfite determination with the ThioGlo 1 method demonstrated good repeatability com-

TABLE III
Repeatability and Reproducibility of the Matrix-Matched ThioGlo 1 Method Compared with the Monier-Williams Method, the *p*-Rosaniline Method, the Institute of Brewing (IOB) Rapid Method, and the 5,5′-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) Method[a]

	Mean	Repeatability		Reproducibility	
Sample	SO_2 (mg/L)	SD_r	RSD_r (%)	SD_R	RSD_R (%)
ThioGlo 1 method[b]					
Beer A	2.14	0.034	1.6	0.034	1.6[c]
Beer F	3.41	0.124	3.6	0.124	3.6[c]
Monier-Williams method[d]					
Level 1	1.84	0.231	12.6	0.810	44.0
Level 2	7.13	1.341	18.8	1.506	21.1
p-Rosaniline method[d]					
Level 1	1.37	0.206	15.0	0.371	27.1
Level 2	6.38	0.503	7.9	0.656	10.3
IOB Rapid method[d]					
Level 1	1.87	0.414	22.1	0.618	33.0
Level 2	6.01	0.749	12.5	1.653	27.5
DTNB method[d]					
Level 1	2.12	0.278	13.1	3.259	153.7
Level 2	8.16	1.197	14.7	6.87	84.2
Enzymatic method[e]					
Unknown sample	4	0.2	5	0.7	17

[a] Different batches of beers A and F were used than for previous experiment, thus the deviation in sulfite content compared with the results in Table 2. SD_r = Standard deviation of repeatability, RSD_r = relative standard deviation of repeatability, SD_R = standard deviation of robustness, and RSD_R = relative standard deviation of robustness.
[b] Each sample prepared in triplets at each day and reproducibility measured over three days, although at identical conditions.
[c] Intermediate precision. Between-days variance was insignificant.
[d] Assessed by the IOB analysis committee, conducted according to the ISO 5725 (14).
[e] Reported by the European Brewery Convention (10).

TABLE IV
Determined Recoveries Based on the Matrix-Matched Calibration Curves[a]

	Analyzed beer, Recoveries (%)					
Beer for Standard[b]	A	B	C	D	E	F
A	104	97	88	87	76	73
B	107	100	90	89	78	75
C	127	118	107	106	93	89
D	125	117	105	105	91	88
E	136	127	114	114	99	96
F	138	128	116	115	101	97

[a] Background sulfite levels were quantified using the standard addition method and the mean values of recoveries were based on SO_2 spike levels of 5, 10, 15, 20, and 25 mg/L.
[B] Beer used for constructing standard curve.

pared with already accepted methods (6,10), with values of 1.6 and 3.6% expressed as RSD for beers A and F, respectively. A variance components analysis using nested analysis of variance showed that the variance component between days was not significant compared with the within-day variance. Thus, the variation between days could simply be accounted for by the random error. Therefore, basic estimations of intermediate precision provided 1.6 and 3.6% RSD by analyzing beers A and F, respectively. The precision of the presented method appeared to be comparable with and possibly better than the recommended methods, including the Monier-Williams, *p*-rosaniline, IOB rapid, DTNB, and enzymatic method. However, it must be stressed that precision estimations for these methods were evaluated under other conditions and in a much larger scale, covering 8 (performed by IOB) or 12 (performed by EBC) different laboratories.

Recoveries were calculated using five different levels of spiking ranging from 5 to 25 mg/L of added sulfite (Table IV). For all of the beers and concentrations tested, several matrix matches with good (90 to 110%) to acceptable (80 to 90 or 110 to 120%) accuracy could be found. The only exception was quantification of sulfite in beer C at low levels. The quantification of sulfite in beers A and B was not accurate when using beers C, D, E, or F as matrix match (i.e., recoveries were lower than 90% or higher than 110%). To the same end, quantifications of sulfite in beers C, D, E, and F were acceptably accurate using beers C, D, E, or F as matrix match. Best results are obtained if a beer of corresponding color is used as matrix match. Not surprisingly, lower sulfite levels were determined with slightly lower accuracy. Because beers A and B were bright beers and beers C, D, E, and F dark beers, the general recommendation is to use light beer as matrix match for quantification of sulfite in light beer and vice versa.

Quantification of Free and Bound Sulfite

The current method is based on standard calibration curves prepared with addition of free sulfite to beer. However, sulfite in beer appears both as free and bound sulfite, where acetaldehyde-sulfite adducts, according to Kaneda et al (17), is the dominating bound form. The ability of ThioGlo 1 to react with bound sulfite was tested by adding acetaldehyde to beer C and, subsequently, quantifying sulfite by the standard addition calibration procedure. Acetaldehyde binds sulfite reversibly as adducts:

$$CH_3CHO + HSO_3^- \leftrightarrows CH_3CH(OH)SO_3^- \quad (1)$$

Addition of acetaldehyde (25 m*M* final concentration) to beer and incubation for 20 min result in complete reaction between acetaldehyde and free sulfite (17). Based on this knowledge, acet-

aldehyde (25 m*M*) was added to a beer containing 5.2 ± 0.3 mg/L of SO_2 as determined by the ThioGlo 1 method. After the addition of acetaldehyde, the level of sulfite in the beer as determined by the ThioGlo 1 method was 5.5 ± 0.1 mg/L of SO_2, which demonstrates that the potential binding of sulfite by acetaldehyde does not affect the measured level of sulfite. This finding was further supported by an experiment where pure crystalline acetaldehyde-sulfite adduct was synthesized and added to a beer similar to beer C. The beer originally contained 4.4 ± 0.2 mg/L of SO_2. After addition of acetaldehyde-sulfite adduct equivalent to 3.0 mg/L of SO_2, the level of sulfite was determined to be 7.4 ± 0.2 mg/L of SO_2, in very good agreement with the value expected. These results confirm that the ThioGlo 1-based method detects both bound and free sulfite and, thereby, quantifies the total level of sulfite in beer.

CONCLUSIONS

Sulfite in different types of beer can be determined by initial derivatization with ThioGlo 1 and subsequent RP-HPLC separation of the fluorescent adducts. The method determines total sulfite in beer and is based on using standard addition for the quantification. A matrix-matched calibration curve was found suitable for the simultaneous analysis of beer of different brands and types.

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Paper VII

Quantification of Protein Thiols using ThioGlo 1 Fluorescent Derivatives and HPLC separation

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Quantification of protein thiols using ThioGlo 1 fluorescent derivatives and HPLC separation†

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A method for quantification of total soluble protein-derived thiols in beer was developed based on the formation of fluorescent adducts with the maleimide compound ThioGlo 1. The problem of interference from fluorescent adducts of sulfite and ThioGlo 1 was solved by HPLC separation of the adducts followed by fluorescence detection. Using standard addition of GSH, a detection limit of 0.028 μM thiols was achieved. The application and validation of the method was demonstrated for beers with different color intensities, and the application range is in principle for any biological system containing thiols. However, the quantification of cysteine was complicated by a lower fluorescence response of its ThioGlo 1 adducts. Based on the studies of the responses of a series of cysteine-derived thiols and ^{1}H NMR studies of the structures of ThioGlo 1 adducts with GSH and cysteine, it was concluded that thiols with a neighboring free amino group yield ThioGlo 1 adducts with a reduced fluorescence intensity.

1 Introduction

The thiol group (–SH) of cysteine is easily oxidized by many oxidants and radicals, and the rate of thiol oxidation in some systems reaches diffusion controlled rates.[1,2] Furthermore the thiolate anion (RS^-) is one of the strongest biological nucleophiles, and consequently, the thiol group of cysteine is one of the most reactive functional groups in proteins.[3] In cells, thiol groups are implicated in the defense against oxidation, where glutathione (GSH) represents the major low molecular weight antioxidant, as well as being part of fundamental redox-signaling systems.[4,5] During oxidation of thiols, a number of different oxidation products can be formed such as disulfides and oxy acids (sulfenic, sulfinic, and sulfonic acids). Disulfides, which are often the major product, can be reduced enzymatically in cells[6,7] and are involved in protein folding, regulation of enzyme activity, redox cell signaling, and other biological and biochemical functions.[6,7] However, when the rate of oxidation exceeds that of repair, a loss of thiols can be detected as observed for various diseases, disorders, and cell ageing, making thiols a quantitative marker for oxidative stress.[8]

Thiol oxidation has also been found to be of high relevance in relation to food quality. For instance, the tenderness of meat has been linked to the formation of disulfide bonds in the major myofibrillar protein, myosin,[9] while in bread production disulfide bond formation is critical for the strength of the dough and its baking properties.[10] In beer, the major antioxidant is believed to be sulfite, but recently, thiols have been suggested to contribute to the antioxidant capacity in beer by removing H_2O_2,[11,12] and the content of thiols has been shown to be correlated with the oxidative stability of beer.[13] Hence, understanding of the thiol redox status in relation to food quality is gaining increasing interest in order to obtain high quality food products with good flavor and texture stability.

The most commonly applied method for thiol determination is based on Ellman's derivatizing reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with spectrophotometric detection of the generated 5-thio-2-benzoic acid at 412 nm.[14] A number of other derivatizing agents has been used for sensitivity improvement,[15,16] but using this methodology for determination of thiols in beer is not straightforward. Some beers contain relatively high concentrations of sulfite produced by yeasts during the fermentation process. Sulfite reacts with the thiol-derivatizing reagents providing similar signals to those of thiols, and sulfite therefore greatly complicates thiol determination in beer. Previously, we developed a method for thiol determination in beer based on derivatization with ThioGlo 1, a maleimide derivative of a naphthopyranone fluorophore that yields a fluorescent adduct when reacted with thiols (Fig. 1). Sulfite gives rise to a fluorescent ThioGlo 1 adduct with similar spectral characteristics to those of the thiol–ThioGlo 1 adducts, and the contribution to the fluorescent signal from the sulfite–ThioGlo 1 adduct must be subtracted by using a separate determination of the sulfite concentration and a standard addition curve based on sulfite and ThioGlo 1.[13] Recently, a method for sulfite quantification based on ThioGlo 1 derivatization was developed, where the problem of interference from thiols was solved by HPLC-separation of the ThioGlo 1

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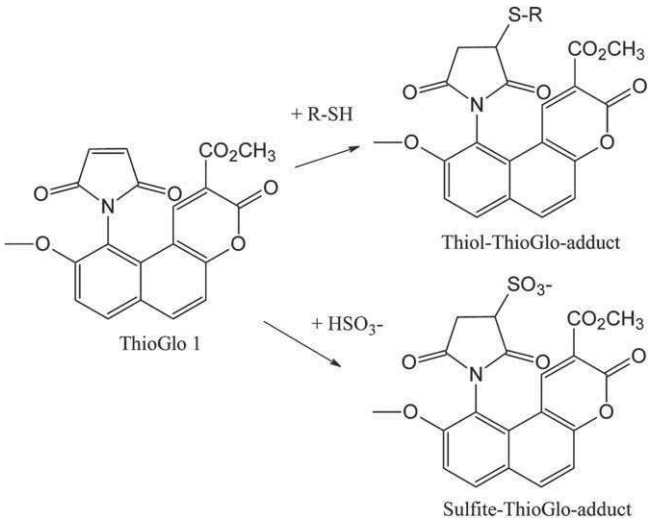

Fig. 1 Reaction between ThioGlo 1 and thiol (R–SH) or hydrogen sulfite (HSO_3^-) yielding a fluorescent adduct.

adducts.[17] In the present study the HPLC based method is further developed to allow quantification of the level of total soluble thiols. Cysteine was found to give lower responses than other thiols, and the origin of this different behavior has been linked to the structural features of the thiol molecules. The method is applied here and validated for beer analysis, but it should be easily adapted to any (biological) system where thiol quantification may be complicated by the presence of sulfite.

2 Materials and methods

2.1 Reagents and chemicals

A stock solution of ThioGlo 1 (10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-mehoxy-3-oxo-3*H*-naphthol [2,1-*b*]pyran-2-carboxylic acid methyl ester) (2.6 mM, Covalent Associates Inc., Woburn, MA) was prepared by dissolving 5.00 mg ThioGlo 1 in 5.07 mL anhydrous acetonitrile (Sigma-Aldrich, St Louis, MO). The solution was kept anhydrous during storage by adding dried molecular sieves (0.3 nm, Metrohm Ltd., Herisau, Switzerland), and the solution was stored at 4 °C and protected from light.[2] A buffer solution (tris-buffer) was made with 0.25 M tris(hydroxymethyl)aminomethane (Merck, Darmstadt, Germany) and the pH was adjusted to 7.5 with hydrochloric acid (HCl, 37%, Sigma-Aldrich, Steinheim, Germany). The tris-buffer was treated with nitrogen gas to preserve it. Stock solutions of glutathione (GSH, Sigma-Aldrich, Steinheim, Germany) and sodium sulfite (J.T. Baker, Deventer, Holland) were freshly prepared every day in Milli-Q water, kept cold, and subsequently diluted to 2.0 μM and 7.81 μM (0.50 mg L^{-1}), respectively, in 0.25 M tris-buffer (pH 7.50). ThioGlo 1 solution was diluted 1 : 100 with tris-buffer to a concentration of 26 μM just prior to use. GSH, cysteine (Merck, Darmstadt, Germany), cysteine ethyl ester (Sigma-Aldrich, Steinheim, Germany), homocysteine (Sigma-Aldrich, Steinheim, Germany) and *N*-acetyl cysteine (Sigma-Aldrich, Steinheim, Germany) were used for calibration curves. 1-Octanol (>99.5%, Fluka, Buchs, Switzerland) was used as a foam controller. Milli-Q water from a Q-plus purification system was used for standard solutions and mobile phases (Millipore Corp., Bedford, MA). Methanol (>99.9%, Merck, Darmstadt, Germany) was of analysis grade and acetonitrile (>99.9%, Sigma-Aldrich, St Louis, MO) was of HPLC-gradient grade. Trifluoroacetic acid (TFA, >99.8%, Merck, Darmstadt, Germany) was of spectroscopy grade.

Beer A was an all-malt bock-type of lager beer (bottle, 7.2% alcohol). Beer B was a brown ale (bottle, 4.7% alcohol). Both beers were obtained from local shops.

2.2 Sample derivatization for the HPLC method

The method for quantification of thiol groups in beer was based on the recently developed method for quantification of sulfite in beer.[17] 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 in tris-buffer (pH = 7.50). GSH was added as a 2.0 μM stock solution in tris-buffer and the total volume was adjusted to 100 μL with tris-buffer providing the final added concentration in the range of 0.1 to 0.5 μM. 100 μL of 26 μM ThioGlo was added to each sample (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. Linear standard addition calibration curves from GSH addition were prepared from the obtained peaks for each beer sample and used for thiol quantification. A blank sample of only ThioGlo 1 and tris-buffer (pH 7.5) was run in parallel on each day and subtracted from the standard addition curves to compensate for background fluorescence from ThioGlo 1. The final dilution factor of beer was 1 : 105 and the concentration of ThioGlo 1 was approximately 13 μM.

Sample derivatization concerning quantification of sulfite was carried out as described for thiol quantification using standard addition calibration curves based on sodium sulfite.[17]

2.3 Instrumentation and chromatographic conditions

An Agilent 1100 Series liquid chromatographic system consisting of 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) was used. Separation was performed on a Jupiter C18 (150 × 2.0 mm, 5 μm particle size, 300 Å pore size) column (Phenomenex, Torrance, CA). Water (mobile phase A) and methanol (mobile phase B) were both acidified with equal amounts of TFA (pH 2.0, 10 mM). The gradient was held at 25% B for 8 min (isocratic), instantly increased to 95% B and kept at 95% B for 6 min. The mobile phase conditions were then returned to starting conditions and re-equilibrated for 7 min resulting in a total run time of 21 min. The injection volume was 20 μL. The flow rate was 0.5 mL min^{-1}, and detection was performed at an excitation wavelength of 242 nm and an emission wavelength of 492 nm. The area of the bulk peak of co-eluting thiol-containing peptides and proteins was used for the determination of thiols. Thiols were quantified as GSH equivalents.

2.4 Thiol quantification using ThioGlo 1 in a microtiter plate

Quantification of thiols using microtiter plates was carried out according to Lund and Andersen[13] by preparing two standard addition curves based on GSH and sulfite, respectively. Sulfite was quantified externally by the HPLC method described by Abrahamsson *et al.*[17] The contribution of sulfite to the fluorescence signals from the beer was subtracted based on the amount of externally quantified sulfite and the standard addition curve based on sodium-sulfite. For experiments testing the influence of SDS on the reaction between ThioGlo 1 and thiols, 1% SDS was added to 0.25 M tris-buffer (pH = 7.5).

2.5 Thiol quantification using Ellman's reagent, DTNB

Quantification of thiols using Ellman's reagent, DTNB, was carried out with standard addition curves of both GSH and sodium sulfite with beer, similar to the experiments using the microtiter plate method, however, the beer was diluted only 12 times. Based on the method described by Jongberg *et al.*,[18] the analysis was carried out by mixing 250 μL of sample, 1.00 mL of 0.25 M tris buffer (pH 7.5), and 250 μL of 10 mM DTNB dissolved in 0.25 M tris buffer (pH 7.5). The mixture was protected against light and allowed to react for exactly 30 min, and absorbance was measured at 412 nm. Blanks without DTNB and blanks without sample were prepared, and the blank values were subtracted from the sample values.

2.6 ^{1}H NMR experiments

Reaction solutions of ThioGlo 1 with cysteine or GSH were analyzed in the final concentration of 1 mM reacting 1 : 1. NMR measurements were performed on a Bruker Avance DRX 500 spectrometer operating at 500.13 MHz for ^{1}H using a double-tuned BBI probe equipped for 5 mm (o.d.) sample tubes. The spectra were recorded at 10 °C on a mixture of 495 μL sample (made from ThioGlo 1 stock solution mixed with either cysteine or GSH in 0.25 M tris buffer) and 55 μL D_2O (containing 5.8 mM TSP-d4). All spectra were recorded using a recycle delay of 5 s (during which the water resonance was pre-saturated), an acquisition time of 1.64 s, a spectral width of 10 kHz and 8 scans. Chemical shifts were referenced to TSP-d4 at 0.0 ppm.

3 Results and discussion

3.1 HPLC separation of ThioGlo 1 adducts and thiol quantification

ThioGlo 1 is a maleimide derivative of naphthopyranone fluorophore that yields a fluorescent adduct upon reaction with thiols or sulfite (Fig. 1). The HPLC separation of ThioGlo 1 adducts was based on the method for sulfite quantification, described by Abrahamsson *et al.*,[17] resulting in baseline separation of two sulfite derivatives and elution of thiol-containing peptide- and protein-derivatives within one single peak enabling quantification of the total amount of thiols (Fig. 2). A matrix effect for quantification of ThioGlo 1 adducts was observed,[13] and it was found to be driven by the presence of sulfite. However, the standard addition procedure, described by eqn (1), was applied in this study for two reasons: (1) since the standard addition procedure is necessary during sulfite quantification, it was also applied for thiol quantification, and (2) for quantification of thiols in beers with different colors, an inner filter effect will cause the ThioGlo 1 adduct to be insufficiently excited in dark beers compared to light beers. The standard addition procedure can be described by eqn (1):

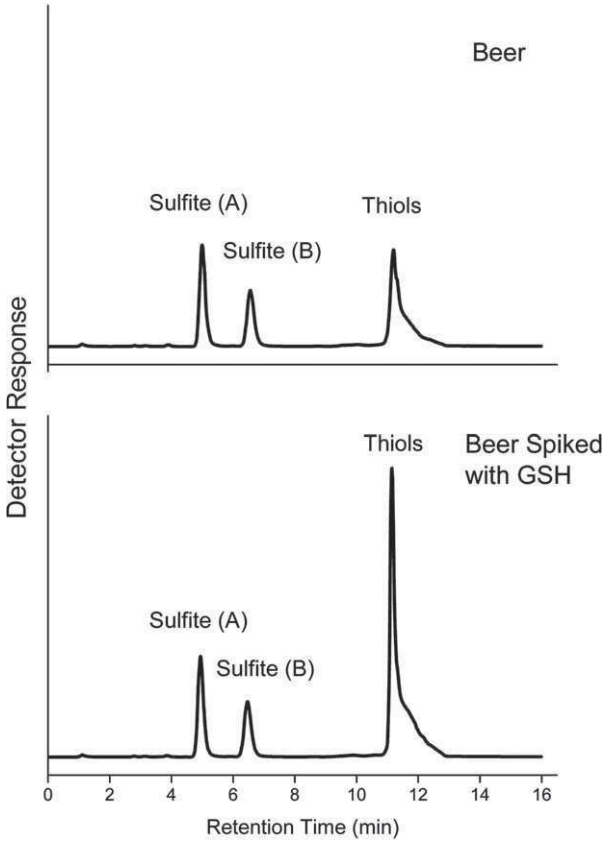

Fig. 2 Chromatogram of beer A before and after spiking with GSH.

$$\frac{[X]_i}{[S]_f + [X]_f} = \frac{I_x}{I_{s+x}} \quad (1)$$

$[X]_i$ is the unknown initial concentration of the analyte, with the signal intensity I_X. $[S]_f$ is the known (final) concentration of the standard, and I_{S+X} is the signal observed for the solution consisting of the solvent and the analyte. The diluted unknown (final) concentration of the analyte is $[X]_f$. Application of the standard addition procedure makes it possible to determine and compare thiol contents in both light and dark samples, which is highly relevant in beer studies. The thiol content was quantified based on the standard addition of GSH and is therefore expressed in terms of the equivalent GSH concentration. The beer subject to analysis was spiked with 4 different concentrations of GSH and each reaction was quenched with HCl, 5 minutes after initiation of the reaction with ThioGlo 1. For each determination, the background signal from ThioGlo 1 and tris buffer (pH 7.5) was measured and subtracted (Fig. 3). The tris-buffer (pH 7.5) was found to be unstable over time, resulting in an increased background signal from the blank sample. However, by purging the tris-buffer solution with

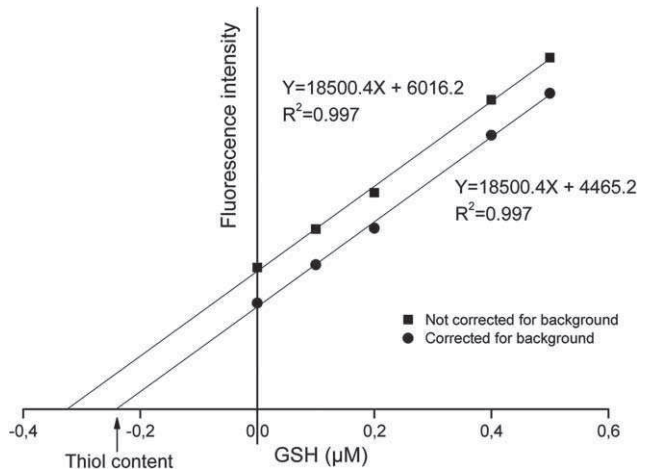

Fig. 3 Standard addition curve of beer A spiked with 4 different concentrations of GSH, showing the standard addition curve with and without correction for background from ThioGlo 1.

nitrogen gas (N_2), carbon dioxide (CO_2) or even atmospheric air, the buffer remained stable with a low background over extended periods. The possible interference of iron was also investigated, but the addition of iron salts had no influence on the buffer stability and background signal.

Thiols are very sensitive to oxidation, and once the beer bottle is opened to the air, the thiols start to oxidize at accelerated rates. Therefore, handling of the beers from the opening of the bottle until the initiation of the reaction with ThioGlo 1 should be highly standardized, including a minimum of oxygen exposure until the derivatisation step.

3.2 Method validation

Determination of LOD and LOQ was based on the standard deviation of the response ($n = 10$) and slope.[19] Beer with ThioGlo 1 added but without standard was injected 10 times, and the standard deviation of the response was based on the integrated thiol HPLC peak, resulting in a LOD of 0.028 μM and a LOQ of 0.085 μM (Table 1). Further method validation was based on beer from different bottles, but from the same batch of brew. The accuracy, expressing the degree of consistency between the measured and the actual concentration in the sample, based on the average recovery of the spiking with 5, 10, 20 and 25 μM of GSH to beer was 117% for beer A and 104% for the dark colored beer B (Table 1).

Determination of repeatability was performed on different days since it was not possible for practical reasons to prepare and analyze standard addition curves for 6 beers on the same day. Stock solutions of GSH and sulfite were freshly prepared every day. For each bottle, both sulfite and thiols were quantified and repeatability was determined for both sulfite and thiols. The Relative Standard Deviation (RSD) value of the thiol quantification was 9.87% and the RSD value for the sulfite quantification was 10.5% (Table 2). Abrahamsson *et al.*[17] found that sulfite could be determined with a high precision of 2.6 RSD% using matrix matched standard addition calibration curves. Therefore the larger RSD value, observed in this study, is likely to be caused by a variation in sulfite content in the beers. As the sulfite content varies between the bottles, it seems likely that also the thiol content is varying. Therefore, the repeatability determination of the thiol content in beer A contains the variation between the bottles, as well as the variation caused by the method.

3.3 Comparison of HPLC and plate methods

A method for thiol determination in beer carried out in microtiter plates using the standard addition procedure and fluorescent detection with ThioGlo 1 was published by Lund and Andersen.[13] As both sulfite and thiols react with ThioGlo 1, application of this method requires that the sulfite content of the beer is known, in order to be able to subtract the contribution from the ThioGlo–sulfite adduct to the fluorescence signal. Beer A was analyzed in triplicate using the plate method, resulting in an RSD of 28.5% and the average thiol content close to the value detected by the HPLC method (Table 2). The fact that the sulfite content needs to be determined separately is a major disadvantage of this method. Furthermore, the sulfite concentration in some beers is up to *ca.* 10 times larger than the thiol concentration, and sulfite therefore contributes greatly to the total fluorescent signal. As can be seen from Table 2, beer A had an average thiol concentration of 28.2 μM, whereas the sulfite concentration was 210.6 μM (13.5 mg L^{-1}), *i.e.* 7.5 times larger. Therefore, a small relative change in sulfite content results in a large variation in the thiol quantification and introduces an uncertainty to the result. In addition to the potential variation in the sulfite content in beers used in the current study and the fact that the sulfite is responsible for a large part of the signal, there is a larger variation in the plate method compared to the HPLC method. However, the result from the plate method shows relatively good compliance with the result from the HPLC method, confirming that the thiols all enter the detector and are not trapped in the column.

3.4 Thiol determination in beer using DTNB

The most commonly used method for thiol determination is based on Ellman's derivatizing reagent, DTNB, with spectrophotometric detection at 412 nm of the released thiolate ion upon reaction with thiols.[14] As observed for ThioGlo 1, DTNB

Table 1 Limit of Detection (LOD) and Limit of Quantification (LOQ) based on 10 injections and the slope and accuracy, based on the average of the addition of 5, 10, 20 and 25 μM GSH

	Beer A	Beer B
Accuracy	117%	104%
LOD $n = 10$	0.028	—
LOQ $n = 10$	0.085	—

Table 2 Precision of the methods for quantification of thiols by HPLC and in microtiter plates, and of sulfite determined by HPLC in beer A

	Average (μM)	RSD (%)
Thiols, HPLC method ($n = 6$)	28.2 ± 2.8	9.87
Thiols, microtiter plate method ($n = 3$)	25.8 ± 7.4	28.5
Sulfite, HPLC method ($n = 7$)	210.6 ± 21.9	10.5

also reacts with both sulfite and thiols, and correction for the sulfite content is required in order to quantify the thiols. Spectrophotometric detection is much less sensitive than fluorescence detection and the DTNB method has been reported to be unreliable for concentrations below 3 μM.[20] In the present study the detection limit using ThioGlo 1 was 0.028 μM resulting in a more than 100 times increased sensitivity compared to the DTNB method. Wright and Viola[21] studied thiol detection using both DTNB and ThioGlo 1 and demonstrated that the detection limit for the DTNB method could be decreased to 0.3 μM by converting it to a titration method and found a detection limit of 0.01 μM when using ThioGlo 1. In the current study, experiments were carried out to test the compatibility between the DTNB and the ThioGlo 1 method. However, results from the DTNB method showed a thiol content close to zero and with a rather large variation between the two repetitions (see ESI†). This is most likely explained by the fact that the contribution from the thiols is too small compared to the contribution from sulfite, the background signal from DTNB, and the background signal from the beer. Furthermore, quantification of thiols in dark beers may complicate the measurement even with the standard addition procedure due to higher background absorption from the beer. Therefore, the DTNB method is not suitable for quantification of thiols in beer containing sulfite.

3.4.1 Influence of SDS. The thiol determination based on DTNB typically includes a denaturing agent such as Sodium Dodecyl Sulfate (SDS) in the buffer with the purpose of improving the accessibility of the thiols to DTNB by unfolding the protein. The influence of SDS was first investigated in the DTNB assay, where it was found to have a positive influence on the amount of thiols quantified in beer. But, as described in Section 3.4 the low sensitivity of the DTNB method still resulted in a thiol content close to the detection limit. SDS is not compatible with the current HPLC separation, and the influence of SDS on the ThioGlo 1 assay was therefore tested using the microtiter plate method.[13] SDS did not result in an increased detection of thiols (Fig. 4A). In contrast, it resulted in a slight decrease in the detected level of thiols possibly caused by the negative influence SDS has on the sulfite standard addition curve (Fig. 4B). The sulfite standard addition curve prepared in the presence of SDS had a very low fluorescence intensity. This may be explained by the generation of SDS-micelles that may trap ThioGlo 1 and thereby make it unavailable for reaction with the water-soluble sulfite. The reason why SDS influenced thiol determination when using DTNB, and not ThioGlo 1, may also be explained by the difference in solubilities of the derivatizing agents. DTNB is a more hydrophilic molecule and it therefore requires protein unfolding by denaturing agents to get access to the more hydrophobic areas of proteins. Addition of denaturing agents is not necessary for ThioGlo 1 due to its larger hydrophobic areas. In agreement with this observation, Wright and Viola[21] found the derivatization with ThioGlo 1 to be rapid, also with thiols that were only partially accessible. Furthermore, due to the boiling step in the brewing process, beer proteins are likely to be denaturated to a certain degree[22] and thiol groups in beer are therefore likely more accessible to ThioGlo 1. Based on these results it can be concluded that SDS does not have a positive influence on the quantification of thiols using ThioGlo 1 and most likely a negative influence on the detection of the sulfite–ThioGlo 1 adduct. Thiol quantification using ThioGlo 1 is therefore carried out without SDS.

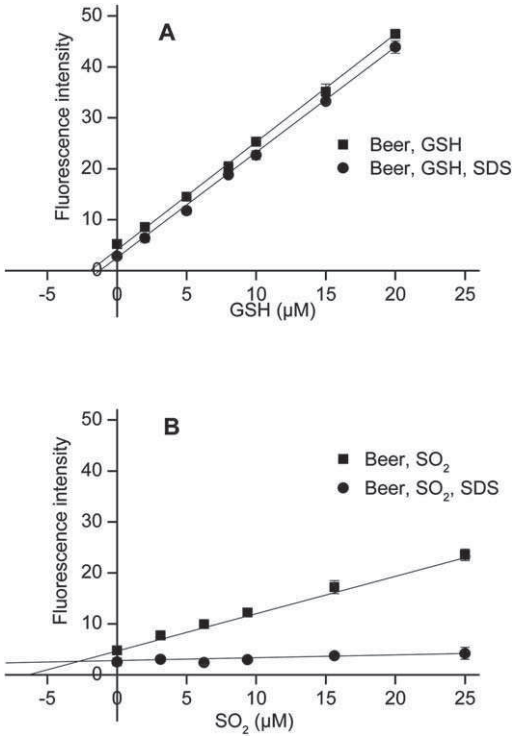

Fig. 4 Background corrected standard addition curves of beer A spiked with GSH (A) and sulfite (B) with and without SDS determined by the microtiter plate method using ThioGlo 1. Values are given as means and standard deviation is shown by error bars ($n = 2$).

3.5 Fluorescence intensity of cysteine–ThioGlo 1 adducts

Attempts were also made to use cysteine instead of GSH for constructing standard addition curves. However the slopes of the linear standard curves were smaller when cysteine was used, demonstrating a lower fluorescence intensity of the cysteine–ThioGlo 1 adducts (Fig. 5). The difference between the signal intensity of GSH–ThioGlo 1 adducts and cysteine–ThioGlo 1 adducts was further examined by generating ThioGlo 1 adducts with three structurally similar small thiol compounds (Fig. 6). The response of *N*-acetyl cysteine, which has a blocked amino-group, yielded the same slope of the standard curves made with GSH. Homocysteine, which has a longer thiol-side chain than cysteine, yielded a slightly lower slope than GSH, whereas cysteine ethyl ester, which has a blocked carboxylic acid group, gave a similar response to that of cysteine. As GSH and *N*-acetyl cysteine gave the same fluorescence intensity, this illustrates that the interference is likely to be caused by the free amino group. In the GSH molecule, the free amino group is located far from the thiol group and does not cause a problem for the binding to the ThioGlo 1 molecule. Furthermore, the signal from homocysteine is closer to the signal from GSH, and the signal from cysteine ethyl ester is the same as the signal from

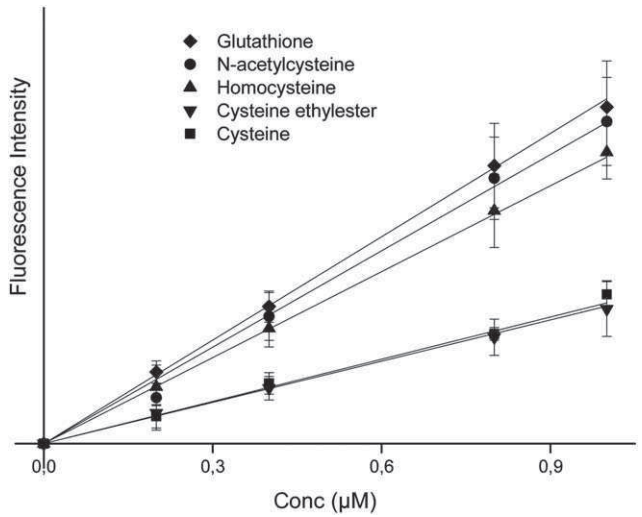

Fig. 5 Standard curves of 5 thiol compounds prepared in buffer (no beer added). Values are given as means and standard deviation is shown by error bars ($n = 4$).

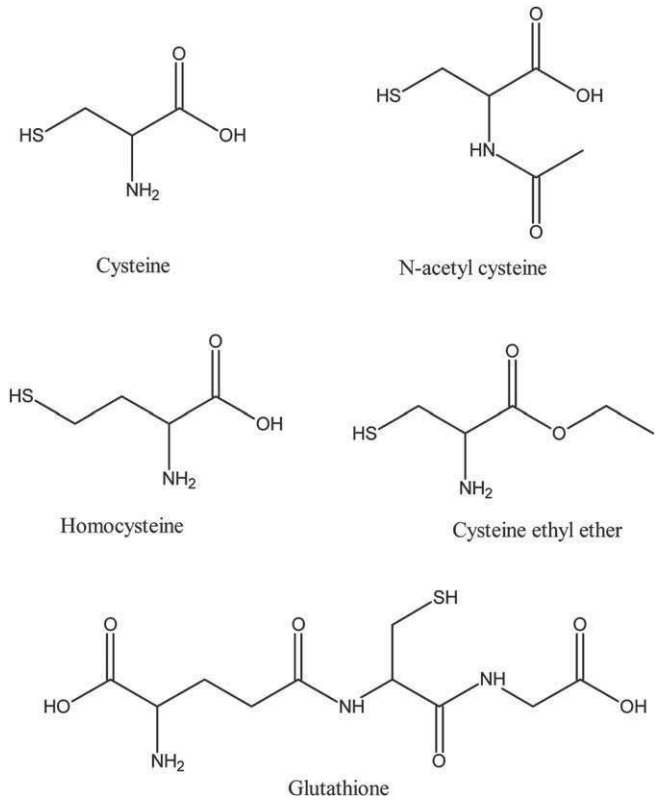

Fig. 6 Molecular structure of the 5 thiol compounds used for the standard curves presented in Fig. 5.

cysteine, explained by the fact that the amine is closer to the thiol group in cysteine ethyl ester than in homocysteine. Based on the molecular structure of the thiol compounds, the fluorescence intensity seemed to decrease when the free amino group was close to the thiol group. Thus proteins and peptides are expected to give the same response as GSH, unless cysteine is found as the *N*-terminal amino acid. Ercal *et al.*[23] studied the related probe ThioGlo 3 with derivatization in a 75% acetonitrile solution and found good agreement between standard curves prepared with cysteine and GSH. Inspired by this, an experiment using ThioGlo 1 was carried out with cysteine and GSH where the derivatization was carried out in 100% acetonitrile. However, this did not result in better agreement between the fluorescence responses of the two thiol–ThioGlo 1 adducts, proving that the differences are not caused by a solvent effect (data not shown).

3.5.1 Investigation of the reaction mechanisms using ^{1}H NMR. A more detailed study of why cysteine–ThioGlo 1 adducts give lower fluorescence intensities than GSH–ThioGlo 1 adducts was carried out using ^{1}H NMR. All compounds were mixed at the same molar concentration (1 mM). Initially ^{1}H NMR spectra of cysteine, GSH and ThioGlo 1 were recorded individually. Focusing on the reaction mechanisms, the aromatic and methoxy hydrogens were of particular interest and in the ^{1}H NMR spectra of cysteine and GSH none of these functional groups were present (data not shown). The reaction products between cysteine, GSH and ThioGlo 1 were studied (1) after the reaction was inactivated with HCl, 5 minutes after activation, matching the HPLC procedure; and (2) after the compounds had been mixed directly in the NMR tube. The latter procedure allowed the cysteine–ThioGlo 1 spectrum to be recorded 5 min and 45 s after the compounds were mixed and the GSH–ThioGlo 1 spectrum to be recorded 4 min and 45 s after the compounds were mixed. Recording of NMR spectra every minute the following hour showed that the reaction was complete after the first measurement. In both cases the disappearance of the resonance at 7.3 ppm demonstrates that either

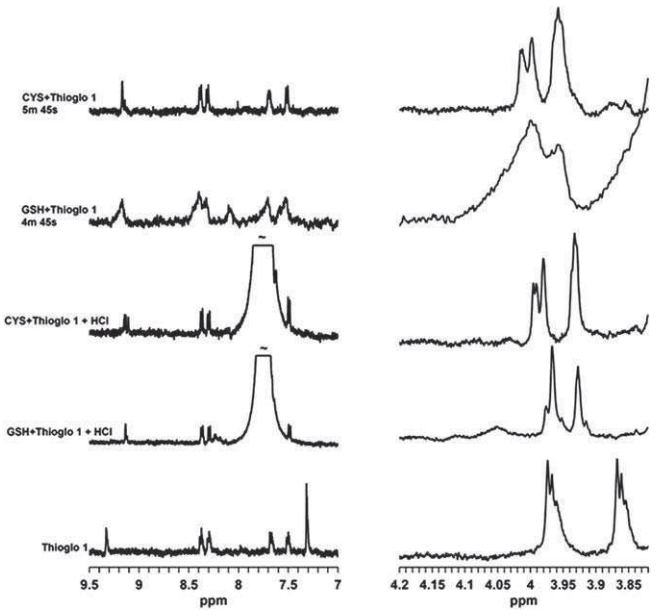

Fig. 7 Selected regions of the ^{1}H NMR spectra of ThioGlo 1, cysteine–ThioGlo 1 adduct and GSH–ThioGlo 1 adduct with and without HCl (after 5 min). The spectra have been normalized vertically relative to the most intense methoxy resonance (3.82–4.20 ppm).

Table 3 Relative integrals of ^{1}H NMR resonances from methoxy groups and selected aromatic hydrogens (8.2–8.5 ppm) in percent (%)[a]

Sample	Methoxy (%)	Methoxy-A (%)	Methoxy-B (%)	Relative ratio of methoxy resonances (%)	Aromatic (%) (8.2–8.5 ppm)
ThioGlo 1	72	37	35	51 : 49	28
Cysteine + ThioGlo 1 (quenched with HCl)	75	35	40	46 : 54	25
Cysteine + ThioGlo 1 (5 min 45 s after mixing)	71	30	41	42 : 58	29
GSH + ThioGlo 1 (quenched with HCl)	70	43	27	61 : 39	30
GSH + ThioGlo 1 (4 min 45 s after mixing)	69	n.a.	n.a.	n.a.	31

[a] n.a.: not applicable.

cysteine or GSH reacts with the double bond in the maleimide ring of ThioGlo 1 (Fig. 7).

Further analysis of the ^{1}H NMR spectra requires assignment of the individual resonances. Two resonances at ~3.97 ppm (denoted A) and ~3.86 ppm (denoted B) in the ThioGlo 1 spectrum are assigned to the methoxy group attached to the aromatic ring and the methoxy group attached to the carbonyl group. The present data do not allow for a unique assignment of the methoxy resonances, but methoxy resonances having a chemical shift above 3.96 ppm will be categorized as A in the following. Likewise, will methoxy resonances with a chemical shift below 3.96 ppm be categorized as B. In Table 3 the relative ratios of both methoxy resonances and resonances from two aromatic protons in the spectral range 8.2–8.5 ppm are tabulated along with the relative ratios of the two types of methoxy resonances. In unreacted ThioGlo 1, the ratio of these integrals should be 6 : 2 (6 hydrogens from two methoxy groups *versus* two aromatic hydrogens) and the two methoxy groups should be in a 1 : 1 ratio, and that is approximately what is observed. Due to severe line broadening in the spectrum of GSH–ThioGlo 1 without HCl, it is not possible to compare its methoxy ratios with those of cysteine–ThioGlo 1, but the corresponding spectra containing HCl can be compared. This comparison demonstrates that two different reaction mechanisms are adopted. For cysteine–ThioGlo 1 the integral of both methoxy groups is approximately 3 times the integral of the two aromatic protons, as expected for a complete reaction, whereas a slightly lower ratio (~2.33) was obtained for the GSH–ThioGlo 1 adduct. In contrast, the relative ratio of methoxy resonances was 46 : 54 for cysteine–ThioGlo 1 and 61 : 39 for GSH–ThioGlo 1. This indicates different reaction pathways, which is also supported by larger line widths in the spectrum of GSH–ThioGlo 1 without HCl, indicating a more dynamic system. Furthermore, the spectrum of cysteine–ThioGlo 1 with HCl contains at least four methoxy resonances, indicating at least three type A methoxy populations of ThioGlo 1 derivatives, while the spectrum of GSH–ThioGlo 1 with HCl contains two main resonances, indicating only two highly populated methoxy sites. These observations showing different reaction pathways for GSH and cysteine are likely to explain the decreased fluorescence intensity observed for cysteine–ThioGlo 1 compared to the GSH–ThioGlo 1 adduct. Sharov *et al.*[24] investigated the reaction between cysteine residues in the protein, calmodulin, and ThioGlo 1 by a proteomic approach and their results show that up to six reaction compounds were formed by hydrolysis and an opening of the maleimide ring occurred together with a release of the methoxy group from ThioGlo 1, resulting in a loss of methanol. In a study by Abrahamsson *et al.*,[17] two sulfite–ThioGlo 1 derivatives were formed by hydrolysis and an opening of the maleimide ring occurred, which was identified by MS. The release of methanol from ThioGlo 1 as shown by Sharov *et al.*[24] could not be confirmed by the present study – either due to very low abundance and/or overlap from very intense buffer resonances in this chemical shift region.

These results show that the decreased fluorescence intensity observed from the cysteine–ThioGlo 1 adduct, and most likely other ThioGlo 1 adducts with thiols having an amine group very close to the thiol group, is caused by different reaction pathways or reaction products than other thiol compounds. This may result in an underestimation of the thiol content in any biological system containing free cysteine when ThioGlo 1 is used as the derivatising agent and the standard addition curves are based on GSH. Regarding beer, the concentration of cysteine will vary with malt, mashing method, yeast strain and age. However, it has been found that sweet wort, a product from the early stage of beer brewing, did not contain any reduced cysteine thiols as they are easily oxidized by other components in the sweet wort.[25] Some of the cysteine may be reduced during the fermentation process and Matsui *et al.*[26] found the amount of free cysteine in beer to be on average 5.8 mg L^{-1} corresponding to 47.7 μM, of which 4.5 μM was in the reduced form. This amount of reduced cysteine may be large enough to cause a minor interference with the current method. To distinguish the thiol-contribution from cysteine compared to the contribution from other thiols chromatographic separation of cysteine–ThioGlo 1 adducts from the bulk thiol peak on the HPLC chromatogram could be a possible way.

4 Conclusion

This paper describes an HPLC method for quantification of the total level of thiols based on reaction with ThioGlo 1 in beer when there is interference from the presence of sulfite. Both sulfite and thiols react with the ThioGlo 1 reagent, but the present HPLC method enables separate determinations of sulfite and thiol quantification simultaneously in the same system. Additionally the present method is 100 times more sensitive than the commonly used DTNB based method, which

was found to be unsuitable for quantification of thiols in beer containing sulfite. ThioGlo 1 shows high accessibility to thiols and no protein unfolding agent such as SDS is needed. Application of the standard addition procedure makes it possible to determine and compare the thiol content in samples with different color intensities, which is highly relevant when working with different beer types. However, a lower fluorescence intensity was detected from cysteine–ThioGlo 1 adducts, most likely due to interference from the amine group located close to the thiol group, which results in underestimation of the thiol concentration in samples containing (high amounts of) reduced cysteine. ^{1}H NMR studies showed that the reaction pathway and the number of reaction products formed between cysteine and ThioGlo 1 compared to GSH and ThioGlo 1 were different, explaining the different fluorescence intensities.

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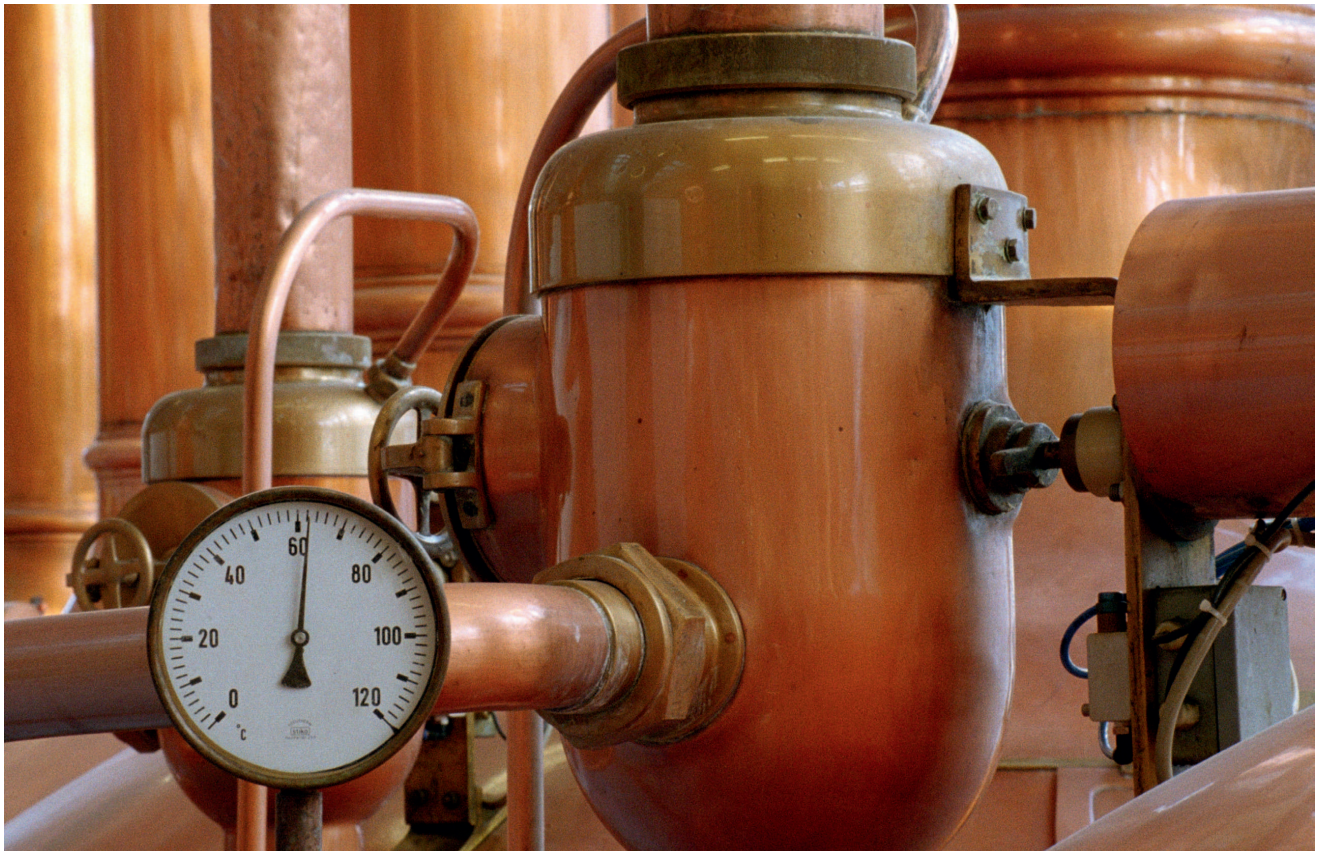
Popular Science Article

Protein-thioler og stabilitet af øl

Signe Hoff, Birthe M. Jespersen, Marianne N. Lund og Mogens L. Andersen

Dansk Kemi, September 2013



Protein-thioler og stabilitet af øl

Det færdige øls mikrobielle holdbarhed var tidligere en af de største udfordringer i forbindelse med ølbrygning. I dag er de mikrobielle udfordringer imidlertid under kontrol, og opmærksomheden er rettet mod fremstilling af øl, der har en stabil høj kvalitet i længere tid efter tapning.

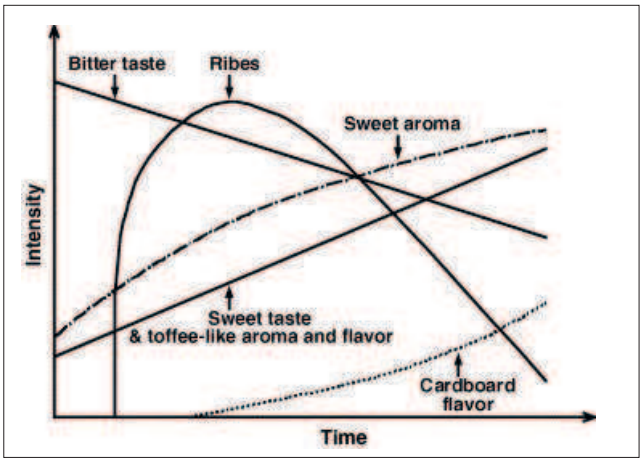
Af Signe Hoff, Birthe M. Jespersen, Marianne N. Lund og Mogens L. Andersen, Institut for Fødevarevidenskab, Københavns Universitet

Tilstedeværelsen af naturlige thiolholdige proteiner i den færdige øl kan gøre øllet mere smagsstabilt pga. den mulige antioxidative effekt af redox-aktive thiolgrupper.

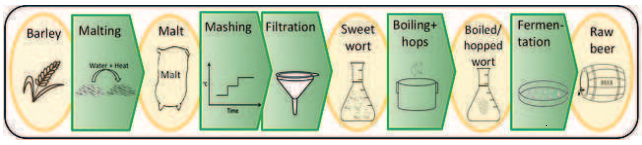
Varme, lys og ilt er alle vigtige faktorer, der fremmer oxidation og begrænser smagsstabiliteten og dermed øllets holdbarhed. Smagen af gammelt øl er kompleks, og mange forskellige typer oxidative reaktioner kan resultere i dannelse af afsmag (figur 1). Især lipidoxidation og dannelsen af trans-2-nonenal er blevet gjort ansvarlig for smagen af gammelt pilsnerøl med dens papagtige smag og lugt.

Forholdet mellem prooxidanter og antioxidanter er afgørende for øllets smagsstabilitet.

Prooxidanterne fremmer oxidation, mens antioxidanterne modvirker oxidation. Når antioxidanterne er brugt op, får prooxidanterne frit spil til at starte de oxidative kædereaktioner, der ender med dannelse af uønskede kemiske smagsforbindelser. En øget mængde af antioxidanter vil kunne modvirke disse oxidative processer og bevare den høje kvalitet i længere tid.

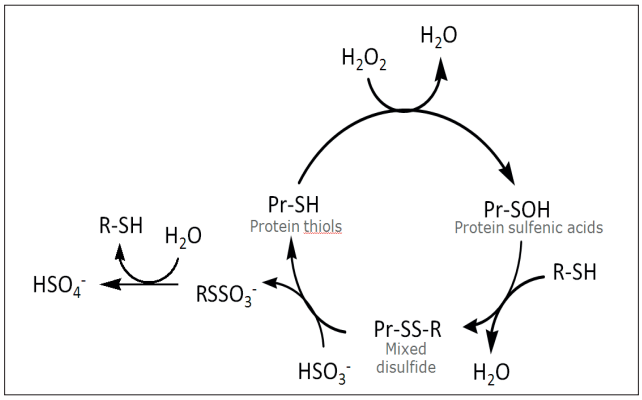


Figur 1. Skitse over udviklingen af afsmag dannet under lagring i lyse øl [9].



Figur 2. Brygprocessen.

Ved Institut for Fødevarevidenskab har vi i længere tid arbejdet med hypotesen om, at de naturligt forekommende thioler virker som antioxidanter i øl. Denne forskning er sket i samarbejde med mikromalter og mikrobrygger Per Kølster (KØLSTER malt og øl), brygmester William Frank, Grauballe Bryghus samt Novozymes A/S. For rationelt at kunne udnytte thiolerne



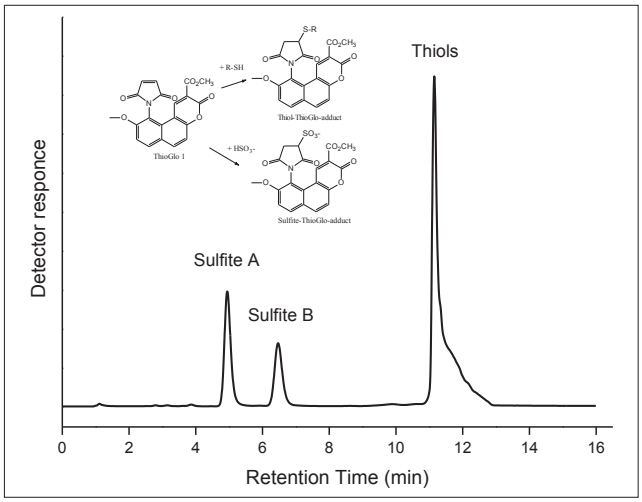
Figur 3. Hypotesen hvorved thioler indgår i den antioxidative kædereaktion med sulfit.

til at øge øllets kvalitet kræves en grundlæggende forståelse af de mange oxidative mekanismer, der finder sted under bryggeprocessen (figur 2).

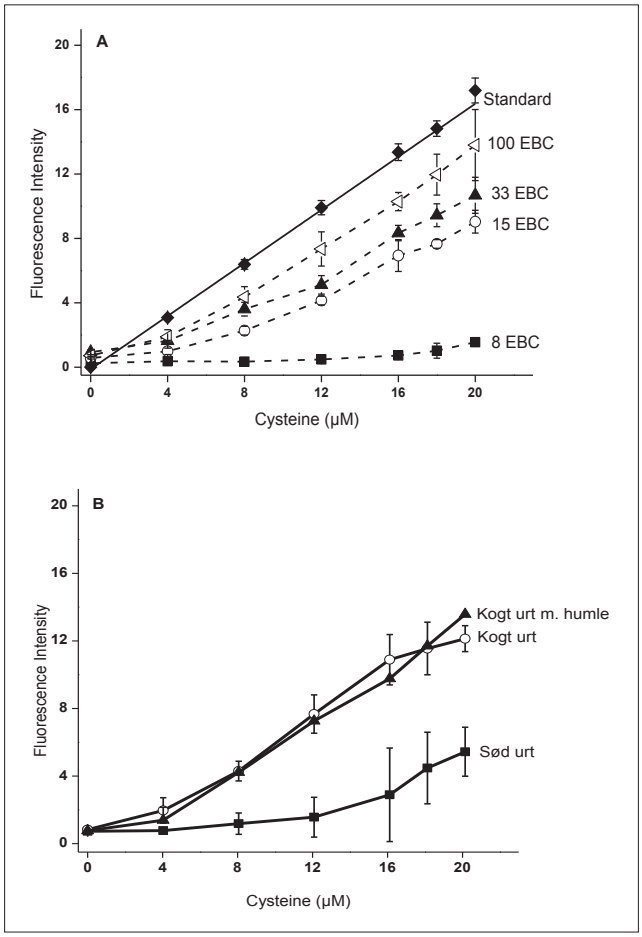
Sulfit, der bliver udskilt af gæren under fermenteringen, er den vigtigste antioxidant i øl. Sulfit reagerer med hydrogenperoxid (reaktion 1), der er en af de vigtigste precursorer for dannelsen af radikaler.

$$HSO_3^- + H_2O_2 \rightarrow HSO_4^- + H_2O \qquad (1)$$

Thioler antages at kunne katalysere reaktionen mellem sulfit og hydrogenperoxid (figur 3). I stedet for sulfit er det en thiol, der reagerer med hydrogenperoxid, hvorved der dannes en sulfensyre under frigivelse af vand. Derefter dannes et blandet disulfid mellem den meget reaktive sulfensyre og en anden thiol. Til sidst reagerer sulfit med disulfidet og gendanner den oprindelige thiol. ▶



Figur 4. Separation af sulfit og thiol på HPLC-kolonne. ThioGlo 1 binder sulfit stereoisometrisk, hvilket resulterer i to sulfittoppe. Alle thioler eluerer i samme top.



Figur 5.
A: Standard-additionskurver med cystein (0-20 µM thiol) tilsat sød urt fremstillet af malt med stigende ristningsgrad (EBC er et farvemål, der udtrykker ristningsgraden).
B: Standard-additionskurver med cystein (0-20 µM thiol) tilsat sød, kogt og humlet urt. Mængden af cystein, der ikke er oxideret af den thioloxidative effekt måles som fluorescensintensitet af cystein-ThioGlo 1 addukter. Øget maltristning fører til nedsat thioloxidation, og urtkogning eliminerer thioloxidationskapaciteten.

Metoder

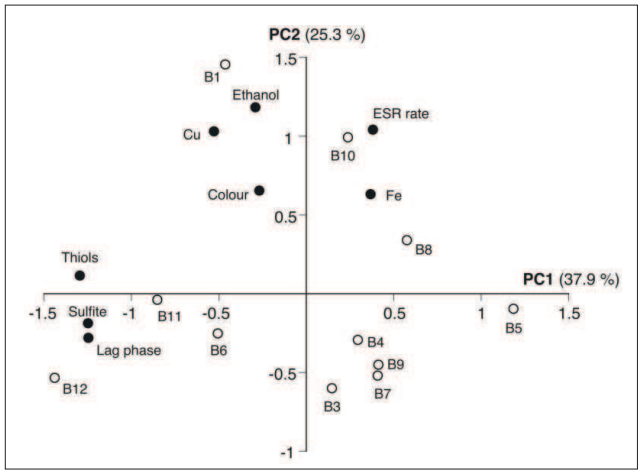
Den mest anvendte metode til thiolbestemmelse er baseret på Ellmans reagens, DTNB[1] og spektrofotometrisk detektion. Den store udfordring ved kvantificering af thioler i øl er tilstedeværelsen af sulfit. Sulfit reagerer nemlig også med DTNB og danner den samme kromofor som thioler. Ved brug af DTNB i øl vil man derfor få et samlet bidrag fra thioler og sulfit.

For at kunne lave en sikker kvantificering af thioler i øl har vi udviklet en metode baseret på det fluorescerende thiolreagens ThioGlo 1 [1,2]. ThioGlo 1 danner addukter med både thiol og sulfit, der fluorescerer ved samme bølgelængde, men som kan adskilles vha. HPLC-separation (figur 4). ThioGlo 1-metoden er ca. 100 gange mere følsom end DTNB-metoden. Ved at anvende standard-additionsprincippet er det muligt at bruge metoden til bestemmelse af thioler og sulfit i øltyper, der spænder fra meget lyse pilsnere til meget mørke portere og stouts.

Thioler igennem brygprocessen

Thiolindholdet varierer indenfor bygsorter og derfor også indenfor malttyper. Derudover påvirker udførelsen af selve mæskeprocessen også indholdet af de oxidationsfølsomme thioler. Thioloxidation og dannelse af disulfidbindinger mellem proteiner under mæskningen medfører nedsat filtreringshastighed af urten. Nedsat filtreringshastighed er problematisk i industriel produktion af øl, og det er derfor allerede i den tidlige brygfase vigtigt at holde thiolerne på deres reducerede form - både for at bevare deres antioxidative effekt og for at undgå længere filtreringshastighed. Thiolindholdet i urten er særlig følsomt overfor tilgængeligheden af oxygen under mæskningen, og udføres mæskningen under atmosfæriske forhold oxideres alle thioler. Større bryggerier mæsker i lukkede systemer, mens mæskning med adgang til atmosfærisk oxygen finder sted på mange mikrobryggerier.

Sød urt viste sig at have en thioloxiderende effekt, som har oprindelse i malten. Øget maltristning resulterede i et fald i den thioloxiderende effekt i sød urt (figur 5, A), mens effekten forsvinder under urtkogningen (figur 5, B). Det virker derfor sandsynligt, at thioloxidationen i sød urt er forårsaget af et enzym, der kan inaktiveres ved høj varme. På trods af denne thioloxiderende effekt kan der stadig detekteres reducerede thioler i sød urt. Det skyldes, at den thioloxiderende effekt primært påvirker lavmolekylære thioler [3,4].



Figur 6. Principal Component Analysis (PCA) af 11 lagrede øl (B1, B3-B12), som viser, at thiolindhold, sulfitindhold og øllets holdbarhed (lag phase), målt med electron spin resonance (ESR) spektroskopi, er korrelerede.

[1] 5,5'-dithiobis(2-nitrobenzoic acid)

De thioler, der findes i det færdige øl, kan være overført fra urten, være dannet ved reduktion af disulfider under fermenteringen eller være frigivet fra gæren. Både selektion af råvare samt minimering af ilt under brygprocessen kan være afgørende for thiolernes tilstedeværelse i øllet. Ved en screening af 12 kommercielle danske øl blev der ikke kun fundet en relativ stor variation mellem thiolindholdene i forskellige øltyper, men også en variation mellem forskellige batches af samme øl [5]. Samme screening viste, at thiolindholdet i øllene korrelerede med sulfitindholdet og øllenes holdbarhed, der blev vurderet vha. accelererede ældningsforsøg (figur 6).

Forskellig gær udskiller varierende mængde af thioler, og positiv selektion kan potentielt være gavnligt for stabiliteten. Under lagring falder sulfitindholdet lineært over tid i takt med, at den forbruges som antioxidant i forebyggelse af oxidative reaktioner [6]. Thiolindholdet derimod ser ud til kun at falde lineært i den første del af lagringsperioden, hvorefter tabet af thioler stagnerer [7]. Det tyder derfor på, at kun en del af thiolerne i øl er vigtige ift. øllets holdbarhed.

Den formodede antioxidative effekt af thioler er også aktuel i andre fødevarer som f.eks. mælkeprodukter, hvor der også forskes i thiolernes betydning. I kød har man fundet, at thioloxidation og dannelse af disulfidbindinger resulterer i at kødet bliver sejt [8].

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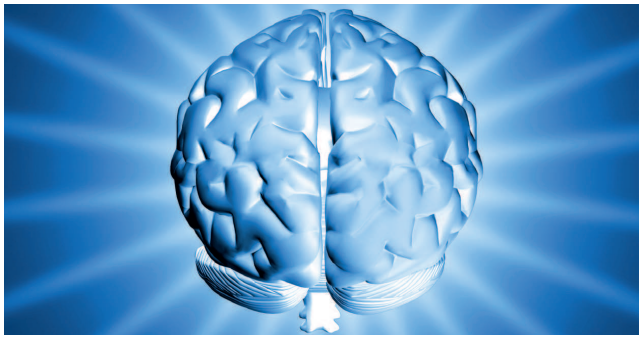
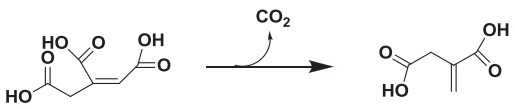
Nyt om …

… Itakonsyre beskytter hjernen



Itakonsyre (methylenbutandisyre), hvis navn er et anagram af akonitsyre, er et industrikemikalie, der hovedsageligt anvendes i plastindustrien. Det produceres af mikrosvampe som



cis-akonitsyre — itakonsyre

Aspergillus terreus ved decarboxylering af *cis*-akonitsyre. Nye undersøgelser viser helt uventet, at itakonsyre også produceres af mikrogliaceller i pattedyrs hjerner. Syren virker antibakteriel ved at blokere enzymer, der er livsvigtige for bakterier. Både væksten af *Salmonella enterica* og *Mycobacterium tuberculosis* hæmmes kraftigt af den substituerede ravsyre.

Carsten Christophersen

Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. A. Michelucci *et al.PNAS* 2013, http://www.pnas.org/content/early/2013/04/18/1218599110.full.pdf+html