ASSESSING THE FLAVOUR STABILITY OF LAGER-STYLE BEERS

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Abstract

Beer, as manufactured, is not at chemical equilibrium and undergoes complex chemical reactions during storage which can lead to undesirable changes in the sensory characteristics of beer. Since beer contains more than 600 volatile compounds, beer flavour change is a complex field of research and the dependable prediction of shelf life remains a top research priority.

Chapter 1 places the work described in this thesis into this context.

Chapters 2 and 3 provide details of the experimental methods used and their development, respectively, to evaluate flavour stability. The methods used and developed include: (i) the determination of one of the major antioxidants present in beer, sulphur dioxide (SO_2), via distillation; (ii) a solid phase micro extraction (SPME)-GC-MS method with on fibre derivatization as a reliable detection method for aldehydes related to off flavours perceived in aged beer; (iii) measurement of the oxidative stability using electron paramagnetic resonance (EPR) spectroscopy.

Chapter 4 describes oxidative stability measurements via EPR spectroscopy for three different lager-style beer qualities pre- and post-filtration. The trial included a large scale Kieselguhr frame filter, and two pilot-scale membrane filtration system. The results illustrate how EPR spectroscopy is sensitive to metal ion pick-up from traditional filter media and oxidative stability measures were evaluated versus oxygen pick up during filtration and sulphur dioxide content of the beer samples.

The effect of a brewhouse addition of gallotannins on the flavour stability of a lager-style beer was investigated in Chapter 5. Pilot scale (16 hl) and large scale production line (1500 hl) experiments with gallotannins additions in the Brewhouse were performed. The effects of the different additions were monitored at key points of the production process and through to the final beer. Despite showing significant

improvements in the pilot scale wort samples, the results for the related packed beer samples did not show clear flavour stability improvements. For the production-scale trials, only a very limited effect of improved flavour stability could be observed.

In Chapter 6 a factorial experimental design was adopted to probe the interactions between seven factors known to impact on beer flavour stability. Chemical additions were made to a bright beer prior to bottling, to vary the following factors: total in pack oxygen, SO_2, total iron, iso-α-acid and α-acid content, (+)-catechin and glutathione. Increased SO_2 concentrations had the largest impact across the entire design space, resulting in reduced radical formation, staling aldehyde concentrations and improved sensory scores. The impacts of increased TIPO levels were rather limited. In contrast, a significant impact regarding the oxidative stability could be observed for increasing Fe concentration, highlighting the significance of pro-oxidative effects of transition metals.

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Abbreviations

%	percent
°C	degrees Celcius
ASBC	American Society of Brewing Chemist
BU	bitterness units
CO_2	carbon dioxide
Cu	copper
DO	dissolved oxygen
EBC	European Brewery Convention
EPR	electron paramagnetic resonance
Fe	iron
g	gram
GC-MS	gas chromatography-mass spectrometry
h	hours
H_2O	water
H_2O_2	hydrogen peroxide
hl	hectolitre
HNO_3	nitric acid
ICP-MS	inductively coupled plasma mass spectrometry
kg	kilogram
l	litre
M	Molar
m/z	mass to charge
MEBAK	Mitteleuropäischen Brautechnischen Analysenkommission
mg	milligram
min	minutes
ml	millilitre
mM	mill molar
mm	millimetre
Mn	manganese
NaOH	sodium hydroxide
O_2	oxygen
OG	original gravity
PG	present gravity
ppb	parts per billion
ppm	parts per million
RO	reverse osmosis
s	seconds
SO_2	sulphur dioxide
SPME	solid-phase micro extraction
TEMPOL	4-hydroxy-2, 2,6,6-tetramethylpiperidine 1-oxyl
TBI	thiobarbituric acid index
TIPO	total in pack oxygen
v/v	volume to volume
v/w	weight to volume
w/w	weight to weight
µl	microlitre

1 Introduction

As multinational brewers export their products into to distant markets, products are exposed to longer and more challenging storage conditions. Beer, as manufactured, is not at chemical equilibrium and undergoes complex chemical reactions during storage which can lead to undesirable changes in the sensory characteristics of the beer. This challenges a major objective of the brewing industry, which is to provide the customer with a consistent product.

1.1 Factors impacting the flavour instability of beer

Singh and Cadwallader (2003) proposed a formula (Equation 1.1) to describe the ageing process in food systems.

Equation 1.1: Formula to describe ageing processes in food, where: rQ = rate of quality degradation, Ci = compositional factors (content of reactive species, catalysts, reaction inhibitors, pH, etc.) Ej = environmental factors (temperature, light, mechanical stress, total pressure, etc.), φ = proportionality constant

$$rQ = \varphi(Ci, Ej)$$

The formula attempts to take into account the various factors that can influence the shelf life including the formulation, processing, packaging and storage conditions. According to Bamforth and Lentini (2009), the formula fails to balance these numerous factors. Most of the factors influencing the shelf life of beer can be separated into intrinsic factors (i. e. compositional factors) and extrinsic factors (i.e. external events and conditions the beer is exposed to). These approaches attempt to include and

assess the various aspects but the complex matrix of beer and the varied chemistry of beer ageing makes it nearly impossible to accommodate all factors into a simple equation.

1.2 Sensory changes

According to Meilgaard (1982) beer flavour is influenced by a number of volatile compounds and the combination of bitterness, sweetness, ethanol and carbonation. These factors also influence the mouthfeel and appearance of the beer (Langstaff and Lewis, 1993). Flavour is perceived by the gustatory, olfactory and trigeminal systems. The flavour perception is based on the interaction between these three systems which can change the sensory perception (Verhagen and Engelen, 2006). The sensory quality of packaged beer is subject to constant changes during its shelf life (Drost et al., 1971, Meilgaard, 1972, Eichhorn et al., 1989, Vanderhaegen et al., 2006, Stewart and Priest, 2011). One of the first detailed descriptions of the sensory changes of beer during storage was published by Dalgliesh (1977). Figure 1.1 shows a generalised description of the sensory changes occurring during beer storage. The absolute intensity and the time scale of these changes differs for different beer styles. The figure can only be seen as a general approach to visualise the trend of different flavour and taste impressions during the ageing.

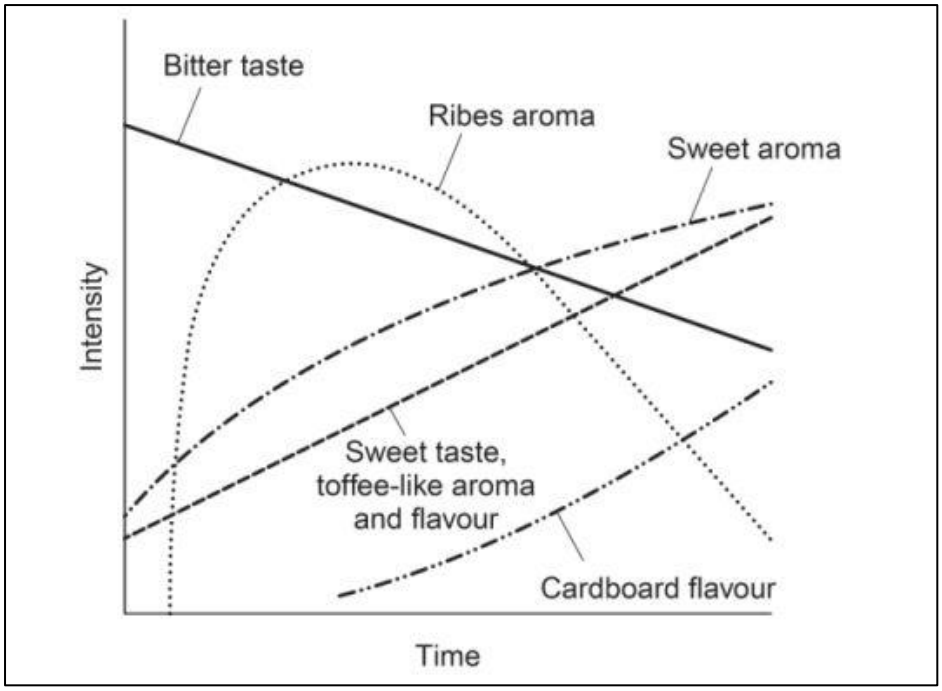


Figure 1.1: Sensory changes during beer ageing according to Dalgliesh (1977).

During beer ageing a continual decrease of the bitterness was detected. A rapid formation of so called "ribes" aromas was observed, followed by a rapid decrease. The formation of the so called "cardboard" flavour developed over a longer time frame. Very similar changes in flavour were presented by Zufall et al. (2005) but they showed an increase of the cardboard flavour over four weeks of storage to a maximum followed by a rapid decrease. Furthermore the bitter taste was replaced by quality of bitterness with a trend of a constant decrease of bitterness quality during storage.

Predominately carbonyl compounds were described to be responsible for the flavour changes during beer storage. The pathways of the formation of these compounds were subject to intensive research but are poorly understood (Meilgaard et al., 1970, Hashimoto, 1972, Drost et al., 1974, Hashimoto and Eshima, 1977, Walters, 1997, Vanderhaegen et al., 2006, Baert et al., 2012). A detailed review of the possible reactions leading to their formation is provided in Section 1.3. Depending on storage conditions and beer type, various other compounds are involved in the formation of

stale flavours during storage. This can include dioxolanes (Vanderhaegen et al., 2003), heterocyclic compounds such as furan and furanone (Lustig et al., 1993), esters and lactones (Bohmann, 1985, Eichhorn et al., 1989, Gijs et al., 2002) and a number of sulphur compounds (Tressl et al., 1980, Schieberle, 1991, Gijs et al., 2002). A total of 624 flavour compounds found in beer are described in detail in the ASBC flavour database (2016a). This database includes flavour thresholds and typical concentrations found in beer. As it would go beyond the scope of this work to describe each of the compounds related to flavour instability, the database can be recommended as a very helpful tool to find information regarding beer flavour compounds. Furthermore a number of reports refer to those compounds being related to the sensory changes during beer ageing (Schieberle, 1991, Kamimura and Kaneda, 1992, Schieberle and Komarek, 2003, Vanderhaegen and Derdelinckx, 2005, Vanderhaegen et al., 2006, Vanderhaegen et al., 2007). The flavour thresholds of the compounds related to stale flavour in beer have been described by Saison et al. (2009). Despite the fact that concentrations of many of the flavour compounds do not exceed the evaluated flavour threshold during storage, the contribution to stale flavour in the beer matrix could be measured (Herrmann, 2008).

Further to the formation of ageing compounds, the degradation of positive beer flavour attributes such as floral, fruity or estery notes is also observed through ageing (Dalgliesh, 1977, Zufall et al., 2005). The degradation of the compounds responsible for those fresh flavours might be just as important as the formation of stale flavours and is mostly related to direct degradation induced by oxidation (Bamforth, 1999c).

The change in the intensity of bitter taste and quality of bitterness during beer storage is another important factor impacting beer flavour stability (De Cooman et al., 2000). The degradation of iso-α-acids during storage and the changes in the cis-trans ratio

(Caballero et al., 2012, Blanco et al., 2014) can lead to an unpleasant, long-lasting harsh bitterness in stored beer samples. Furthermore the polymerisation of polyphenols can negatively impact the bitterness perception of aged beer (McMurrough et al., 1996).

The impact of staling on the taste of different beer types shows considerable diversity. According to Vanderhaegen et al. (2006) aged lager beers show a very strong cardboard flavour whereas cardboard or metallic flavours play no role in aged dark ale beers, as those flavours are masked by the strong initial flavour of the beer (Preuß, 2001). Similar findings were described by Malfliet et al. (2009). Additionally, the consumer preference is significantly influenced by gender, age, drinking occasion and blind or informed tasting conditions and is not correlating with the quality ratings of an expert panel (Guinard et al., 2000). Therefore a separate evaluation of sensory changes is needed for each beer style and brand or in extreme cases even every different beer batch, as different production batches of the same beer might show different ageing characteristics.

1.3 Chemical changes during ageing

De Schutter et al. (2009) characterise beer as an ethanol-water-solution with a pH of 4 to 4.5 and a large number of different other compounds, for example a broad spectrum of compounds from very small volatile compounds like methional to high-molecular-weight non-volatile substances such as polyphenols. During the beer ageing process countless reactions take place resulting in formation and degradation processes of various compounds. These include but are not limited to the oxidation of higher alcohols, Maillard reactions, the Strecker degradation of amino acids, aldol condensation, the degradation of iso-α-acids and the oxidation of unsaturated fatty

acids (Vanderhaegen et al., 2006, Bamforth and Lentini, 2009, De Schutter et al., 2009).

A comprehensive classification of the different reactions based on the impact on beer staling is nearly impossible. A good example for this complexity is the disagreement about the role of (E)-2-nonenal as a key component in stale character development. According to Wang and Siebert (1974) and Sakuma et al (1994), (E)-2-nonenal plays a very important role in staling. On the other hand Foster et al. (2001), Narziss et al. (1999) and Vanderhaegen et al. (2003) question the status of (E)-2-nonenal as a key component as in some experiments the amount decreases during storage. The disagreement about the role of one compound illustrates the complexity of the chemistry of beer ageing and therefore the following paragraphs can only provide an overview over the mechanisms involved in flavour changes during beer ageing.

1.3.1 The impact of oxygen

It is widely known that oxygen plays a very important, maybe the most important role in flavour degradation during beer ageing. Oxygen in its ground state ($^3O_2$) is relatively non-reactive. The ground state oxygen must be activated by either increased temperature, light or a catalytic activity to be converted into singlet oxygen ($^1O_2$), superoxide (O_2^-), hydroperoxyl radical (●OOH), hydroxyl radical (●OH) and hydrogen peroxide (H_2O_2). The pathways of this activation are illustrated in Figure 1.2. The higher reactivity of the reactive oxygen species (ROS) is due to a reduced coherence of the bonds between the two oxygen atoms and increases with their reduction status (Bamforth and Lentini, 2009).

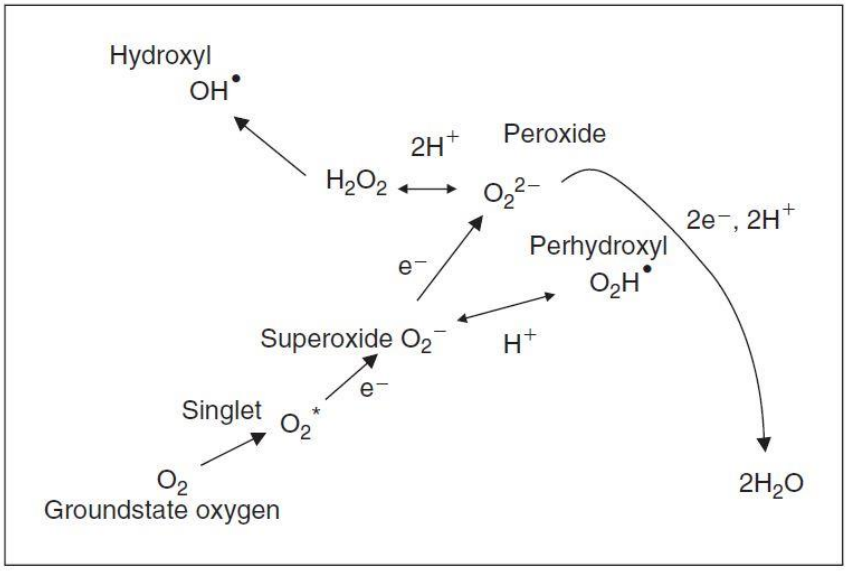


Figure 1.2: The activation of oxygen through the addition of electrons according to Bamforth and Lentini (2009)

Bamforth and Parson (1985) were the first to describe the reactive species of oxygen and their influence on beer flavour stability. The high reactivity of the oxygen-based radicals leads to reactions with different components that are present in the beer. The implication of reactive oxygen species (ROS) in the formation of staling characteristic of aged beer has been discussed by different authors (Dadic, 1985, Kaneda et al., 1988, Uchida and Ono, 1996, Andersen and Skibsted, 1998). The initial reaction pathways of ROS in beer are shown in Figure 1.3.

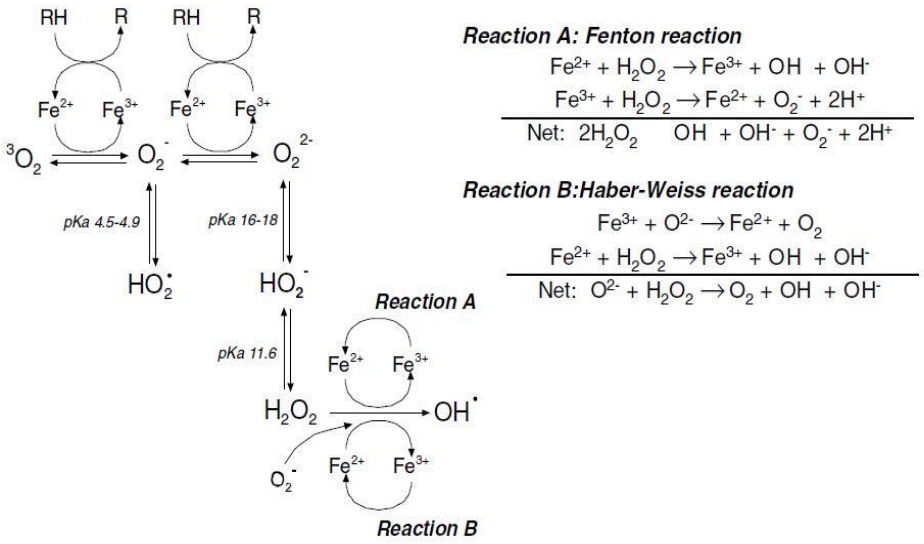


Figure 1.3: Reactions producing reactive oxygen species (ROS) in beer (Kaneda et al., 1999)

The formation of O_2^{2-} from ground state oxygen during the storage of beer is catalysed by the oxidation of Fe^{2+} to Fe^{3+} and Cu^{+} to Cu^{2+}. Fe and Cu are present in sufficient quantities of up to 300 µg/l in beer (Zufall and Tyrell, 2008). The low pH of beer and the high pKa values involved in the formation of O_2^{2-}/HO_2^{-} and HO_2^{-}/H_2O_2 suggest that H_2O_2 is formed principally in beer (Kaneda et al., 1999). Furthermore Chapon and Chapon (1979) pointed out the requirement of an electron donor like ethanol in this mixed function oxidation system. Brezova et al. (2002) reported the contribution of ascorbic acid and the involvement of polyphenols, isohumolones and melanoidins has been suggested by Irwin et al. (1991). The metal-catalysed Fenton and Haber-Weiss reactions are the next steps in the formation of the ●OH radical. The ●OH radical is highly reactive towards alcohols, sugars, isohumolones or polyphenols and can be the starting point for reactions responsible for the formation of staling compounds. Zufall and Tyrell (2008) investigated the influence of heavy metals in beer staling and their role in radical reactions. They demonstrated that Cu has the largest effect on the

promotion of radical reactions. Fe and Mn have a lower impact. According to Bamforth (2001) the most efficient strategy to minimise oxidative deterioration of beer is to prevent the formation of ROS. By keeping the oxygen level as low as possible, by reducing the temperature impact and by introducing antioxidants the staling of beer could be decelerated.

1.3.2 Maillard reactions

The non-enzymatic browning reactions of reducing sugars and amino-compounds and all consecutive reactions are summarised as the 'Maillard reaction'. In the brewing process these reactions predominantly occur during wort boiling (Tressl et al., 1979), but will continue, albeit at greatly reduced rates, throughout storage even at ambient temperatures (Bravo et al., 2001). Hodge (1953) elucidated the early pathways of the Maillard chemistry, noting the complexity of the reactions and the resulting products. Figure 1.4 illustrates the complex and interacting pathways and shows the two different stages at which Maillard reactions occur in the malting and wort boiling processes respectively, as well as during beer storage.

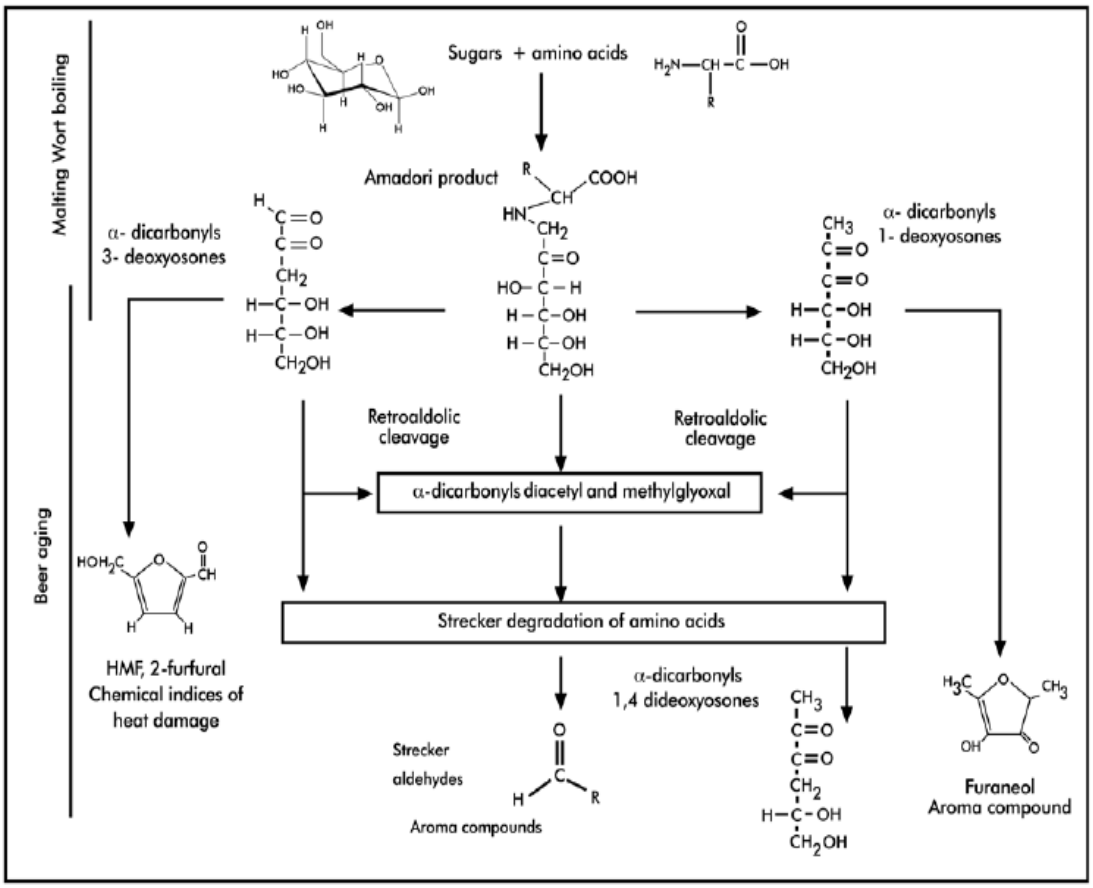


Figure 1.4: The Maillard reaction pathways (Bravo et al., 2002)

During the mashing process starch is broken down into different fermentable sugars including glucose, maltose and maltotriose. The large concentrations of sugars and amino compounds present in the wort and the higher temperatures during wort boiling lead to an increased formation of Maillard reaction products. 5–hydroxymethylfurfural (HMF) (originate from a hexose sugar) and furfural (product of reaction of pentose sugar) are the most important Maillard monomers in wort on the basis of relative concentrations. The reaction of the reducing sugar (hexose for HMF and pentose for furfural) and the amino acid is initiated by the start of the formation of a Schiff base between the carbonyl group of the sugar and an amino group leading to an imine. This imine undergoes an Amadori rearrangement to form the more stable 1-amino-1-deoxyketose. The pH of the wort or beer determines the enolisation of the 1-amino-1-

deoxyketose and a release of an amine. So the reactive α-carbonyl-compound, 3-deoxy-2-hexsulose (3-DH), is formed and this compound can give rise to HMF or furfural, respectively. HMF and furfural can be seen as intermediates on the pathway of the formation of melanoidins, brown pigments of a high molecular weight. Condensation, dehydration, cyclisation and isomerisation reactions are involved in the formation of the melanoidins (Vanderhaegen et al., 2006).

The effect of Maillard reaction products on flavour stability is described controversially in the literature. Bolvin (2001) showed the antioxidant potential of Maillard reaction products during malt and beer production. Melanoidins showed to ability to act as a hydroxyl ethyl radical scavenger in a Fenton-system (Morales, 2005). The reducing activity is related to the enediol structure and depended on the size of the molecules, increasing with the size of the molecule (Lee and Shibamoto, 2002). On the other hand, a pro-oxidative behaviour of melanoidins could be described in a model solution (Drost et al., 1974). The direct oxidation of different alcohols in the presents of melanoidins was described by Hashimoto (1972). Noddekaer and Andersen (2007) used EPR spectroscopy to evaluate the effect of Maillard reaction products on oxidative reactions in beer. A clear correlation between the addition of Maillard reaction products and increased generation of radicals could be detected by reduced EPR lag times. Additionally irreversible bonding of SO_2 with Maillard reaction products lead to decreased antioxidant potential of the beer and the degradation of SO2 during storage was pronounced.

1.3.3 Oxidation of higher alcohols

The main higher alcohols in beer are 2-methyl-propanol, 2-methyl-butanol, and 2-phenyl-ethanol and are produced mainly during the fermentation process

(Vanderhaegen et al., 2006). The yeast also has the ability to reduce carbonyls to higher alcohols. These carbonyls are formed during the wort production process. Nevertheless a reverse reaction can take place and an oxidation of the higher alcohols leads to the formation of the corresponding carbonyls. According to Hashimoto (1972) melanoidins play the key role in the oxidation processes of higher alcohols. The formation of aldehydes is directly related to the melanoidin content. During the forced ageing of beers with reduced melanoidin content the formation of aldehydes was reduced. In melanoidin-free model solutions there were no detectable aldehydes. An increased in-pack oxygen concentration in the melanoidin free model solutions resulted also in very low carbonyl concentrations. Therefore a reaction mechanism was proposed in which alcohols transfer electrons to reactive carbonyl groups of melanoidins. A higher amount leads to a forced formation of carbonyl compounds by a transformation of the reactive groups of the melanoidins. This correlation was questioned by Devreux et al. (1981). They showed that low concentrations of polyphenols can inhibit the pathway and light is required during the oxidation process. Irwin et al. (1991) also discuss the relevance of the pathway in the formation of aldehydes. Andersen and Skibsted (1998) reported the importance of the 1-hydroxyethyl radical in stale beer. The reaction of this quantitatively most significant radical with ethanol to a main degradation product acetaldehyde is shown in Figure 1. 5.

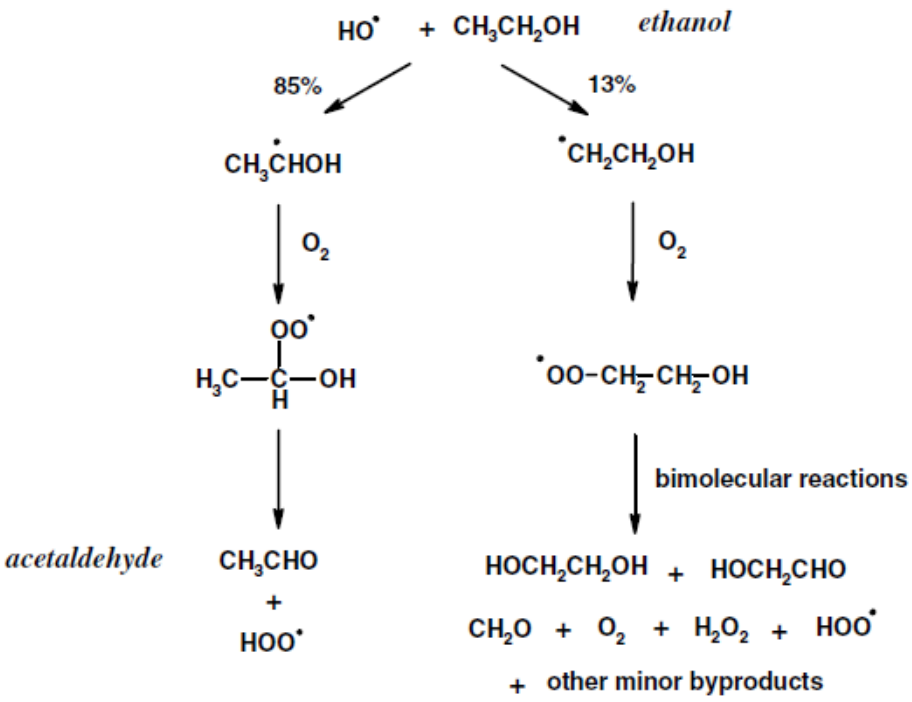


Figure 1. 5: Reaction of ethanol with hydroxyl radical in beer (Andersen and Skibsted, 1998)

According to Vanderhaegen et al. (2006) radical oxygen species may react in a similar way with the other higher alcohols present in beer. Therefore the reaction mechanisms observed by Hashimoto (1972) are a generalization of the reaction of ROS with alcohols.

1.3.4 Strecker degradation reactions

Despite hardly exceeding the flavour threshold during beer ageing (Thum et al., 1995), the Strecker aldehydes, acetaldehyde, 2-methyl propanal, 2-methyl butanal, 3-methyl butanal, methional, phenyl acetaldehyde and benzaldehyde, are commonly examined to evaluate the effects of changes to the brewing parameters or the storage conditions (Methner et al., 2003). They are described as good analytical markers correlating with the sensory evaluation of flavour degradation during storage (Guedes de Pinho and

Silva Ferreira, 2006). The Strecker degradation reaction has been described by Hoffmann et al. (2000) as an oxygen-dependent reaction. The reaction includes a transamination, followed by a decarboxylation and hydrolysis yielding a Strecker aldehyde. Another possible reaction mechanism was suggested by Hidalgo et al. (2005). The Strecker aldehyde is produced through the corresponding imine, which is then decarboxylated and hydrolysed. Suda et al. (2007) stated up to 85 % of the Strecker aldehydes in aged beer are formed during wort boiling and only 15 % are produced during the storage time of the packed beer. Therefore most of the Strecker aldehydes must originate from the brewing process. Similar findings were reported by Baert et al. (2015). Additionally Bravo et al. (2008) reported only negligible amounts of Strecker degradation products formed during beer ageing, regardless of high amounts of α-dicarbonyls present in fresh beer.

1.3.5 Oxidation of unsaturated fatty acids

Two different pathways for the formation of unsaturated fatty acids take place during beer production. The enzymatic oxidation (Drost et al., 1971) occurs during the mashing process. The malt-enzymes lipase and lipoxygenase are able to break down linoleic and linolenic acid, lipid compounds present in malt, into carbonyl compounds (Kobayashi et al., 1993).The second pathway is the auto-oxidation of fatty acids described by Lindsay (1973). Since enzymes are fully inactivated during the boiling process, reactive oxygen species are responsible for the auto-oxidation of the fatty acids later on in the process. The reactive oxygen species attack the double bonds of the unsaturated fatty acids and form predominately 9- and 13-hydroperoxides. The further breakdown of these hydroperoxides leads to the formation of (E)-2-nonenal (Saison et al., 2009). According to Noël et al. (1999), all (E)-2-nonenal present in beer

is formed during wort by autoxidation. Enzymatic activity during mashing can lead to the formation of an imine with amino acids and proteins through Schiff's base formation (Lermusieau et al., 1999). According to Noël and Collin (1995), this formation is not reduced to nonenol by yeast during fermentation.

During mashing and wort boiling, (E)-2-nonenal In general the unsaturated fatty acid oxidation plays an important role. Nevertheless as this process only occurs significantly during mashing and wort boiling, low enzymatic activity and a low oxygen impact during mashing and wort boiling can reduce the potential impact.

1.3.6 Aldol condensation

Hashimoto and Kuroiwa (1975) illustrated the formation of carbonyl compounds by the reaction of separate ketones or aldehydes. This is a plausible route for the formation of, for example, (E)-2-nonenal by the reaction between acetaldehyde and heptanal. According to de Schutter (2009) this pathway leads to a transformation of substances with a medium flavour threshold to substances with a very low flavour threshold. In contrast Bamforth (1999b) questioned the importance of the aldol condensation during beer storage. The amount of compounds formed by the pathway of aldol condensation during storage is presumably not high enough to reach the flavour threshold.

1.3.7 Role of sulphur dioxide (SO_2)

The SO_2 found in beer is formed during fermentation as an intermediate product of sulphur-containing amino acid synthesis (Brewer and Fenton, 1980, Nordloev, 1985, Thomas and Surdin-Kerjan, 1997). The yeast cells assimilate sulphate out of the wort and release excessive sulphite back into the fermented beer. The origin can be sulphur

containing amino acids or organic sulphur containing compounds. During fermentation, a set of two sequential reactions take place. The first reaction transfers the adenosyl-phosphoryl moiety of ATP to sulphate, yielding adenylyl sulphate (APS) with ATP sulphurylase as catalyst. The APS is transferred into phosphorylated to yield phosphoadenylyl sulphate (PAPS). The enzymes catalyzing this reaction is APS kinase. The activated sulphate is reduced to sulphite, followed by a further reduction by sulphite reductase to sulphide. The sulphide is in cooperated in the sulphur containing amino acids cysteine, methionine and S-adenosylmethionine (Boulton and Quain, 2001). The formation of SO_2 during fermentation can be described in four stages (Dufour, 1991):

- Stage 1 - Early fermentation: Synthetic pathway of sulphite formation is supressed by high concentrations of methionine and threonine
- Stage 2 – yeast growth: all sulphite is consumed in the formation of sulphur containing amino acids for the production of biomass
- Stage 3 – mid to late fermentation: due to reduced yeast growth, excessive amounts of sulphite are synthesised and released extracellular
- Stage 4 – end of fermentation: reduced yeast activity leads to reduced sulphite synthesis and stops accumulation

The formation of SO_2 during fermentation is depending on a large number of factors. So yeast health, pitching rate, pitching and fermentation temperature, wort aeration, wort gravity, lipid concentrations and amino acid concentrations can impact the SO2 formation during fermentation (Brewer and Fenton, 1980, Nordloev, 1985, Ohno and Takahashi, 1986, Van Haecht and Dufour, 1995, Boulton and Quain, 2001, Wurzbacher et al., 2005). Additionally SO_2 can be added as a preservative during the brewing process. In beer the SO_2 is predominantly present as HSO_3^- due to the pH of

beer (*ca.* 4.3) (Nyborg et al., 1999) in concentrations ranging from < 1 mg/l to 30 mg/l (Ilett, 1995). The proposed roles of SO_2 in beer are as one of the main antioxidants (Andersen et al., 2000, Nakamura et al., 2001), it can reduce the staling flavour impact of carbonyl compounds (Kaneda et al., 1994, Bushnell et al., 2003) and in high concentrations SO_2 has an antibacterial effect (Ilett, 1995).

The antioxidant effect has been described (Klimovitz and Kindraka, 1989) and is related to the reaction of SO_2 with oxygen to produce sulphate. An exact pathway of this reaction in food systems has not been proven and only possible schemes of the reaction pathways are assumed (Ilett, 1995). Presumably SO_2 reacts with hydrogen peroxide and prevents the Fenton reaction. A further possibility could be the direct reaction with molecular oxygen, thereby influencing the lag time determined using EPR spectroscopy (Hoffmann and Edwards, 1975, Wedzicha, 1984, Uchida and Ono, 1996, Andersen and Skibsted, 1998, Uchida and Ono, 1999, Andersen et al., 2000). However, Noddekaer and Andersen (2007) showed different correlations between lag time and SO_2 content, suggesting additional factors like melanoidins could affect the radical formation.

Bushnell et al. (2003) described the effect of SO_2 reducing the formation of carbonyl compounds during ageing. Furthermore SO_2 has the ability to bind flavour active aldehydes through the formation of aldehyde-bisulfite adducts (Dufour, 1991, Barker et al., 1983). The formation of these hydroxyl sulfonates is reversible and the reaction equilibrium is towards the reaction product (Wedzicha, 1984). The amount of free SO_2 was specified by Kaneda et al. (1994) as below 2 % of the total SO_2 present in beer. Most of the SO_2 in beer is bound to acetaldehyde. This is due to the chemical affinity of SO_2 to carbonyl compounds decreasing with the molecular weight of the carbonyl compounds. Furthermore smaller compounds are able to replace larger carbonyl

compounds in the hydroxyl sulfonate binding (Burrough and Sparks, 1973, Dufour, 1991, Kaneda et al., 1994, Lustig, 1994). The antioxidant properties of SO_2 are not affected by the hydroxyl sulfonate binding (Kaneda et al., 1994, Ilett, 1995, Andersen et al., 2000). The reducing effect on carbonyls of the yeast during fermentation is likewise most likely unaffected by the hydroxyl sulfonate binding (Lustig, 1994).

The concentration of SO_2 in packaged beer declines during storage at a rate determined by the storage conditions. Elevated temperatures increase the degradation (Ilett and Simpson, 1995, Kaneda et al., 1996). Up to 3 mg/l of SO_2 can be oxidised per month (Back, 2005). Nevertheless the correlation between SO_2 concentration and the rate of decrease is described differently by different researchers. Lustig (1994) illustrated a direct correlation of SO_2 concentration and rate of decrease suggesting an exponential reaction relation. Ilett and Simpson (1995) demonstrated a correlation but only up to a certain extent. This was further investigated by Ilett et al. (1996) using different modelling approaches for the total loss of SO_2 during storage, showing that the chemical composition of the beer sample controls the stability of SO_2 in beer and the initial concentration is not the key factor.

1.3.8 Protein thiol groups and beer flavour stability

The antioxidant properties of protein thiol groups have been reported for different biological systems (Nordberg and Arner, 2001). A positive effect on health related to the antioxidant properties of glutathione connected to its thiol group was presented by Wu et al. (2004). The positive effects of protein thiol groups in regards to improved flavour stability and possible reaction pathways were first reported by Rogers and Clarke (2007). Thiol groups were described to react with H_2O_2 to form protein sulfenic acids. The protein sulfenic acids may react with other protein thiol groups to form

mixed disulphides that would be reduced by either sulphate or other educing compounds. These reactions were further investigated by Lund and Andersen (2011), demonstrating a correlation between the antioxidant potential of beer evaluate by EPR spectroscopy and the protein thiol content. A very similar finding was presented by Wu et al. (2011), showing improved antioxidant activity in beer samples with protein thiol groups present in comparison to beer samples without active thiol groups.

1.3.9 Polyphenols and their antioxidant activities

As polyphenols are not primarily involved in plant growth, they are classified as secondary plant metabolites. They can impact scent, colouring or aroma of plant material to protect the plant from natural enemies such as insects or worms, fungal infestation or UV irradiation (Grace, 2005).

In beer the two main sources of polyphenols are barley malt (70-80%) and hops (20-30%) (Knorr, 1977, Vanderhaegen et al., 2006). The concentration of polyphenols in the final beer is largely influenced by the raw materials and the brewing procedure.

The antioxidant behaviour of polyphenols is well documented (Walters et al., 1997a, Lermusieau et al., 2001, Nakamura et al., 2001, Lugasi, 2003) but assessed differently by Andersen et al. (2000), discounting the effect of polyphenols on the formation of free radicals during wort production and storage of beer, as addition of polyphenols did not affect the radical formation assessed by EPR spectroscopy. The antioxidant potential is described to be dependent on a number of factors, including the pH of the analysed sample (Lingnert et al., 1982, Nakamura et al., 2001) and the number of hydroxyl groups on the phenol ring (Thumann and Herrmann, 1980, Shahidi et al., 1992). Further the molecular weight impacts the antioxidant potential (Buggey, 2001). Smaller molecules (< 5 kDa) have a higher antioxidant potential and are responsible

for up to 80 % of the antioxidant potential (Fantozzi et al., 1998, Pascoe et al., 2003). Polyphenols may demonstrate three different properties in beer. They can react with free radicals to produce phenoxy radicals, chelate transition metals (Vanderhaegen et al., 2006) and they may behave as pro-oxidants by transferring electrons to transition metal centres (Bamforth, 1999c). Thus, these varied potential roles make it difficult to evaluate the net influence of polyphenols in the reaction pathways of beer flavour instability.

1.3.10 Degradation of hop compounds

The involvement of hop compounds in flavour change during beer ageing was observed by Hashimoto et al. (1979). Unhopped beers showed a significantly lower level of ageing flavour compounds than the normally hopped control beers. The rapid degradation of iso-α-acids in the presence of ROS was shown by Kaneda et al. (1989). Huvaere et al. (2003) described the ability of iso-α-acids to transfer electrons to suitable acceptors. Reactive oxygen species are not essential for the electron transfer. Therefore the presence of oxygen is not necessary for the oxidative degradation of hop compounds. The deterioration of hop bitter compounds can be inhibited by the use of reduced isomerized hop extracts. De Cooman et al. (2000) illustrated the resistance of tetrahydro-iso-α-acids to oxidative degradation and a constant bitterness over beer storage. Furthermore a clear difference between the stability of trans-iso-α-acid and cis-iso-α-acid during beer ageing was presented. The trans-iso-α-acids deteriorate much faster and are less stable during the course of beer ageing. The ratio of concentrations of trans and cis isomers has been described as an indicator for beer flavour degradation (Araki et al., 2002).

1.4 Research aims and objectives

The most appropriate methods to monitor flavour stability, the underlying factors that cause instability, and the best ways in which to improve stability in the supply chain continue to be debated. A large number of different contenders were described (section 1.3), that can have a positive (+) or negative (-) effect on beer flavour stability:

- Sulphur dioxide (+)
- Oxygen (-)
- Hop compounds (+)
- Transition metals (-)
- Polyphenols (+/-)
- Protein thiol groups (+/-)

The aim of this research was to assess and enhance different chemical and instrumental methods to further investigate and possibly predict flavour (in)stability of lager-style beers. Additional work was focused on applying these methods in combination with sensory evaluation to assess the influence of changes in the production process and composition of beer on flavour stability.

The availability and application of a wide range of sophisticated chemical methods is indispensable for the examination of chemical changes related to flavour degradation during beer ageing. In Chapter 3 different techniques to chemically and instrumentally evaluate flavour stability were established and developed. As sulphur dioxide (SO_2) is regarded as one of the major antioxidants in beer (Andersen et al., 2000), a reproducible and reliable quantification in beer samples was indispensable for this work. Methods based on the Monier-Williams Method via distillation were refined to achieve this goal. Different aldehydes are strongly associated with known off-flavours

in aged beer and the concentration of these aldehydes increases during storage (Saison et al., 2009). Therefore aldehyde concentrations in aged beer samples are a potentially important indicator of flavour stability. A solid phase micro extraction (SPME)-GC-MS method with on fibre derivatization was established as a reliable detection method, based on previously described methods (Ortiz and Cornell, 2009), to monitor the concentration of these aldehydes during this work. Electron paramagnetic resonance (EPR) spectroscopy is a technique used to measure the oxidative stability of beer (Kaneda et al., 1995) and to evaluate the effects of different brewing process modifications on beer flavour stability (Uchida and Ono, 2000). However, the predictive power in regards to flavour degradation during storage, especially in correlation to sensory evaluation, is still debated (Wurzbacher and Back, 2007). Thus, EPR spectroscopic assays to evaluate oxidative beer stability were investigated and one of the aims was to examine the ability of EPR spectroscopy to probe beer flavour degradation during storage. Furthermore, to improve understanding of how EPR measurements relate to other commonly used metrics for flavour stability and how each may best be used to predict the ultimate arbiter of flavour stability: sensory flavour degradation.

Kieselguhr or diatomaceous earth is the most common filter aid for the filtration of beer. However, the Kieselguhr filtration has come under increased criticism due to issues with waste disposal, the hazards associated with the use of diatomite powder and a potential increase in arsenic and iron concentration in the filtered beer (Freeman and McKechnie, 1995, Bernstein and Woods, 2013). Furthermore alternative filtration systems are associated with less impact on beer flavour stability (Broens et al., 2011). In Chapter 4, as part of a direct comparison of the performance of a production scale Kieselguhr filtration system and two pilot scale membrane filtration systems, the

impact on flavour stability was evaluated. The goal was to contribute to the performance evaluation of the different systems with regards to the impact on flavour stability.

The positive effect of gallotannin additions in the brewing process on flavour stability has been reported (Aerts et al., 2004, Methner, 2015). Nevertheless the amounts added exceed the manufacturer recommendations by a factor of up to three times. In Chapter 5, gallotannins were added in the brewhouse in quantities in line with the recommendations of the manufacturer. The aim was to evaluate the possible positive effects on flavour stability of a cost-effective addition in contrast to previous studies, where the amounts of gallotannins added were based on maximising the flavour stability improvement.

The main factors influencing the rate of flavour change during ageing are widely acknowledged. This includes but is not limited to dissolved oxygen, SO_2, transition metal ions, hop acids, polyphenols and thiols. However, much less is understood about the relative importance of each factor and the ways in which the factors interact to determine the product stability. In Chapter 6, a factorial experimental design was adopted to probe the interactions between seven factors known to impact on beer flavour stability. Chemical additions were made to a commercial lager prior to bottling, to vary the following factors: total in pack oxygen, SO_2, total iron, iso-α-acid and α-acid content, (+)-catechin and glutathione. With the evaluation of instrumental and sensory measures of flavour stability as the factors were varied across a defined design space, the aim was to study interactions between factors and to evaluate the relative significance of single factors on flavour stability of the approached lager-style beer.

2 Material and Methods

2.1 List of chemicals

Table 2.1: List of chemicals used including purity and supplier

Chemical	Purity	Supplier
2-methylpropanal	> 99.5 %	Sigma Aldrich, UK
2-methylbutanal	98 %	Sigma Aldrich, UK
3-methybutanal	98 %	Sigma Aldrich, UK
Furfural	99 %	Sigma Aldrich, UK
Benzaldehyde	99.5 %	Sigma Aldrich, UK
Phenyl acetaldehyde	98 %	Sigma Aldrich, UK
(E)-2-nonenal	97 %	Sigma Aldrich, UK
3-fluoro benzaldehyde	97 %	Sigma Aldrich, UK
Ethanol	> 99.5 %	Fisher Scientific, UK
Sodium chloride (NaCl)	> 99 %	Sigma Aldrich, UK
N-tert-Butyl-α-phenylnitrone (PBN)	> 98 %	Fisher Scientific, UK
4-hydroxy-2, 2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL)	> 98 %	Sigma Aldrich, UK
o-(2,3,4,5,6-Pentafluorobenzyl-hydroxylamine hydrochloride (PFBOA)	> 99 %	Sigma Aldrich, UK
Carboxymethylcellulose (CMC)	95 %	Fisher Scientific, UK

Ethylenediamine tera acetic acid (EDTA)	> 90 %	Sigma Aldrich, UK
Ortho phosphoric acid	85 v/v %	Fisher Scientific, UK
Sodium hydroxide	95 to 100.5 %	Fisher Scientific, UK
Hydrogen peroxide	30 to 32 %	Fisher Scientific, UK
Glacial acetic acid	> 99.7 %	Fisher Scientific, UK
Thiobarbituric acid	98 %	Alfa Aesar, UK
Nitric acid	70 %	Fisher Scientific, UK
Hydrochloric acid	37 %	Fisher Scientific, UK
Isooctane	HPLC grade	Fisher Scientific, UK
Ammonia concentrate	Laboratory grade	Fisher Scientific, UK
Ammonium ferric citrate	Laboratory grade	Fisher Scientific, UK

2.2 Analysis of volatile aldehydes in beer by solid phase micro extraction (SPME) with GC-MS

The highly volatile compounds 2-methylpropanal, 2-methylbutanal, 3-methybutanal, furfural, benzaldehyde, phenyl acetaldehyde, and trans-2-nonenal were determined based on the method presented by Ortiz and Cornell (2009).

2.2.1 Preparation of stock solutions

2.2.1.1 *Standard stock solution (SSS)*

A 200 ml volumetric flask was filled with ethanol (*ca.* 50 ml) and placed on a digital balance. A positive displacement pipette was used to transfer the following volumes of reagent into the 200 ml volumetric flask: 400 µl 2-methylpropanal, 400 µl 2-methylbutanal, 400 µl 3-methylbutanal, 1200 µl furfural, 300 µl benzaldehyde, 600 µl phenyl acetaldehyde and 20 µl trans-2-nonenal.

As these compounds are extremely volatile, the weight of each reagent was additionally measured using the digital balance. The obtained mass of each compounds was used to calculate the concentration of each reagent in the standard solution. After the addition of the reagents, the flask was made up to volume with ethanol. The SSS was stored at -18˚C and prepared fresh for each independent project.

2.2.1.2 *Calibration solution (CALS)*

Two different calibration solutions were prepared. For CALS B the SSS (200 µl) was transferred into a 100 ml volumetric flask half filled with ethanol. After the addition it was made up to volume using ethanol. For CALS A, CALS B (10 ml) was transferred into a 100 ml flask half filled with ethanol and made up to volume after the addition. Both CALSs were stored at -18˚C, brought up to 4˚C prior to analysis and prepared fresh for each set of samples obtained from the different trials for GC analysis.

2.2.1.3 *Internal standard solution (ISS)*

In a 200 ml volumetric flask half filled with ethanol, 3-fluorobenzaldehyde (120 µL) was added and made up to volume. This solution was stored at -18˚C. 8 ml of this solution were transferred into to a 200 ml volumetric flask half filled with ethanol and made up to volume to obtain the ISS. The ISS was stored at -18˚C, brought up to 4˚C prior to analysis and prepared fresh for each set of samples obtained from each independent trial for GC analysis.

2.2.1.4 *Derivatization Solution (DS)*

o-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBOA, 150 mg) was transferred into a 25 ml volumetric flask. Reverse osmosis (RO) water (*ca.* 10 ml) was added and the flask was vortexed. After the PFBOA fully dissolved, the flask was made up to volume with RO water. The DS was divided into four portions and stored at -18˚C. The DS was brought up to 4˚C prior to analysis and a fresh portion was used for each analysis.

2.2.2 Sample preparation

All samples were stored at 2˚C prior to analysis. For degassing the beer samples, the cool beer sample (*ca.*100 ml) were poured into a 500 ml baffled flask. The beer was swirled around by hand and discarded. This procedure was repeated to cool the inner surface of the flask. Then the beer sample (*ca*. 100 ml) was poured gently down the sidewall of the flask and shook for 5 min using an orbital shaking incubator set at 175 rpm and 10˚C. Immediately after degassing, a 10 ml bulb pipette was rinsed with the beer sample and then the beer sample (10 ml) was pipetted into a 20 ml headspace vial and 3-fluorobenzaldehyde (50 µl) as internal standard was added. Sodium chloride

(3.5 g) was added and the vial was sealed immediately with a crimp cap lined with a PTFE/silicone septum. Prepared samples were stored at 4˚C prior to analysis and no sample was prepared more than 12 h prior to analysis.

2.2.3 Preparation of derivatization agent solution

For each beer sample a separate derivatization agent solution was prepared, pipetting RO water (10 ml) into a 20 ml headspace vial with DS (100 µl) added and sealed with a crimp cap lined with a PTFE/silicone septum. The prepared derivatization agent solutions were stored at room temperature for no longer than 12 h.

2.2.4 Preparation of calibration standards for calibration curve

For each new set of samples run on the GC and for each new fibre, a 6 point calibration curve was obtained. To a fresh beer sample, prepared in Section 2.1.2, the following volumes of CALS were added before the addition of the ISS and the NaCl before the vial was crimped and treated the same as the beer samples afterwards:

0 µl (blank), 20 µl of CALS A, 40 µl of CALS A, 80 µl of CALS A, 160 µl of CALS A and 40 µl of CALS B, respectively.

2.2.5 GC sample run

The derivatization agent vial was pre-incubated at 50°C for 1 min with the agitator speed set to 250 rpm. Afterwards the headspace was exposed to a solid-phase micro extraction (SPME) fiber (65 µm PDMS-DVB, 23 gauge needle), and equilibrated for 10 min at 50°C.

This was followed by a pre-incubation (5 min at 50°C) of the beer sample. Then the headspace was exposed to the SPME fibre and equilibrated for 60 min at 50°C.

Samples were analysed by GC-MS using a Trace GC Ultra Chromatograph with a DSQ mass spectrometer (Thermo, Manchester, UK). The equilibrated SPME fibre was exposed to the injector port (250°C) and desorbed for 3 min. The GC was operated in splitless mode (splitless time: 0.1 min, split flow: 53 ml/min) with an initial oven temperature of 40°C that was increased at a rate of 10°C/min to 140°C and from 140°C to 250°C at 7°C/min (transfer line: 250°C). Volatiles were separated on a DB-5 column using a helium carrier gas (8.32 psi). The DSQ mass spectrometer was operated in selected-ion mode scanning for ion m/z 181 from 4 min to 20 min and from 22 min to the end of the run, ions m/z 301, 319 from 20 min to 22 min.

All samples were run in duplicate.

If after a number of runs, the area of the internal standard reached less than 50 % compared to the area of the internal standard achieved with the newly installed fibre at the beginning of the measurement, the fibre was changed and a new calibration curve was obtained.

2.2.6 Approximate retention times of aldehyde geometrical isomers

2-Methylpropanal	12.54 and 12.56 min
2-Methylbutanal	14.09 and 14.18 min
3-Methylbutanal	14.34 and 14.45 min
Furfural	17.48 and 17.78 min
3-Fluorobenzaldehyde	20.32 and 20.58 min

Benzaldehyde	20.58 and 20.73 min
Phenylacetaldehyde	21.55 and 21.68 min
Trans-2-nonenal	22.73 and 22.98 min

2.2.7 Calculation of concentrations of calibration standards

Depending on the different aldehyde standard, the concentration was calculated and the purity of the regents was accounted for, based on the purity stated by the supplier of the aldehyde standards.

To calculate the concentration of each analyte in the SSS, Equation 2.1 was used.

Equation 2.1: Concentration of each analyte in the calibration standard SSS, where M = Mass of analyte added into the solution in mg, P = purity of analyte solution (%), SSS = concentration of analyte in SSS stock solution

$$\frac{M * P * 1000 * 1000}{0.2} = SSS(ppb)$$

The concentration of the reagents in the two calibration standard solutions CALS A and CALS B was calculated using Equation 2.2 for CALS A and 2.3 for CALS B.

Equation 2.2: Concentration of each analyte in the calibration standard CALS A, where SSS (ppb) = concentration of analyte in calibration standard stock solution SSS, CALS A (ppb) = concentration of each analyte in calibration standard A (CALS A)

$$SSS\ (ppb) * \frac{\frac{0.2}{100}}{10} = CALS\ A\ (ppb)$$

Equation 2.3: Concentration of each analyte in the calibration standard CALS B, where SSS (ppb) = concentration of analyte in calibration standard stock solution SSS, CALS B (ppb) = concentration of each analyte in calibration standard B (CALS B)

$$SSS\ (ppb) * \frac{0.2}{100} = CALS\ B\ (ppb)$$

The next equation leads to the concentration of each analyte after addition of the calibration standards CALS A or CALS B in the beer sample used for calibration (Equation 2.4).

Equation 2.4: Added concentration of each analyte in the beer sample used for calibration, where CALS added (µl) = amount of CALS A or CALS B added for calibration, Concentration CALS (ppb) = Concentration of each analyte in calibration standard A (CALS A) or calibration standard B (CALS B), A (ppb) = concentration of added analyte in beer calibration sample

$$CALS\ added\ (\mu l) * Concentration\ CALS\ (ppb) * \frac{1}{10000} =\ A\ (ppb)$$

2.2.8 Calculation of concentrations of aldehydes in the samples

The concentration of the different aldehydes, 2-methylpropanal, 2-methylbutanal, 3-methybutanal, furfural, benzaldehyde, phenyl acetaldehyde, and trans-2-nonenal, in the sample was calculated using the area of the peaks obtained in the gas chromatogram relative to the peak area of the internal standard.

As the method of standard additions was used, the measured values for the peak area must be corrected to take into account the concentration of the aldehydes already present in beer used to create the calibration curve. The correction is shown in the following example.

Table 2.2: Concentration (ppb) of aldehyde added to the fresh beer in correlation the peak area relative to the IS

Conc. (ppb) added	Peak area relative to IS
0	1
0.2	2
0.4	4
0.8	7.9
1.6	16.5
4	40.2

Table 2.2 shows the concentration (ppb) for each calibration level and the corresponding peak area relative to the internal standard. The data set above generated Figure 2.1 and the corresponding linear equation.

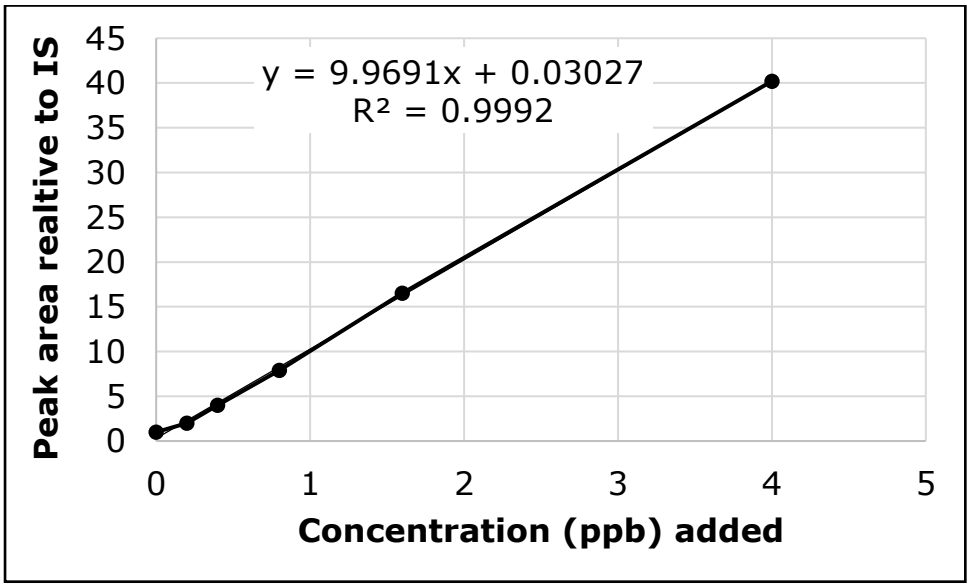


Figure 2.1: Concentration (ppb) for each level of calibration for the over addition plotted against the peak area relative to the IS

The value for the y-intercept or no addition equals the concentration of the aldehyde already present in the fresh beer sample used to obtain the calibration curve. This value

needs to be added to achieve the concentration of the aldehyde of each calibration level. The values are shown in the Table 2.3.

Table 2.3: Concentration (ppb) of the aldehyde present in correlation to the peak area relative to the IS

Conc. (ppb)	**Peak area relative to IS**
0.03	1
0.23	2
0.43	4
0.83	7.9
1.63	16.5
4.03	40.2

The data set produced Figure 2.2 and the obtained linear equation was used to calculate the concentration of the aldehyde present in the analysed samples. The peak area relative to the IS was directly correlated to the concentration of the aldehyde.

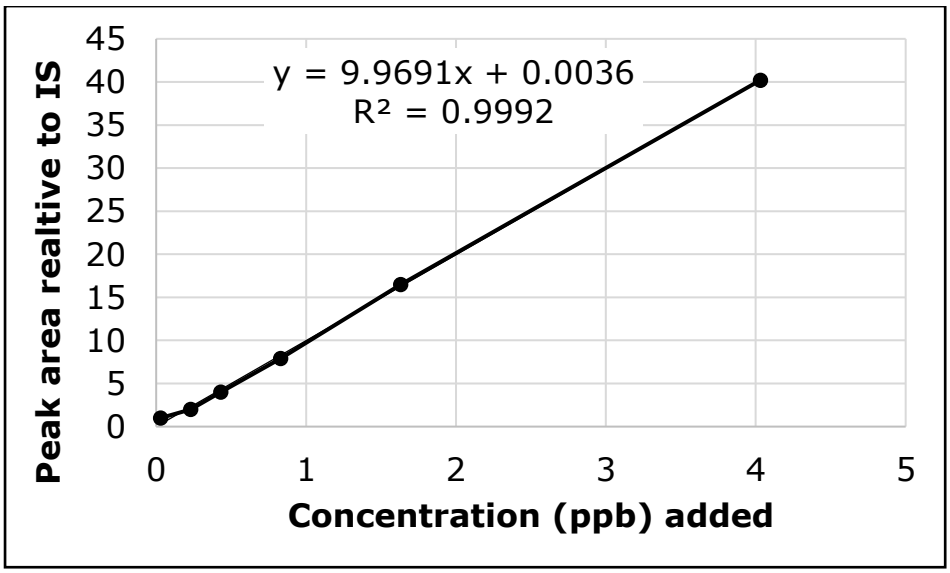


Figure 2.2: Concentration (ppb) for each level of calibration plotted against the peak area relative to the IS and obtained linear equation to calculate the concentration of the aldehyde present in the analysed sample

2.3 Total sulphur dioxide (SO_2) by distillation method

The determination of total SO_2 was based on the Monier-Williams method described by MEBAK (2002) and EBC (2005). After the acidification of the beer sample, the SO_2 was distilled and collected in a neutralized hydrogen peroxide solution where the SO_2 was oxidized to sulphuric acid.

2.3.1 Preparation of solutions

2.3.1.1 *Phosphoric acid (30% v/v) solution*

A 1000 ml volumetric flask was filled with RO water (*ca*. 600 ml). Phosphoric acid (360 ml, 85 % v/v) were added carefully. The solution was stirred and cooled down, and after it reached ambient temperature, the volume was made up with RO water.

2.3.1.2 *Sodium hydroxide (NaOH) solution (0.01 M)*

Sodium hydroxide (400 mg) was added to a volumetric flask (1000 ml) containing RO water (500 ml). After all of the sodium hydroxide dissolved, the volume was made up to 1000 ml with RO water.

2.3.1.3 *Neutralized hydrogen peroxide (H_2O_2) solution (1% v/v)*

Hydrogen peroxide (34 ml, 300 g/l) were mixed with RO water (966 ml) in an Erlenmeyer flask (1000 ml). Universal indicator (15 drops) was added to form a red coloured solution (pH 4). This solution was titrated to yellow (pH 6) using sodium hydroxide solution (0.01 M). The solution was prepared fresh every day.

2.3.2 Distillation

Figure 2.3: Distillation apparatus used during determination of total sulphur dioxide content

The neutralized H_2O_2 solution (100 ml) was transferred into a Dreschel bottle (250 ml) and connected to a delivery tube connected to the top of a double surface condenser (200 mm length). The end of the delivery tube was submerged in the neutralized H_2O_2 solution.

The beer sample was stored at 2˚C before the analysis. Immediately after the beer sample was opened, a 50 ml glass bulb pipette was rinsed with the beer sample to cool the inner surface of the pipette. Afterwards the beer sample (50 ml) was transferred into a 250 ml 2-neck round bottom flask containing phosphoric acid (30% v/v, 5 ml) and instantly connected to the bottom of the double surface condenser. To the second neck of the round bottom flask, a nitrogen gas delivery tube was connected. The gas

delivery tube ended underneath the surface of the solution in the flask. The round bottom flask was placed in an 180˚C heating block. The sample was boiled under reflux and a constant flow of nitrogen of 2 to 3 bubbles per second regulated with a valve for 15 min. After 15 min the Dreschel bottle was disconnected and the delivery tube was rinsed with RO water. The sulphuric acid that formed was titrated back to yellow (pH 6) with sodium hydroxide solution (0.01 M).

2.3.3 Calculation of total SO_2

Equation 2.5: Calculation of total SO_2, where V = volume in ml of NaOH (0.01 N) solution used

$$Total\ SO_2 \left(\frac{mg}{l}\right) = 6.4 * V$$

2.4 Thiobarbituric acid index (TBI)

2.4.1 Preparation of solutions

2.4.1.1 *Glacial acetic acid solution (90% v/v)*

In a 1000 ml volumetric flask, glacial acidic acid (100 % v/v, 900 ml) were added and made up to volume with RO water.

2.4.1.2 *Thiobarbituric acid solution (0.02 M)*

To a 200 ml brown glass volumetric flask filled with glacial acetic acid solution (90% v/v, 100 ml), thiobarbituric acid (456 mg) was added and the solution mixed for 2 min and placed in a water bath (70˚C) for 10 min. The heated flask was mixed until all thiobarbituric acid dissolved. After the solution was cooled to ambient temperature,

the solution was made up to volume with glacial acetic acid solution (90% v/v). The thiobarbituric acid solution was prepared fresh every day.

2.4.2 Sample preparation

The beer samples were filtered through a cellulose filter (Whatman Grade 1) prior to analysis to degas. All wort samples were filtered utilizing a glass fibre filter (Whatman Grade GF/A) to remove any turbidity.

All samples, wort and beer, were diluted 1/11 before the analysis. Thus, the sample (5 ml) was pipetted into an Erlenmeyer flask (100 ml) and RO water (50 ml) was added using a bulb pipette (50 ml). The Erlenmeyer flask was swirled to mix the sample.

Diluted sample (10 ml) and thiobarbituric acid solution (0.02 M, 5 ml) were added to a brown glass vial (20 ml), which was capped and mixed for 1 min.

For the determination of the blank value, diluted sample (10 ml) and glacial acidic acid solution (90% v/v, 5 ml) were added to a brown glass vial (20 ml), capped and mixed for 1 min.

Sample for blank value determination and beer/wort samples were placed in a water bath (70˚C) for 60 min. Immediately after the 60 min all vials were cooled down to 20˚C in an ice bath and removed instantly after reaching 20˚C.

2.4.3 Spectrophotometer measurement

The spectrophotometer (Jenway 7315) was switched on 30 min before the analysis to warm up and set to read the absorbance at a wavelength of 448 nm using a visible light source.

RO water was used to zero the spectrophotometer.

Directly after the samples had reached a temperature of 20˚C, the samples were transferred into 2.5 ml macro UV plastic disposable cuvettes and measured utilizing the spectrophotometer. One measurement for the blank and three independent measurements for a sample were obtained. All samples were measured no longer than 15 min after they reached the 20˚C.

2.4.4 Calculation of TBI

Equation 2.6: Calculation of thiobarbituric acid index (TBI), where AS = absorbance of sample, AB = absorbance of blank, F = dilution factor

$$TBI\ (no\ unit) = (AS - AB) * 10 * F$$

2.5 Metal content analysis via Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

2.5.1 Preparation of solution

All standards and solutions used were already prepared at the ICP-MS laboratory.

2.5.2 Sample preparation

Beer samples were degassed by sonication (5 min) and wort samples were clarified by centrifugation at a speed of 400 g for 5 min. All samples were diluted (1:10) with nitric acid (2 %) by pipetting the sample (1 ml) and nitric acid (2%, 9 ml) into a 10 ml plastic sample tube. Sample tubes were capped and inverted three times. Diluted samples were stored at 2˚C pending elemental analysis.

2.5.3 ICP-MS analysis

A multi-element analysis (Fe, Cu, Mn) of diluted beer sample was undertaken by ICP-MS (Thermo-Fisher Scientific X-SeriesII) with a 'hexapole collision cell' (7 % hydrogen in helium) to remove polyatomic interferences. Samples were introduced from an autosampler (Cetac ASX-520 with 4 x 60-place sample racks) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific; 1 ml/min).

Internal standards were introduced to the sample stream via a T-piece and included Scandium (100 ng/ml), Rhodium (20 ng/ml) and Iridium (10 ng/ml in 2% nitric acid. External multi-element calibration standards (Claritas-PPT grade CLMS-2, Certiprep/Fisher) included Fe, Cu and Mn in the range 0-100 µg/l.

Sample processing was undertaken using Plasmalab software (version 2.5.4; Thermo-Fisher Scientific) set to employ separate calibration blocks and internal cross-calibration where required.

2.6 Oxidative stability by electron paramagnetic resonance (EPR) spectroscopy

The technique is based on the direct measurement of the free radical formation during a forced ageing test.

2.6.1 Preparation of solutions

2.6.1.1 *α-Phenyl-t-butylnitrone (PBN) solution (2.5 M)*

To a glass vial (8 ml) containing PBN (1.77 g), ethanol (2 ml) and RO water (2 ml) were added. The vial was capped and vortexed till all PBN crystals were dissolved. The solution was prepared fresh every day.

2.6.1.2 *4-Hydroxy-2, 2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) solution (1 µM)*

First a 1 mM TEMPOL stock solution was prepared by adding TEMPOL (172 mg) to a volumetric flask (1000 ml) filled with RO water (500 ml). After the TEMPOL was fully dissolved, the volume was made up using RO water.

To achieve the final concentration of 1 µM TEMPOL, TEMPOL solution (1 mM, 1 ml) was added to a volumetric flask (1000 ml) filled with RO water (500 ml). After mixing, the volume was made up with RO water. This solution was used as reference standard.

The 1mM TEMPOL stock solution and the 1 µM TEMPOL solution were stored at 2˚C. For each set of independent samples, new stock solutions were prepared.

2.6.2 Sample preparation

Beer samples were degassed by sonication (60 s) and wort samples were clarified by centrifugation (5 min at 400 *g*).

The sample was added to a vial with a glass bulb pipette and PBN solution (2.5 M) was added to achieve a 50 mM PBN concentration in the sample solution. After the

addition, the vial was capped and vortexed for 60 s. After mixing, the vial was vented, capped and vortexed again for 30 s. After venting again, a hole was pierced into the lid of the vial using an injection needle to allow gas exchange but prevent increased evaporation during the forced ageing at 60˚C.

Depending on the required measuring points, 2.5 ml of sample were prepared for each measuring point. Between 8 and 11 measuring points were required.

2.6.3 EPR spectroscopy measurement

EPR spectra were obtained with an EMX X-Band spectrometer (Bruker) (microwave frequency, 9.75 GHz) equipped with an ER 4102 ST cavity and a 19 tube Aqua-X flow system.

The instrument settings were as follows:

- Microwave power, 20 mW
- Modulation amplitude, 3 G
- Modulation frequency, 100 kHz
- Conversion time, 20.48 ms
- Time constant, 20.48 ms
- Number of scans, 8

The settings were adjusted so that no changes in the profile of the signals were detected whilst maximising the signal-to-noise ratio.

Prepared samples were heated to 60°C with a water bath during the oxidative stability measurement and EPR spectra were obtained for a time period of 200 min or 210 min respectively.

3 ml luer lock syringes were used to inject the sample into the Aqua X. The syringe was filled with sample (*ca*. 2 ml) and air (1 ml). The air was injected first to purge out the previous sample. The sample was injected with a pulsating motion to achieve a bubble-free injection. After the injection, the EPR spectrometer was tuned and the measurement was started. EPR spectra were obtained every 10 min to 20 min depending on the required number of measuring points over 200 min or 210 min, respectively.

Before and after each full measurement run, a TEMPOL (1 µM) standard was used to evaluate the performance of the EPR spectrometer and to check if the required signal intensity was achieved. An RO water sample was measured to record the shift of the cavity at the beginning of each run.

2.6.4 Data analysis

EPR data analysis was carried out with the EasySpin Toolbox (Stoll and Schweiger, 2006) incorporated into Matlab R2012a. Each EPR spectrum was analysed using two different methods to determine the signal intensity, peak height determination and double integration to calculate the area of the spectrum. In addition, each spectrum was simulated in an approach to exclude noise in the experimental EPR spectra. The simulated spectra were analysed by peak height determination.

The different Matlab codes used can be found in the appendix. The data analysis included the following steps:

- EPR data input.
- Base line correction by subtracting the RO water spectrum from each sample spectrum to correct for the shift of the cavity

- The determination of peak height using the first feature of the spectrum
- Double integration of the full spectrum (all three features) to determine the area
- The simulation of each spectrum to remove the noise associated with the experimental spectrum
- The determination of peak height and double integration each simulated spectrum
- Export of the data as an Excel spreadsheet including values for time, height and area for baseline corrected and for simulated spectra

QtiPlot software was used to calculate the area under the curve by integration of the given data points over 200 min or 210 min for the data handling approaches described above.

Additionally the Boltzmann function was used to fit a curve through the data points and determine x0 lag time and dx value (Equation 2.7).

Equation 2.7: Boltzmann function used to fit curve through the data points of the EPR measurement and determine x0 lag time and dx value

$$y = A_2 + \frac{A_1 - A_2}{1 + e^{\frac{x - x_0}{dx}}}$$

The standard lag-time was determined by an algebraic approach. The linear expression for the first flat part of the curve and the linear expression for all the points in the propagation stage were defined. The intersection point of the two lines equals the lag-time.

T_{150} and T_{160} values were directly given data points, if an EPR spectrum was obtained at 150 min or 160 min, respectively. If no spectrum was obtained at the required time points, interpolation was used to calculate the T_{150} or T_{160} value.

2.7 Determination of Bitterness units (BU) with manual isooctane extraction

2.7.1 Preparation of solutions

2.7.1.1 *Hydrochloric acid solution (HCl) (3 N)*

To a volumetric flask (1000 ml) filled with RO water (500 ml), hydrochloric acid (37 % w/v, 246 ml) were added carefully and after cooling to ambient temperature made up to volume with RO water.

2.7.1.2 *Isooctane*

HPLC grade isooctane without any further purification was used for the analysis.

2.7.2 Sample preparation

Beer samples were degassed by sonication (5 min). The beer sample (5 ml) was transferred into a 50 ml centrifuge tube and HCl (3 M, 0.5 ml) were added. After the addition of isooctane (5 ml), the tube was closed tightly, inverted three times and shaken for 15 min on a roller bed at a speed of 60 rpm.

Afterwards the tubes were centrifuged for two times 5 min at a speed of 400 *g*. The layer formation was checked after each centrifugation. If the separation of the layers

was not sufficient enough, an additional centrifugation run of 5 min at 400 g was added.

A sample to obtain the blank value was prepared using the same procedure omitting the beer sample volume.

2.7.3 Spectrophotometer measurement

The spectrophotometer (Jenway 7315) was warmed up for 30 min prior to the measurement and the absorbance was determined at a wavelength of 275 nm

A plastic pipette was used to transfer the upper clear isooctane layer into a 10 mm, 2.5 ml, plastic disposable macro UV cuvette. The blank value was used to do a zero reading and was followed by measuring the absorbance at a wavelength of 275 nm. All samples were measured in triplicate.

2.7.4 Calculation of bitterness units (BU)

Equation 2.8: Calculation of bitter units (BU), where Absorbance = Absorbance sample corrected by blank value

$$BU = Absorbance\ x\ 50$$

2.8 Total polyphenols

The red colour formed by the reaction of ferric iron with polyphenols was used to determine the total polyphenol content.

2.8.1 Preparation of solution

2.8.1.1 *Carboxylmethylcellulose (CMC) solution (1 % w/v) with ethylenediamine teraacetic acid (EDTA) (0.2 % w/v)*

CMC sodium salt (10 g) and EDTA (2 g) were slowly added under stirring to a glass beaker (1000 ml) filled with RO water (800 ml). The solution was stirred for approx. 2.5 h until everything was dissolved. The solution was transferred to a volumetric flask (1000 ml) and made up to volume with RO water.

2.8.1.2 *Ferric iron solution (0.56 % w/v)*

In a volumetric flask (100 ml) filled with RO water (50 ml), ammonium ferric citrate (0.35 g) were added and after dilution made up to volume with RO water. The solution was prepared fresh every week.

2.8.1.3 *Ammonia solution (33 % v/v)*

To a volumetric flask (100 ml), concentrated ammonia (33 ml) was added and made up to volume with RO water.

2.8.2 Sample preparation

Beer samples were degassed by sonication (5 min) and wort samples were centrifuged for 5 min at 400 G for clarification.

To a volumetric flask (25 ml) the prepared beer/wort sample (10 ml) was added together with the CMC solution (8 ml) and inverted three times to mix the two solutions. The ferric iron solution (0.5 ml) was added and after mixing again, ammonia

solution (0.5 ml) was added and the flask was inverted again three times to mix all solutions thoroughly. The volume was made up with RO water and mixed again.

To obtain the blank value, the same procedure was repeated apart from the addition of the ferric acid solution.

2.8.3 Spectrophotometer measurement

The spectrophotometer (Jenway 7315) was warmed up for 30 min prior to the measurement and the absorbance was determined at a wavelength of 600 nm

After the prepared blank and sample had rested for colour development for 10 min, they were transferred into a 10 mm, 2.5 ml, plastic disposable macro cuvette and the absorbance was measured. Each sample was prepared in triplicate and one blank accordingly.

2.8.4 Calculation of concentration of total polyphenols (mg/l)

Equation 2.9: Calculation of total polyphenol concentration (mg/l), where Absorbance = Absorbance of sample corrected by blank value

$$Concentration\ total\ polyphenols\ \left(\frac{mg}{l}\right) = Absorbance * 820$$

3 Method development for the determination of total sulphur dioxide content, staling aldehyde concentration and oxidative stability of beer samples

3.1 Introduction

Due to its complexity, the assessment and evaluation of flavour stability requires a wide range of different chemical methods, suitable to determine the factors influencing flavour stability and shelf life of beer. The required methods had not been established previously at Nottingham and therefore new protocols had to be developed. These included protocols for the determination of sulphur dioxide via a distillation method, the detection of free staling aldehydes utilizing PFBOA on fibre derivatization and an assessment of the oxidative stability of wort and beer samples by electron paramagnetic resonance (EPR) spectroscopy.

3.2 Determination of total sulphur dioxide (SO_2) in beer

3.2.1 Introduction

Sulphur dioxide plays an important role in the flavour stability of beer. It reacts with carbonyl compounds to form non-flavour active α-hydroxysulphonates and inhibits oxidative reactions which are a major cause of flavour instability (Ilett, 1995). The form the SO_2 in solution depends on the pH of the solution. For beer (pH = 3.8-4.2), SO_2 is mostly present as HSO_3^- (bisulphite or hydrogen sulphite anion) and sulphur

dioxide is regarded as the major antioxidant in beer (Andersen et al., 2000). Legislation requires that the concentration of total SO_2 is limited to 20 mg/l in packaged beer and levels over 10 mg/l must be labelled according to the European Parliament and the Council Directive No 95/2/EC (1995). Therefore the application of SO_2 to improve flavour stability is restricted and a reliable and reproducible identification of the SO_2 concentration is indispensable in research into the flavour stability of beers.

Several different methods for the identification of the SO_2 content in beer have been described (Dvorak et al., 2006, Almeida et al., 2003, Munar et al., 1994, Anonymous, 2005). The most common methods are the three methods recommended by the European Brewery Convention (EBC). These are the determination via distillation based on the Monier-Williams method, a method utilizing an enzyme kit and a method based on the reaction of between SO_2, p-rosaniline and formaldehyde. All three methods are included in the relevant collections of analytical methods for the brewing industry from American Society of Brewing Chemists (ASBC), European Brewery Convention (EBC) and by Mitteleuropäische Brautechnische Analysen Kommission (MEBAK). Further advanced techniques to quantify the amount of total SO_2 present in beer have been described. This includes a flow injection analysis (FIA) method (Fernandes et al., 1998), ion exclusion chromatography with electrochemical detection (Wagner and McGarrity, 1991), headspace gas chromatography using a flame photometric detector (FPD) (Manur et al., 1994), spectrophotometric determination with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Guido, 2005) and an inline technique based infrared spectroscopy (Skands et al., 2003). All methods have advantages and disadvantages regarding their sensitivity and reproducibility (Ilett, 1995, Dvorak et al., 2006, Kunz et al., 2009). Due to the availability of the required measuring devices,

issues with the waste disposal for the p-rosanillin method and archiving of the enzymatic method by EBC and ASBC, only the distillation method could be considered for this work.

3.2.2 Development of the distillation method

The distillation method presented by EBC (2005) and MEBAK (2002) are arranged differently despite the MEBAK method being described as an EBC method. The differences are displayed in Table 3.1.

*Table 3.1: Difference between distillation methods for total SO_2 MEBAK 2.25.2 and EBC 9.25.1 (*excluding time to reach increased temperature of distillation bridge)*

	MEBAK method 2.25.2	**EBC method 9.25.1**
Concentration NaOH (N)	0.01	0.02
Beer volume (ml)	100	50
Concentration H_3PO_4 (% vol.)	25	5
Volume added H_3PO_4 (ml)	10	50
Boling time (min)	20	15*

While each method is based on the same principle and described as the same method, they have significant differences.

The concentration of NaOH in the test samples has a significant influence on the accuracy of the titration. One drop of stock NaOH solution from a 2 ml micro burette corresponds to a volume of 0.05 ml of NaOH solution. With a sample volume of 50 ml and a NaOH concentration of 0.02 N, this correlates to 0.64 mg/l of SO_2 for the

EBC method. For the MEBAK method, the same volume of 0.05 ml NaOH corresponds to 0.16 mg/l of total SO_2.

The volumes and concentrations of phosphoric acid added to lower the pH of the beer sample prior to the distillation vary between the MEBAK and EBC methods: 10 ml and 25 % vol. and 50 ml and 5 % vol. for the MEBAK and EBC methods, respectively. Thus, while the number of moles of phosphoric acid added is the same for the two methods, the amount of water added and therefore the amount of volume to be boiled off differs.

The boiling time shows a noticeable discrepancy between the two methods. For the MEBAK method, 20 min of boiling time are indicated, whereas for the EBC method only 15 min of boiling are required. Further to the 15 mins, the water supply for the condenser needs to be switch off and the boil should be continued until the distillation bridge gets hot.

In this work we used a combination of the MEBAK and EBC methods (Material and Methods 2.2.5) for the determination of total SO_2 in beer. The concentration of the NaOH solution was 0.01 N in combination with a beer volume of 50 ml. The combination of 100 ml beer volume and a NaOH concentration of 0.01 N required volumes of over 2 ml of NaOH. As a 2 ml burette was used for the titration to realise an accurate titration, exceeding a volume of 2 ml required additional attention during the titration step, as the burette could run empty. A H_3PO_4 concentration of 30 % and an addition of 5 ml was used to reduce the volume required to boil. The boiling time was set to 15 min. Additional boiling time of 5 min to 10 min did not result in any differences in the detected SO_2 concentrations. The EBC method requires an additional boiling time until the distillation bridge gets hot after the water supply to

the cooler was switched off. However even after 60 min of additional boiling time the temperature of the distillation bridge did not increase.

3.2.3 Regular performance testing

A benchmark beer was used to monitor the quality and reproducibility of the method. A benchmark beer analysed by Campden BRI, Nutfield, with a known SO_2 concentration was used to validate the accuracy of the presented method, followed by the introduction of an independent benchmark beer. The benchmark beer was a single batch of a large scale production lager style beer. The beer was stored at 2 °C to minimize ageing and to retain the original SO_2 content for as long as possible. To determine the SO_2 content of the benchmark beer, six independent SO_2 measurements were performed. The average of these measurements was used as the sample mean. The upper and lower control limited was based on the coefficient of variation of +/- 10 % specified in MEBAK (2002).

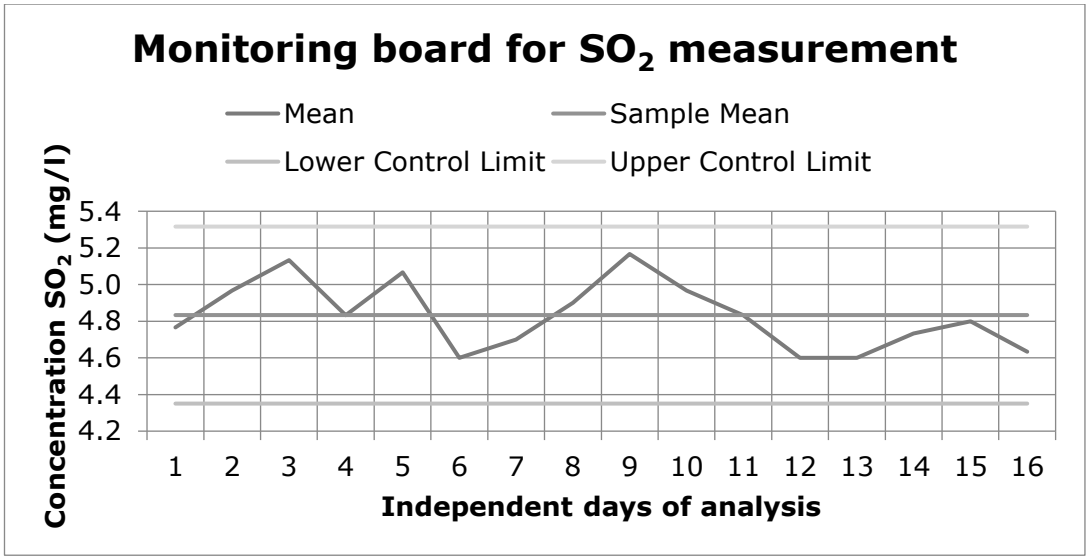


Figure 3.1: Monitoring board for the determination of the total SO_2 content of the canned benchmark beer (sample mean derived 6 independent measurements in triplicate, mean results determined in triplicate before each measurement run)

Figure 3.1 shows the results for the regular measurement of the benchmark beer in relation to the sample mean determined in six independent measurements. The measurement of trial samples was only commenced after the SO_2 content of the benchmark beer was determined within the range of lower and upper control limits. This system was very helpful to avoid variation in the SO_2 determination and identify circumstances affecting the measurement. Using this approach the adhesion of alkaline detergent on glassware taken out of the dishwasher was identified as having an effect on the measurement. As the method is based on the neutralization of very low concentrations of sulphuric acid with sodium hydroxide, any alkaline residue can affect the measured SO_2 concentration. Hence all glassware was double rinsed using demineralized water before the analysis. Furthermore the first run of each set of measurements was rejected due to the lower levels of SO_2 typically measured. This might have been related to larger amounts of dioxygen still present in the distillation apparatus. Another factor influencing the measured SO_2 levels was the flow rate of the

nitrogen carrier gas. A too low or a too high dinitrogen flow rate disturbed the concentrations detected. Therefore the flow rate was adjusted until the control limits set for the control beer were met.

3.2.4 Conclusion

The coefficient of variation of +/- 5 % stated in the analytica-EBC (2005) could not be achieved, despite the improvements of the method. It appears that such a low variation is difficult to achieve, especially when using a NaOH concentration of 0.02 mol/l, as one drop of NaOH already equals 0.64 mg/l SO_2 in the sample and most beers range between 3 mg/l and 7 mg/l total SO_2. Furthermore the influence of the total in pack oxygen on the SO_2 concentration has to be considered as a possible source of error for the same beer batch tested from two different bottles. Therefore the coefficient of variation of MEBAK (2002) was applied for the adopted method.

3.3 Staling aldehydes quantification using SPME-GC/MS with on fibre derivatization

3.3.1 Introduction

Typical 4 % ABV UK-lager style beers are subject to undesirable changes in flavour through shelf-life. Due to their relatively low amount of primary flavour in comparison to other beer styles like ales, the undesirable staling off-flavours are much more apparent and thus of significance. Some of these off-flavours can be directly related to different aldehydes present in beer. The extremely low flavour thresholds and accumulative effects produce undesirable flavour changes during storage. These flavours are attributed to the aldehydes and can be described variously as cardboard,

papery, stale, oxidised, astringent, almond and bready (Rodrigues and Almeida, 2009, Hayase et al., 1996, Harayama et al., 1994, Malfliet et al., 2009). To monitor and further understand the role of different aldehydes related to beer flavour changes during storage, the reliable quantification of these aldehydes is required. The starting point for the investigation of such compounds was the introduction of gas-chromatography in the early 1950s (James and Martin, 1952, Bartle and Myers, 2002). Nevertheless, to quantify such low concentrations of aldehydes present in beer, a further development of the gas chromatographic technique was necessary. The use of solid phase micro extraction (SPME) in combination with on fibre derivatization and GC/MS has been reported as the most suitable technique to quantify aldehydes related to beer staling (Baert et al., 2012).

3.3.2 Enhancement of a preliminary SPME GC/MS method

A method for SPME GC/MS with on fibre derivatization was presented to the ASBC Technical Committee to evaluate this method for the determination of volatiles in beer (Ortiz and Cornell, 2009). This method was established as a foundation to develop a suitable method for the volatile determination. Nevertheless, following collaborative inter-laboratory trials, the initial method was not adopted due to several reasons.

The initial internal standard cis-11-hexadecenal (Sigma-Aldrich) had a retention time of over 35 min. As all relevant aldehydes are highly volatile and have a significantly lower retention time (< 25 min), over 10 min of additional run time were required to detect the internal standard and it is not good GC practice to choose an internal standard with a retention time which differs substantially from the analytes of interest. Therefore, in the present work, 3-fluoro benzaldehyde (Sigma Aldrich) was used as internal standard. With a retention time around the 21 min mark, the peaks of the

internal standard could be detected in between the relevant aldehydes. Unfortunately this standard had a different disadvantage as it interfered with the detection of benzaldehyde. The second isomer of derivatized benzaldehyde and the first isomer of the derivatized internal standard 3-fluoro benzaldehyde overlapped with the MS response in SIM 181 (Figure 3.2, chromatogram 1).

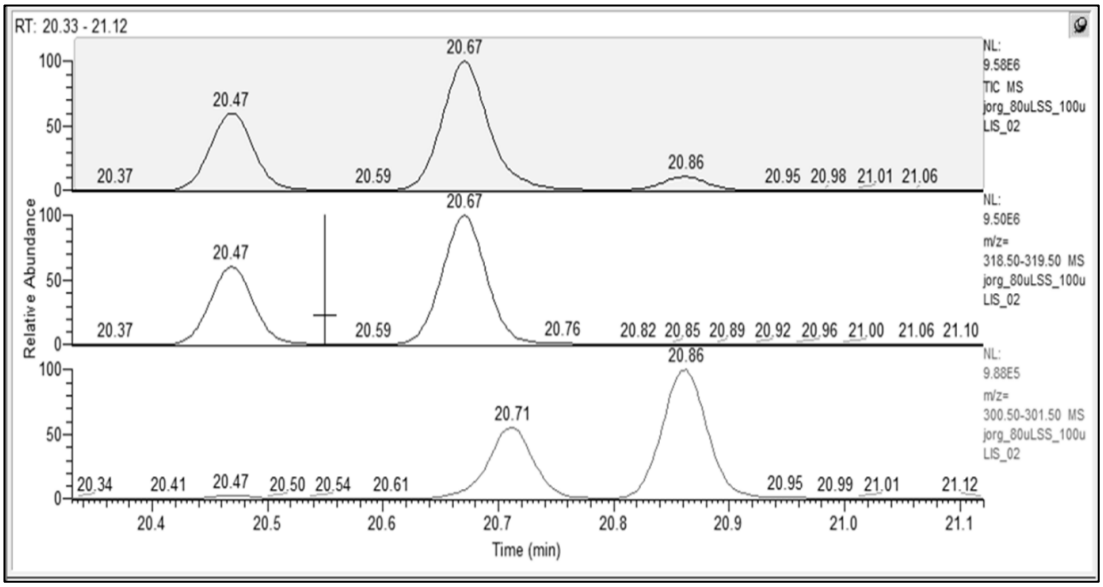


Figure 3.2: Overlapping of benzaldehyde and internal standard 3-fluoro benzaldehyde in SIM 181 and separation in SIM 319 (chromatogram 2, benzaldehyde) and SIM 301 (chromatogram 3, 3-fluoro benzaldehyde)

To separate the peak at 20.67 min (Figure 3.2, chromatogram 1), the ion masses of derivatized benzaldehyde and derivatized 3-fluoro benzaldehyde were investigated. For derivatized benzaldehyde a mass of 319 and for derivatized 3-fluoro benzaldehyde a mass of 301 could be identified as unique for the two analytes, respectively. To detect the two unique masses, the setup of the MS was changed from SIM 181 to SIM 301 and 319 from run time 20 min to 22 min. This still resulted in an overlapping of the two peaks of the second isomer of derivatized benzaldehyde and the first isomer of derivatized 3-fluoro benzaldehyde. To finally separate the two peaks, the data analysis

of the chromatograms utilizing the Xcalibur software (Thermo Scientific) between 20 min and 22 min was analysing mass 301 and mass 319 separately. As these two masses are unique for the derivatized benzaldehyde and derivatized 3-fluoro benzaldehyde, two peaks for each of the two analytes could be obtained (Figure 3.2). This allowed a reliable identification and quantification of benzaldehyde and the internal standard 3-fluoror benzaldehyde.

In addition, the concentrations of the aldehydes in the standard solutions used for the calibration required additions between 2 µl and 40 µl into the beer sample for calibration. Such low amounts of a high percentage ethanol solution (> 95 % vol.) are very difficult to pipette with a good consistency and reproducibility. Despite taking special care during the pipetting steps (by filling the head space of the pipette tip with ethanol vapour and by emptying the pipette tip with the ethanol solution three times), no acceptable linearity could be achieved using the very small volumes of standard solution for the calibration. To improve the linearity of the calibration, the concentration of the measured aldehydes was decreased in the standard solution and two different concentrated standard solutions A and B were prepared (see Section 2.1). This resulted in an addition of 20 µl up to 160 µl for standard A and 40 µl for standard B to cover the entire working range.

Table 3.2: Linearity of the calibration for the analysed aldehydes

Analyte	Linearity of calibration (R^2)
2-Methylpropanal	> 0.98
2-Methylbutanal	> 0.98
3-Methylbutanal	> 0.98
Furfural	> 0.98
Benzaldehyde	> 0.97
Phenyl acetaldehyde	> 0.98
(E)-2-nonenal	> 0.99

The linearity for the calibration of the different aldehydes achieved with the adapted concentration for the aldehyde standards is presented in Table 3.2. For such low concentrations of highly volatile compounds, a linearity of $R^2 > 0.97$ is considered acceptable and despite substantial effort, no better linearity could be accomplished.

The above mentioned enhancements of the initial method resulted in a considerable improvement in the quality of the aldehyde analysis.

3.3.3 Preparation of the mass spectrometry (MS)to ensure required sensitivity

For the detection of staling aldehydes, MS is a commonly used detector (Ochiai et al., 2003, Vesely et al., 2003, Barker et al., 1989, Bravo et al., 2008). To be able to use the full potential of the MS as a reliable and highly sensitive detector, it needs to be maintained to a high standard. To achieve these conditions, the MS needed to be cleaned before each set of measurements. After 24 h of settle down time after the cleaning of the MS and a re-tune, the sensitivity increased significantly. To evaluate the sensitivity, the tuning gas feature of the MS was used and the cleaning increased the signal intensity for the tuning gas by at least a factor of ten. Unfortunately for the

runs directly after the cleaning procedure, the peak area of the internal standard dropped successively for each run, as shown in Figure 3.3.

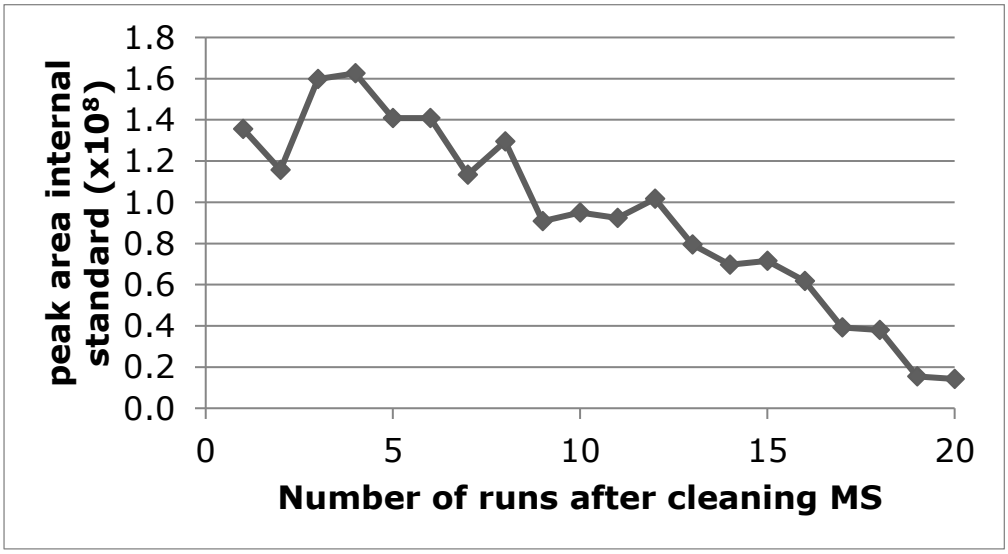


Figure 3.3: Decrease of peak area of internal standard for runs directly after cleaning of MS

The decrease was very difficult to explain, as the sensitivity was very high based on the tuning gas signal intensity after the cleaning procedure. This phenomenon had not been identified previously, as for standard maintenance, the MS was only cleaned if the signal intensity of the tuning gas dropped considerably. After the runs finished showing the decrease in the peak area of the internal standard (Figure 3.3), the MS was re-tuned and further runs were performed. The runs following the re-tune of the MS showed a consistent peak area for the internal standard inside the range of +/- 20 %. This finding resulted in the following procedure before each set of aldehyde measurements:

1. Cleaning and tune of the MS
2. Running the same beer sample for 24 h to settle MS

3. Re-tune and evaluation of the MS
4. Start of aldehyde measurements

The above procedure ensured a consistent and reliable quantification of the aldehyde concentrations via the MS detector.

3.3.4 Conclusion

The described method of SPME GC/MS with on fibre derivatization for the staling aldehydes quantification is a suitable method to determine aldehydes in the ppb and ppm range in the matrix beer. Nevertheless to ensure a consistent and reliable detection, special care has to be taken with respect to calibration, sample preparation and GC-MS maintenance, especially under the given circumstances of an instrument used by multiple users (and not dedicated to this specific method). Further developments of the described method have already been realised (Ortiz, 2015) and the use of tandem MS will further improve sensitivity and reliability of the aldehyde detection (Schmarr et al., 2008). The use of tandem MS for the matrix beer will be a very interesting topic for future work.

3.4 Oxidative stability via EPR spectroscopy

3.4.1 Theoretical aspects of EPR spectroscopy

EPR spectroscopy can probe the electronic structures of compounds containing unpaired electrons such as free radicals and many transition metal ions (Andersen and Skibsted, 1998). The method is based on the interaction of these unpaired electrons with an external magnetic field and the absorption of microwaves associated with the change in orientation of electro spin in the external magnetic field.

Electrons possess an intrinsic angular momentum. This is the so called ‘spin‘ of the electron and results in a self-rotation of the electron. The self-rotation creates a small magnetic field and induces a magnetic moment. In a magnetic field, $B_{o,}$ the magnetic moment behaves like a compass (Figure 3.4).

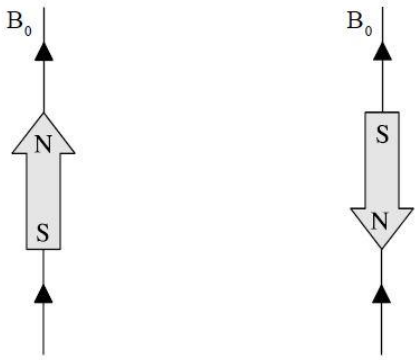


Figure 3.4: Minimum and maximum energy orientations of μ with respect to the magnetic field Bo (Weber et al., 1998)

In the presence of an external magnetic field the electrons can orientate in two different ways (Figure 3.5), either aligned with, or opposed to the applied magnetic field. These two states are separated by an energy $\Delta E = g_e * \beta_e * B_o$ where g_e is the g-value of the electron (g_e = 2.0023 for a free electron), β_e is the Bohr magneton and B_o is the magnitude of the external field. The effect is called the Zeemann effect (Rohn and Kroh, 2005)

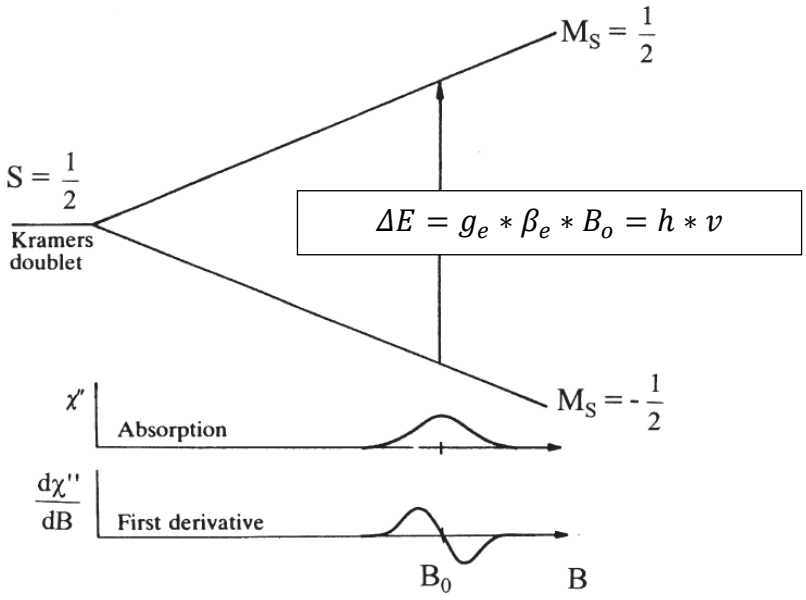


Figure 3.5: Energy level diagram for an isolated electron in a magnetic field B and a corresponding absorption spectrum and first derivative ESR spectrum (Weckhuysen et al., 2004)

The two energy levels are almost equally populated. If energy is supplied to the system by electromagnetic radiation, then the system can undergo excitation from a lower to a higher energy state by absorption. Usually a fixed frequency of microwave radiation is used and the external magnetic field B_o is varied. Resonance occurs when the energy of the electromagnetic radiation $\Delta E = h * v$ (v = frequency, h = Planck's constant) matches $g_e * \beta_e * B_o$ resulting in the absorption of microwaves. Usually the first derivative of the absorption is reported in EPR spectra.

3.4.1.1 *Hyperfine splitting*

Hyperfine splitting results from the interaction of the electron spin with the magnetic moment of a nucleus in the vicinity of the electron spin. Interactions with protons ^{1}H (nuclear spin I = 1/2) or nitrogen nuclei ^{14}N (nuclear spin I = 1) can be observed. The hyperfine interaction effectively amplifies or attenuates the external magnetic field.

The active magnetic field B_{eff} results from the addition of the magnetic field generated by the nuclear spin B_{loc} and the external magnetic field $B_{0.}$ A nucleus with a nuclear spin, I, can interact with the external magnetic field. In the simplest case with the interaction of a nuclear spin I = ½, generating two different effective magnetic fields. B_{eff} the electron resonance arising from the Zeemann effect is split (Figure 3.6).

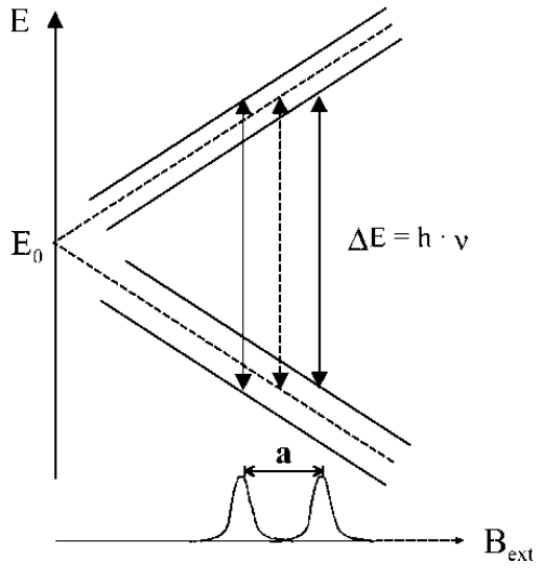


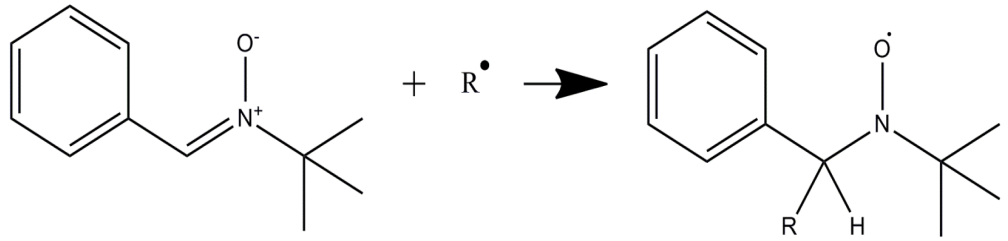
Figure 3.6: Hyperfine splitting through the interaction of the unpaired electron with the magnetic moment of the nuclei (Kunz et al., 2002)

The hyperfine structure thus results from two energy transitions and leads to a corresponding number of 2nI+1 EPR lines, dependent on I and the number of interacting nuclei, n. The separation between the EPR lines, the hyperfine coupling constant, is characteristic for each substance.

3.4.1.2 *Spin trapping*

According to Rohn and Kroh (2005) the half-life time of free radicals ranges between nanoseconds and seconds. For example a hydroxyl radical has a half-lifetime of 1 ns and a nitric oxide radical up to 10 s. These short life-times result from the reactivity

of free radicals. Therefore the spin trapping technique was developed to overcome difficulties in observing free radicals (Lagercrantz, 1971). Spin traps are organic molecules with the ability to interact with free radicals to form a spin trap radical reaction product. The spin labelling effect of these spin traps is based on the reaction of a nitroso compound with the free radical to form persistent nitroxyl radicals as spin adducts (Gerson and Huber, 2003). The reaction of PBN with a free radical to form a spin adduct in shown in Figure 3.7.



N-tertiary-butyl-alpha-phenylnitrone (PBN)

Figure 3.7: Reaction of PBN with free radical species to form a spin adduct

Spin traps can be divided into two different groups, specific and nonspecific spin traps. Non-specific spin traps show a higher reaction velocity and form more stable adducts but have no ability to trap specific compounds. Specific spin traps react with radicals at a double bond and show a specific EPR signal characteristic.

3.4.2 EPR spectroscopy in brewing

Over the last three decades, the use of electron paramagnetic resonance (EPR) spectroscopy to investigate the complex reactions occurring during the storage of beer has been established (Kaneda et al., 1989, Kunz et al., 2002, Uchida et al., 1996, Uchida and Ono, 2000, Andersen et al., 2000, Forster et al., 1999, Frederiksen et al., 2008, de Almeida et al., 2013, Foster, 2009). The method is based on the indirect detection of short lived radicals formed during accelerated beer ageing at 60 ˚C in the

presence of air to reveal the potential antioxidant capacity. A spin trap reagent is required to form long-lived spin trap adducts to detect and quantify the concentration of radicals formed during the accelerated ageing. These adducts are detectable via EPR spectroscopy and the concentration of radicals formed can be directly related to beer flavour stability. Different techniques may be used to process the acquired data. The determination of lag-time is most commonly used to evaluate the flavour stability of the finished beer and T_{150} in wort to describe the potential shelf life of the resulting beer. Both measures have been found to correlate with the ageing of the beer sample (Ono and Uchida, 1998, Forster et al., 1999). Other approaches including a sigmoidal fitting of the data have been presented by Foster (2009). Here an assessment of the different data handling approaches and findings acquired over a diverse range of EPR measurements including the identification of additional features in the EPR spectrum was made.

3.4.3 Quantification of radical formation: Peak to peak height approach reviewed

The determination of the peak height for the quantitative analysis of EPR spectra is a very common approach and is used in a range of different research areas (Swartz and Glockner, 1989, Basly et al., 1997, Jung and Lee, 2002). The method has been used to quantify the hydroxyl radical formation in beer and may now be described as the established method (Huvaere and Andersen, 2009). Nevertheless, quantitative EPR spectroscopy may be subject to many causes of error (Nagy, 1994). For example EPR-active impurities can result in additional features being detected that can interfere with the measured peak heights (Kocherginsky et al., 2005). The origin of these additional features is discussed in section 3.4.5. In addition, the peak broadening observed in a

typical EPR spectrum, and the resulting lower peak height, is usually not considered when the peak-to-peak height approach is used. Figure 3.8 displays a simulated spectrum for an EPR measurement of a beer sample using N-tert-Butyl-α-phenylnitrone (PBN) as the spin trap reagent. In this simulated spectrum, all six features that result from the electron-nuclear hyperfine coupling possess the same height and line width.

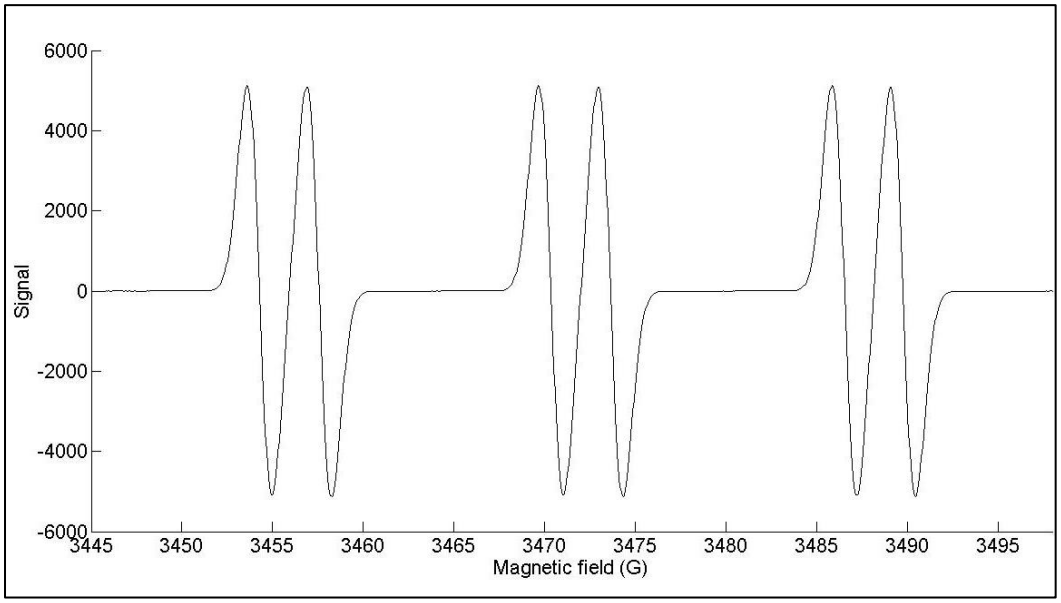


Figure 3.8: Simulated spectrum obtained from EPR spectroscopy of beer utilising a spin trap reagent

In contrast, in an experimental spectrum these six features are usually not of the same profile as a result of second order hyperfine effects and molecular motion (Figure 3.9).

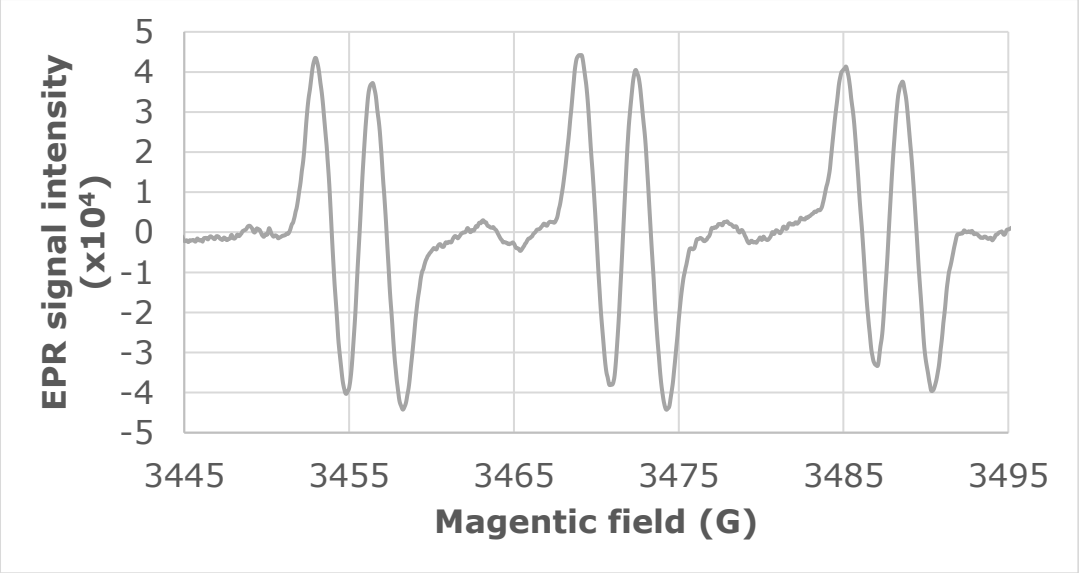


Figure 3.9: EPR spectrum of a lager beer sample after 150 min of forced ageing at 60 ˚C, PBN concentration 50 mM

The peak height approach was compared with two other data handling approaches. The first approach simulates each recorded spectrum using the EasySpin Toolbox for Matlab R2014a software (Stoll and Schweiger, 2006) through a least-squares fitting routine.

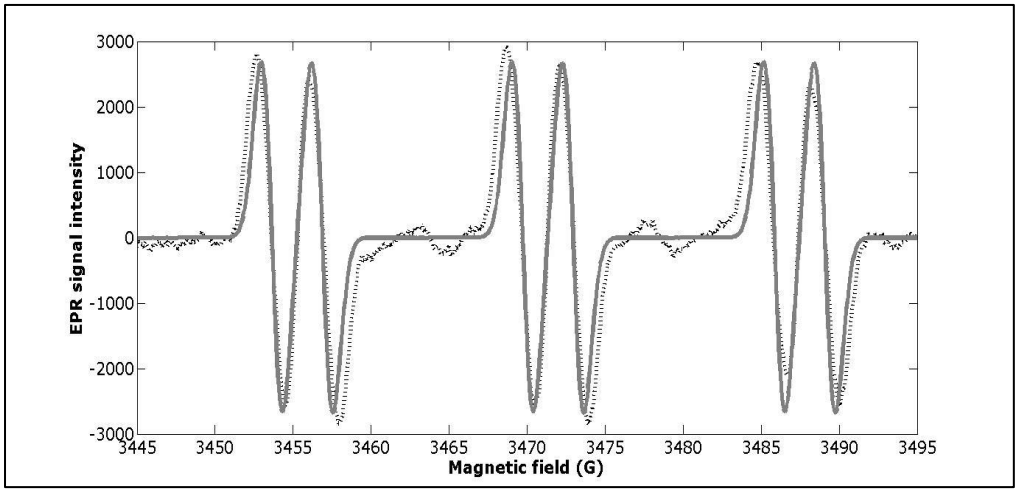


Figure 3.10: Simulated EPR spectrum overlaid on EPR spectrum of lager beer sample after 150 min at 60 ˚C, PBN concentration 50 mM.

Figure 3.10 displays the best-fit simulated EPR spectrum (as determined by EasySpin) overlaid on the experimental EPR spectrum of a lager beer sample.

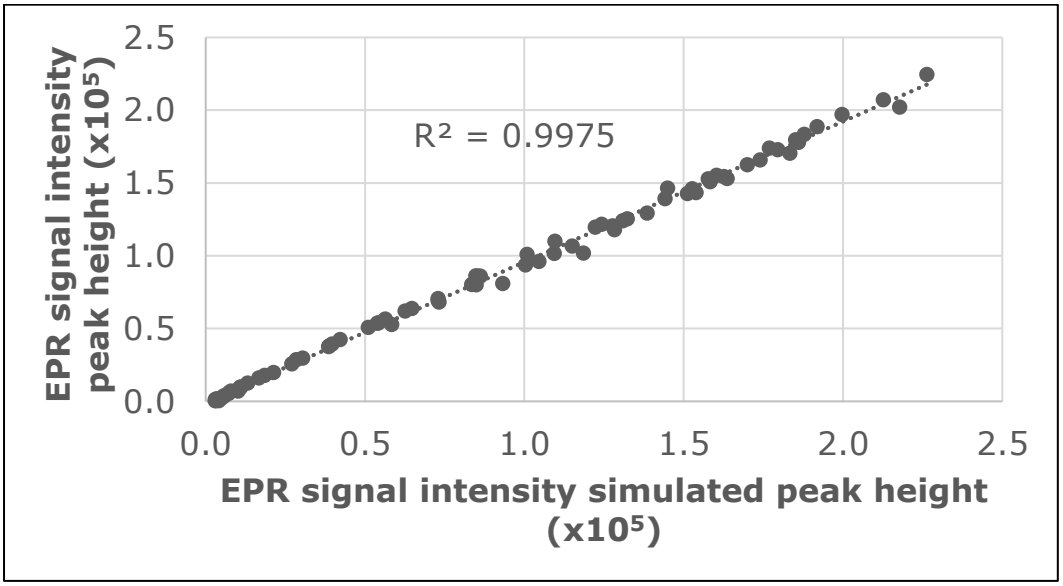


Figure 3.11: Correlation of EPR signal intensity based on peak height for standard peak height approach compared to peak height of simulated spectrums.

Figure 3.11 displays the correlation of the EPR signal intensity based on peak height for the standard peak height approach compared to the peak height of the simulated spectra. Thus, based on the correlation displayed (R^2=0.9975), the peak heights derived from spectral simulation and those measured directly from the recorded spectra are essentially identical.

The second approach to determine free radical concentration is based on the determination of the area of the EPR spectrum and is a standard procedure in quantitative EPR spectroscopy (Eaton et al., 2010). EPR spectra are usually recorded as first derivatives of the absorption intensity and therefore in order to determine the area of an EPR spectrum a double integration is required. The first and second integrations of an EPR spectrum are shown in Figure 3.12 and Figure 3.13, respectively.

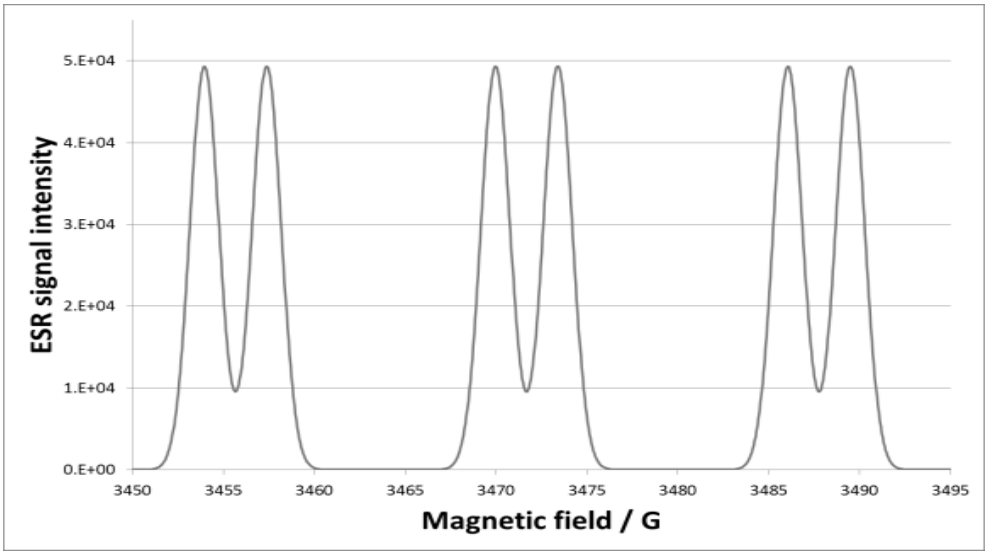


Figure 3.12: First integral of simulated ESR spectrum to determine the EPR signal intensity

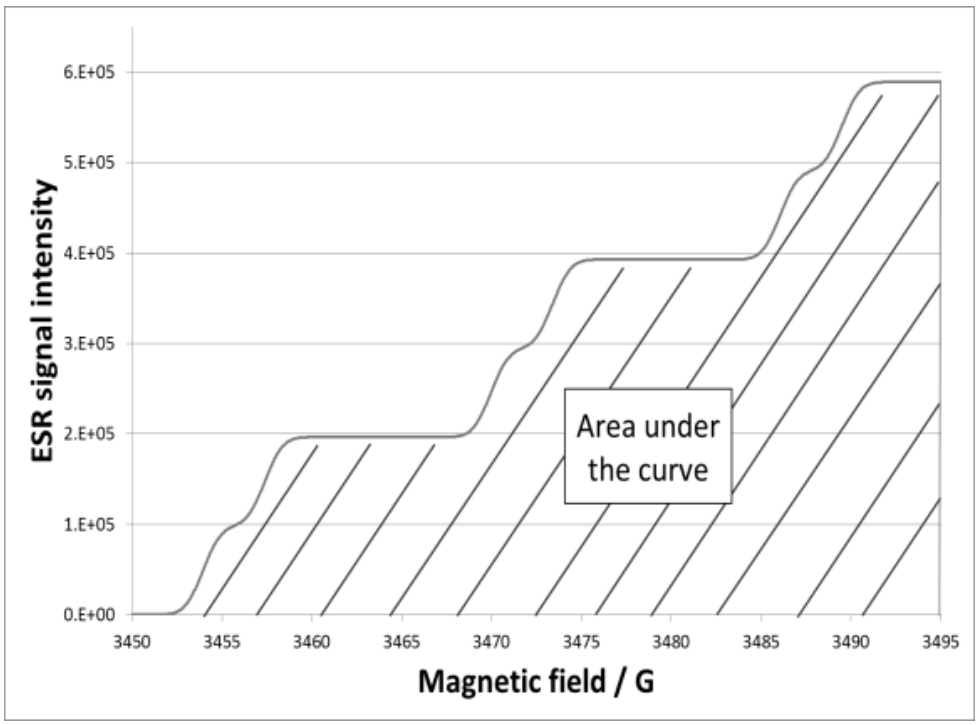


Figure 3.13: Second integral of the simulated ESR spectrum and determination of the area under the curve to determine the EPR signal intensity

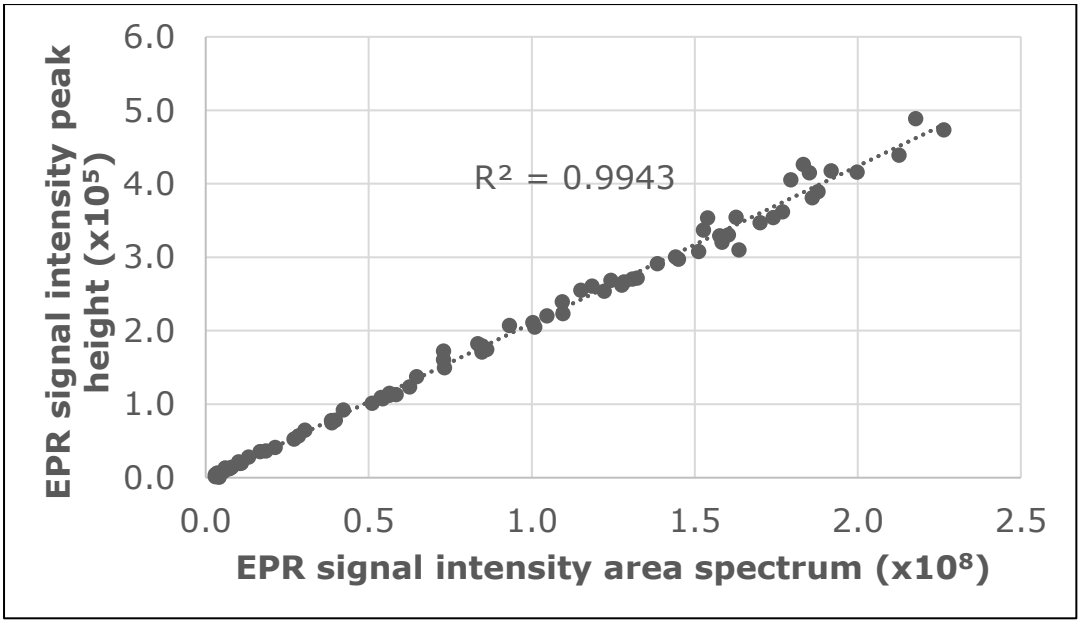


Figure 3.14: Correlation of standard peak height approach in comparison to area approach to determine the EPR signal intensity.

The area obtained by double integration was correlated to the EPR signal intensities obtained via the standard peak height approach (Figure 3.14). Based on the high correlation (R^2=0.9943), the results obtained via the determination of the area and the peak height can be described as almost identical for this set of samples (EPR data obtained for three different lager style beers pre- and post-filtration as described in Chapter 4).

Peak height determination after simulation and the area of the spectrum after double integration show high correlations with the standard peak height approach. Therefore the standard peak height methodology to access the radical formation in a beer sample via EPR may be considered sufficient, as described previously (Kunz et al., 2002, Kocherginsky et al., 2005). Nevertheless to avoid any issues with the impact of additional features or variations within the experimental EPR spectra, our approach of spectral simulation by a least squares fitting routine and the determination of the peak height was used for the work described in this thesis.

3.4.4 Determination of traditional lag time vs x0 lag time approach

The use of lag time values obtained via EPR spectroscopy was first introduced by Uchida et al. (1996) and is a very useful tool to evaluate the oxidative stability of beer.

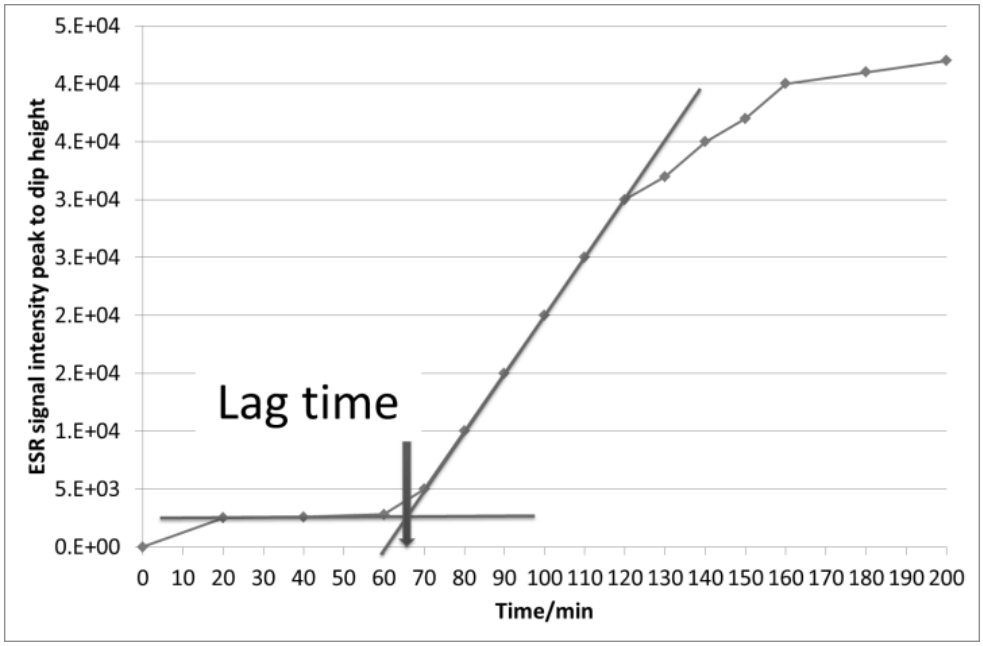


Figure 3.15: Lag time determination by intersection point of two fitted lines of an idealised EPR measurement

Figure 3.15 displays the determination of the lag time. Two best fit straight lines are fitted through the two linear sections of a graph of EPR signal intensity with time. The intersection of the two lines is used to calculate the lag time of the beer sample. The first line is fitted through the phase where no or only low radical formation may be detected. The second line is fitted through the points showing an increase in radical formation. For an ideal measurement (Figure 3.15), the fitting of the two lines does not usually present any issues. For real beer measurements, however, the determination can be difficult and subject to error as described by Kunz et al. (2002). Recommendations include the exclusion of data points around the inflection point of the two lines to avoid an influence on the lag time. It can be difficult to decide which

of the data points should be included or not, or assigned to either of the two fitted lines. Furthermore when processing a significant number of EPR measurements, it can be very time consuming to evaluate each lag time determination individually. Therefore a different approach to determining the lag time has been introduced by Foster (2009) in which the data are fitted with a sigmoidal curve. In this procedure the lag time is defined as the inflection point of the curve, where the slope of the curve changes.

The sigmoidal fitting approach also shows an advantage compared to the traditional lag time determination in that data analysis can be partially automated using the data fitting software QTI plot 2013. No further evaluation of each lag time was necessary, as the software displays the error for the fitting and the inflection point (= lag time) in +/- min.

3.4.5 Additional peaks between the main features

During the EPR studies additional features in between the six main peaks were be observed (Figure 3.16). These additional peaks have already been described by Kocherginsky et al. (2005). The origin of the peaks was speculated to be most likely related to the degradation of PBN during the forced ageing test but no evidence for this has been presented previously.

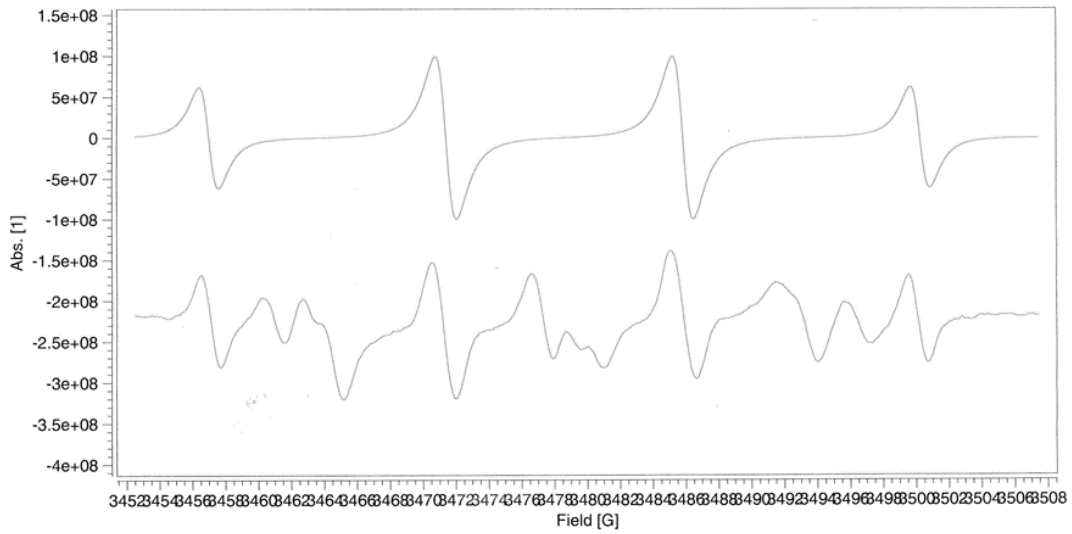


Figure 3.16: EPR spectrum (2nd spectrum) of a strong wort (16 ˚P) sample after 200 min at 60 ˚C. 1st spectrum displays a simulation of the four main features of the 2nd spectrum obtained with XSophe-Sophe-XeprView software.

The additional features and the simulation of the EPR spectrum for the principal features are shown in Figure 3.16. Figure 3.17 illustrates a possible degradation pathway for PBN during the forced ageing test.

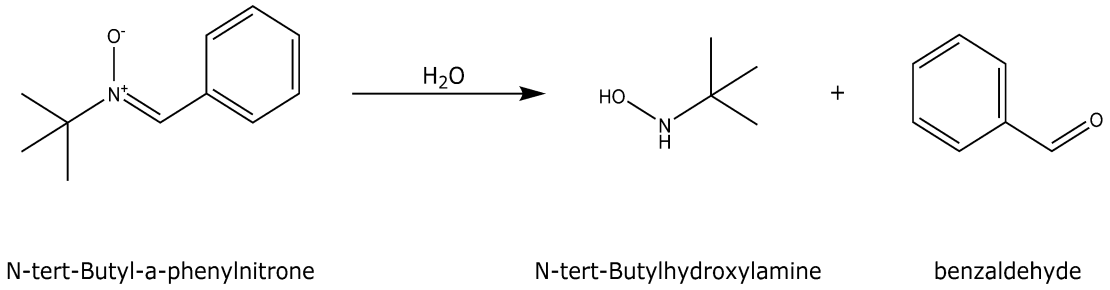


Figure 3.17: Hydrolysis of PBN during EPR measurement of forced aged beer sample

The formation of N-tert-Butylhydroxylamine may result in an EPR signal that exhibits ^{14}N and ^{1}H hyperfine coupling. Thus, the nuclear spin for ^{14}N is I= 1 and for ^{1}H I = ½ and the nuclear hyperfine interaction may result in a spectrum with six peaks similar to the spectrum obtained for the PBN spin adducts and the overlapping of these features with those of PBN may generate the experimental spectrum shown Figure

3.16. The degradation pathway theory shown in Figure 3.16 is also supported by the strong almond smell (associated with the formation of benzaldehyde) of the samples during forced ageing at 60 ˚C. With continuing forced ageing, the intensity of the peaks associated with PBN degradation and the almond odour increased but this increase was not uniform for the different samples.

3.4.6 Effects of spin trap reagent qualities

The quality of the PBN employed as a spin trap in the EPR experiment can have a strong influence on the measurement of radical concentrations in a beer sample (Foster, 2013a). The direct comparison of two batches of PBN from different suppliers (Alfa Aeser, purity 98 % and Fisher Scientific, purity 98 %) is presented in Figure 3.18.

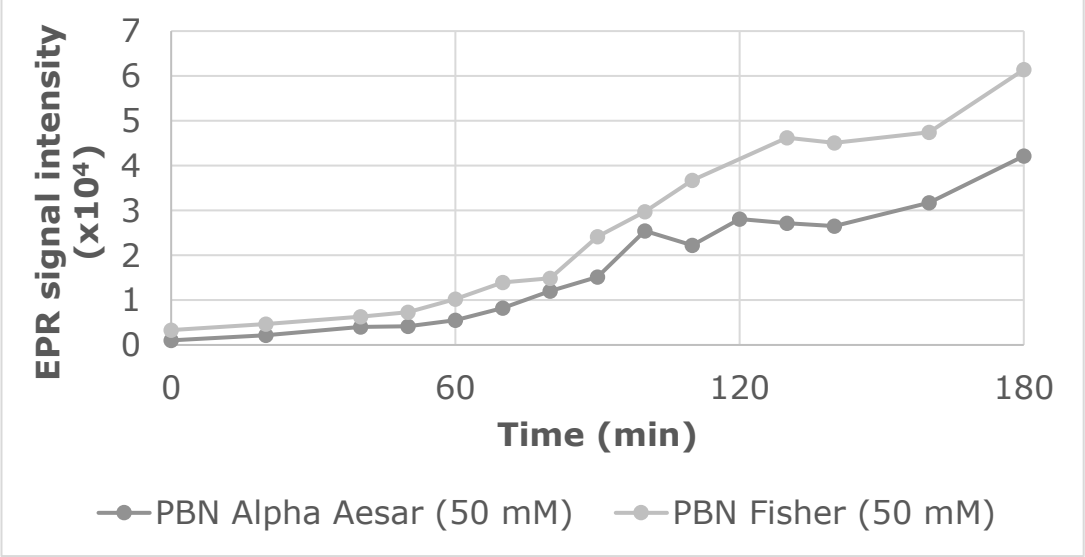


Figure 3.18: Comparison of the EPR measurements of the same lager style beer sample with two different PBN qualities (Alpha Aesar, 98 % purity; Fisher Scientific, 98 % purity). (Forced ageing at 60 ˚C)

Despite the same specifications and purity of the PBN from Fisher and Alpha Aesar, a difference in the radical formation detected via EPR spectroscopy was observed. The EPR signal intensity for the Fisher PBN was consistently higher over the entire measurement range. This experiment clearly shows that the source and purity of PBN can impact the experimental results. Visual inspection of batches of PBN showed clear differences in their appearance: PBN sourced from Fisher appeared as white, fluffy crystals, whereas that from Alpha Aesar had a yellow tint and a gummy consistency. The Alpha Aesar PBN had a strong almond odour whereas the Fisher PBN had no obvious smell. TO ensure consistency during EPR experiments, the same batch of PBN was used for the entire experiment and if multiple batches were delivered, then these were mixed before EPR experiments were undertaken.

An attempt was made to synthesise PBN based on method II described by Hinton and Janzen (1992). In this method, benzaldehyde (10 mmol, 1.016 g, Sigma-Aldrich), 2-methyl-2-nitropropane (20 mmol, 2.06 g, Sigma-Aldrich) and zinc (30 mmol, 1.96 g, Fisher Scientific) were transferred into a round bottom flask together with 75 ml of ethanol (95 % vol.), mixed and cooled to 2 ˚C. To the mixture acetic acid (60 mmol, 3.79 g, Fisher Scientific) was slowly added with stirring. The mixture was stirred for 2 h and stored at 2 ˚C for 12 h. The solution was filtered to remove the $Zn(OAc)_2$ and concentrated using a Rotavap. The procedure resulted in a yellow liquid with an oil-like consistency and a strong almond odour. Despite several further purification steps, no white, pure solid PBN could be produced.

An increase in pH can lead to an increase in radical formation (Kunz et al., 2011) and the pH of the solution may be affected by the addition of a radical spin trap such as PBN. Ideally the spin trap employed should have no effect on the radical formation of the sample. Therefore N-tert-butyl-α-(4-pyridyl) nitrone N-oxide (POBN) has been

introduced as an alternative spin trap to PBN. The concentration of POBN is much lower (< 10 mM, PBN = 50 mM) and any effect on the pH may be minimised. However, possible effects of POBN on the reduction of Fe(III) during the detection of hydroxyethyl radicals via EPR measurement have been reported (Reinke et al., 1994). The comparison of the effects of different spin traps on the radical formation in beer during the oxidative stability measurement of beer would be a very interesting topic for further work. For this work PBN was used as a spin trap, as for the entire Molson Coors Brewing Company PBN is used for all beer related EPR measurements.

3.4.7 Conclusion

Despite the use of EPR to measure the oxidative stability of beer and wort samples for over two decades, this method of analysis is far from being routine. The accuracy of the results can be affected by the quality of the spin trap and the data work-up. However EPR spectroscopy has significant potential as a research tool to help to further understand the mechanisms that impact the flavour stability of beer. A detailed description of the method used during this work can be found in Section 2.6.

4 A comparison of three different filtration systems regarding their impact on flavour stability indices

4.1 Introduction

Haze-free and bright are required attributes for the widespread consumer acceptance of the vast majority of beers produced globally (Bamforth, 1999a). Following fermentation, colloidal matter, for example yeast cells and large proteins, is present in the beer, and a separation step is usually required to achieve clarity. This separation aims to remove the unwanted material to produce the visual appearance and the pleasant taste the customer demands from the final product (Siebert, 2006). Commonly in the brewing industry this separation employs a deep bed filtration with diatomaceous earth (DE), or so-called Kieselguhr, as a filter aid. Membrane filtration processes have been used in the brewing industry for several years (Daufin et al., 2001) and recently cross-flow membrane filtration has received a lot of attention as an alternative to the standard Kieselguhr approach (Ambrosi et al., 2014). A number of advantages of cross-flow filtration over conventional filtration have been reported including the elimination of the filter aid Kieselguhr and the associated issues with the possible carcinogenic effect of inhaled dust during handling of Kieselguhr, disposal of the Kieselguhr waste (Freeman and McKechnie, 1995), reduced beer losses (Leeder and Girr, 1994), the ability to handle high amounts of solid material, increase in the quality of the product (Rizvi, 2010), high flexibility and low running costs (Gan et al., 2001, Fillaudeau et al., 2006).

However, commercial scale cross-flow filtration units are not yet widely accepted by the industry due to the high investment costs, variable life span of the membranes and

the higher energy consumption compared to a Kieselguhr filtration system (Leeder et al., 2011, Zuber, 2012).

The study reported in this chapter, focuses on the effects on flavour stability of cross-flow membrane filtration as compared with traditional Kieselguhr filtration. The pickup of iron from DE during Kieselguhr filtration is well known (Rizvi, 2010, Foster, 2013b, Uchida and Ono, 2000) and the negative effects of a high Fe content on beer shelf-life have been reported (Zufall and Tyrell, 2008). However, very little attention has been paid to the flavour stability impacts of rough beer filtration with Kieselguhr as compared with cross-flow filtration and therefore barely any independent scientific publication can be found discussing this topic. The only publications available are supported by large manufactures of membrane filter systems (Broens et al., 2011) or are promotional material for membrane filtration systems highlighting the advantages of the membrane systems. The present trial gave a rare opportunity to directly compare a large-scale Kieselguhr frame filtration system (KF) with two cross-flow membrane filtration (MF) systems at a large scale brewery from a customer perspective. Three continental lager-style high gravity beers were analysed pre- and post-filtration to investigate the influence of the different filtration systems on different indices related to flavour stability.

4.2 Material and methods

4.2.1 Filtration systems

4.2.1.1 *Plate and frame kieselguhr filter (filtration capacity 400 hl/h)*

A full-scale Kieselguhr plate and frame filter located in a Molson Coors Brewing Company brewery was used for the trials. The filtration trials were performed following the standard filtration procedure of the brewery.

4.2.1.2 *Cross-flow membrane filter GEA-Pall PROFI-System (filtration capacity 20 hl/h)*

The GEA-Pall PROFI-system used during the trials combines a high performance centrifuge with a cross-flow membrane filtration. The incorporation of the centrifuge reduces the amount of solid matter transferred onto the membrane.

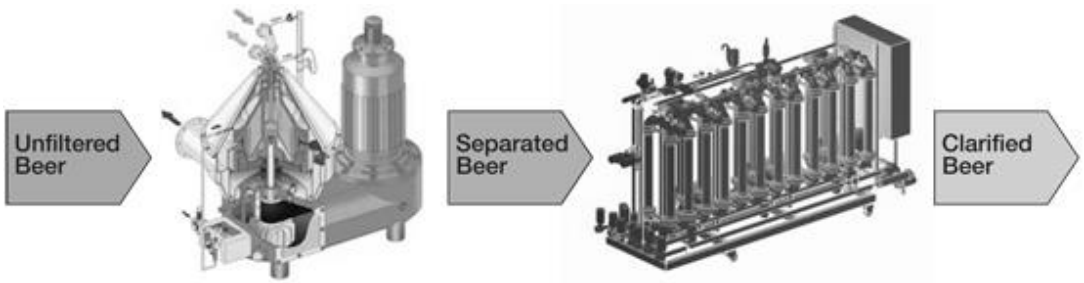


Figure 4.1: Schematic of the filtration process of the PROFI-system performed during the trials from Pall Cooperation (2009b)

Figure 4.1 shows the flow of the unfiltered beer through the filtration system. To verify the impacts of the cross-flow filtration process on flavour stability, beer samples were taken before the centrifuge and after the membrane filtration as the system is marketed

as a complete unit and the centrifugation step is required to ensure the required filter performance.

4.2.1.3 *Cross-flow membrane filter Pentair Beer Membrane Filtration (BMF) system (filtration capacity 8 hl/h)*

Figure 4.2: Picture of the cross-flow membrane filter Pentair BMF used during the filtration trials (8 hl/h) by Pentair (2012)

Compared to the PROFI-system, the Pentair BMF system (Figure 4.2) does not require an additional centrifugation of the rough beer before the filtration, as it is able to manage larger quantities of solid material. The membrane design and a different filtration cycle time makes this possible.

4.2.2 Brewery trial setup and design

Both membrane systems were connected to the same conditioning tank (CT) via hose connections through a beer distributer. This set up allowed for both membrane filter systems to be fed with the exact same unfiltered beer.

As the Kieselguhr plate and frame filter is the permanently installed large scale filtration unit in this brewery, the unfiltered beer was pumped to that filter through permanently installed stainless steel pipes. A buffer tank was used to feed the Kieselguhr filter to avoid any pressure surges. To minimize the impact of the different compositions of the filter cake at the beginning compared to during the filtration, all samples were taken as close as possible to the mid-point of the filtration cycle.

In the following sections the membrane filtration systems will be designated as membrane filters (MF) A and B and no direct identification of the two MF systems will be given to avoid any commercial bias regarding the two cross-flow membrane filtration systems. This was a requirement of one of the suppliers for the membrane filter systems to use the obtained data without further consultation and review before publication outside of Molson Coors Brewing Company.

In total three different, high gravity lager-style beers (Beers A, B and C) were filtered through the three different filtration systems (Kieselguhr (KF), MF A and MF B). For each of the beers A, B and C, one filtration run was conducted for each of the three filter systems.

The trial setup was a very restricted element and could only be influenced to a limited extent. In addition to the flavour stability trials, the entire filtration performance including, for example, the filtration cycle volumes after different times of maturation, colloidal stability tests and energy and consumable consumption of the two MF were tested at the same time. As for the brewery, flavour stability played only a secondary role during these trials, the logistics required to receive samples of the same brand for filtration by all three filtration systems was somewhat complex. Furthermore, a direct comparison between the standard Kieselguhr filter system and the two MF systems

was not required for the assessment of other performance measures. This led to the following trial setup for the three different beers: Beer A was brewed on two different brew streams (brewery 1 and brewery 2), each following the same recipe. Brewery 1 for KF and brewery 2 for both MFs A and B. This was necessary, as for the specific brand only one CT was used for the MF filtration trials. Unfortunately the beer filtered with the two MF A and B was transferred back into the initial CT and therefore mixed with the remaining unfiltered beer. This CT was then blended away and filtered on the second filtration stream in a different part of the brewery. At this point is was not foreseeable, when the next batch of this brand from the same brewing stream 1 would have been available. To be able to have any samples of this brand available and to avoid adding an additional Kieselguhr filtration system into the trial with only limited availability for sampling, the only available option was to take the samples of the same brand brewed on a different brew stream and conditioned separately from the Kieselguhr filter. Beer B was brewed on the same brew stream only in Brewery 1 but two different batches of beer (Batch I and Batch II) were filtered. Batch I through the KF and batch II through MFs A and B. This was necessary as the beer was filtered during a nightshift without further notice and without any chance of sampling. Therefore a second batch had to be used. For Beer C the initially planned trial setup could be realised, as it was brewed in Brewery 1 and filtered out of the same conditioning tank for all three filtration systems. All the above stated issues with trials on production scale had to be accepted to be able to make use of such a rare opportunity to compare three different filtration system at the same time.

Unfiltered samples were taken out of the conditioning tank (CT) directly before filtration and filtered beer samples directly after the filtration for the two MFs.

For the KF, unfiltered samples were taken out of the KF buffer tank (BT) and filtered samples directly after the filtration. All samples were filled in CO_2-purged plastic bottles with minimal headspace and stored at -20°C prior to analysis.

4.2.3 Oxidative stability of beer

For all three filter systems, all samples taken before and after filtration for beer A, B and C were analysed. For all performed oxidative stability tests, the same batch of spin trap was used to avoid any influence of the spin trap quality and improve the comparability and reproducibility of the obtained results (N-tert-Butyl-alpha-phenylnitrone, purity 98%, Fisher Scientific, stored at -20 ˚C). The samples were defrosted for 24 h at 2 ˚C before the analysis. The sample preparation and measurements were performed following the analysis specified in section 2.5. For each sample, two individual bottles were utilized.

4.2.4 Metal content analysis via Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

All samples were defrosted for 24 h at 2 ˚C before the analysis and evaluated following the method described in section 2.4. The analysis was performed for all samples taken before and after filtration for all three filtration systems for all three beers A, B and C.

4.2.5 Total sulphur dioxide by distillation method

Before the analysis, the samples were defrosted for 24 h at 2 ˚C. The analysis was performed for all samples taken before and after filtration for all three filtration systems for beer A, B and C, as stated in section 2.2. To obtain consistent results and eliminate any possible issues during sampling affecting the SO_2 content, three

individual bottles per sample were analysed to obtain three technical replicates of the analysis.

4.2.6 Dissolved oxygen measurement

The oxygen content was measured ‘at-line’ using an ORBISPHERE Portable ppb EC O_2 analyser. The oxygen meter was calibrated at the Molson Coors Brewing Company s laboratory every day prior to the analyses. Measurements were taken pre- and post-filtration for each of the three filtration systems. After connecting the oxygen meter to a sample point, a constant flow of beer was adjusted and retained until a stable reading could be achieved for *ca*. 30 s.

4.3 Results and Discussion

4.3.1 Results and Discussion for Beer A

The beer for the KG filtration was brewed in brewery 1 and for the membrane filtration in brewery 2. Both breweries reside very close to each other but were historically two independent breweries. Due to a transfer of ownership, both breweries are now interconnected and combined to one brewing site. Nevertheless the water supply and the two brewhouses of the two breweries operate independently from one another. The trial setup of the production procedure is shown in Figure 4.3.

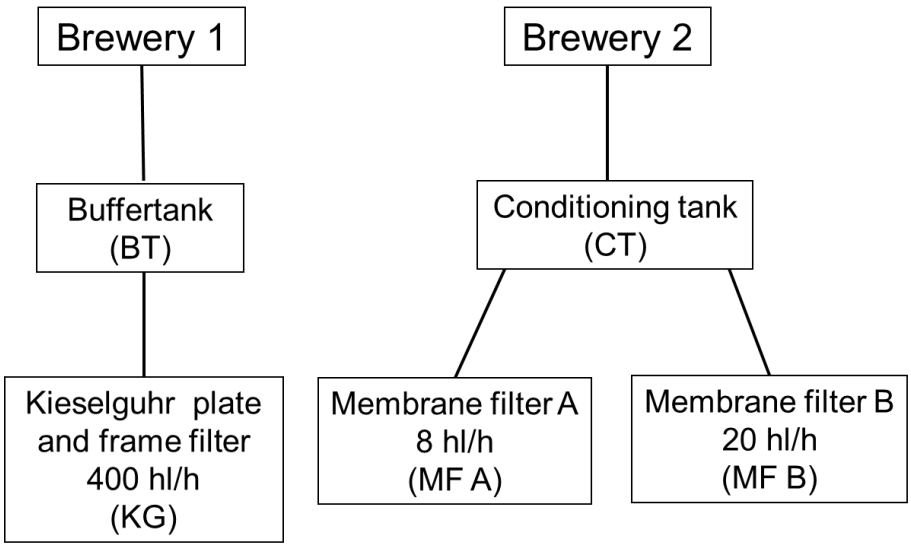


Figure 4.3: Trial setup for beer A

Due to the issues with available batches of beer A already explained (see Section 4.2), the trial setup could not be altered and was performed as stated.

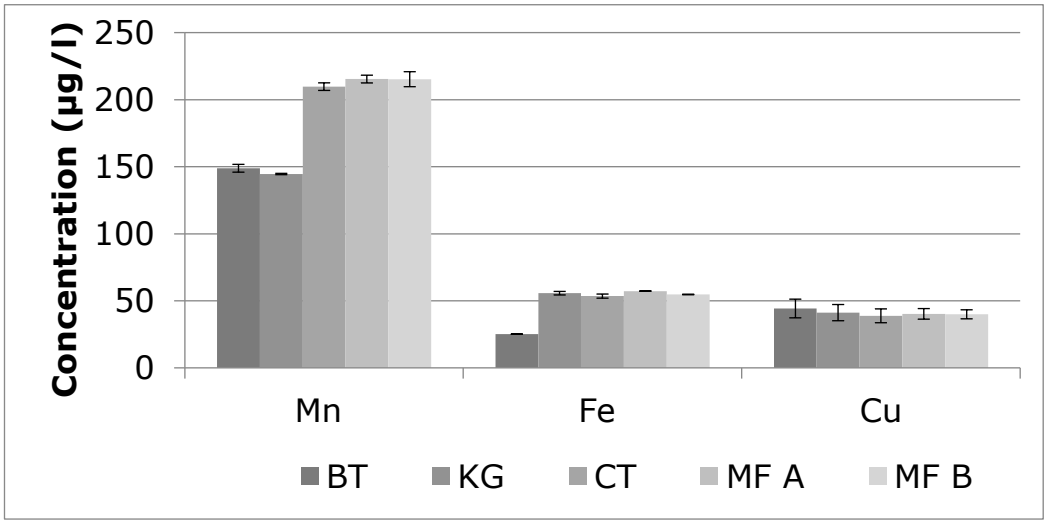


Figure 4.4: Concentration of Mn, Fe and Cu for beer A, (error bars represent standard deviation of technical replicates, n=3, BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

The results for the metal analysis based on the ICP-MS results for beer A are shown in Figure 4.4.

A clear difference of around 60 µg/l in the Mn content could be observed for the unfiltered beer brewed in brewery 1 (BT) compared to the unfiltered beer brewed from brewery 2 (CT). The type of filtration system used had no influence on the concentration of Mn in the filtered beer since no differences between the pre- and post-filtration samples could be detected. According to Zufall and Tyrell (2008) the main sources of Mn in beer are the cereal raw materials and wheat contains higher amounts of Mn than barley. A possible explanation for the different Mn-levels could be addressed to different batches of cereal raw materials used in the two breweries during the trial. Nevertheless such a significant alteration in quality is very unlikely, as all of the barley used in both breweries is malted in the brewery-owned maltings, located a short distance away. Both breweries are supplied with the same batches of malt and therefore variations in the Mn-level by the barley malt supplied are suggested to be negligible. Another source of Mn are hops with up to 70 mg/kg (Poreda et al., 2015). A noticeable effect on the Mn content of the final beer by the hop addition is very unlikely, as the two breweries are supplied with the same batches of hops. As the two pre-filtration samples were brewed in two different breweries under large scale production conditions, no further control other than the brewery standards for the wort quality and the status of the yeast, especially viability and re-pitching cycles, could be achieved. Mochaba et al. (1996), described the ability of yeast to accumulate and release Mn during fermentation with different levels of accumulation and release depending on yeast generation and wort quality. This indicates that the different conditions during fermentation could have had an influence on the Mn-levels of the pre-filtration samples. Nevertheless such a significant difference of 60 µg/l (Figure 4.4) remains unexplained.

The Cu content of both unfiltered beers and of the three filtered beer samples did not differ significantly from one another (Figure 4.4). No differences between the pre- and the post-filtration samples were observed and thus none of the three filtration systems influenced the Cu-level of the beer samples.

A large Fe pickup of more than 30 µg/l was observed during the Kieselguhr filtration. The increase in the Fe-level of beer during Kieselguhr filtration is well established (Rizvi, 2010, Foster, 2013b). Due to the continuous dosage of Kieselguhr during the filtration, a constant Fe-pickup during filtration was expected because of the low pH of the beer (*ca*. pH 4), Fe is released from the Kieselguhr into the filtered beer. Similarly to the variation noted in the Mn-levels, the Fe-levels of the pre-filtration beer samples from brewery 2 were higher as compared to the pre-filtration beer samples from brewery 1 (by more than 30 µg/l). As already discussed, the cereal raw materials, the hops and the yeast may influence metal-ion levels in the finished beer. The difference of more than 30 µg/l Fe between the two breweries led to a further investigation of the brewery water supply as a potential source of the increased Fe-levels. The two breweries use different water supplies but all brewing liquor is demineralised using reverse osmosis and the mineral content required for brewing is adjusted during the brewing process. An analysis of the RO-water did not show any difference as the levels for Fe, Cu and Mn were negligible (< 1 µg/l). The standard testing procedure for the Fe content of the water supply performed by the MolsonCoors Brewing Company laboratory is less sensitive than the applied ICP-MS measurements. The limit of quantification is around 100 ppb and this value was set as critical value for the Fe content. Further investigation of the brewing liquor supply in brewery 2 led to the discovery of a number of rusty tanks in the supply system and showed the importance of a more sensitive detection method for the Fe content

determination. The rust in the brewing liquor supply for brewery 2 could have directly resulted in the increase in Fe-levels. The increased Mn levels could be related to this fact as well. The metal ion content of the water after the rusty water tanks was not measured, as these findings resulted in the discovery of the issues with the water supply.

The effect on dissolved oxygen levels of the three filtration systems measured pre- and post-filtration are presented in Table 4.1.

Table 4.1: Dissolved oxygen and dissolved oxygen pickup during filtration for beer A (BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

Beer A	**BT**	**KG**	**CT**	**MF A**	**MF B**
Dissolved O_2 (ppb)	36	90	28	125	280
Dissolved O_2 pickup during filtration (ppb)	-	Δ54	-	Δ97	Δ252

The increase in dissolved oxygen was in the expected range for a large scale KG filter but already at the upper end of the tolerated pickup. For MF A the oxygen pickup could still be seen as acceptable for a small scale operation but for MF B the increase of over 250 ppb was too high for a modern filtration system. According to the operator of MF B, the membrane filter requires a constant CO_2 gas supply providing a constant gas pressure to achieve the required dissolved oxygen levels. Due to the trial location, no CO_2 gas supply with the required flow and pressure could be provided, and an available nitrogen gas supply was used. According to the operator, the N_2 gas supply

was not sufficient enough for the filtration rack to achieve the required dissolved oxygen levels. Therefore the targeted dissolved oxygen levels could not be achieved.

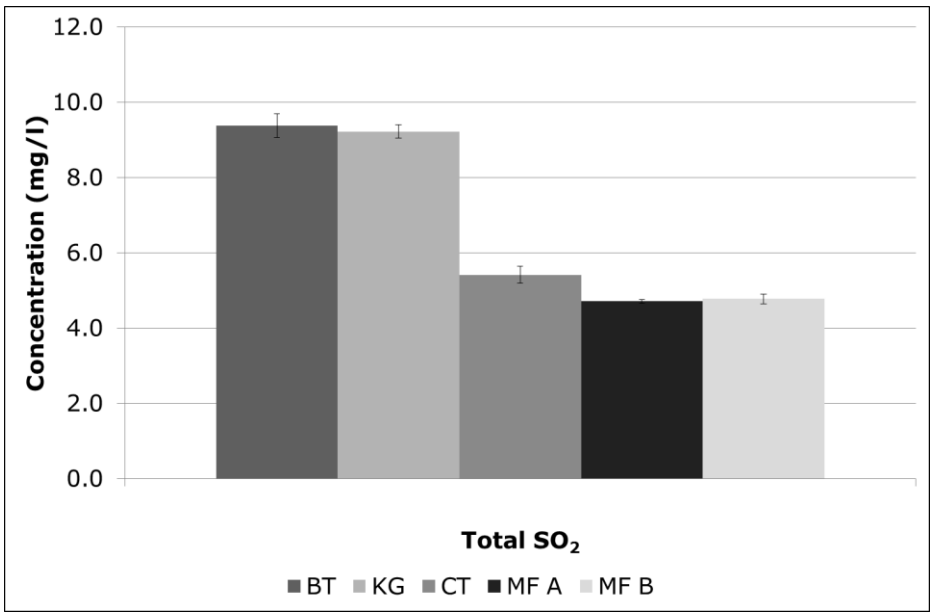


Figure 4.5: Total SO_2 content in beer A (error bars represent standard deviation of technical replicates, n=3, BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

Figure 4.5 shows the total SO_2 content for the different samples of beer A. A clear difference of more than 4 mg/l could be observed between the beers from brewery 1 and brewery 2, whereas only a marginal difference from pre-filtration to post-filtration could be observed for any of the filtration systems. The total SO_2 level derives primarily from the yeast during fermentation and is influenced by numerous factors like yeast viability and generation number, wort composition or fermentation temperature (Boulton and Quain, 2001). As the two beer samples were fermented at different brewery sites, the different total SO_2 levels derived most likely from the different conditions during fermentation.

In the next paragraph, the influence of the different filtration systems on the oxidative stability, determined by EPR measurements, are presented. Table 4.2 displays the data obtained by EPR measurement for lag-time, T_{150} value and the area under the curve.

Table 4.2: Lag-time, T_{150} and area results (peak height) (Samples analysed in duplicate, BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

Beer A	**BT**	**KG**	**CT**	**MF A**	**MF B**
Lag-time (min)	80	82	25	32	28
T_{150} (/10^5)	0.51	1.01	1.85	1.58	2.18
Area (/ 10^7)	0.72	1.1	2.5	2.1	2.3

The KG clearly outperformed the two MF systems in terms of oxidative stability on the basis of the EPR results for all three parameters, area under the curve, lag-time and T_{150} (Table 4.2). The difference between pre- and post-filtration KG samples with regard to area under the curve are significantly higher and the T_{150} value is twice as high post-filtration. In contrast, for MF A and MF B a small decrease of the area value could be observed across filtration. However the T_{150} values did not show this trend for both MF. MF A showed a decreased and MF B an increased T_{150} value. A clear correlation (R^2=0.96) between the lag-time and SO_2 values of all samples was observed. The lag-times for the BT and KG samples did not correlate with the corresponding results for area and T_{150}, as no decrease for the lag-time was observed. For both MF a small increase in lag-time followed the filtration, although the difference was very small. For the KG, a difference for area under the curve and T_{150} between pre- and post-filtration samples indicates the influence of Fe on the EPR

results, as the dissolved oxygen pickup was still acceptable and the total SO_2 level was not influenced by the Kieselguhr filtration. The higher total SO_2 level in brewery 1 most likely caused the longer lag-time for the KG sample as compared to the MF samples.

4.3.2 Results for Beer B

All beer was brewed in brewery 1. Two different batches (I and II) of beer (brewed following the same recipe but fermented and conditioned in different tanks) were filtered through the three systems. Batch I with pre-filtration sampling out of the Kieselguhr filter buffer tank (BT) was filtered through the Kieselguhr filter. Batch II with pre-filtration sampling out of the conditioning tank (CT) was split before filtration and filtered with membrane filter A and membrane filter B respectively. The trial setup is shown in Figure 4.6.

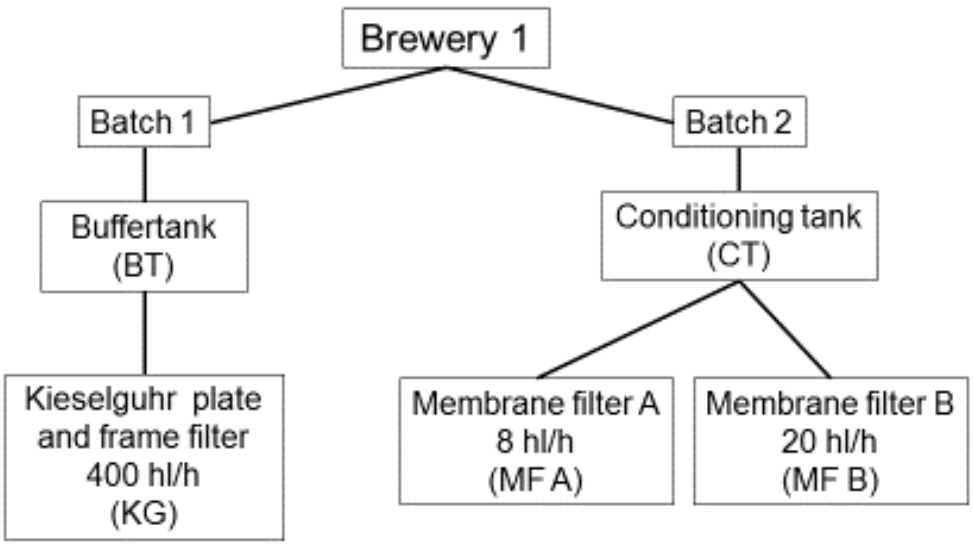


Figure 4.6: Trial setup for beer B

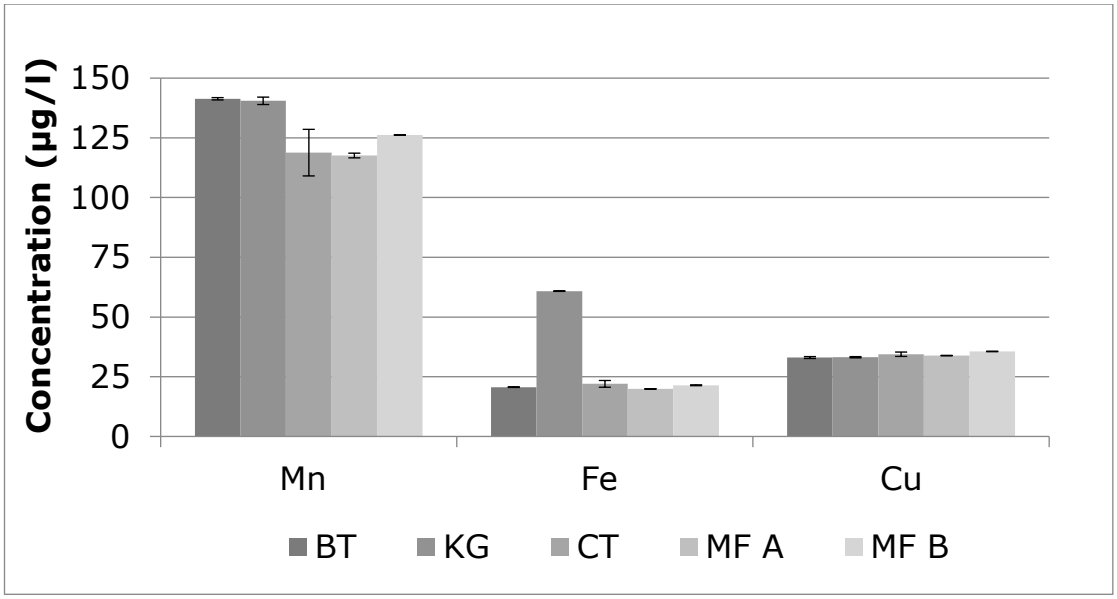


Figure 4.7: Concentrations of Mn, Fe and Cu analysed in beer B (error bars represent standard deviation of technical replicates, n=3, BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

The results for the metal ion analysis determined via ICP-MS are presented in Figure 4.7.

The initial Mn content was *ca.* 20 µg/l lower in the sample pre-membrane filtration than in the sample pre-kieselguhr filtration. As already discussed (Section 4.3.1), this could have been influenced by the use of different grist compositions or by the yeast. Nevertheless the difference between the two samples was much smaller than for beer A. Furthermore only a negligible difference between the pre- and post-filtration samples for all three filtration systems could be perceived.

The initial Fe content of the unfiltered beer samples (BT and CT) were very similar. An uptake of 40 µg/l Fe during Kieselguhr filtration could be detected but there was essentially no increase in either of the MF systems. This was most likely related to the

Fe leaching out of the Kieselguhr during beer filtration as already discussed in section 4.3.1.

The Cu content did not appear to vary across any of the beer samples and there were no obvious impacts of any filtration process on dissolved Cu levels.

Table 4.3 displays the impact of the three filtration systems on the oxygen level of the beer samples before and after filtration.

Table 4.3: Dissolved oxygen and dissolved oxygen pickup during filtration for beer B (BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

Beer B	**BT**	**KG**	**CT**	**MF A**	**MF B**
Dissolved O_2 (ppb)	34	63	38	51	263
Dissolved O_2 pickup during filtration (ppb)	-	Δ29	-	Δ13	Δ225

Only a low increase of dissolved oxygen could be observed during KG filtration and an even lower pickup for MF A was measured. Similarly to the results obtained when filtering beer A (Section. 4.3.1), a large oxygen pickup for MF B was detected. This again related to the insufficient gas supply for MF B.

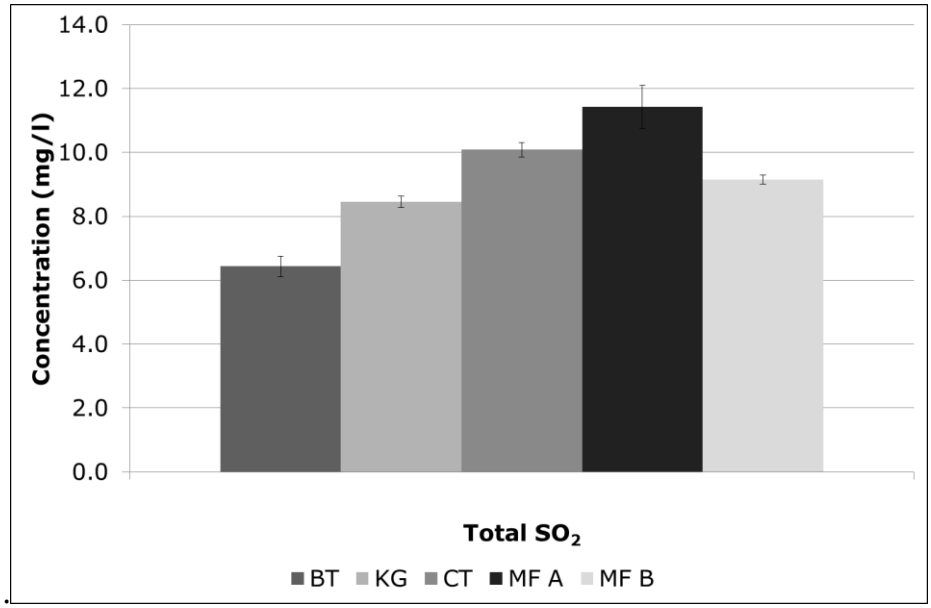


Figure 4.8: Total SO_2 content in beer B (error bars represent standard deviation of technical replicates, n=3, BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

Figure 4.8 presents the total SO_2 for samples of beer B before and after filtration with the three systems. An increase of approximately 2 mg/l from pre- to post-filtration was observed for the KF run. A similar increase (2 mg/l) from pre- to post-filtration was monitored for MF A. In contrast the total SO_2 value for MF B decreased (1mg/l) during the filtration. The difference between the two pre-filtration samples can again be related to the different conditions during fermentation as the two batches were fermented separately using different yeast generations. The increase of SO_2 during the Kieselguhr filtration is not easily explained, as the oxygen uptake during filtration should rather result in a small decrease of the SO_2-level, due to the oxidation of SO_2. The variation in the results for the pre- and post-membrane filtration samples, especially for MF A, did not allow a clear statement about the trend of the SO_2 values,

particularly with only single filtration runs being undertaken in these brewery scale trials.

The results for the determination of the three parameters for the oxidative stability are presented in Table 4.4.

Table 4.4: Lag-time, T_{150} and area results (peak height) (Samples analysed in duplicate, BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

Beer B	**BT**	**KG**	**CT**	**MF A**	**MF B**
Lag-time (min)	126	90	104	111	110
T_{150} ($x10^4$)	2.04	6.91	4.33	2.48	2.74
Area ($x\ 10^6$)	2.43	6.94	4.31	3.48	2.79

For the Kieselguhr filtered beer stream, the lag-time values substantially dropped (- 30 %) across filtration. Accordingly the T_{150} and area values increased by more than 300 %. As for beer A, this was most likely due to the increased Fe content of the sample post Kieselguhr filtration. The CT sample filtered through MF A and B had an initially 22 min shorter lag-time and approximately double T_{150} and area value in comparison to the BT sample. This can be directly related to the not ideally balanced experiment with two different batches used for testing the different filter systems. Nevertheless this highlights how difficult it is to compare different batches, despite being brewed on production scale. Overall, the lag-times for beer B did not correlate with the measured amounts of SO_2 (R^2= 0.13). The values for total SO_2 did not show

a clear trend. The increase of total SO_2 for the KG in comparison to BT and MF A in comparison to CT could not be explained. As the beer is exposed to oxygen pick up, a minor decrease due to oxidation of SO_2 would have been expected. Despite the higher oxygen pick up for MF B (Table 4.3), the EPR data for that stream were not all that different to MF A post-filter.

4.3.3 Results for Beer C

Beer C was brewed in brewery 1 and filtered out of the same conditioning tank for all three filtration systems. Therefore only one pre-filtration sample (CT = BT) will appear in the following results section. The trial setup is shown in Figure 4.9. This setup gave the best opportunity to compare the performance of the different filtration systems directly to each other. This was the preferred and initially planned trial setup for all three beer samples A, B and C but do to the organisational difficulties during the trials, it could only be performed for beer C.

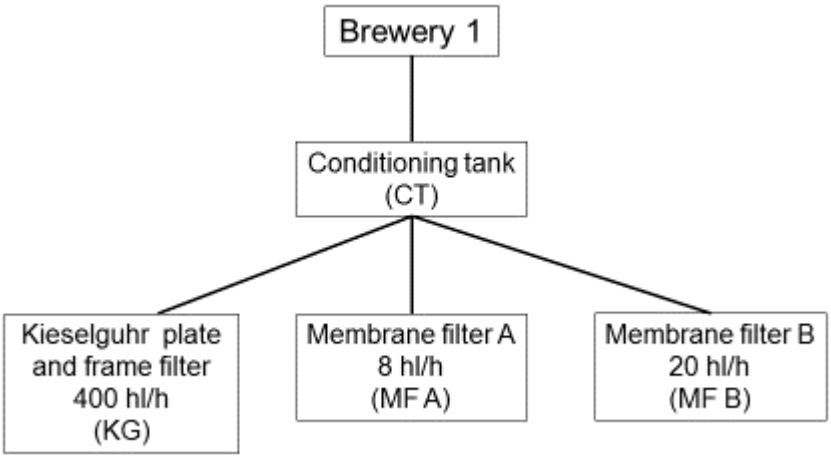


Figure 4.9: Trial setup for filtration of beer C

Figure 4.10 displays the results for the ICP-MS analysis of Fe, Cu and Mn for the pre- and post-filtration samples.

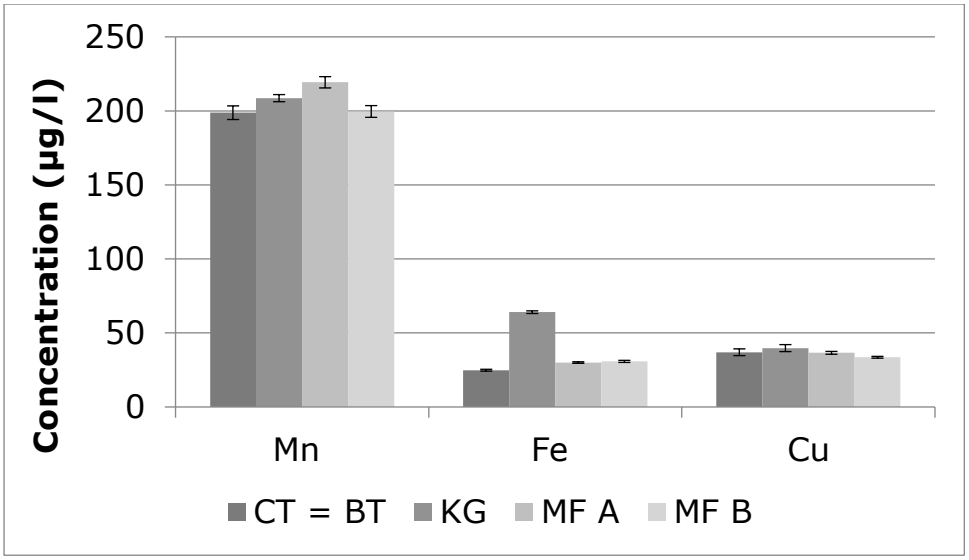


Figure 4.10: Concentrations of Mn, Fe and Cu analysed in beer C prior to and post-filtration (error bars represent standard deviation of technical replicates, n=3, BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

No clear differences in the Mn and Cu content of the samples could be observed across filtration (Figure 4.10). As all beer was filtered from the same conditioning tank, no differences were expected. A clear increase of the Fe-content (*ca.* 40 µg/l) during the Kieselguhr filtration was identified, as with the results obtained for Kieselguhr filtration of beers A and B.

Table 4.5: Dissolved oxygen and dissolved oxygen pickup during filtration for beer C (BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

Beer C	**CT**	**KG**	**MF A**	**MF B**
Dissolved O_2 (ppb)	22	65	113	254
Dissolved O_2 pickup during filtration (ppb)	-	Δ43	Δ91	Δ232

The dissolved oxygen levels were of a similar order to those analysed in beer A as presented in Table 4.5. A reasonable increase for KF and for MF A could be measured but a massive oxygen pickup for MF B was observed. As discussed for beers A and B, this was related to the provided gas supply for MF B.

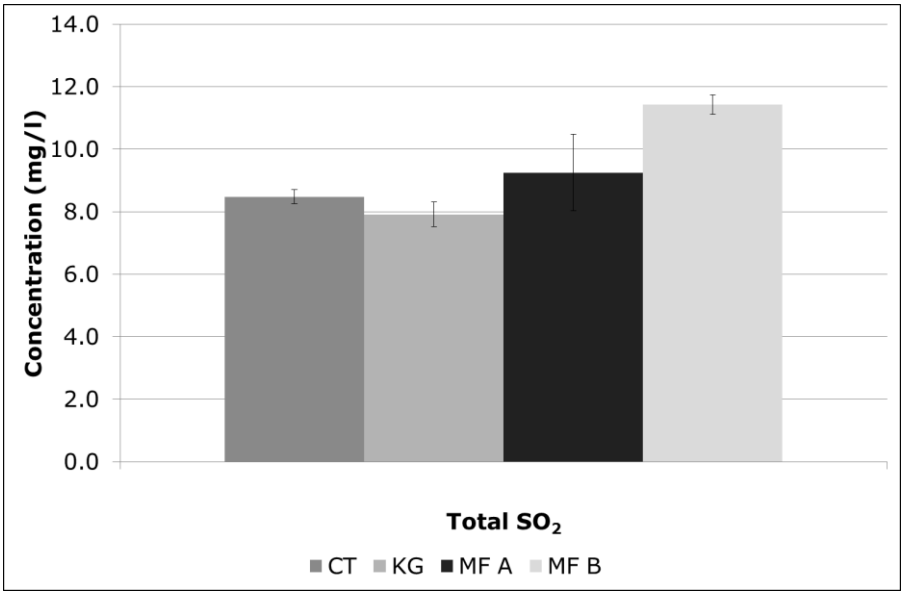


Figure 4.11: Total SO_2 content in beer C (error bars represent standard deviation of technical replicates, n=3, BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

No clear picture could be observed for the total SO_2 levels of the samples of beer C (Figure 4.11). For CT (8.5 mg/l), KG (7.9 mg/l) and MF A (9.2 mg/l) only marginally different amounts of total SO_2 could be detected. The value for MF B (11.4 mg/l) was higher compared to the results for the other three measurements. The results for CT, KG and MF A are in the range of the error of the distillation method, as stated in MEBAK (2002) with 10 % and EBC Analytica (2005) with 5 %. The difference to the result for MF B exceeds these values. As the beer was filtered out of the same CT and no SO_2 was added during filtration, the difference cannot readily be explained.

Table 4.6: Lag-time, T_{150} and area results (peak height) for beer C (BT = buffer tank pre-kieselguhr filtration, KG = post-kieselguhr filter, CT = conditioning tank pre-membrane filtration, MF A = post-membrane filter A, MF B = post-membrane filter B)

Beer C	**CT**	**KG**	**MF A**	**MF B**
Lag-time (min)	>200	75	88	98
T_{150} (10^4)	1.24	9.94	3.75	3.09
Area (x 10^6)	1.89	9.94	4.25	4.22

The results for the oxidative stability measurement via EPR for beer C are displayed in Table 4.6. A lag-time determination for sample CT was not possible because the increase in radical formation over 200 min for 4 replicates was very small and therefore no log-phase could be observed. The lag-times for the post-filtration samples were lowest for KF (75 min), 88 min for MF A and highest for MF B (98 min). A correlation of the lag-time with the total SO_2 value could not be confirmed for the CT sample in comparison to the post-filtration samples, as presented previously (Kunz et al., 2002). This might be related to the very high lag-time value obtained. Such a difference between lag-time for pre- and post-filtration samples indicates that additional factors influenced the lag-time for the pre-filtration sample. An acceptable correlation (R^2= 0.95) of the lag-time and values for total SO_2 for the post-filtration samples could be determined. For all three filtration systems an increase in T_{150} and the area value was monitored across filtration. For the KF, the T_{150} value increased by 800 % and the area value by 525 % in comparison to the values for the pre-filtration sample CT. For the MF systems, MF A post-filtration sample showed an increased T_{150} value (300 %) and area value (225 %). For MF B, the filtration step resulted in an increased T_{150} (250 %) and area (225 %) values. The difference between the two MF

systems was small for the T_{150} value and negligible for the determined area under the curve despite the higher oxygen pick up of 141 ppb for MF B (Table 4.5). Comparing the KF to the two MFs, a clear difference for the area (up to 100 %) and the T_{150} value (300 %). For the same beer sample filtered with the three different systems, the MF systems retain much more of the oxidative stability of the beer sample based on the EPR results.

4.3.4 Combined discussion of trial results for beers A, B and C

Due to the production requirements of a large scale brewery, the trial setup was not ideal. The same batch of beer filtered out of the same CT would have been the ideal setup. Nevertheless the three different trial setups resulting in samples for beers A, B and C showed some interesting results.

For all three beer samples, an increase in Fe-levels of around 30 to 40 µg/l was noted for the Kieselguhr filtration. This results from the filtered beer at pH 4 extracting iron out of the Kieselguhr which is continuously dosed during filtration. Interestingly for all three Kieselguhr filtrations (Beer A, B and C), the iron pick-up remained broadly similar in each case.

The Cu level was not significantly different from pre- to post-filtration and therefore it was apparent that the Cu content of beers was not influenced by the filtration step in these trials.

The difference in the absolute Mn levels (125 µg/l to over 200 µg/l) between the different beers could be attributed to the different recipes for beers A, B and C. All three beers were continental-style lager beers; nevertheless beer A was brewed with 100 % malted barley resulting in the lowest Mn content, whereas the grist of beer A

and beer C contained raw wheat resulting in higher total Mn levels. This is in agreement with the findings of Zufall and Tyrell (2008). Furthermore all three beers were brewed using different yeast strains under different fermentation conditions which could influence the total Mn levels as discussed in Section 4.3.1.

A low dissolved oxygen level is widely accepted to be beneficial for an extended shelf life. Interestingly, the very high oxygen pick-up across filtration using MF B for all three filtration runs did not affect any of the flavour stability indices measured using EPR spectroscopy.

The decrease in EPR area values for beer A and beer B filtered with the MF systems suggests a positive effect of the filtration step on flavour stability and was confirmed by Foster (2013b). He showed decreasing T_{150} values for a membrane filter system from pre- to post-filtration.

4.4 Conclusions

The initial filtration setup at the time of the trials made the evaluation of the performance of the three different filtration systems more complicated. As all trials were performed in a large scale brewery environment, reasonable comprises were needed to make the trials accomplishable. Nevertheless the results obtained showed clear differences in the performance of the filtration systems. The modified trial design for beer A showed clear differences between the two brew streams producing the same beer, revealing the impact of the brewing water supply and providing insights to inform any proposed refinements of the brewing facilities. For filtration of beer C, where the trial design was optimal, the cross-flow filtration systems showed a clear advantage over the KG filter regarding the EPR results and hence the flavour stability

of the filtered beer. A better performance regarding the impact on flavour stability could also be shown for beer B but not as clearly as was the case for beer C. These results support the findings of Broens et al (2007) and can be explained by the avoidance of substantial iron pick-up during membrane filtration and the resulting lower total Fe content (Zufall and Tyrell, 2008) and hence an increased flavour stability. The Fe pickup during kieselguhr filtration was consistently between 30 and 40 µg/l for all three beers.

The negative effect of high dissolved oxygen levels in beer is widely accepted. Therefore the oxidative stability following filtration using MF B in comparison to the values obtained for MF A was a surprise, as the high levels of oxygen pickup during filtration should have had a negative effect on the oxidative stability and hence on flavour stability. The reduced impact could be related to the sample handling during the filtration trials. As the beer was filtered at a temperature of around 1 °C and the sample bottles were transferred into a freezer directly after sampling, the impact of the oxygen pickup could have been suppressed.

The total SO_2 values varied for the different samples and could not be correlated to all of the EPR measurements, especially for beer B. The error for the distillation method with up to 10 % in MEBAK (2002) is high and it is not the most precise technique for SO_2 analysis in comparison to Continuous Flow Analysis (CFA) (Kunz et al., 2009). Nevertheless for this work no other technique was available and the distillation method is a very cost effective and well established method. For further work the analytical method was investigated. The use of a reference beer in combination with a monitoring system to evaluate the method on a daily basis to improve the reproducibility was introduced for the SO_2 determination, as described in Section 3.1.

The results for beer A showed the requirement for an appropriate control sample for the EPR measurements. The initial high Fe content in beer A from brewery 2 was most probably related to rusty tanks in the brewing water supply. The trials resulted in an awareness of higher Fe levels in the beer from brewery 2. Nevertheless the results are difficult to compare with one another and could not be used to make a statement about the influence of the filtration systems on the oxidative stability and related flavour stability. A direct comparison of beer from brewery 2 filtered with all three filtration systems would have been necessary but it demonstrates one limitation of applying EPR spectroscopy in this way. Without appropriate control samples only limited information is given by the EPR measurements.

The results show evidence of a better performance of both membrane cross-flow filtration systems regarding the analysed flavour stability indices but further investigation in final pack beer after bottling and pasteurisation needs to be performed. This was planned for this trial but due to the lack of a functional small-scale bottling device at the time of the trials, no further investigations could be performed. For all three trial beers, samples were transferred into 20 l keg containers with an autoclaved sampling device and stored at 20˚C for three month. Unfortunately the microbiological requirements for sterile sampling were not fulfilled and most of the sample kegs got infected due to the impossibility of a heat treatment to achieve microbiological stability. Therefore no further chemical or sensory analysis could be performed on aged beer samples.

5 Assessing the impacts of brewhouse gallotannin addition on beer flavour stability

5.1 Introduction

Gallotannins belong to the group of hydrolysable tannins and can be found in higher plants as contributors to their chemical defence mechanisms (Haslam, 1989, Bi et al., 1997, Pohl, 2009). Commercially used gallotannin products are sourced from various plants, including Chinese gallnuts, sumac leaves and Turkish Aleppo gall nuts. Gallotannins sourced from Chinese gallnuts have traditionally been used in the food industry as a preservative due to their antioxidant and antimicrobial properties (Power, 2006). Gallotannins have a long history in beer making as a traditional precipitating agent in the cold stabilisation of beer (De Clerck, 1970). The commercially used products consist of a glucose core surrounded by gallic acid moieties in varying structures (Figure 5.1). They can be purchased in powdered form and are dissolved in water before their addition into the beer production stream.

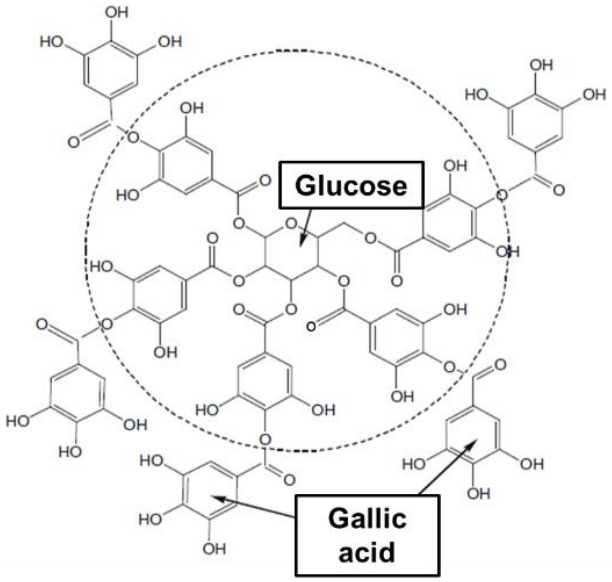


Figure 5.1: Gallotannin molecule: glucose core surrounded by gallic acid fragments

Recently the brewing industry has 'rediscovered' the use of gallotannins as a process aid to improve flavour stability and prolong shelf-life. This is based on the ability of gallotannins to act as metal-chelating agents, reducing agents and radical scavengers (Lopes et al., 1999, Gülçin et al., 2010). Their ability to bind aldehydes and coagulate with thiol-containing proteins can also have a positive effect on retaining the fresh beer flavour for a longer period of time, as reported by Anonymous (2009a).

Brewhouse operations are reported to have a significant influence on the flavour stability of finished beer (Lustig et al., 1999, Herrmann et al., 2010). Negative effects on flavour stability are reported to be influenced by the dissolved oxygen levels in mash and wort, and can be related to oxidative enzymatic and non-enzymatic reactions (Bamforth, 1999b, Wackerbauer et al., 2003).

The addition of gallotannins in the brewhouse has already been reported to have a positive effect on flavour stability based on a pilot scale trials (Aerts et al., 2004). In that trial, the added levels were up to 15 g/hl. Similar findings were reported by Methner (2015), with additions of over 10 g/hl required to achieve improved flavour

stability. The positive effect on flavour stability relative to the costs of the addition were not investigated for both papers. The properties of gallotannins should result in a positive effect on flavour stability but to be economical on a production scale, only relatively low levels of addition can be incorporated on these scales. The manufacturer recommends additions between 1.5 g/hl to 4 g/hl to help improve flavour stability (Anonymous, 2016b). To investigate the anticipated positive effects of a gallotannin addition in the brewhouse, a set of pilot scale and full-scale trials were performed, and the effects of the addition during mashing and at the end of wort boiling were evaluated. The assessment of the effects on flavour stability included the analysis of both wort and beer samples.

5.1 Materials and Methods

5.1.1 Pilot scale trials

The trials were performed at the Molson Coors Brewing Company pilot brewery (18 hl) in Burton-on-Trent. A lager-style beer was produced using a standard recipe provided by the brewery. The trial included three trial brews: a control brew and two trial brews featuring gallotannin addition at different steps in the brewhouse process: either mash conversion vessel addition (3 g/hl finished beer) or wort kettle addition (2.5 g/hl finished beer). The added amounts were based on the recommendations by the supplier of the gallotannin product (Anonymous, 2009a). As the beer was produced using high-gravity brewing, the amounts of gallotannins added were calculated on the anticipated final volume of beer as stated in Table 5.1.

Table 5.1: Estimated brewing volumes, estimated original gravities and added amounts of gallotannins for pilot scale tannic acid trial brews (OG = original gravity)

Estimated final wort volume (hl)	17.7
Estimated final beer volume (hl)	31.1
Estimated OG pitching wort	64
Estimated OG beer	36.5
Resulting Gallotannin additions (g per brew) Control Mash CV addition Wort kettle addition	 0 93 (3 g/hL finished beer) 78 (2.5 g/hL finished beer)

For the control brew, the standard recipe was used without any alteration. For the mash conversion vessel addition, the gallotannins were added during the mashing-in process. The addition to the wort kettle was performed 12 min before the end of the boiling process separated by 2 min from the copper fining addition. For both additions, the accurately measured amount of gallotannins were mixed into hot water (70 ˚C) in a plastic bucket (25 l) and stirred until all gallotannins were dissolved. The mixture was added immediately following its preparation. For all three pilot-scale brews, part of the cooled and aerated wort (8 hl) was transferred into a conical fermentation vessel (10 hl volume).

Wort samples for analysis were taken at key points in the production process (Table 5.2).

Table 5.2: Wort sampling regimes during pilot scale tannic acid trials (x = sample taken, - = no sample taken)

Sampling point	**Brewing Trial**		
	Control	Gallotannin addition at mashing-in	Gallotannin addition during the boil
Strong wort (Sample tap mash filter)	X	X	-
10 min before end of boil (sample tap wort kettle)	X	-	X
End of boil (sample tap wort kettle)	X	X	X
Cooled clarified wort (sample tap wort chiller before aeration)	X	X	X

All three brews were pitched using an equal pitching rate (15 million cells per ml) of the same yeast strain and fermented and conditioned under matching regimes. After fermentation and conditioning, a CO_2 purged keg (50 l) was filled for each trial brew. The keg was filtered utilizing a sheet filter and the filtered beer was collected in another CO_2 purged keg (50 l). The beer was bottled into flint glass bottles (330 ml) operating the single filling station pilot plant bottling line. All bottles were pasteurised (22 PU) and stored at 2 ˚C prior to further processing.

To simulate the ageing process of the bottled beer samples, bottles of each product were transferred into a warm room (30 ˚C) and stored for 10.5 days, 21 days and 31 days, respectively, prior to further analysis.

5.1.2 Full-scale trials

The full-scale trial was performed in the Burton brewery South (brew length 1500 hl). The same recipe and addition regime as for the pilot scale trials was used (control brew, mash CV addition (3 g/hl finished beer), wort kettle addition (2.5 g/hl finished beer). Three consecutive trial brews for each addition regime (control, mash addition, wort addition) were produced and spilt into two fermenters with the whole first brew and the first half of the second brew transferred into one fermenter and the second half of the second brew and the whole third brew fermented in the another fermenter (total volume per fermenter 2250 hl).

Table 5.3: Estimated brewing volumes, estimated gravities and added amounts of gallotannins for large scale brewing trials

Estimated final wort volume (hl)	1500
Estimated final beer volume (hl)	2630
Estimated OG pitching wort	64
Estimated OG beer	36.5
Resulting Gallotannin additions (kg per brew) Control Mash CV addition Wort kettle addition	 0 7.9 6.6

For both additions, the accurately measured amount of gallotannins was dissolved in hot deaerated brewing liquor (70 ˚C) in plastic buckets (25 l). The required gallotannin

mixture was prepared at the beginning of each run of three consecutive trial brews and stored (20˚C) for up to 5 h prior to the addition.

For the mash addition, the dissolved gallotannins were added through the manhole of the mash CV directly after the mashing in process was finished. The required amount of gallotannin solution was added through the wort kettle manhole 10 min before the end of the boil for the wort kettle addition at least 2 min before the copper fining addition.

All trial beers were fermented, processed and packaged following the standard production procedure for the particular lager style beer. For the Kieselguhr filtration, special care was taken to avoid filtering the trial beers at the beginning of a filtration cycle. This was executed to eliminate the issues of higher iron pick-up at the beginning of a Kieselguhr filtration cycle. All beers were packaged in aluminium cans and stored at 2˚C prior to further processing. The aging of the samples was performed at a controlled temperature (20°C) for 3, 6 and 9 months, respectively.

Due to production issues beyond our control during one of the wort kettle addition trials, two control brews, two mash CV addition brews but only one wort kettle addition brew could be collected.

All fresh and aged beer samples were stored at 2°C prior to analysis.

5.1.3 Thiobarbituric acid index

For the description of this analysis see Section 2.3. All wort samples as stated in Table 5.2, all fresh and aged beer samples of the pilot scale and large scale trial were analysed. The analysis included three technical replicates of each sample.

5.1.4 Total sulphur dioxide via distillation method

The analysis is described in Section 2.2. All fresh and aged beer samples of pilot scale and large scale trials were analysed. The analysis included three technical replicates of each sample.

5.1.5 Metal analysis via ICP-MS

For the description of this analysis see Section 2.4. All wort samples as stated in Table 5.2 and all pilot scale and production scale fresh beer samples were analysed. Due to the costs of the analysis, a single runs of each sample were performed. Three samples were run in triplicate to evaluate the error of the analysis.

5.1.6 Oxidative stability by EPR spectroscopy

For the description of this analysis see Section 2.5. All wort samples as stated in Table 5.2 and all fresh and aged beer samples of pilot scale and production scale trails were analysed. The analysis was performed in duplicate. The evaluation of the results included the determination of the area under the curve, x0-lag time, and T_{150} values.

5.1.7 Analysis of volatile aldehydes in beer by solid phase micro extraction (SPME) with GC-MS

For the description of this analysis see Section 2.1. All fresh and aged beer samples of pilot scale and production scale trials were analysed. The analysis was performed in duplicate.

5.1.8 (E)-2-nonenal potential of cooled wort

Wort samples were analysed using the method described in analysis of volatile aldehydes in beer by SPME with GC-MS (Section 2.1). Degassing was not required, because unfermented wort contains only negligible amounts of CO_2. The DSQ mass

spectrometer was operating in selected-ion mode scanning for ion m/z 181 from 20 min to the end of the run. 3-fluoro benzaldehyde was used as internal standard.

The trans-2-nonenal potential was measured by determining the amount of trans-2-nonenal present in untreated and treated cooled wort samples. For the treated wort sample, the pH was adjusted to pH 4 with ortho-phosphoric acid (5 %) and the sample transferred to a 50 ml conical-based glass tube and purged with nitrogen to exclude as much oxygen as possible before sealing with a PTFE lined cap. The sample was heated for 2 h in a boiling water bath and then plunged into an ice bath to cool and stored at 2 °C prior to SPME-GC/MS analysis.

5.1.9 Sensory analysis

A sensory evaluation was performed by the sensory expert panel of the Molson Coors Brewing Company brewery in Burton on Trent. The sensory evaluation included parameters to evaluate the overall flavour (estery, hoppy, malty, grainy, sweet, bitter, astringent, sour, caramel/toffee, burnt) for the fresh beer samples and additional assessment of the stale character (oxidised, leathery, papery and catty) in the fresh and aged beer samples from pilot scale and production scale trials. All sensory parameters were rated on a scale of 0 to 10.

5.2 Results and Discussion

5.2.1 Pilot scale trials

5.2.1.1 Wort analyses: Thiobarbituric acid index (TBI)

The TBI has traditionally been used as a reliable, uncomplicated and inexpensive indicator to evaluate the brewhouse operations in reference to heat impact and the resulting formation of Maillard reaction products. During the TBI assay a large number of the Maillard reaction products and other aldehydes are measured and TBI

values can be used to quantify the extent of beer staling. Lower TBI values will indicate a lower impact on flavour stability.

In Figure 5.2 the results for the TBI measurement are displayed. Whilst each of the trial brews was only conducted once at pilot plant scale (hence no index of variability was obtained) the differences in TBI resulting from gallotanin addition were proportionately large. For the strong wort samples a difference between the control (TBI = 8.4) and the mash CV addition (TBI = 5.6) could be observed.

For the samples taken 10 min before the end of boil, before the wort kettle addition, the difference between control (TBI =12.3) and wort kettle (TBI =10) was not expected, as both trial brews were produced using the same batch of raw materials, the same brewing liquor and following the same recipe. At the end of boil, a clear difference in the TBI values between control (TBI =16.3) and the two additions mash CV (TBI =10.6) and wort kettle (TBI =9.8) was detected, whereas the two treated brews showed very similar TBI results. The same could be observed for the cooled wort samples, as the TBI value for the control (TBI =20.8) was approx. twice as high as for both treated brews (mash CV TBI =10.9 and wort kettle TBI =12) but the treated brews showed very similar results for the cooled wort samples.

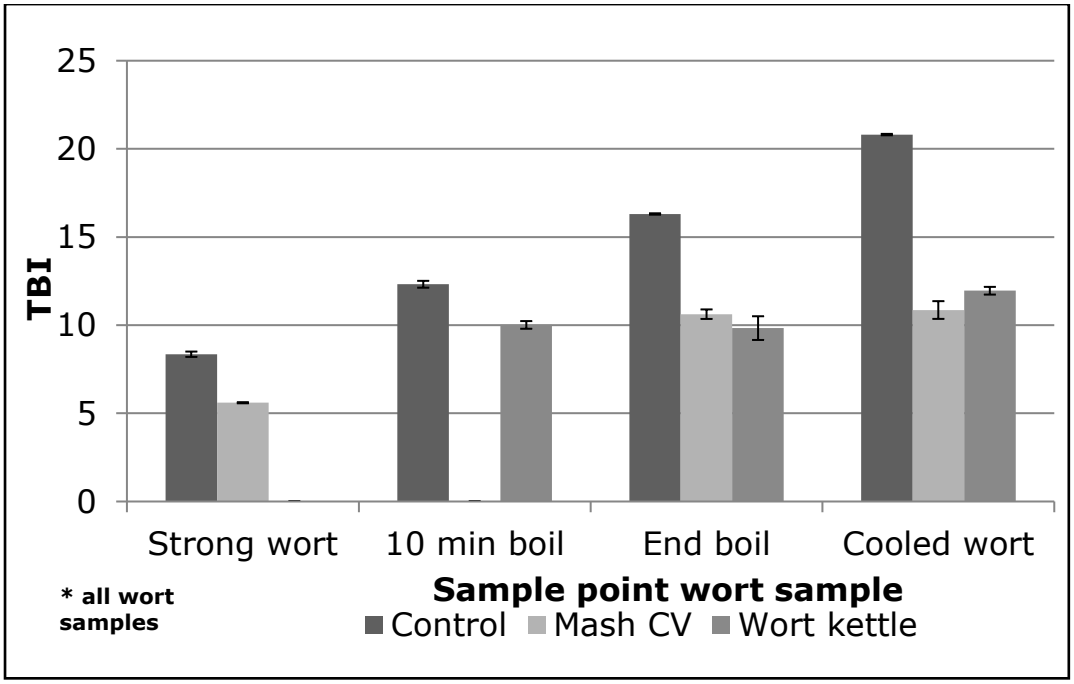


Figure 5.2: TBI (448 nm) of pilot scale wort samples (all wort samples normalized to gravity 1.048; error bars show standard deviation of technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

At the end of the brewhouse operations, both treated brews showed a substantially lower TBI value (*ca*. 50 %) compared to the control in the cooled wort sample. This indicates a much lower formation of Maillard reaction products and aldehydes significant to beer staling. Hence the resulting beers should in theory have improved flavour stability.

5.2.1.2 Wort Metal-ion contents

Through the ICP-MS analysis the amounts of Fe, Cu and Mn present in the wort samples were determined. It was important to assess the concentrations of metal ions because of their pro-oxidative impact in beer and to determine whether gallotanins were modifying free metal-ion concentrations through the process by chelation.

Due to the expense of analysis, each sample was only analysed once. To evaluate the repeatability of the analysis, three samples were selected to be run in triplicate, and this resulted in a standard deviation of under 1% for the three technical replicates (data not shown).

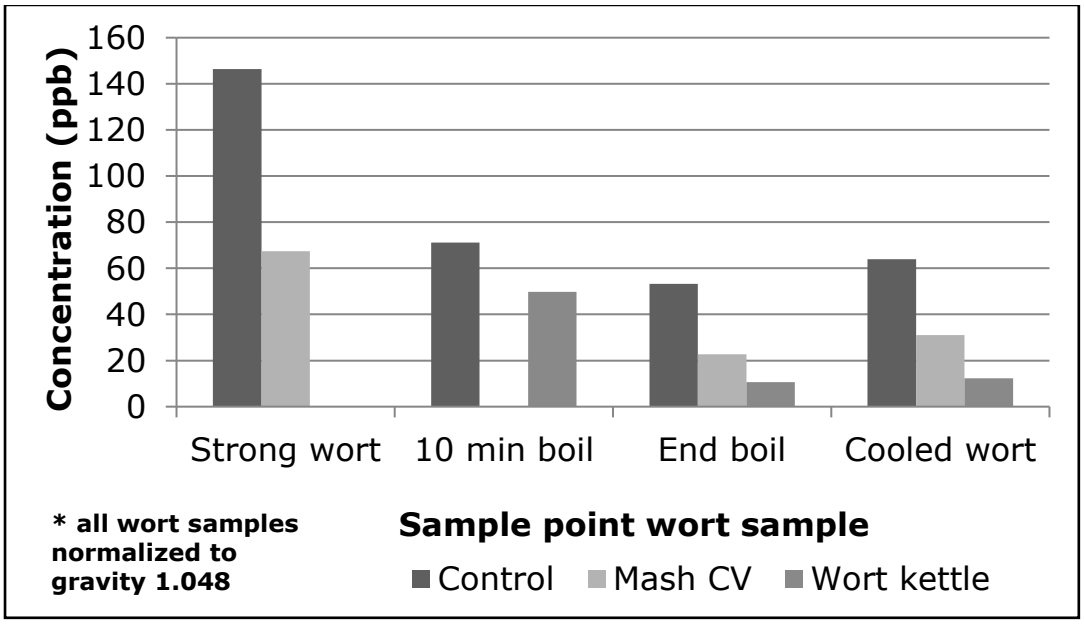


Figure 5.3: Fe content of pilot scale wort samples (all wort samples normalized to gravity 1.048; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

Figure 5.3 displays the Fe levels measured in the taken wort samples. For the two strong wort samples, a significant difference in the total Fe levels between control (146 ppb) and mash CV (67 ppb) was observed. For the two samples taken 10 min before the end of the boil, control (71 ppb) and wort kettle (50 ppb) show only a small difference but as both brews were untreated, a similar Fe-concentration was expected. At the end of boil, a clear difference in the Fe concentrations for all three treatments was apparent. Compared to the two gallotannin-treated samples, the control sample (53 ppb) had a much higher Fe content. The Fe content of the mash CV sample (23

ppb) had only half the amount of Fe present compared to the control and an even lower amount (11 ppb) was detected in the wort kettle addition sample. A similar result was obtained in the cooled wort samples, as control (64 ppb) had by far the highest amount of Fe present, the mash CV sample (31 ppb) contained only half the amount of Fe and the value for the wort kettle sample (12 ppb) was even lower.

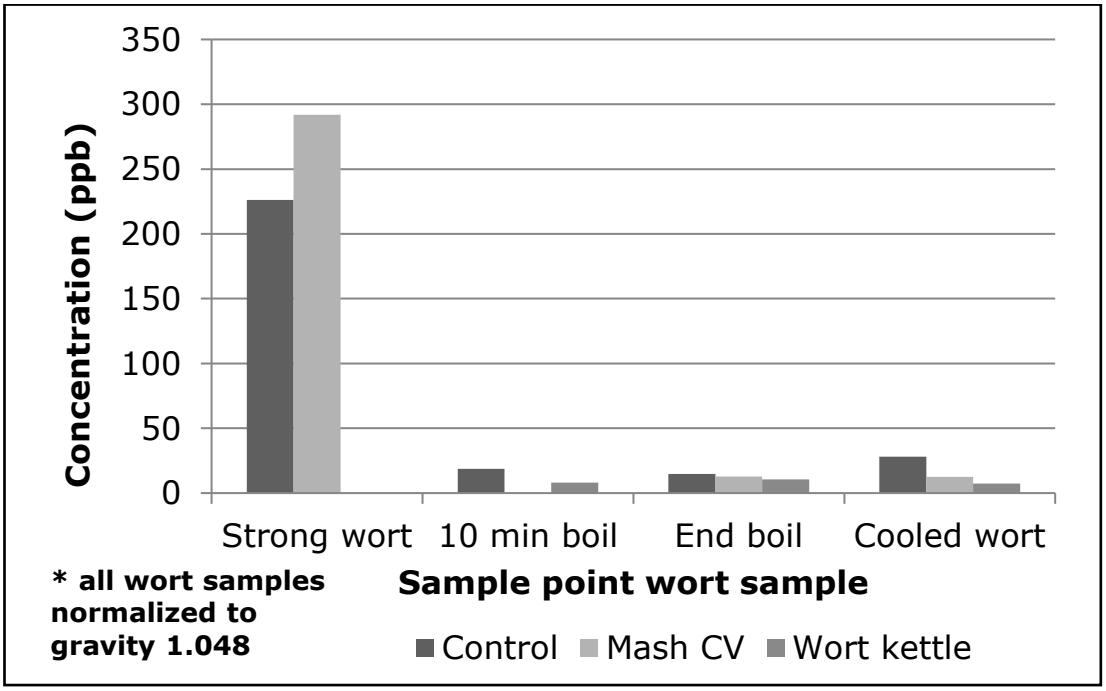


Figure 5.4: Cu content of pilot scale wort samples (all wort samples normalized to gravity 1.048; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

Figure 5.4 shows the concentrations of Cu measured in pilot scale wort samples. Substantial concentrations of Cu could be detected in the strong wort samples for control (226 ppb) and an even higher concentration in the mash CV (292 ppb). The high levels could be related to the Cu-input by the malt as high levels of Cu in malt have been reported (Krüger and Anger, 1992, Zufall and Tyrell, 2008). The brewing water could be eliminated as a source of Cu as all water used was RO-water. These

amounts of Cu were heavily reduced during mash separation and boiling, resulting in almost equal Cu amounts at the end of boil for all three samples, control = 15 ppb, mash CV = 13 ppb, wort kettle = 10 ppb. As Cu is lost with the trub during boiling, a reduction in the Cu content was expected. The cooled wort showed a slightly higher Cu level in the Control (28 ppb) compared to the end of boil control samples and compared to the other two cooled wort samples (Mash CV = 13 ppb, wort kettle = 7 ppb).

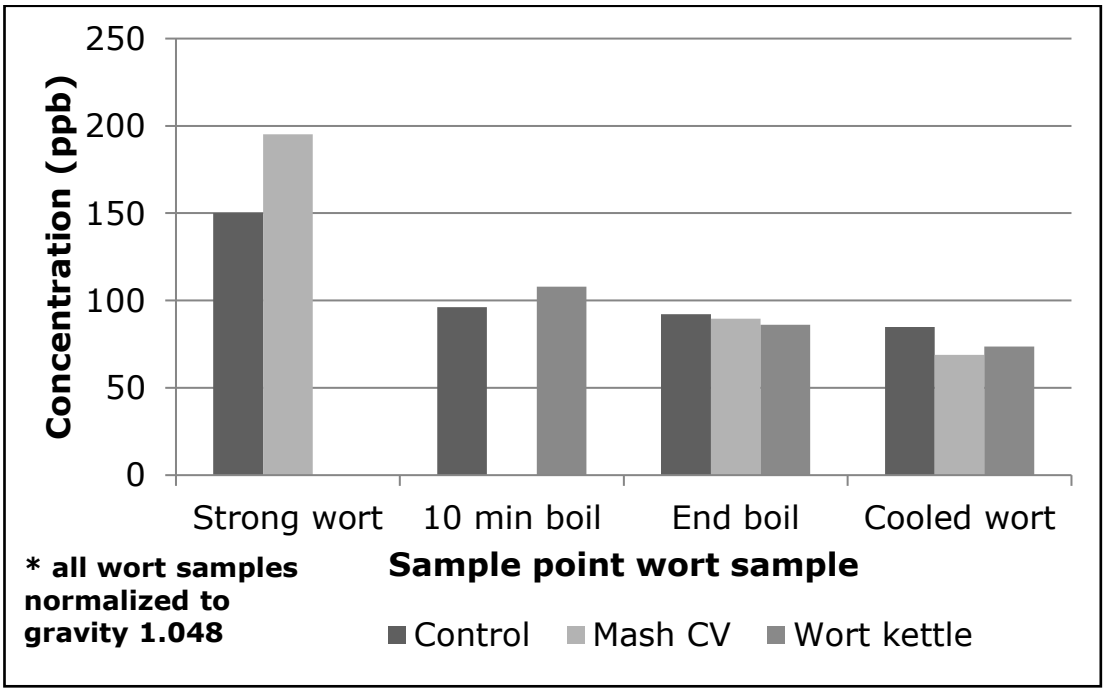


Figure 5.5: Mn content of pilot scale wort samples (all wort samples normalized to gravity 1.048; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

For Mn a reduction across mash separation and boiling was detected (Figure 5.5) but not to the extent that was observed for Cu. All samples had similar levels of Mn present at the end of boil and a slight drop could be detected for the cooled wort. This correlates with the data reported by Bromberg (Bromberg et al., 1997), as the

brewhouse processes should only have limited influence on the Mn levels. For Fe and Cu, a chelation by the gallotannins was expected. For Cu, the results were unclear, as the Cu amounts at the end of boil for all three samples were very similar whereas based on the cooled wort samples, the control had a significantly higher Cu-level. The increase from the end boil sample to the cooled wort sample could not be explained. Based on these results, a clear ability of gallotannins to reduce the Cu-levels is difficult to demonstrate. The results for the Fe content show a substantial effect of the gallotannin addition for the treated samples compared to the control brew with constantly lower levels for the treated brews throughout the entire brewhouse process. This indicates the ability of gallotannins to chelate Fe and reduce the amount of Fe in treated wort. The mash addition had significantly lower Fe levels in the strong wort (50 % lower). This indicates the improved chelation of Fe in the mash and the separation from the wort with the spent grains. The kettle addition gave the lowest cooled wort Fe levels and it seems likely that adding gallotannins in the kettle was most effective at incorporating Fe into trub. Assumedly where the gallotannins were added earlier in the process, the potency at removing Fe in the kettle was lowered, resulting in slightly higher levels at the end of boil and in the cooled wort compared to the kettle addition.

5.2.1.3 Wort (E)-2-nonenal potential

With its very low flavour threshold of about 0.1 mg/l and its unpleasant cardboard-like flavour, (E)-2-nonenal formation can have a negative impact on the flavour character of lager-style beers in particular (Saison et al., 2009). The (E)-2-nonenal potential has been reported to be a good indicator to predict the release of and the formation of (E)-2-nonenal in the finished beer sample (Drost et al., 1990). Lower

levels of (E)-2-nonenal and it precursors in a wort sample should result in a better flavour stability of the finished beer.

The initial content of (E)-2-nonenal in the cooled wort samples (Figure 5.6) was very similar for the control (0.7 ppb) and the wort kettle addition (0.68). The initial (E)-2-nonenal content of the mash CV addition (0.5 ppb) was lower. Nevertheless the standard deviations of the measurements, especially for the control and wort kettle addition, were very high and so the observed difference due to the mash CV gallotannin addition cannot be described as significant. The high standard deviation is due to the complexity of the analytical method and the extremely low amounts of (E)-2-nonenal present in the different samples.

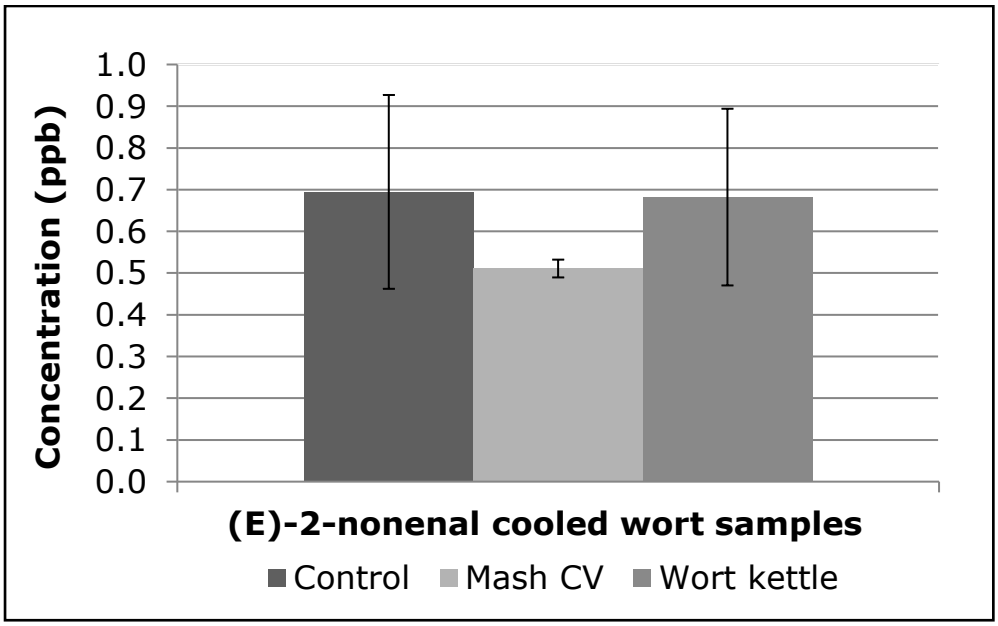


Figure 5.6: (E)-2-nonenal content (ppb) of cooled wort samples (error bars signify standard deviation of technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The results for the (E)-2-nonenal potential after the treatment of the wort samples (Figure 5.7) showed a similar trend as the results for the initial (E)-2-nonenal content

of the wort samples. Control (19.7 ppb) and wort kettle addition (20.2 ppb) had a very similar (E)-2-nonenal potential whereas mash CV addition (16.3 ppb) had a lower potential. Again the rather high standard deviation for especially control and mash CV made it impossible to verify a significant difference but a trend for a lower initial and potential value for (E)-2-nonenal could be detected for the mash CV addition.

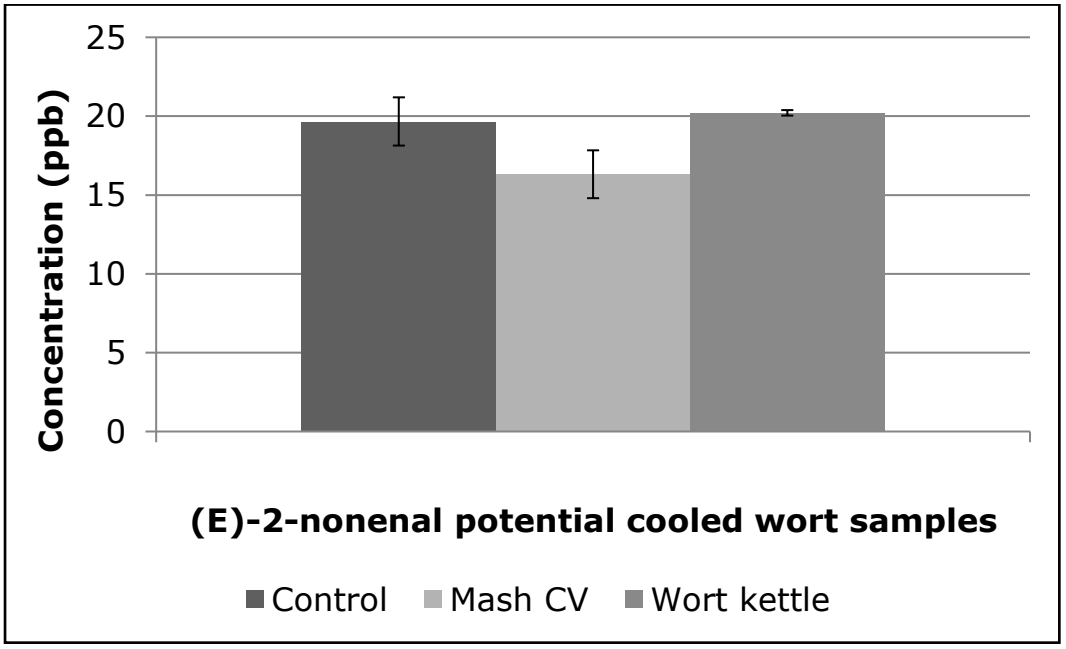


Figure 5.7: (E)-2-nonenal potential (ppb) of cooled wort samples (ph = 4.0 and heating time = 120 min), (error bars signify standard deviation of technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The formation during beer production and the release during beer storage of (E)-2-nonenal is still not fully understood. Nevertheless there is some strong evidence that the non-enzymatic and enzymatic oxidative processes during wort boiling have a significant influence on the level of (E)-2-nonenal in the finished beer (Noël et al., 1999). The above presented results might show some evidence of the potential of gallotannins to reduce the oxidative processes and have an influence on the

lipoxygenase (LOX) activity during mashing but the results are based on two single trials and the error associated with the analytical assay implies that the noted differences were not statistically significant. Furthermore an influence during wort boiling could not be observed, as the results for the wort kettle addition were equal to the results for the control brew for both, the initial and the potential (E)-2-nonenal values.

In general, the determination of (E)-2-nonenal is complex as the amounts present are in the low ppb range and therefore the detection pushes the sensitivity limit of the GC-MS system. In addition, the preparation of the samples for the (E)-2-nonenal potential without or with a limited oxidation of the samples was very difficult. Despite taking all precautions to avoid an increased oxygen contact, during the pH adjustment and the transfer of the samples an oxygen uptake was unavoidable. Therefore the noted variation in the results might be linked to the sample preparation rather than to the actual GC/MS run, as the calibration for (E)-2-nonenal showed little variance (R^2 = 0.991). Similar issues regarding the value of the analysis have been reported by Wackerbauer et al. (2004).

5.2.1.4 EPR spectroscopy measurements of wort oxidative potential

The results for the oxidative potential, based on the area under the curve of the EPR signal forcing test of wort samples are shown in Figure 5.8. The area under the curve includes all measuring points of an EPR spectroscopic experiment whereas the T_{150} value only reports a single measuring point.

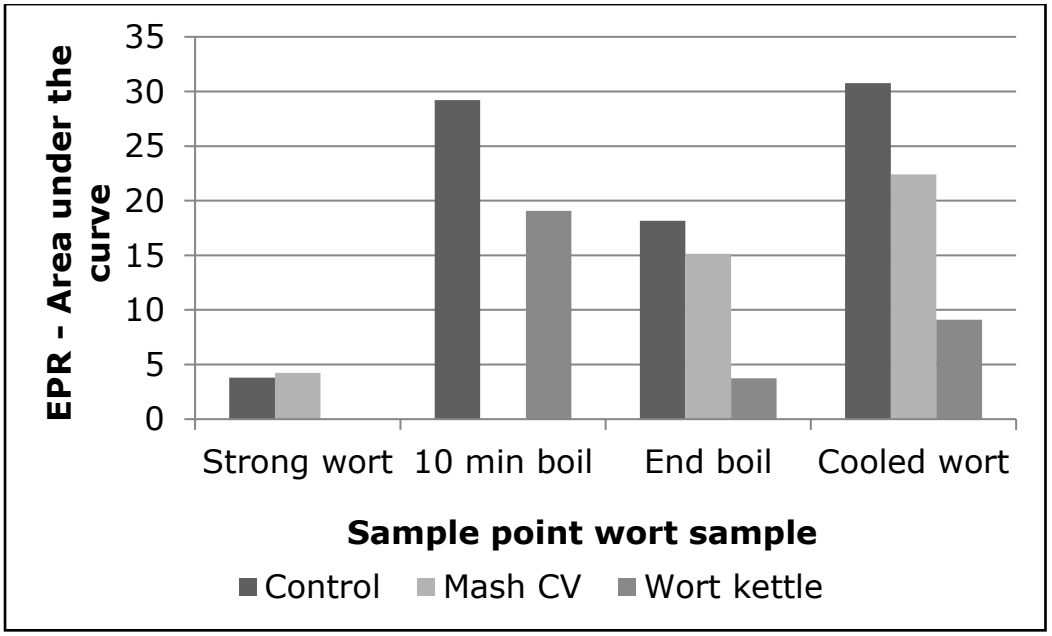


Figure 5.8: Area under the curve for pilot scale wort samples (peak height determination; two technical replicates per sample point; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

For the strong wort samples for control (area = 3.8) and for the mash CV addition (area = 4.2) the results were very similar. Despite the addition of gallotannins during mashing, no significant difference could be detected between the two strong wort samples. Nevertheless the detected radical formation was very low for both samples and even after 200 min of forced ageing (60˚C), the signal intensities of the EPR spectra for both strong wort samples were very low and so were the resulting area under the curve values. The strong wort of the mash filter had a very high gravity (< 25 ˚P), resulting in a very high concentration of natural antioxidants and radical scavengers present in both wort samples. Therefore the potential effect of the mash CV addition might have been obscured by the high natural antioxidant concentration already present in the very high gravity wort samples. For the wort sample taken 10 min before the end of the boil just before the addition of the gallotannins, control (area

= 29) and wort kettle addition (area = 19) gave rather different results. Using the same batch of raw materials, brewing liquor and following the same recipe, the significant difference between control and wort kettle before the addition was not expected (Figure 5.8). The cooled wort samples showed a substantially lower area for both treated samples compared to the control. The mash addition resulted in a 30 % lower and the wort kettle addition in a 70 % lower area value compared to the control. The EPR area values correlate very well (R^2 = 0.92) with the Fe levels of the samples, suggesting a strong relationship between radical formation and Fe-concentrations.

5.2.1.5 Standard analysis of finished beers (pilot scale)

The results in Table 5.4 validate reasonable consistency over all three trial beers considering that they were produced on a pilot scale.

Table 5.4: Analytical parameters of pilot scale bottled beers: ABV (%), pH, total polyphenols (ppm) and TIPO (ppb) (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

	Control	Mash CV	Wort kettle
ABV (%)	3.85	3.88	3.8
pH	4.51	4.28	4.28
Total polyphenols (ppm)	56	75	84
TIPO (ppb)	198	174	188

The ABV values for all three beers were almost equal. The pH for the control (pH = 4.51) was higher compared to that of both trial beers (pH = 4.28). The total in pack oxygen (TIPO) levels were very good for a pilot scale bottling operation and should

not have any dissimilar influence on the ageing of the beer samples. Only the total polyphenol levels showed some difference between the samples. The control had the lowest level (56 ppm), whereas both treated beers had an increased total polyphenol level. As gallotannins were added during these trials, a slight increase in the total polyphenol level could be expected, as otherwise, the raw materials were the same for all three brews.

5.2.1.6 Analysis of metal ions in finished beers (pilot scale)

The results of the metal ion analysis of the fresh beer samples are shown in Figure 5.9.

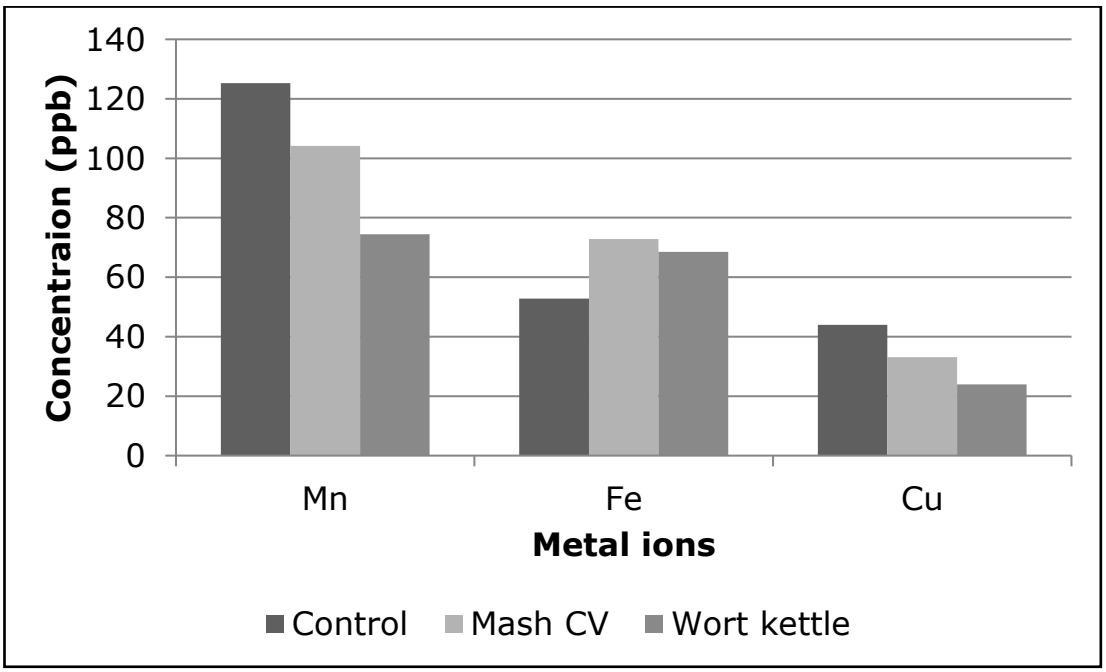


Figure 5.9: Content of Fe, Cu and Me in ppb present in fresh pilot scale beer samples (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The Fe content of the control (53 ppb) was approximately 20 ppb lower than the Fe levels of both treated brews (mash CV 73 ppb and wort kettle 69 ppb). There was no direct correlation between the metal ion measurements in the cooled wort samples

(Figure 5.3) and the levels in the bottled beer. For the control, the Fe pick up during the production processes after the wort cooling resulted in a small decrease (11 ppb). On the other hand, for both treated samples, mash CV (42 ppb) and wort kettle (57 ppb), the Fe content increased considerably. This increase in the treated brews compared to a slight decrease in the control brew is difficult to explain. Due to the nature of small scale operations, the probability of Fe pick up through metal hose connecters, pipes or kegs is always present, but the increase only occurred in the two treated brews, despite all beers having been produced in the same environment. The filtration with the plate and frame filter could have had an influence as the filter sheets were made up out of a mixture of cellulose and kieselguhr. Due to the pH drop from the rinsing water to the filtered beer, an increased release of Fe out of the filter sheets into the first litres of filtered beer could have occurred but this should have limited the high Fe pick up to one of the samples, the one filtered first.

The amount of Cu present in the control (44 ppb) exceeded the amounts present in the mash CV (33 ppb) and the wort kettle (24 ppb). In correlation to the Cu levels in the cold wort, an increase of approx. 20 ppb was observed for all three samples.

The Mn content showed a similar picture as the Cu content with the highest amount detected in the control (125 ppb) compared to slightly lower levels present in mash CV (104 ppb) and substantially lower in the wort kettle (74 ppb). Compared to the cooled wort samples, an increase was detected for all three samples. Compared to the data presented in the literature, a Mn uptake of up to 60 ppb by the yeast during fermentation could be expected but could not be confirmed based on the results shown (Mochaba et al., 1996a).

Compared to levels of Fe, Cu and Mn in commercial British beers (Pohl, 2009), the obtained values are well within the range of large scale production beers.

5.2.1.7 Thiobarbituric acid index (TBI) finished beers (pilot scale)

The results for TBI (448 nm) of fresh and aged pilot scale beer samples are displayed in Figure 5.10.

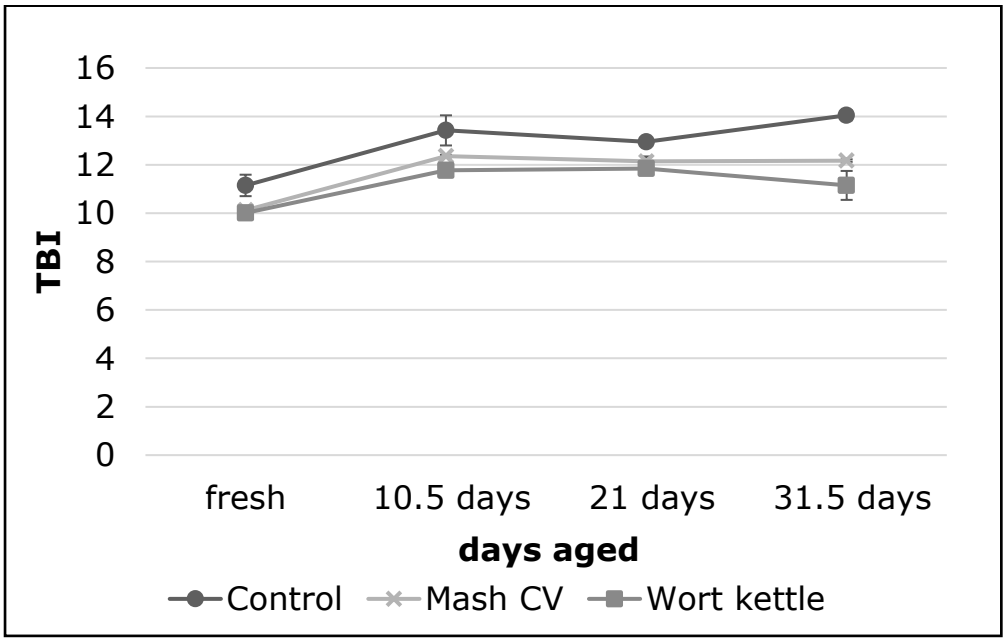


Figure 5.10: TBI (448 nm) of fresh and aged pilot scale beer samples (error bars represent standard deviation of technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The results of the TBI analysis (Figure 5.10) showed only marginal differences between all the different samples, fresh and aged. The values for the control are significantly higher for fresh and aged samples, by exceeding the values for the treated samples mash addition and wort kettle addition by 1 for the fresh, 10.5 days and 21 days aged samples and 2 for 31.5 days aged samples. Nevertheless a difference of only

1 or 2 between TBI values of beer samples is not a significant indicator for the resulting flavour stability. As Maillard reactions play a key role in the formation of dicarbonyl compounds and Strecker aldehydes and these compounds influence the TBI value (Fegredo et al., 2009), a higher increase of the TBI values during storage might have been expected.

Interestingly the TBI values for the wort samples (Figure 5.2) showed a significant difference between the treated brews and the control brew (Control = 21, mash CV = 11, wort kettle = 12), whereas a significant but marginal difference could be observed in the resulting beers. It could be suggested that the reducing power of the yeast during fermentation had a major influence on the TBI values and could explain the discrepancy between the wort and beer TBI values.

5.2.1.8 Total sulphur dioxide finished beers (pilot scale)

Figure 5.11 illustrates the total sulphur dioxide detected in the fresh and aged pilot scale beer samples. In the fresh samples a difference of *ca.* 2 mg/l was observed between the control (5.8 mg/l) and both treated samples (mash CV = 4 mg/l, wort kettle 3.7 mg/l). During the ageing procedure a trend for a decrease in the total sulphur dioxide content could only be observed for the control sample (1 mg/l over the 31.5 days). No trend for a decrease for both treated samples could be observed.

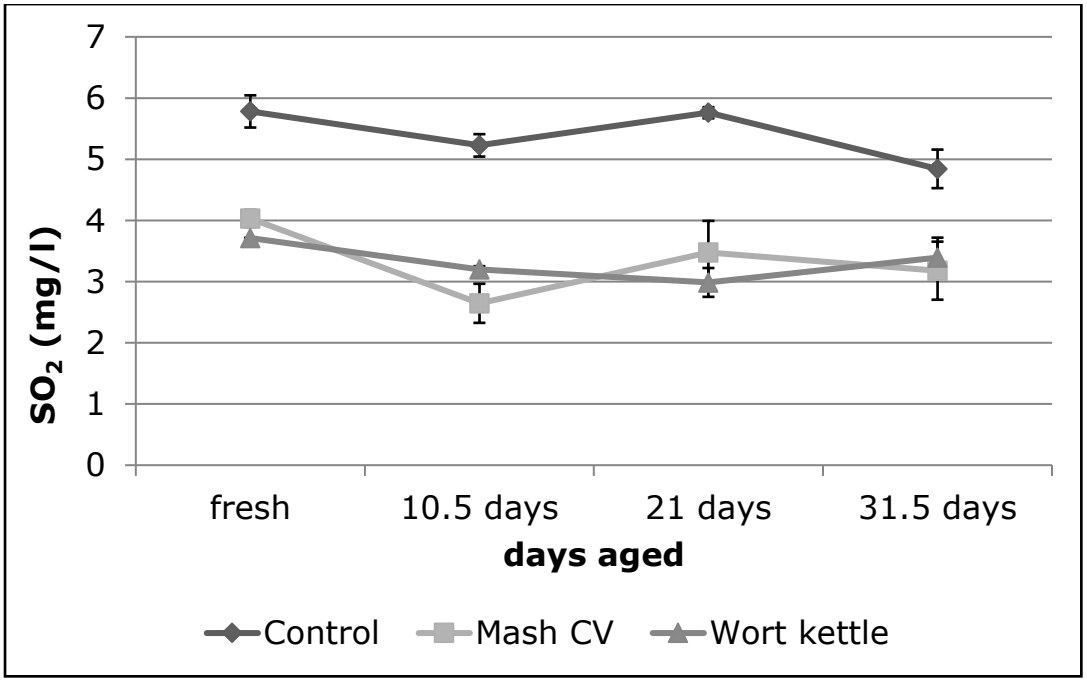


Figure 5.11: Total sulphur dioxide level in fresh and aged pilot scale beer samples (error bars represent standard deviation of technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

A decrease in SO_2 during ageing was expected but the decrease was somewhat limited (Control) or inexistent (Mash CV and Wort kettle). The difference in total of nearly 2 mg/l for the control compared to the two treated brews, was not expected as the same yeast at the same pitching rate was used for the fermentation. The fermentation profile did not give any evidence of issues with the fermentation, all three brews reached the final degree of attenuation between 120 and 140 hours.

5.2.1.9 EPR spectroscopic measurements of beer oxidative potential (pilot scale)

The results for the x0 lag-time presented in Figure 5.12 indicated a longer lag-time of around 35 to 40 min for the control as compared to the trials involving mash or wort kettle addition of gallotannins. The decrease during ageing is very similar for all three

brews resulting in a lag-time reduction of close to 10 min for all three samples. The lag-time values correlate very well with the measured SO_2 levels ($R^2 > 0.94$) for all samples over the entire ageing period.

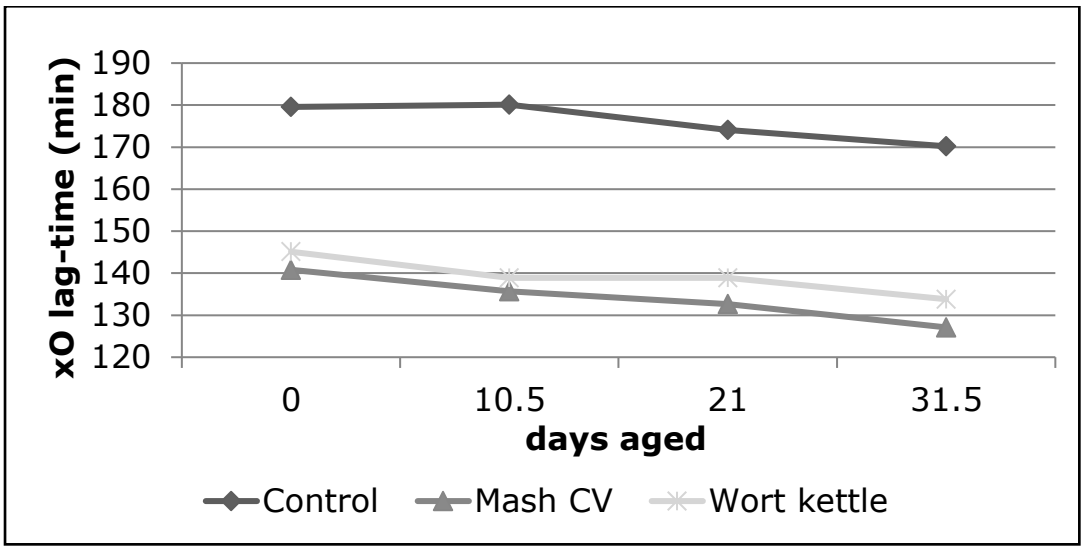


Figure 5.12: x0 lag-time of fresh and aged pilot scale beer samples (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

As longer lag-times indicate a higher anti-oxidant capacity, no positive effect of the gallotannin additions could be observed. The additions actually appeared to have a negative effect on the oxidative stability due to reduced SO_2 levels.

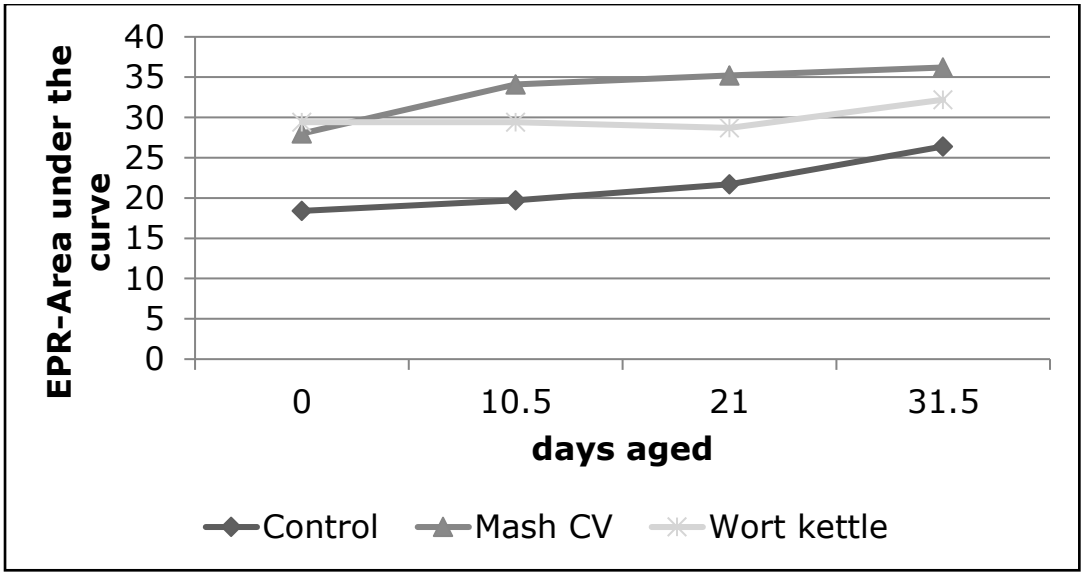


Figure 5.13: Area under the curve of fresh and aged pilot scale beer samples (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The area under the curve values corresponded with the lag-time results by indicating a lower resistance to oxidative forcing (greater radical generation) for the two trial brews relative to the control (Figure 5.13). The increase in area values during storage were not as high for the wort kettle addition (increase of 3 units from fresh to 31.5 days) relative to the control and mash addition beers (increase of 8 from fresh to 31.5 days). This might indicate a greater oxidative stability over time for the wort kettle samples but overall, the values still exceeded those for the control brew even after 31.5 days ageing (control = 26, wort kettle addition = 32).

Based on the EPR spectroscopic results for x0 lag-time and the area under the curve, no evidence of a positive effect of the gallotannin addition on the oxidative stability of packaged beers could be verified. Similar to the pilot scale wort samples, the Fe-

level and the EPR-area for the beer samples showed a high correlation with one another (R^2 = 0.99).

5.2.1.10 GC-MS SPME measurement of staling aldehydes in finished beer samples (pilot scale)

The concentration of aldehydes associated with typical staling off-flavours (papery, bread, almond) generally increase during storage and they play a very important role in flavour degradation during storage. They can be newly formed during storage as a result of Strecker degradation or Maillard reactions as well as be released during storage from the bisulfite bound form (Baert et al., 2015).

Data for Strecker aldehyde concentrations (2-methyl-pronanal, 2-methyl-butanal and 3-methyl-butanal) have been pooled together, to simplify the presentation of the results. The concentrations for furfural, Strecker aldehydes and (E)-2-nonenal after 31.5 days of storage are shown in Figure 5.14.

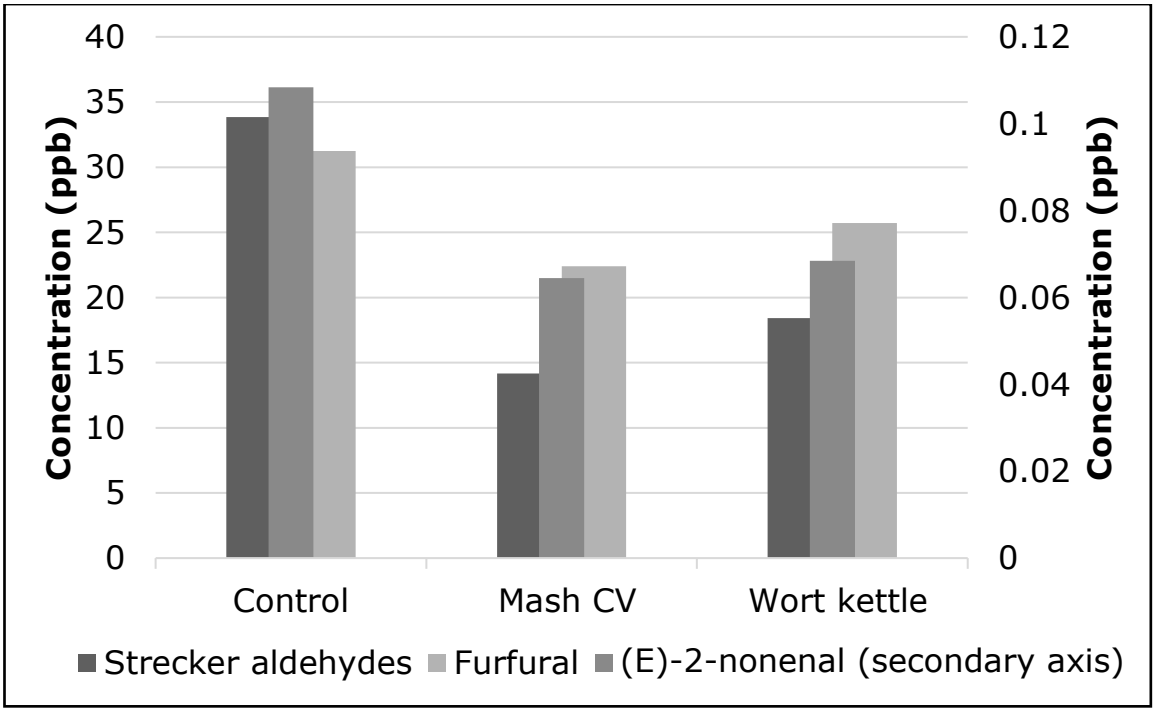


Figure 5.14: Concentration of Strecker aldehydes (total concentration of 2-methyl-propanal, 2-methyl-butanal and 3-methyl-butanal), furfural and (E)-2-nonenal after 31.5 days of storage in pilot scale beer samples (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The aldehyde contents of the control were generally higher compared to the two treated samples. The Strecker aldehyde concentration should be highlighted, as the control contained the highest amount of SO_2 and had *ca.* double the concentration of Strecker aldehydes. During a further ageing procedure, a substantial release could have occurred, due to the predominant binding of the Strecker aldehydes to SO_2 in fresh beer samples. So for the control the concentration of Strecker aldehydes was significantly higher in comparison to the two treated samples after 31.5 days of ageing. The differences between the furfural and (E)-2-nonenal concentrations for the trials versus control were of lower magnitude but still a trend of lower staling aldehyde formation in the gallotannin treated samples was observed.

The lower levels for all staling aldehydes in the treated samples support the positive properties of gallotannins regarding flavour stability. The upstream formation and release as well as the new formation during storage was limited in the treated samples. Nevertheless the low levels of all aldehydes after 31.5 days (below or just above the flavour threshold in beer) made it difficult to draw a clear picture regarding any positive effects on flavour stability.

5.2.1.11 Sensory evaluation of finished beer samples (pilot scale)

The sensory evaluation of fresh and aged beer samples play a very important role in the evaluation of flavour stability. The organoleptic evaluation is often used as a borderline for the determination of flavour stability and shelf life.

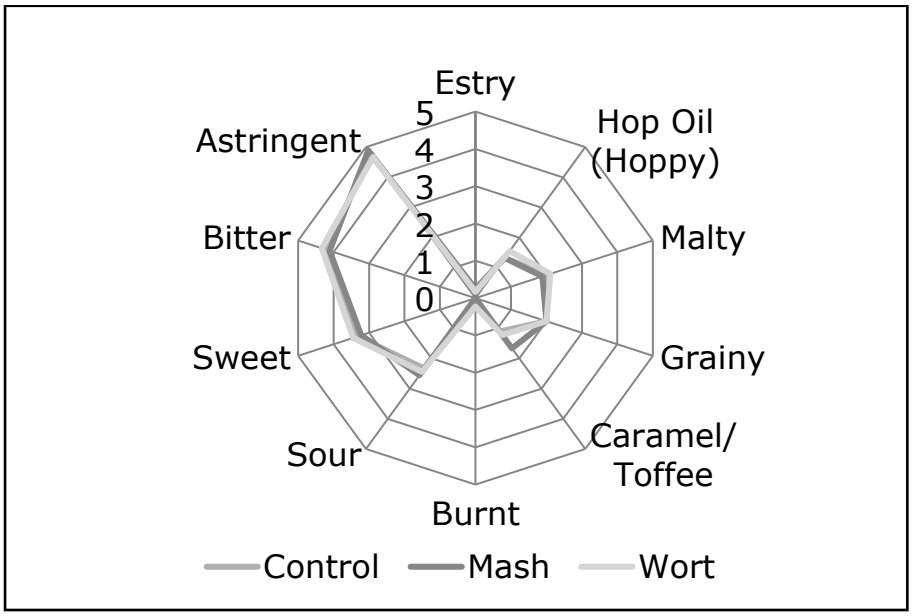


Figure 5.15: Sensory evaluation of flavour profile of fresh pilot scale beer samples (tasters n=9; gallotannin addition: Control = no addition, Mash= 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort= 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

Figure 5.15 compares the fresh flavour profile of the three trial beers. All three samples displayed the same flavour profile and were evaluated similarly. The addition of

gallotannins did not result in any kind of flavour difference in the finished beer samples.

The sensory evaluation of the staling flavours in fresh and 31.5 days aged samples are shown in Table 5.5.

Table 5.5: Sensory evaluation of pilot scale beer samples for staling off-flavours in fresh and 31.5 days aged samples (scale 0-10; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

Sample	Stale flavour (Scale 0-10)			
	Oxidised	Papery	Leathery	Catty
Fresh samples, Taster n=9				
Control	0.8	0.2	0	0
Mash CV	0.1	0.6	0	0
Wort kettle	0.2	0.4	0	0
31.5 days aged samples, Taster n=7				
Control	3.1	1.3	0	0.1
Mash CV	2	0.7	0	0
Wort kettle	2.6	1.3	0	0

The levels of staling aldehydes in the fresh samples for all three trial brews were very low or not present at all. This indicates no effect of the pilot scale production process on the stale flavours and an equal starting point for the sensory evaluation of the three different samples.

The sensory evaluation of the 31.5 days aged samples for leathery and catty showed these characters were not detected by the tasting panel. The papery flavour slightly increased during storage but the very low scores did not indicate a considerable increase. This observation correlates with the measured (E)-2-nonenal levels after 31.5 days, as the flavour threshold was not or only barely exceeded (Figure 5.14) in the three samples. Only the oxidised stale flavour scores significantly increased during storage and are presented in Figure 5.16 including the standard deviation of the sensory evaluation.

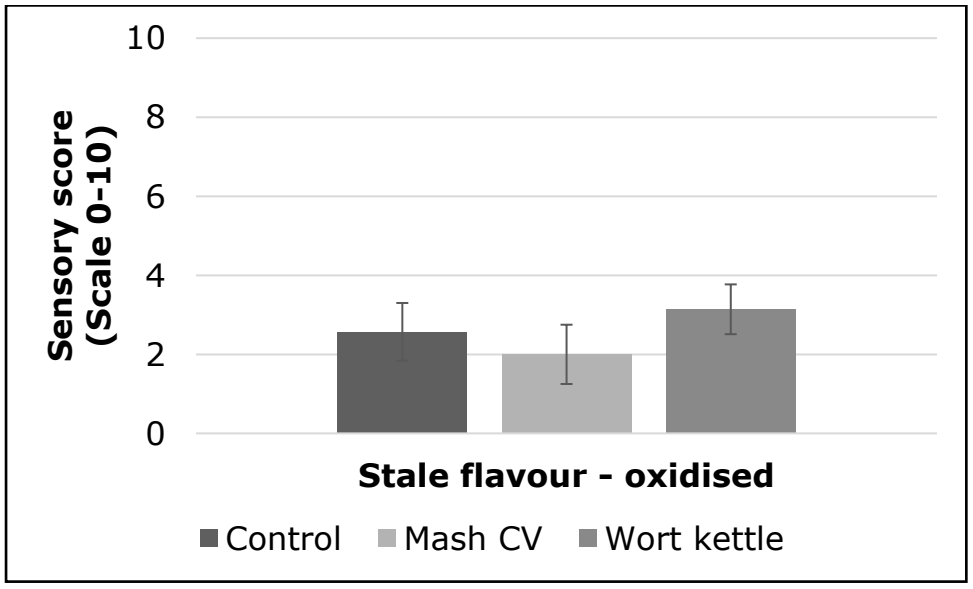


Figure 5.16: Sensory score for stale-oxidised after 31.5 days of ageing on a sensory scale of 0-10 (error bars represent standard deviation between individual tasters, n=7; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The difference between the scores for 'oxidised flavour' were not significant between the three sample treatments. The addition of the gallotannins did not show any positive effect on the sensory evaluation of beers through ageing.

5.2.1.12 Summary: pilot scale brewing trials with/ without the addition of gallotannins

For both mash CV and wort kettle gallotannin additions an improved oxidative stability in the wort could be observed. Both additions showed essentially the same benefits in cooled wort as measured by TBI and EPR metrics. Furthermore gallotannin addition substantially reduced the Fe levels in cooled, clarified wort. This indicated a potential benefit of brewhouse gallotannin addition on flavour stability. Nevertheless results showed that this potential was not realised in terms of enhanced flavour stability metrics in finished beer.

The low sensory scores and the very limited reduction of total SO_2 levels during storage results led to an assessment of the warm room used for the forced ageing test. Unfortunately the warm room was not able to uniformly provide the required temperature of 28 ˚C to 30 ˚C throughout the entire warm room. The temperature probe was mounted directly under the ceiling (15 cm below ceiling), at a similar height as the heating device (30 cm below ceiling). As the heating system had no ventilation system attached, the air inside the warm room was not agitated and the heater stopped as soon as the air temperature around the temperature probe in only a limited area under the ceiling reached 30 ˚C. Therefore only 20 ˚C to 22 ˚C were reached at the bottom of the warm room, where the beer samples were stored. Consequently the different samples were not aged at the required temperature of 28 ˚C to 30 ˚C. This could explain the relatively modest staling aldehyde concentrations and rather high SO_2 levels in the aged samples. Nevertheless all samples were aged following the same temperature regime.

For the pilot scale trial the influence of the gallotannin addition on beer flavour stability was thus somewhat inconclusive and it was decided to repeat trials at

production scale to further investigate the influence of the gallotannin addition on beer flavour stability.

5.2.2 Production-scale results

The results of the pilot scale trials are presented as averaged results for the different control, mash addition and kettle addition trial brews.

5.2.2.1 Standard analysis of finished beers (production scale)

The results for the general beer analysis (Table 5.6) showed consistency across all three addition points for production scale trials.

Table 5.6: General beer analysis of large scale beer samples (OG = original gravity, PG = present gravity; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

	Control	Mash CV	Wort kettle
ABV (%)	4.01	3.98	3.92
pH	4.21	4.2	4.33
Total polyphenols (ppm)	139	163	159
OG (calculated with Balling)	35.11	33.65	35.55
PG	4.25	2.7	5.1
TIPO (ppm)	< 250	< 250	< 250

Only the beer produced from the wort kettle addition trial had a higher pH as compared to the other samples and the degree of attenuation of the mash addition exceeded the other samples resulting in a slightly lower OG and PG. The total polyphenol content

was in the expected range and showed a general increase for the gallotannin-treated samples.

5.2.2.2 Thiobarbituric acid index (TBI) finished beers (production scale)

The TBI values for all samples are shown in Figure 5.17.

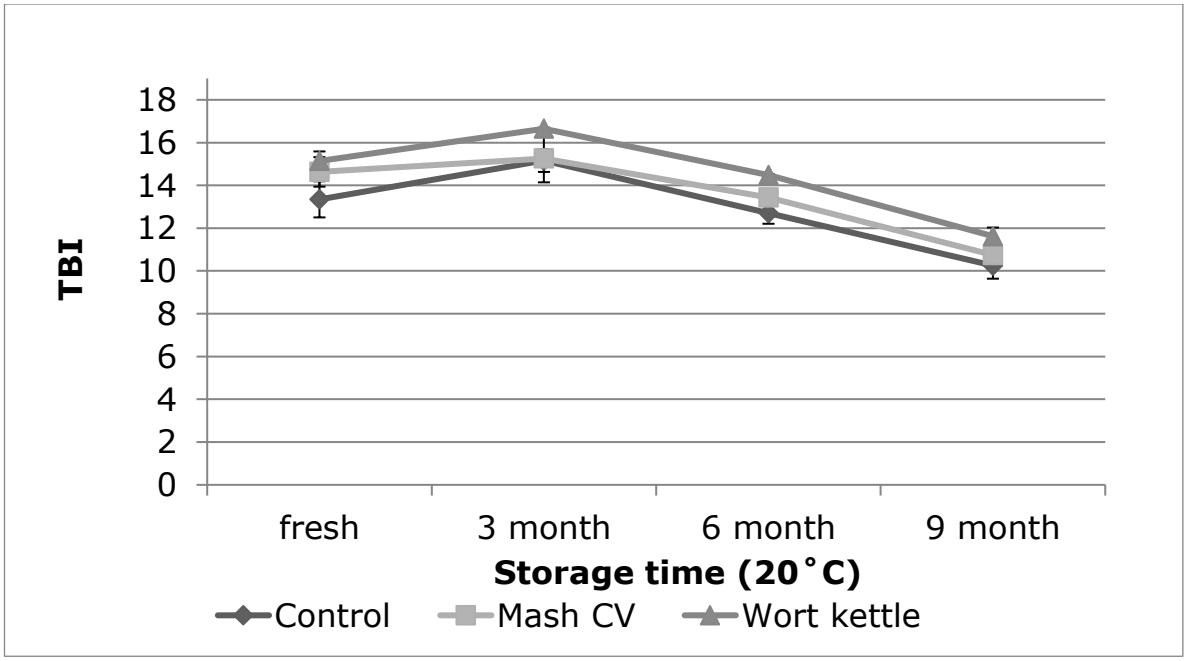


Figure 5.17: TBI (448 nm) of fresh and aged large scale beer samples (error bars represent standard deviation technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The TBI values for all samples (Figure 5.17) were very similar for all three samples. A difference in TBI of 2 was detected in the fresh samples and no significant difference between the samples based on the TBI values could be observed. The trend over the entire storage period was very similar for all samples showing a small increase after three month but decreasing values after 6 months and 9 months. This was an unexpected finding as the increased formation of Maillard reaction products and the release of Strecker aldehydes during storage should result in an increase of the TBI

value (Li et al., 2015). A repeated analysis of all samples utilizing a different batch of thiobarbituric acid confirmed this trend for all samples.

5.2.2.3 Analysis of metal ions in finished beers (production scale)

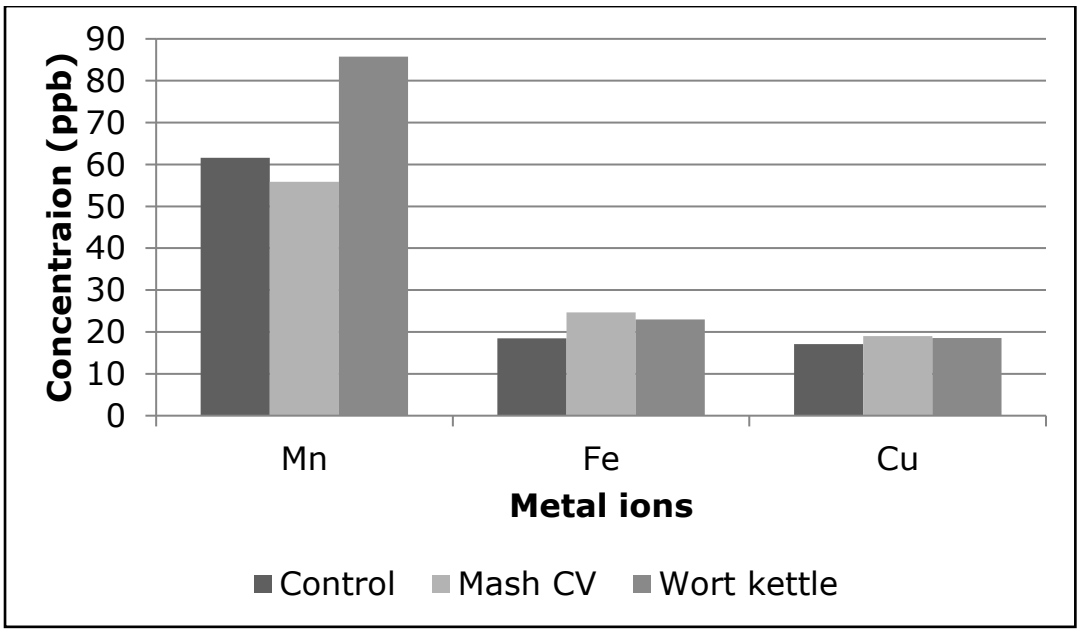


Figure 5.18: Metal ion content (Fe, Cu, Mn) present in fresh production-scale beer samples (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The Mn-levels for control and mash addition were very similar at *ca*. 60 ppb but the wort kettle addition had a significantly higher level of 85 ppb. The Mn content is largely influenced by the raw materials used as well as the fermentation process (Zufall and Tyrell, 2008),. As the brewery has a malting facility directly on site and the batches of raw materials used are very large, the influence of raw materials variation should have been limited. In this case the uptake during fermentation might have had a bigger influence. The correlating lower SO_2 value (Figure 5.19) for the wort kettle addition suggests a different performance of the yeast during fermentation that could somehow have influenced the Mn level in the finished beer.

The Cu levels for all samples were very similar to one another and in the region of 17 to 19 ppb. The gallotannins addition did not make any difference in the resulting Cu levels in the finished beer sample.

The Fe content for all samples ranged between 19 ppb and 25 ppb. The very modest difference between the Fe levels did not indicate an influence of the addition of gallotannins. In general the Fe-levels were low when compared to other commercially available British beers which have been reported to contain between 67 - 500 ppb (Pohl, 2009).

5.2.2.4 Total sulphur dioxide in finished beer samples (production scale)

The results for the total SO_2 levels (Figure 5.19) displayed a very similar trend for control and mash addition.

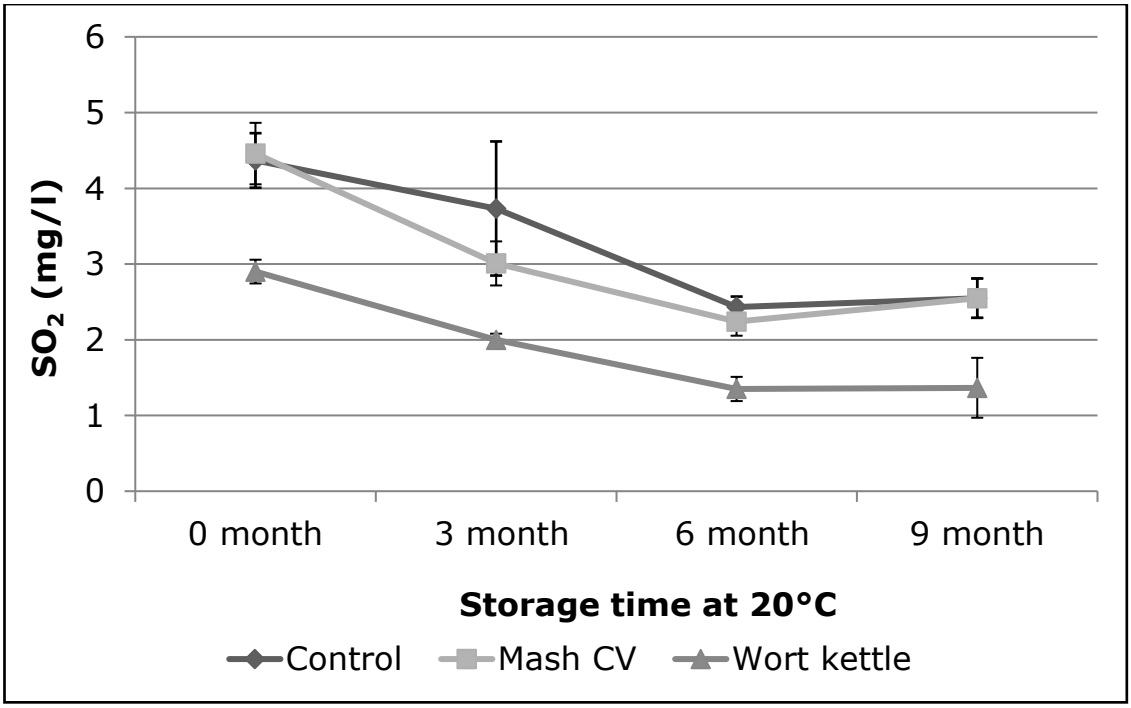


Figure 5.19: Total sulphur dioxide level (mg/l) present in fresh and aged large scale beer samples (error bars represent standard deviation of technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

A constant decrease over the first 6 months storage, of approx. 45% levelling out from 6 to 9 months could be detected. The wort kettle addition trial had a much lower amount of SO_2 present in the fresh beer sample but the trend over 9 months showed a very similar pattern compared to the other two samples.

As the SO_2 level is predominantly defined by the amounts released by the yeast during fermentation, the initial lower content in the fresh sample of wort kettle suggests a difference in the fermentation performance. Nevertheless based on the monitoring of temperature and gravity during fermentation, no inconsistency could be reported.

5.2.2.5 EPR spectroscopy measurements of beer oxidative potential (production scale)

The results for the oxidative stability are displayed in Figure 5.20.

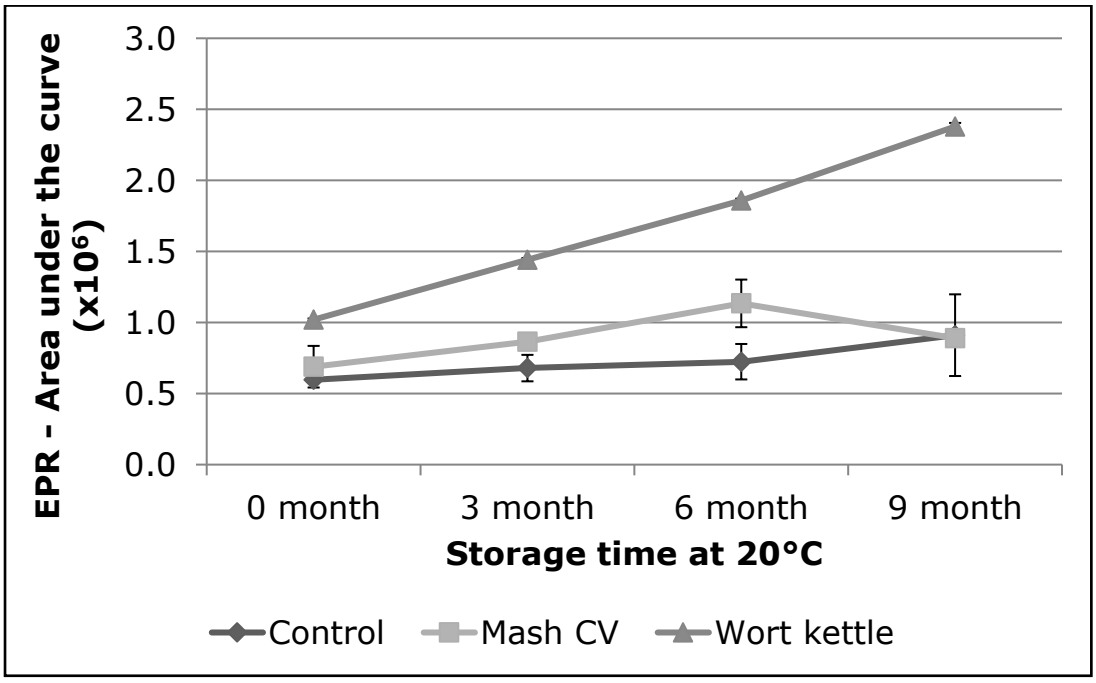


Figure 5.20: Oxidative stability evaluated by EPR (area under the curve) for large scale beer samples (error bars represent standard deviation of technical replicates, n=3, gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The EPR spectroscopic results for beer from the wort kettle addition trial had the highest area under the curve and therefore the lowest oxidative stability of the trial beers measured in this study. This trend was present in the fresh beer and continued through storage, resulting in an increase of nearly 300% after 9 months. These findings correlated with the low SO_2 level (3 mg/l) and a relatively high Mn level in this beer, compared to the other two samples (30 % higher). The lower SO_2 level directly results in a lower resistance against oxidative processes. The involvement of Mn in radical generation in beer has not been investigated as thoroughly as for Fe and Cu, but Mn is

capable of catalysing reactions that produce ROS with a consequent impact on the EPR area values. Furthermore Mn is reported to act synergistically along with Fe and Cu to catalyse oxidative staling reactions (Mochaba et al., 1996b, Kaneda et al., 1999). This could explain the lower oxidative stability of the wort kettle addition sample. Furthermore the pH was slightly higher for the wort kettle addition sample in comparison to the other two samples. A higher pH was described to result in increased radical formation (Kunz et al., 2011).

The EPR area under the curve metric for the control beer increased by *ca.* 20% over the entire storage time. This indicates an excellent oxidative stability and could be attributed to the very low Fe level (19 ppb) and the moderate SO_2 content (4.4 mg/l). Nevertheless the low decrease of the oxidative stability over 9 months is very difficult to explain, as the level of one of the main antioxidants, SO_2, decreased by over 2 mg/l and hence the area values should increase accordingly. For the control and mash addition samples the initial EPR area values in fresh beer were very similar and after 9 months storage time, the area values were in the approx. the same range. This is in accordance with the very similar amounts of Fe, Cu and Mn and the total SO_2 level present in both samples.

Figure 5.21 displays the results for the x0 lag-time of the production-scale beer samples.

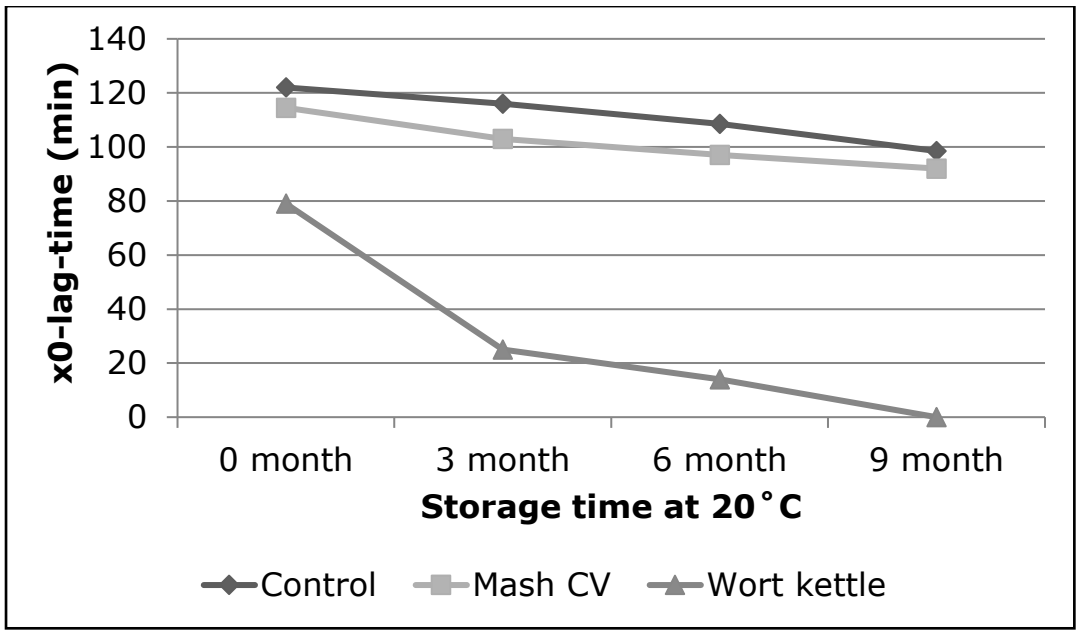


Figure 5.21: Oxidative stability evaluated by EPR (x0-lag-time) for large scale beer samples (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

A very similar trend as for the EPR area could be discerned. Control and mash addition samples had a significantly longer lag-time compared to the wort addition sample. The wort kettle sample had the shortest lag-times of all samples resulting in essentially no lag-time after 9 months of storage. Lag-time values for the control were slightly higher compared to the mash addition.

As the lag-time is reported to be typically related to the SO_2 level (Kunz et al., 2012), Table 5.7 shows the correlation between lag-time and SO_2 over the 9 month storage period.

Table 5.7: Correlation between the xO-lag-time and the SO_2 levels of the large scale beer samples (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

Correlation x0-lag-time and SO_2 level	R^2 - values
Control	0.8
Mash CV	0.88
Wort kettle	0.94

For wort kettle and mash addition a very high correlation was identified. The control samples showed a lower but an acceptable correlation between lag-time and SO_2 level.

5.2.2.6 GC-MS SPME measurement of staling aldehydes in finished beer samples (production scale)

The combined results for the three measured Strecker aldehydes, 2-methyl-propanal, 2-methyl-butanl and 3-methyl-butanal, are presented in Figure 5.22.

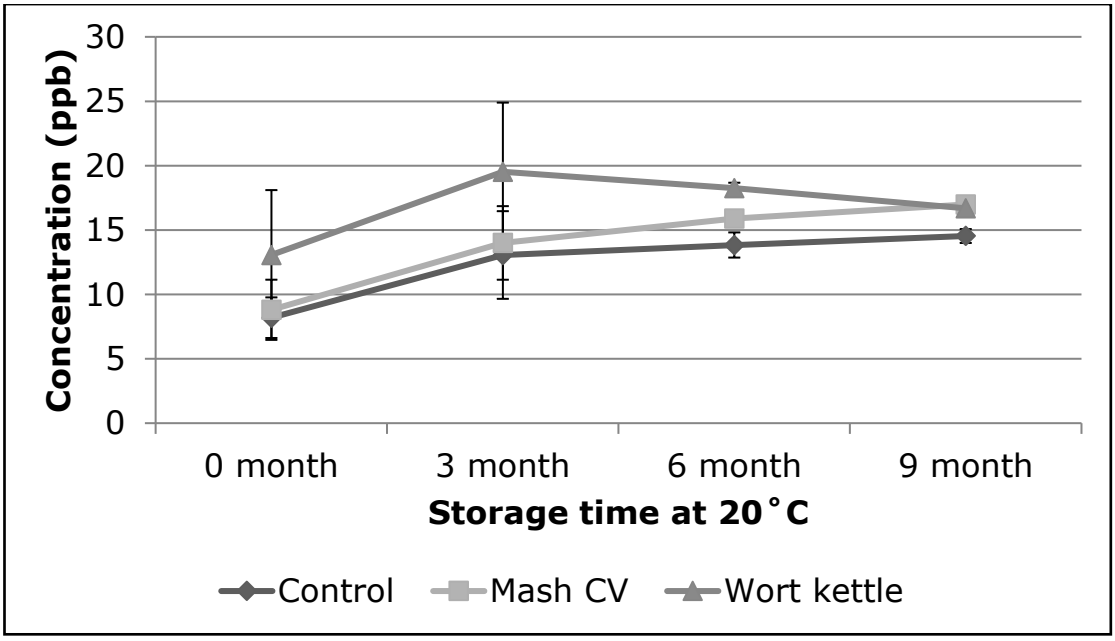


Figure 5.22: Summed concentration (ppb) of Strecker aldehydes (2-methyl-propanal, 2-methyl-butanl and 3-methyl-butanal) present in fresh and aged large scale beer samples (error bars represent standard deviation of technical replicates, n=3, gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The combined results for the three measured Strecker aldehydes, 2-methyl-propanal, 2-methyl-butanl and 3-methyl-butanal, showed a similar trend over 9 month for the Control and Mash CV samples: A rapid increase in concentration during the first three months and a very slow concentration rise over the next six month storage period. The concentration in the fresh beer for the wort kettle sample was higher (4 ppb) compared to the other two samples. The overall trend is difficult to explain, as an increase after three month is followed by a decreasing concentration after 6 month and 9 month. A constant increase would have been expected, as reported previously (Methner et al., 2003). The high standard deviation for the fresh and three month old samples made it impossible to confirm a statistical difference between the sample treatments.

The combined Strecker aldehyde concentration data correlate well with the SO_2 levels measured in the samples (Table 5.8). This corresponds with the belief presented in the literature; Strecker aldehydes are predominately bound to SO_2 in fresh beer samples and released during storage (Dufour et al., 1999, Baert et al., 2012, Baert et al., 2015).

Table 5.8: Negative correlation between the concentration of the Strecker aldehydes and the SO_2 levels of the large scale beer samples (gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

Sample	Negative correlation (R^2)
Control	0.97
Mash CV	0.94
Wort kettle	0.88

Figure 5.23 displays the furfural concentration present in fresh and aged beer samples obtained during the production-scale trials.

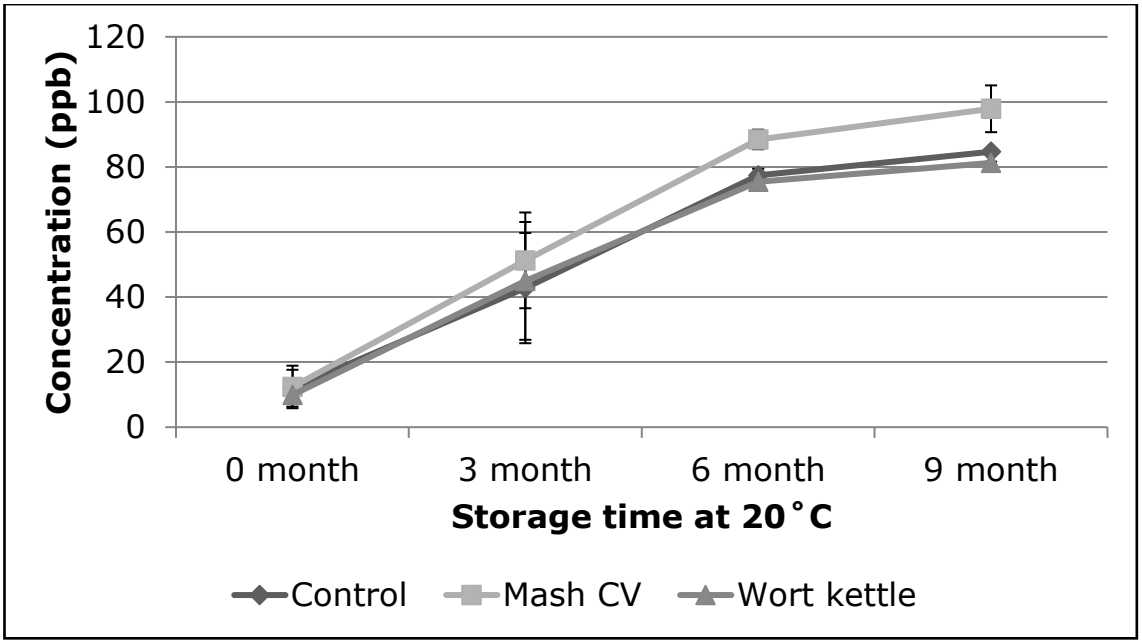


Figure 5.23: Furfural concentration (ppb) present in fresh and aged production-scale beer samples (error bars represent standard deviation of technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

For all samples, the initial furfural concentration (ppb) detected in the fresh beer samples, increased *ca*. 4 to 5-fold during the storage time of 9 months (Figure 5.23). The rate of increase was consistent for the first 6 months with a slightly lower increase during the last three months of storage. The overall concentrations did not differ between the samples over the first three months. For the mash addition samples higher levels of furfural compared to the other two samples could be detected (approx. 15 ppb) after 6 and 9 months of storage.

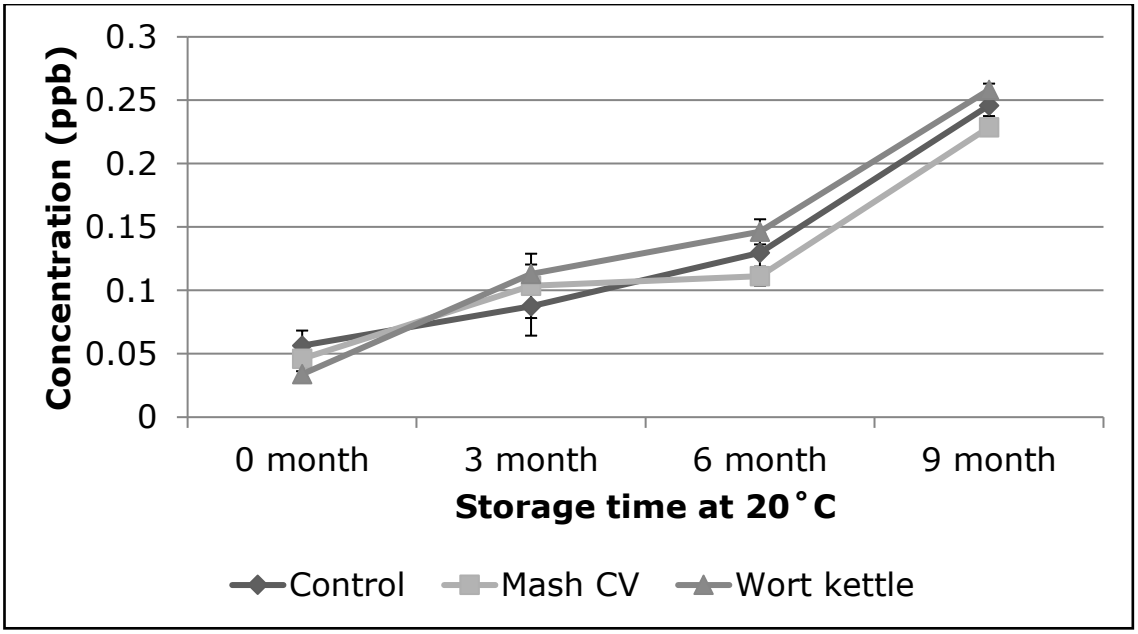


Figure 5.24: (E)-2-nonenal concentration (ppb) present in fresh and aged large scale beer samples (error bars represent standard deviation of technical replicates, n=3; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The (E)-2-nonenal concentrations (ppb) detected are presented in Figure 5.24. For all samples a clear increase in concentration over the 9 month storage period could be observed, with a moderate increase over the first 6 months (100 %) and a steep increase of over 0.1 ppb compared to 6 month during the last three months of storage. No clear differences in the formation of (E)-2-nonenal were observed between the different trial conditions.

5.2.2.7 Sensory evaluation of finished beer samples (production scale)

The flavour profile of the fresh production scale beer samples is presented in Figure 5.25.

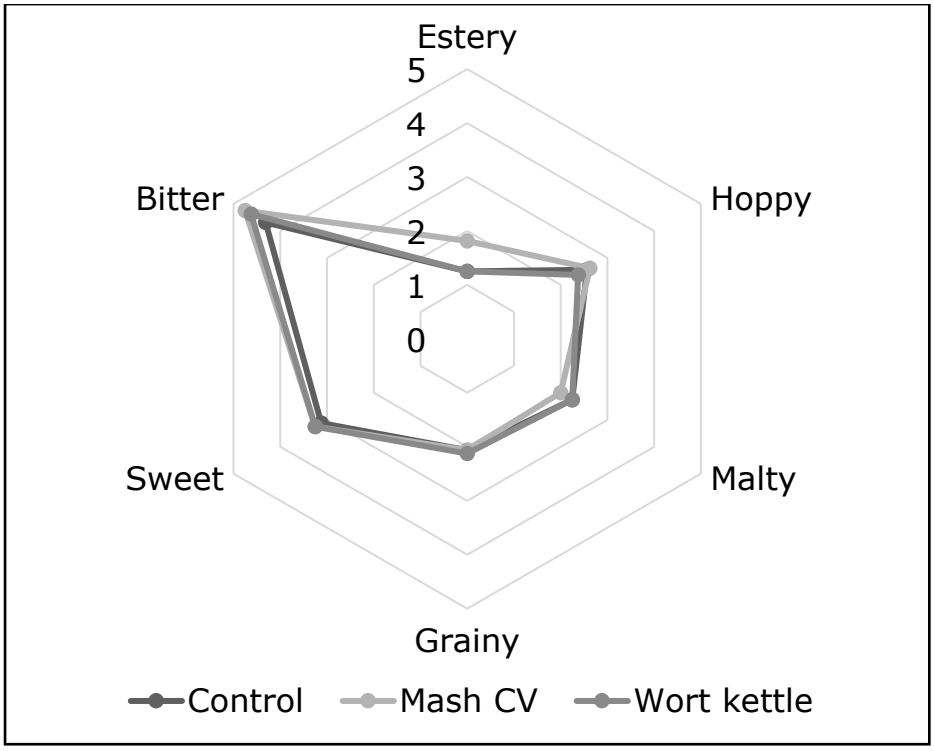


Figure 5.25: Sensory evaluation of flavour profile of fresh pilot scale beer samples (tasters n=8; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

The flavour profiles for all three fresh beer samples were very similar. A slightly higher estery flavour was detected for the mash addition samples. Nevertheless the addition of the gallotannins had no obvious effect on the flavour profile.

The sensory assessment of aged beers was focused on four stale off-flavours, stale-leathery, stale catty, stale-papery and stale-oxidised. Over the ageing period of 9 months (storage temperature 20 ˚C), for catty and leathery off-flavours, a score of 1 was not exceeded, even after 9 months. Therefore according to the results of the tasting sessions, both off-flavours did not occur during the ageing period.

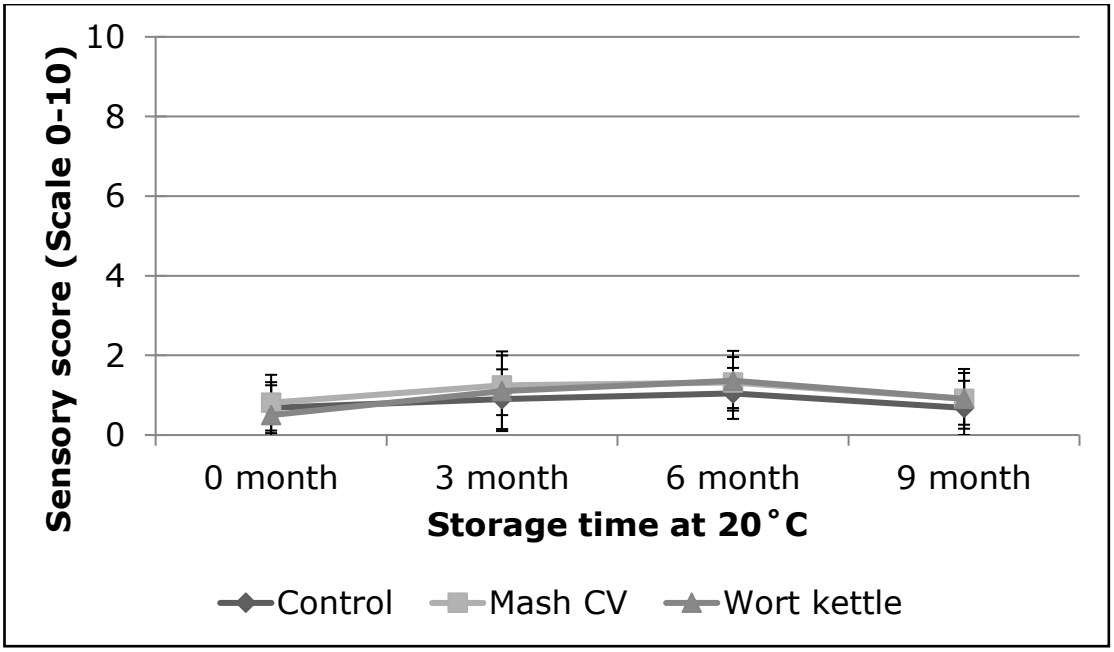


Figure 5.26: Sensory score (scale 0-10) for attribute stale-papery for production-scale beer samples (error bars represent the standard deviation between the individual tasters n=8, gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

Based on the sensory evaluation of the stale-papery off-flavour (Figure 5.26), no significant increase was detected during the storage trial. For all samples the sensory score only marginally exceeded a score of 1 resulting in no difference between the samples for the papery off-flavour. Furthermore the results showed no detectable papery off-flavour even after 9 month of storage.

Since the papery off-flavour is commonly associated with the amounts of (E)-2-nonenal present in the sample (Rodrigues and Almeida, 2009, Saison et al., 2009), no correlation occurred between the up to 5-times increased amounts of (E)-2-nonenal measured (Figure 5.24) and the sensory evaluation for papery.

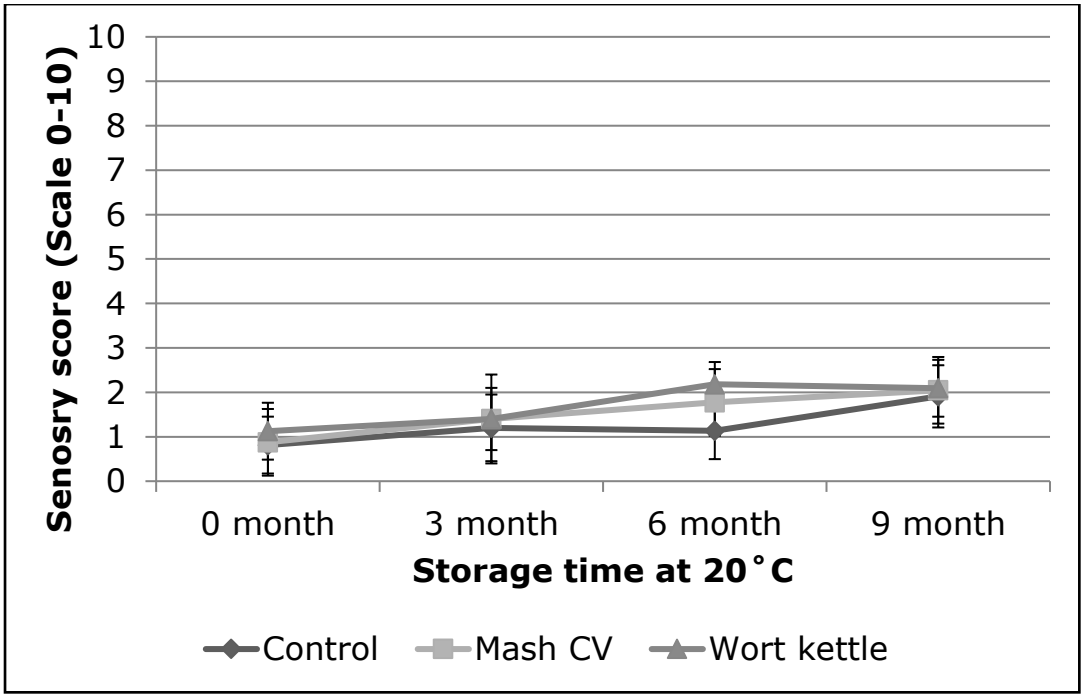


Figure 5.27: Sensory score (scale 0-10) for attribute stale-oxidised for large scale beer samples (error bars represent the standard deviation between the individual tasters n=8; gallotannin addition: Control = no addition, Mash CV = 3 g per hl finished beer mash conversion vessel addition during mashing in, Wort kettle = 2.5 g per hl finished beer wort kettle addition 10 min before the end of boil)

Figure 5.27 illustrates the sensory scores for stale-oxidised. Similarly to the other three investigated stale flavours, the scores for all samples were low even after 9 months of storage. For all samples, the score only increased by 1 over the 9 month period. Based on a scale of 0 -10, this increase could not be considered as significant, especially with the deviation associated with sensory evaluation.

In general the sensory evaluation did not correlate well with the measured staling aldehydes. For all aldehydes a significantly increased concentration exceeding the flavour threshold for all measured aldehydes (Saison et al., 2009) could be detected whereas the sensory scores only marginally increased. For future work, an adapted set of staling attributes for the sensory panel to describe the off-flavour might be useful

as the attributes are not directly correlated with the flavour attributes of the analysed aldehydes. In addition no clear correlation between the stale-oxidised flavour and the oxidative stability (Figure 5.20) could be identified. For all samples a correlation between oxidative stability and sensory scores of $R^2 = 0.7$ was not exceeded, a direct connection was questionable. Overall the sensory results were very good for 9 month old samples, with beers not showing real signs of any formation of stale flavours during storage. As the Molson Coors Brewing Company sensory expert panel is well trained, the sensory results would not be challenged, however almost not detecting any flavour degradation was very surprising and somehow difficult to explain, as the chemical assays displayed clear evidence of reduced flavour stability of the 9 month of storage.

5.2.2.8 Summary: large scale trials

Overall the three produced beer samples were similar. The general beer analysis and evaluation of the flavour profile showed great consistency in the large-scale production process. This displayed the benefits of production scale environment: increased consistency and less variation.

The TBI values for the beer samples did not indicate any positive effect regarding the formation of Maillard products or other aldehydes for any of the two gallotannin additions in comparison to the control brew.

Lower SO_2 levels were reported for the wort kettle addition. A direct explanation for the lower level could not be given as pitching rate and fermentation profile were very similar for all trial brews.

Both EPR metrics, area and lag-time, showed similar results for control and mash addition sample whereas the wort kettle addition trial performed significantly worse

in terms of EPR flavour stability metrics. To a certain extent the lag-time correlated with the SO_2 levels, nevertheless R^2 values of 0.85 could suggest the influence of other antioxidants on the lag-time values. A strong correlation between the Fe levels in the beer samples and the area values was detected. Furthermore the low SO_2 level and the higher Mn content for the wort kettle addition sample could have influenced the oxidative stability. In general the levels of Fe, Cu and Mn were very low for all samples.

The aldehyde formation was very similar for all three samples and a good correlation between Strecker aldehyde levels and SO_2 levels was identified. This finding agrees with the current state of scientific knowledge relating to beer staling.

The sensory analysis of oxidised, papery, leathery and catty did not reveal any significant differences between the samples. Even after 9 months of storage no significant difference for any of the attributes between the samples could be described as well as no substantial increase of any of the attributes was recognised. Other sensory attributes to describe stale flavours might have been better able to identify differences between the samples over the storage period. In general all beer samples tasted surprisingly good after a storage time of 9 month at 20˚C.

5.3 Conclusion

The presented results make it difficult to confirm the ability of gallotannins added in the brewhouse to improve the flavour stability of finished beer samples. A remarkable improvement of the analytical results for the wort samples was described for both additions, proving the ability of gallotannins to reduce the formation of Maillard products and other aldehydes in wort and demonstrating a significant increase in the oxidative stability of wort. The EPR measurements showed strong evidence for the

radical scavenging and metal chelating properties of gallotannins, regardless of the brewhouse addition point. The more traditional thiobarbituric acid index (TBI) displayed the positive effect of gallotannin addition on reducing the formation of aldehydes used to evaluate the brewhouse performance and overall the wort produced using gallotannin additions complied with all requirements to result in an increased flavour stability in the finished beer in comparison to the control brew. However, the improvements for both addition points in the wort did not clearly transfer to the finished pilot scale beer samples. Oxidative stability measures via EPR spectroscopy as well as TBI, aldehyde formation and sensory results did not result in a conclusive improvement in the finished beer.

The production scale beer samples showed a similar result with no significant improvement in flavour stability for the treated beer samples. The used chemical flavour stability indices and the sensory evaluation of a specially trained expert panel were not able to report any differences in flavour degradation for the different samples. The samples were aged for 9 month at 20 ˚C up to the end of the best before date and showed only very limited flavour degradation for all three samples, control and two samples with gallotannin addition (sensory scores for stale flavours below 2 on a scale of 0-10). Therefore any influence of the gallotannin addition could not be depicted. Using a different brand for these trials might have had a better chance of discerning a positive impact of gallotannin addition on beer flavour stability. Based on the presented results, an addition of gallotannins in the brewhouse, either in the mash or wort did not improve the flavour stability of the finished beer samples.

6 Modelling beer flavour stability as a function of its key factors: interactions between SO_2, dissolved oxygen, metal ions, hop acids, polyphenols and thiol concentrations in a commercial lager

6.1 Introduction

One of the most important quality control problems faced by the brewing industry is the flavour instability of the finished product. To improve the flavour stability and to enable the brewer to extend the shelf life of packed beer, there is a need to understand the underlying mechanisms that may cause flavour changes during beer aging. The main factors influencing the rate of flavour change during ageing are widely acknowledged (Vanderhaegen et al., 2006, Bamforth and Lentini, 2009, De Schutter et al., 2009). These factors include the dissolved oxygen (DO) levels of wort and beer throughout the production process and the oxygen levels found in packed beer. The oxidative reactions that are implicated in flavour stability are not related to ground state oxygen, rather to reactive oxygen species (ROS) (Bamforth and Parsons, 1985). Pro-oxidants such as transition metal ions play a very important role in the formation of these ROS (Kaneda et al., 1989, Zufall and Tyrell, 2008) and therefore can directly reduce flavour stability. On the other hand, anti-oxidants play an important role in retaining the freshness of beer. Deriving from the conversion of sulphate or sulphur containing amino acids as intermediate product of sulphur-containing amino acid synthesis by yeast during fermentation, the main anti-oxidant present in a packed beer sample is SO_2 (Ilett, 1995). The high anti-oxidant potential is related to a two-electron

reaction with peroxide (Andersen et al., 2000) that yields non-radical products. Furthermore SO_2 reduces the staling flavour impact of carbonyl compounds by forming α-hydroxy-sulfonates (Kaneda et al., 1999). Due to their anti-oxidant properties (Pietta, 2000), the positive effect of polyphenols on beer flavour stability has been reported (Walters et al., 1997b, Kaneda et al., 1995, Krofta et al., 2008, Aron and Shellhammer, 2010). Furthermore an improvement of flavour stability correlated to protein thiol group containing compounds has been described, based on the reduction capacity during the ageing process (Lund and Andersen, 2011, Wu et al., 2011). Thus many factors that may influence flavour stability have been examined but much less is understood about the relative importance of each factor and the ways in which the factors interact with one another to determine product stability. To study the relative significance of single factors and the interplay between different factors, an experimental design approach has been used. The levels of total in-pack oxygen (TIPO), Fe, SO_2, iso-α and α-acids, (+)-catechin and glutathione in a commercial lager have been varied to investigate the different factors (and interactions between them) which significantly impact on the flavour stability of a lager beer across a defined design space.

6.2 Material and methods

6.2.1 Experimental design

Design Expert 6 Software (Stat-Ease, Mn, USA) was used for the experimental design. A two factorial design with centre points was utilized to determine the design space. The design included 5 factors for two separate models (Models A and B) in the initial design space. For Model A the 5 factors included TIPO, total SO_2, Fe level, iso-α-acid

level and α-acid level and for Model B, TIPO, total SO_2, Fe, glutathione and (+)-catechin levels were varied. Forced ageing (30 ˚C and 3 time points: fresh, 30 and 60 days aged) was added into the design as an additional factor during the processing of the data. Forced ageing was not considered as an initial factor of the design space, as each sample in the design space was aged in an identical manner. To achieve a resolution V factorial design, 22 samples were prepared per model approach (16 samples, 4 centre points and two base beer samples). The modelling of the data was based on progressive factor reduction. Factors which were of least significance ($p > 0.05$) were excluded from the model until the included factors were significant ($p < 0.05$) and the model R^2 was maximised. If a factor was not significant ($p > 0.05$) but was involved in a significant ($p < 0.05$) two factor interaction, the factor was incorporated into the model but the single factor was not assessed. The applied design allowed the investigation of interactions where two factors combined produced an effect beyond the additive effect of each factor alone. The use of centre points allows for tests for linearity across the design space and to determine the error of the system.

6.2.2 Preparation of the base beer prior to compound addition

The trial design included the preparation of the same base beer for the two Models A and B. The aim was to obtain a base beer with very low Fe content, low bitterness and a low total polyphenol content to study the effects of the additions of the different compounds. The base beer was a high-gravity lager-style beer produced in a full-scale brewing process up to pre-conditioning. The base beer for the trials was taken out of the full-scale production process directly after centrifugation between the transfer of the batch from fermentation tank to the conditioning tank. The beer was filled in CO_2-purged 50 l kegs and conditioned (2˚C) for 4 days. The conditioning was followed by

a filtration step with iron-free cellulose filter sheets from the 50 l kegs into a pilot plant conditioning tank (8 hl), equipped with a cooling jacket and a mixing device. The beer was diluted in the conditioning tank to 4 % ABV utilizing RO-water from the full-scale production process to minimize any Fe and oxygen uptake from the water used for dilution. The dissolved oxygen level of the finished base beer in the pilot-scale conditioning tank was reduced from 25 ppb to 5 ppb directly after the filtration by gas washing with CO_2 (20 min at 2 ˚C). The beer was stored cooled (2˚C) prior to further processing.

6.2.3 Trial design

6.2.3.1 *Trial design Model A*

The trial design for Model A is shown in Table 6.1. The values of the factors were agreed in conjunction with the Molson Coors Brewing Company research and development department.

Table 6.1: Trial design for Model A: Concentrations of different factors in Model A.

Factor	Low	Centre Points	High
TIPO (ppb)	65 ± 20	65 ± 20 and 370 ± 30	370 ± 30
SO_2 (mg/l)	4	7	10
Fe (ppb)	30	75	120
Iso-α-extract (IBU)	3	7.5	15
α-extract (mg/l) (addition)	0	2	4
Ageing (30°C)	Fresh, 30 and 60 days for all samples		

For the TIPO, the low value of 65 ppb was the lowest achievable value utilizing the pilot plant bottling line. The high value of 340 to 400 ppb was chosen based on the quality criteria for large scale production currently operated at Molson Coors Brewing Company. If TIPO levels exceed 350 ppb in a particular batch then this batch will be investigated and potentially not used for distribution. For the centre points for TIPO, two samples with low TIPO level (65 ± 20 ppb) and high TIPO level (370 ± 30 ppb) were prepared. This was necessary as for the TIPO no constant level of ca. 220 ppb could be achieved with the utilized bottling facilities. The design space was adopted accordingly to guarantee the validity of the design by doubling the number of centre points in the design. All required centre point samples were prepared with a low TIPO level (65 ± 20 ppb) and high TIPO level (370 ± 30 ppb).

The range for the SO_2 concentration was centred on the large scale production values for this brand. Thus, the maximum concentration of 10 mg/l is the legal limit of total SO_2 in a beer without declaration in the UK, 7 mg/l is the target specification and 4

mg/l would be considered a low value for this parameter to ensure an increased flavour stability.

The variation of bitterness was based on the levels of iso-α-acids typically present in commercially available lager beers with around 10 to 20 BU.

The amounts of α-acids added were judged relative to those present in dry-hopped pale beers, which would contain higher quantities of the non-isomerised α-acids between 2 mg/l to 7 mg/l (Mitter and Cocuzza, 2013).

6.2.3.2 *Trial design Model B*

The trials design for Model B is shown in Table 6.2. The values of the factors were agreed in conjunction with the Molson Coors Brewing Company research and development department.

Table 6.2: Trial design for Model B: Concentrations of different factors in Model B.

Factor	Low	Centre Points	High
TIPO (ppb)	65 ± 20	65 ± 20 and 370 ± 30	370 ± 30
SO_2 (mg/l)	4	7	10
Fe (ppb)	30	75	120
(+)-catechin (mg/l) Addition	0	25	50
Glutathione (mg/l) Addition	0	20	40
Ageing (30°C)	Fresh, 30 and 60 days for all samples		

The same levels for TIPO, SO_2 and Fe as for Model A (Table 6.1) were used.

The addition of (+)-catechin was used to mimic an increased polyphenol level derived from malt and hops. The trial beer had an initial total polyphenol content of 90 ppm. The level of phenolic compounds ranges from 150 to 350 ppm with the lower values found in lager style beers (Zhao et al., 2010). As (+)-catechin is present in beer and was commercially available, made it a suitable phenolic compound to increase the total polyphenol level of the beer in the model.

The levels of glutathione added were based on the levels of glutathione equivalents present in a beer sample reported in the literature (Lund and Andersen, 2011).

6.2.3.3 *Preparation of stock solutions for compound addition for Model A and B*

To obtain the required levels of the different compounds in the different beer samples, the chemicals shown in Table 6.3 were added depending on the model design.

Table 6.3: Compounds added to achieve required factor concentrations in model A and B

Compound	Supplier
H_2O_2 (30 %)	Sigma-Aldrich, UK
Potassium bisulfite (> 99 %)	Sigma-Aldrich, UK
Fe(II) sulfate heptahydrate (> 99 %)	Sigma-Aldrich, UK
Isohop® (30 %)	Botanix Ltd, UK
Pure α-extract (65 %)	Botanix Ltd, UK
L-Glutathione, reduced (> 98 %)	Sigma-Aldrich, UK
(+)-Catechin hydrate (> 98 %)	Sigma-Aldrich, UK

To achieve a consistent and accurate addition of the different compounds, stock solutions containing the different compounds were prepared before their addition into the beer samples.

Hydrogen peroxide (H_2O_2) solution (Model A and B)

A volumetric flask (1000 ml) was filled with RO-water (500 ml). H_2O_2 (30 %, 25.5 g) was added to the volumetric flask and mixed. The flask was made up to 1000 ml volume with RO-water.

5 ml of this solution were added as required by the sample matrix derived from the Design Expert model to lower the total SO_2 level by ca. 4 mg/l. For the centre points, no H_2O_2 was added.

The addition of H_2O_2 to lower the total SO_2 level is based on the oxidation of SO_2 by H_2O_2 to sulphuric acid.

Potassium bisulfite solution (Model A and B)

RO-water (ca. 500 ml) was added to a volumetric flask (1000 ml). Potassium bisulfite (23.4 g) was added. The solution was mixed until the potassium bisulfite dissolved and the solution was made up to volume (1000 ml) using RO-water.

5 ml of this solution were added as required by the sample matrix to increase the total SO_2 level by ca. 3 mg/l. For the centre points, no potassium bisulfite solution was added.

Fe(II) sulfate heptahydrate solution (Model A and B)

Fe(II) sulfate heptahydrate (1.44 g) was added to a volumetric flask (1000 ml) half filled with RO-water. After all Fe(II) sulfate heptahydrate was dissolved by mixing, the volume was made up using RO-water.

5 ml of this solution were added as required by the sample matrix to increase the total Fe level of the samples by ca. 80 ppb. For the centre points, 2.5 ml of the solution were added.

Iso-α-extract solution (Model A)

To a volumetric flask (1000 ml) half filled with ethanol (99.5 %,), Isohop pre-isomerised hop extract (30 %, 257 g) was added. After the Isohop extract was fully dissolved by mixing, the volume was made up with ethanol (99.5 %).

The amounts added were based on an estimated utilisation of the iso-α-acids of 70 % presented by Kostelecky and Garden (2008), in operating losses of iso-α-acids during the sample preparation.

5 ml of this solution were added as required by the sample matrix to increase the bitterness to ca. 15 IBU. For the centre points, 2.5 ml of the solution were added to achieve 7.5 IBU bitterness.

α-extract solution (Model A)

Pure α-extract (65 %, 16.74 g) was added to a volumetric flask (1000 ml) half filled with ethanol (99.5 %). A vortex mixer was utilised to mix and dissolve the pure α-extract in the ethanol. After all pure α-extract was dissolved, the volume was made up using ethanol (99.5 %).

5 ml of this solution were added as required by the sample matrix to increase the α-acid level by 4 mg/l. For the centre points, 2.5 ml of the solution were added to increase the α-acid level by 2 mg/l.

(+)-Catechin hydrate solution (Model B)

To a volumetric flask (100 ml) half filled with RO-water, (+)-Catechin hydrate (> 99 %, 9 g) was added and mixed until all (+)-Catechin hydrate was dissolved. The volume was made up using RO water.

10 ml of this solution were added as required by the sample matrix to increase the catechin level by 50 mg/l. For the centre points, 5 ml of the solution were added to increase the catechin level by 25 mg/l.

L-Glutathione, reduced, solution (Model B)

L-Glutathione, reduced, (> 99 %, 7.2 g) was added to a volumetric flask half filled with RO water. After all L-Glutathione, reduced, was dissolved by mixing, the volume was made up using RO water.

10 ml of this solution were added as required by the sample matrix to increase the glutathione level by 40 mg/l. For the centre points, 5 ml of the solution were added to increase the glutathione concentration by 20 mg/l.

6.2.3.4 *Preparation of sample beers*

For each model, 22 samples (16 samples, 4 centre points, 2 base beer samples) were prepared. For each sample, a keg (20 l) was purged with CO_2. After purging, 5 kg of base beer were added to the keg. After the addition of the beer, the CO_2 pressure of the keg was released and a fitting equipped with a funnel was attached. Using a Gilson pipette (5 ml), the required amounts of the different compound stock solutions were added into the keg through the fitting. The order of the addition was the same for each sample (Model A: H_2O_2/SO_2, Fe, iso-α-acid, α-acid, Model B: H_2O_2/SO_2, Fe, Glutathione, (+)-catechin). The solutions were added directly through the fitting to minimise the contact of the solutions with the funnel surface to avoid a direct mixing

of the different solutions. If some of the added solution made contact with the surface of the funnel, 5 ml of RO-water were used to rinse the surface and transfer all of the added stock solution into the keg. Each addition of a solution was followed by a short agitation of the keg. After all required solutions were added, the headspace of the keg was purged with CO_2 and further 13 kg of base beer were added. Afterwards the keg was mixed by rolling for ca. 5 min and stored at 2 ˚C prior to bottling.

After preparation of all of the sample kegs, the beer was bottled utilizing the Molson Coors Brewing Company pilot plant bottling line. The bottling line consisted of a single headed filler and a hand bottle crowner. Brown glass bottles (330 ml) were rinsed with RO-water prior to bottling. If a TIPO of 65±20 ppb was required, the bottles were purged with CO_2 for 45 s prior to their entering the filling head. If the high level of TIPO (370 ± 30 ppb) was required then the bottles were not purged with CO_2. After the filling process, the bottom of the bottles were hit with a spanner to obtain an over foaming of the bottles and closed with a standard crown cap (no scavenger). The bottles were pasteurized (20-22 PU) in a box pasteurizer and all samples were stored cooled (2 ˚C) prior to further processing.

For the forced ageing, the samples were stored at 30 ˚C for 30 and 60 days. After the forced ageing, the samples were stored cooled (2 ˚C) prior to further analysis.

6.2.4 Thiobarbituric acid index

For the description of this analysis see Section 2.3. All fresh and aged beer samples of Model A and B were analysed. The analysis included three technical replicates of each sample.

6.2.5 Total sulphur dioxide via distillation method

For the description of this analysis see Section 2.2. All fresh and aged beer samples of Model A and B were analysed. The analysis included three technical replicates of each sample.

6.2.6 Metal analysis via ICP-MS

For the description of this analysis see chapter 2: Material and Methods, all fresh beer samples of Model A and B were analysed. Due to the costs of the analysis, one run of each sample was performed. To evaluate the reproducibility of the anlysis, two samples of each model were analysed in triplicate.

6.2.7 Oxidative stability by EPR spectroscopy

For the description of this analysis see Section 2.6. All fresh and aged beer samples of Model A and B were analysed. The analysis was performed in duplicate. The evaluation of the results included the determination of the area under the curve by integration and the x0-lagtime and the dx value by using the Boltzmann Function.

6.2.8 Analysis of volatile aldehydes in beer by solid phase micro extraction (SPME) with GC-MS

For the description of this analysis see Section 2.2. All fresh and aged beer samples of Model A and B were analysed. The analysis was performed in duplicate.

6.2.9 Sensory analysis (10 am tasting) of fresh and aged samples

All fresh and aged beer samples of Model A and B were tasted. In two independent tasting sessions, the sensory evaluation of each sample was performed by three to four

trained tasters for each independent session. The tasters were all trained tasters, certified to evaluate the product quality of the Molson Coors Brewing Company products on a daily basis. The samples were presented in randomised order at a temperature between 8˚C and 10 ˚C in standard tasting glasses. The samples were marked on a scale of 1 to 4, with 1 being the lowest and 4 being the highest score. A beer rated with a 4 would be marketable without any constraints, whereas a 3 shows a slight defect. Beers rated with a 2 would be considered having a major defect and the marketability would need to be assess separately. A rating of 1 would mark the beer having major defects and would be considered not for sale.

6.3 Results and discussion

6.3.1 Results for Model A

A summary of the significant factors ($p < 0.05$) and their impacts (+/-) on the measured flavour stability indices, the significant two factor interactions ($p < 0.05$) and model statistics for Model A are presented in Table 6.4. In the following sections each index will be discussed and the effects of significant single factors and significant two factor interactions will be presented.

Table 6.4: Summary table of significant factors and their impacts on the flavour stability indices, two factor interactions and model statistics for Model A (x = no significant interaction, $p > 0.05$)

Flavour stability indices	Factors						Two factor interaction		Model statistics	
	Age	TIPO	SO_2	Fe	Iso-α-extr.	α-extr.	Factors interacting	p-value	Model R^2	Model significance (p-value)
SO_2	< 0.0001 (-)	< 0.0001 (-)	< 0.0001 (+)	x	x	x	x	X	0.95	< 0.0001
EPR Area	< 0.0001 (+)	0.0003 (+)	< 0.0001 (-)	< 0.0001 (+)	0.0024 (-)	x	TIPO – SO_2 Age – SO_2 SO_2 - Fe	0.0008 0.0027 0.011	0.93	< 0.0001
EPR x0 lag time	< 0.0001 (-)	0.0208 (-)	< 0.0001 (+)	x	x	x	x	X	0.83	< 0.0001
EPR Dx	0.0011 (+)	0.2444 (+)	< 0.0001 (-)	0.0115 (+)	0.0146 (+)	x	TIPO – Age TIPO – SO_2 TIPO – iso-α-extr. Age – SO_2	0.0247 0.0039 0.0176 <0.0001	0.66	< 0.0001
TBI	< 0.0001 (-)	x	x	x	x	x	x	X	0.25	< 0.0001
2-MP, 2-MB, 3-MB	< 0.0001 (+)	x	< 0.0001 (-)	x	x	x	x	X	0.92	< 0.0001
Benzaldehyde	x	x	x	x	x	x	x	X	x	x
Phenyl acetaldehyde	< 0.0001 (+)	x	< 0.0001 (-)	x	x	x	x	X	0.45	< 0.0001
Furfural	< 0.0001 (+)	x	< 0.0001 (-)	x	x	x	Age – SO_2	0.0005	0.89	< 0.0001
(E)-2-nonenal	< 0.0001 (+)	x	< 0.0001 (-)	x	x	x	Age – SO_2	0.014	0.51	< 0.0001
Sensory	< 0.0001 (+)	x	0.0046 (-)	x	x	x	x		0.42	< 0.0001

6.3.1.1 *Model SO_2 concentration in comparison to analysed concentration of SO_2 for Model A*

SO_2 is regarded as the main antioxidant in beer (Andersen et al., 2000) but the concentration of SO_2 deceases continuously during storage, especially at elevated temperatures (Ilett and Simpson, 1995). Therefore the SO_2 concentration was monitored during the entire storage trial. For the modelling of the data, the defined SO_2 concentrations (4/7/10 mg/l) were used. This was necessary to incorporate the effects of the other factors on the actual SO_2 concentration in the sample. To evaluate the actual SO_2 concentrations in the samples, these concentrations have been analysed as a response in the model.

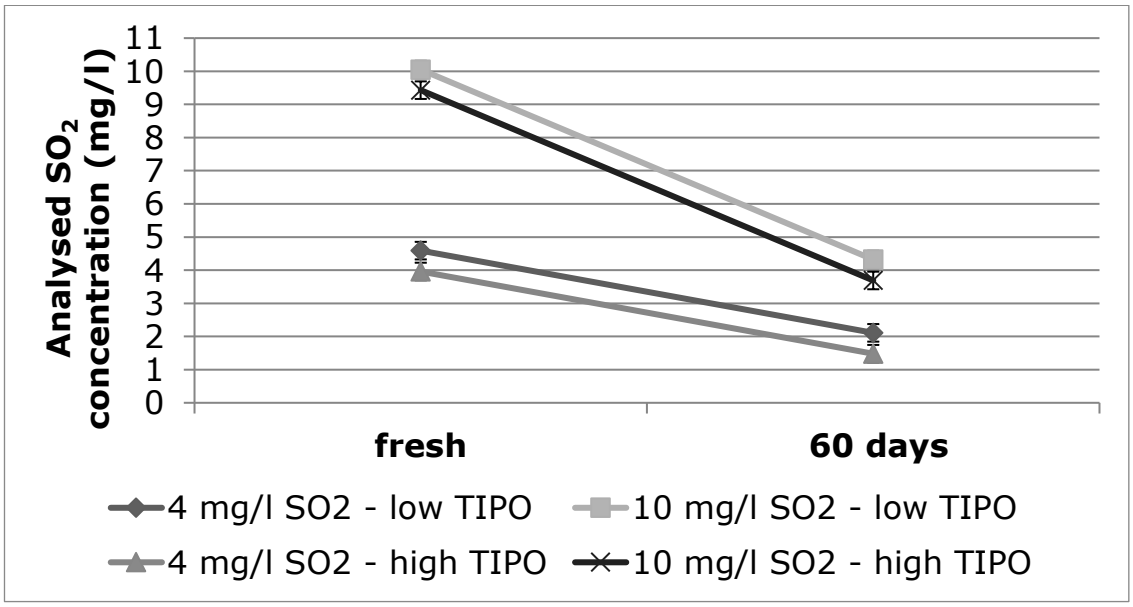


Figure 6.1: Effect of TIPO on analysed SO_2 concentration (mg/l) during ageing. Errorbars represent LSD. Modelled data, Model R^2 = 0.95. Model predicts linear relationship over the 60 days of ageing.

Figure 6.1 shows the effect of TIPO on analysed SO_2 concentration (mg/l) during ageing. For both model SO_2 concentrations (4 and 10 mg/l), the effect of an increased

TIPO resulted in a decrease in the actual concentration of SO_2 of ca. 0.5 mg/l in the fresh sample. For all samples, the percentage decrease was very similar with ca. 40 % of the initial concentration remaining after 60 days of ageing.

The difference of ca. 0.5 mg/l SO_2 in the fresh samples indicates the effect of the increased TIPO and could be related to the reaction of SO_2 with H_2O_2, the formation of which is accelerated by increased temperatures during pasteurisation (Uchida and Ono, 1996, Kaneda et al., 1999, Andersen et al., 2000).

Interestingly none of the other factors significantly influenced the SO_2 concentration in the model, despite the antioxidant properties of the hop acids and the pro-oxidant effects of Fe. This reinforces the suggestion that SO_2 is the major antioxidant present in beer.

6.3.1.2 *EPR spectroscopy measurements of beer oxidative potential for Model A*

The use of the Boltzmann fitting and the evaluation of the different EPR matrixes, area, x0-lag time and dx, should give the opportunity to extract more relevant information from the obtained EPR measurements apart from only using the standard lag time and T_{150} values (Foster, 2009).

6.3.1.2.1 **Area under the curve matrix**

The area under the curve integrates across all measuring points of an EPR experiment whereas the T_{150} value only reports a single measuring point. Both indices may show similar trends across a data set because they are related to one another.

The area under the curve values derived from EPR spectroscopic measurements for the lowest and highest value for a significant single factor are presented in Figure 6.2.

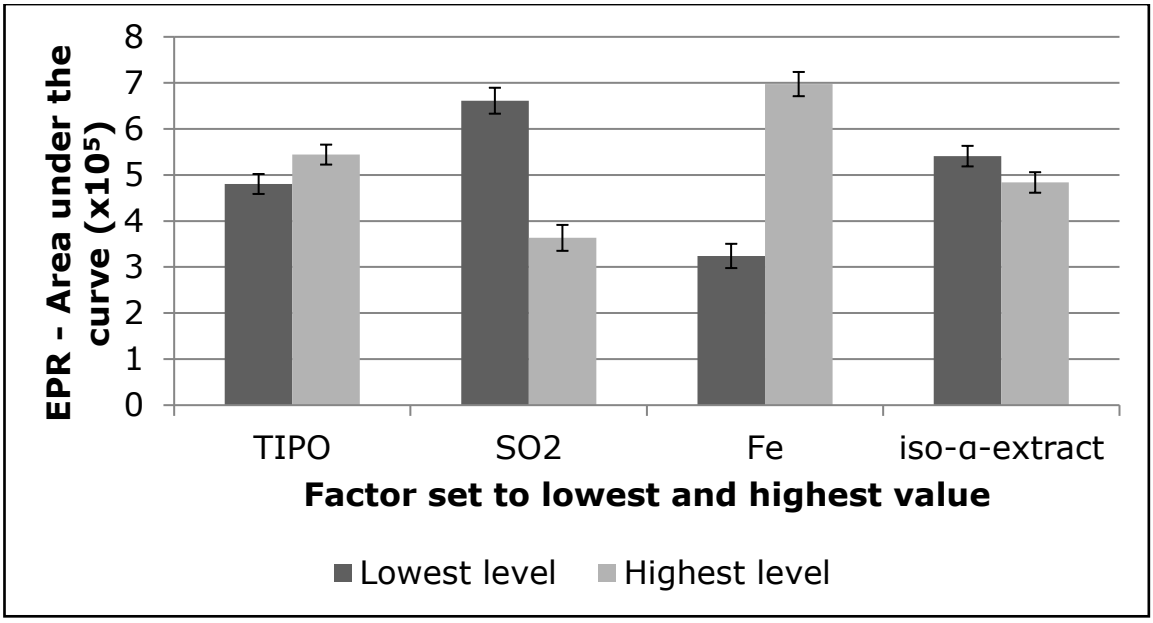


Figure 6.2: Comparison of area under the curve values for lowest and highest values of significant single factors TIPO, SO_2, Fe and iso-α-extract for Model A (TIPO = low, SO2 = 7 mg/l, Fe = 75 ppb, iso-α-extract = 7.5 mg/l, α-extract = 2 mg/l if factor not set for lowest and highest value). Errorbars represent LSD. Modelled data, Model R^2 = 0.93

To determine these values, the reviewed factor was set to its lowest and highest value, for example to 4 mg/l and 10 mg/l for SO_2. The other factors were unchanged at TIPO = low, Fe = 75 ppb, iso-α-extract = 7.5 mg/l, α-extract = 2 mg/l. An increase in TIPO of ca 300 ppb resulted in an increase in the EPR-determined area of 13 %. A difference of 6 mg/l from 4 mg/l to 10 mg/l in the total SO_2 level caused a decrease in the EPR-determined area of 45 %. The largest effect on the area value resulted from an increase in the Fe level from 30 ppb to 120 ppb (+ 115 %). A decrease of 10 % in the area was observed on increasing the iso-α-acid level from 3 mg/l to 15 mg/l. Adding α-acids to the samples did not significantly influence the EPR area in the model.

In Figure 6.3 a 3D-plot of the two factor interaction of SO_2 (mg/l) and TIPO (ppb) on the EPR area data for a fresh sample of Model A are presented.

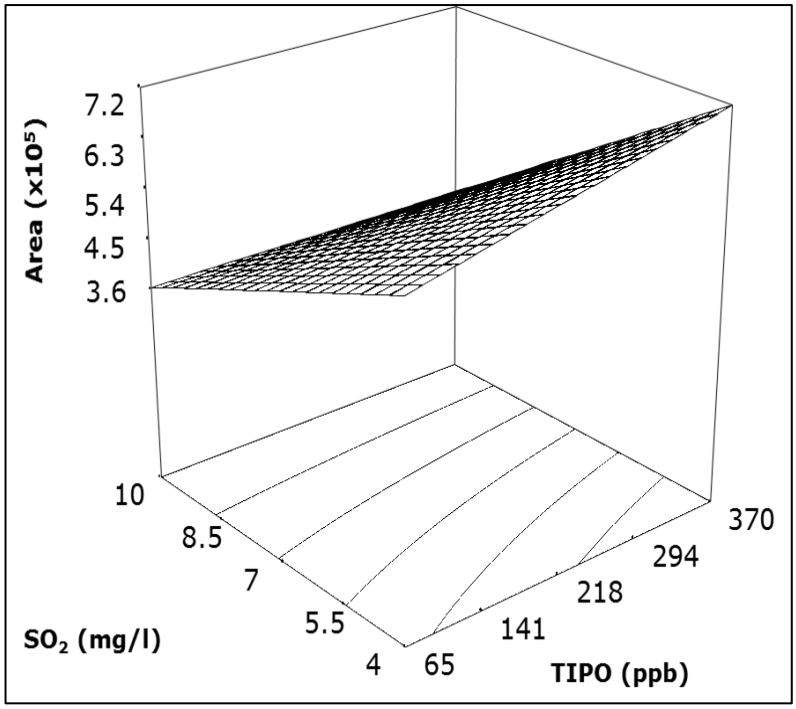


Figure 6.3: 3D-plot of two factor interaction of SO_2 (mg/l) and TIPO (ppb) on the EPR area data for a fresh sample of Model A. Actual factors: days aged: fresh sample, Fe = 75 ppb, α-extract = 2 mg/l (addition), Iso-α-extract = 7.5 mg/l, Model R^2 = 0.93, p = 0.008 for two factor interaction.

A significant two factor interaction (p = 0.008) between the SO_2 and TIPO was observed (Figure 6.3). At low SO_2 levels (4 mg/l) an increased TIPO concentration resulted in an increase in the EPR-determined area value (+ 19 %). Interestingly with increasing SO_2 levels, the influence of an increased TIPO concentration declined. Thus, for the highest SO_2 level (10 mg/l), the increased TIPO concentration had no detectable effect on the EPR-determined area (EPR area = $3.6x10^5$, 0 % increase).

A 3D-plot of the significant two factor interaction of SO_2 (mg/l) and TIPO (ppb) in the 60 days aged samples of modelled EPR area data are presented in Figure 6.4.

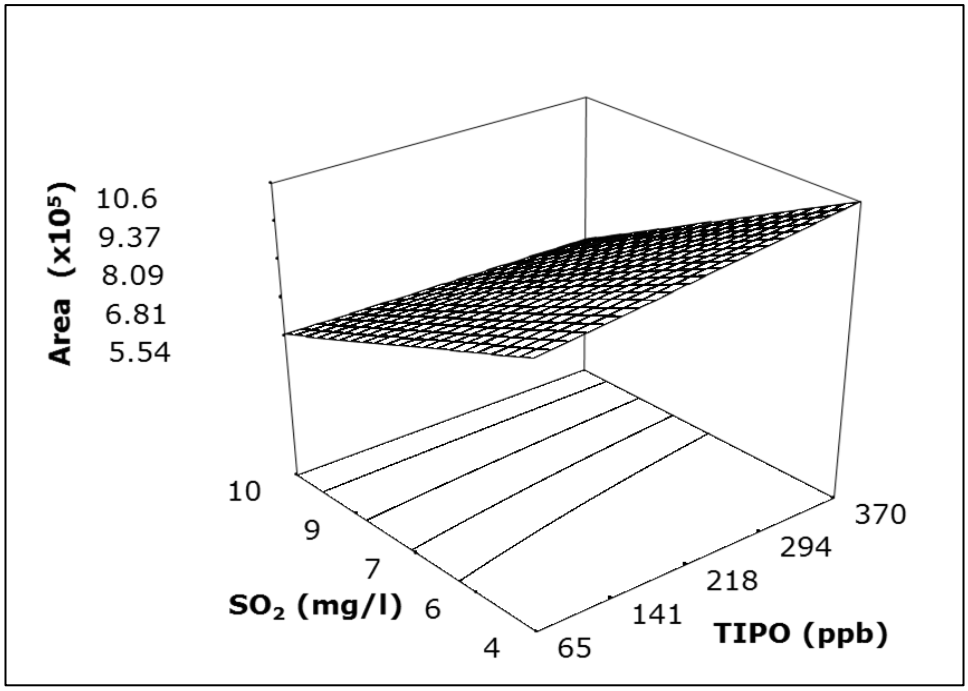


Figure 6.4: 3D-plot of two factor interaction of SO_2 (mg/l) and TIPO (ppb) in 60 days aged samples of modelled EPR area data. Actual factors: days aged: 60 days, Fe = 75 ppb, α-extract = 2 mg/l (addition), Iso-α-extract = 7.5 mg/l, Model R^2 = 0.93

Forced ageing of 60 days at 30 ˚C did not result in a trend change in the interaction between SO_2 and TIPO (Figure 6.4). Despite the forced ageing of the samples, an increased TIPO level for those samples high in SO_2 (10 mg/l) did not result in an increase in the EPR-determined area value (EPR area = $5.6x10^5$, 0 % increase). Lower SO_2 levels, combined with increased TIPO concentrations led to increased EPR area values. For samples with the lowest SO_2 level (4 mg/l), increasing the TIPO concentration from 65ppb to 370 ppb resulted in an increase in the EPR-determined area of 12 %. For fresh and aged samples, increasing the SO_2 level to 10 mg/l resulted in a complete suppression of the effect of an increased TIPO concentration on the EPR-determined area.

Figure 6.5 shows the significant two factor interaction between Fe and SO_2 ($p < 0.011$).

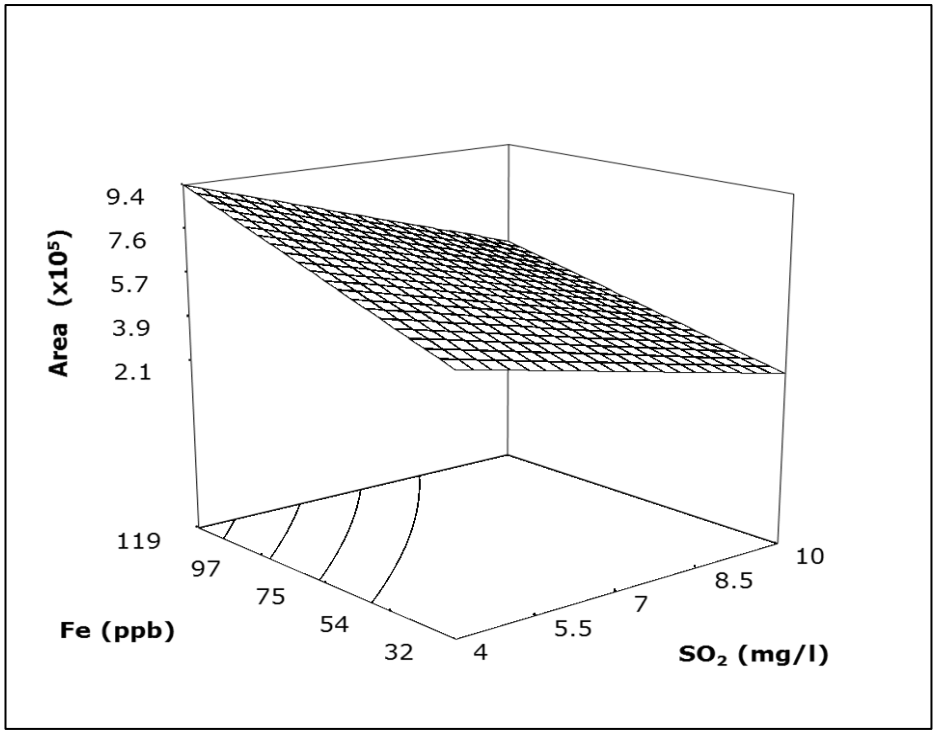


Figure 6.5: 3D-plot of two factor interaction of SO_2 (mg/l) and Fe (ppb) of modelled EPR area data. Actual factors: TIPO = 218 ppb, days aged: fresh sample, α-extract = 2 mg/l (addition), Iso-α-extract = 7.5 mg/l, Model R^2 = 0.93

In comparison to the samples with Fe and SO_2 at their lowest levels (EPR area = $4.3x10^5$), increasing the SO_2 concentration, resulted in a decrease in the area value by a factor of up to 50 % for those samples containing 10 mg/l of SO_2. In contrast, an increase in the Fe concentration led to an increase in EPR area values by up to 100 % for the maximum Fe level (120 ppb). Increasing both factors, Fe and SO_2, in combination, had an opposing effect on the EPR area values. Thus, for the highest Fe and SO_2 concentrations (120 ppb and 10 mg/l, respectively), the EPR area value (EPR area = $5.1x10^5$) was very similar (+15 %) to the value for both factors at their minimum (30 ppb and 4 mg/l, respectively).

Figure 6.6 shows the third two-factor interaction between the length of ageing (at 30 ˚C) and the SO_2 concentration.

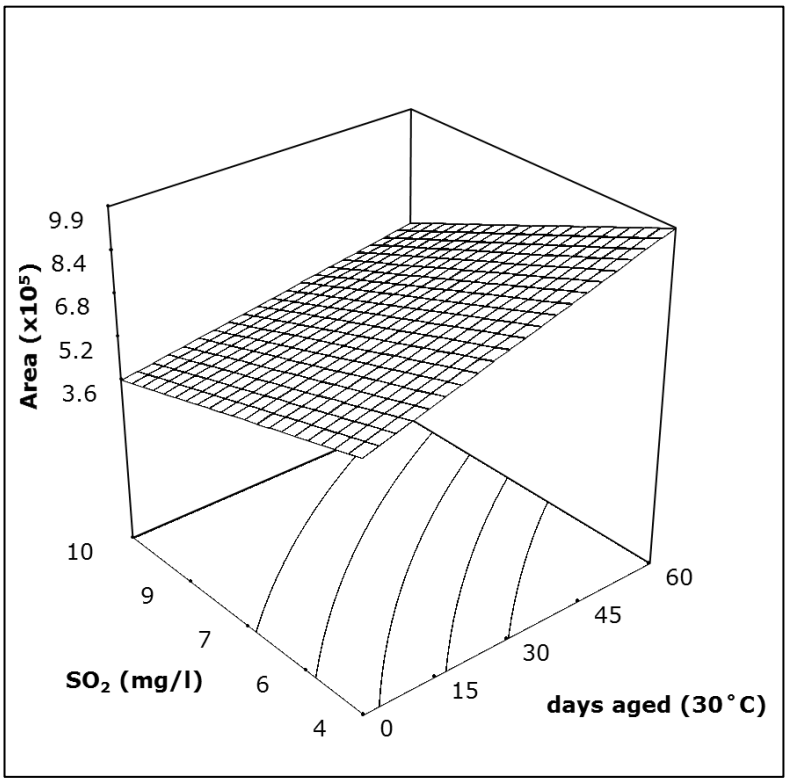


Figure 6.6: 3D-plot of two factor interaction of SO_2 (mg/l) and days aged (30 ˚C) of modelled EPR area data. Actual factors: TIPO = 218 ppb, Fe = 75 ppb, α-extract = 2 mg/l (addition), Iso-α-extract = 7.5 mg/l, Model R^2 = 0.93

Over the entire design space, the forced ageing procedure resulted in increases in EPR area values. For all samples, fresh and aged, increasing the SO_2 level caused decreasing EPR area values over the entire design space. Interestingly the percentage increase in EPR area value over the 60 days of ageing was very similar (ca. 33 %) irrespective of the SO_2 concentration. From 3.6 $x10^5$ to 4.7 $x10^5$ for the low concentration of SO_2 (4 mg/l) and increasing for the high SO_2 concentration (10 mg/l) from 4 $x10^5$ to 9.9 $x10^5$.

The concentration of free radicals, identified by the EPR area matrix, increased with increasing Fe and TIPO concentrations and prolonged storage time, and decreased with greater SO_2 concentrations. In general these findings agree with the findings presented in the literature (Kaneda et al., 1992, Kaneda et al., 1989, Andersen et al., 2000), nevertheless the relative importance of the factors in the model was contrary to expectations. Despite the high profile of TIPO levels in modern brewing, the effect of increased TIPO concentrations on EPR area was relatively minor in the current beer and experimental design. Increased TIPO concentrations should result in an increased formation of ROS or consume a part of the initial antioxidant potential, causing the oxidation of antioxidants and substances present in beer. The relatively high SO_2 concentrations and the forced ageing procedure might have influenced the effect of TIPO on the measured EPR area. Based on the results presented, SO_2 could be identified as one of the key factors influencing the EPR area value. This can be directly related to the strong antioxidant properties of SO_2. The strong influence of increased Fe concentration on EPR area values supports the observation that metal ions catalyse the formation of ROS (Kaneda et al., 1999). The strong influence of the Fe concentration could also be related to the way the EPR measurement is performed in an oxygen rich atmosphere (air). The higher Fe concentration results in an increased reaction rate of the Fenton reaction and therefore in a faster formation of ROS during the EPR measurement.

6.3.1.2.2 **x0-lagtime matrix**

The x0 value represents the inflection point where the slope of the EPR curve changes. As the determination is based on Boltzmann fitting, a defined mathematical determination is guaranteed. Compared to the traditional lag time matrix, the x0-lag

time takes not only account for true antioxidant class compounds but also includes the possible effects of retardant-class antioxidants (Foster, 2009).

Figure 6.7 shows the comparison of x0-lag time values for lowest and highest values of the significant single factors TIPO and SO_2 for Model A in fresh samples.

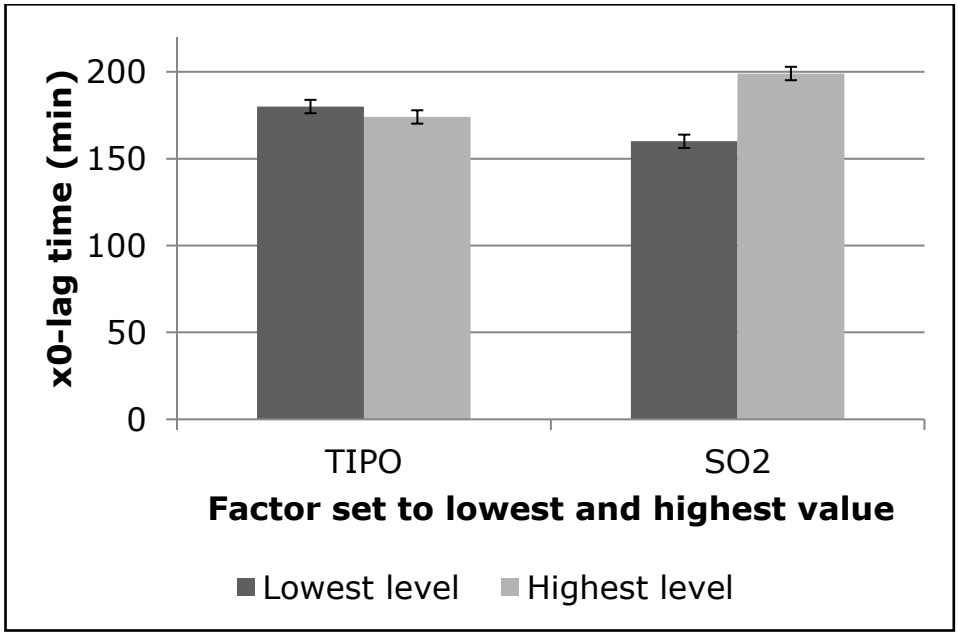


Figure 6.7: Comparison of x0-lag time values for lowest and highest values of significant single factors TIPO and SO_2 for Model A in fresh samples (TIPO = low, SO2 = 7 mg/l, Fe = 75 ppb, iso-α-extract = 7.5 mg/l, α-extract = 2 mg/l if factor not set for lowest and highest value). Errorbars represent LSD. Modelled data, Model R^2 = 0.83

The SO_2 and TIPO concentrations have a significant influence on the x0-lag time for Model A (Figure 6.7). An increase in the SO_2 concentration from 4 mg/l to 10 mg/l, increased the lag time value of 160 min by ca. 40 min. In contrast, an increase in the TIPO concentration, reduced the lag time by ca. 6 min in the fresh sample. The forced ageing (60 days at 30 ˚C) resulted in a decrease in x0-lag time of 33 min.

For the SO_2 and TIPO levels, the lag time results showed a similar trend, compared to the EPR area results (Figure 6.2). Interestingly, the Fe concentration did not

significantly influence the lag time values, as it did for the EPR area (Figure 6.2). The lag time has been reported as a phase of accumulation of H_2O_2 before the formation of the hydroxyl radical takes place induced by the Fenton reaction (Uchida and Ono, 1999). This might reduce the influence of the Fe concentration on the lag time. On the other hand, the reduction of ground state oxygen to H_2O_2 also involves the oxidation of Fe (Bamforth and Lentini, 2009). Therefore the non-significant effect of Fe concentration on the lag time in comparison to the EPR area could not be explained.

A significant effect of increased hop polyphenol concentration on the x0-lag time as reported previously (Foster, 2009), could not be detected. The effect might have been overlapped by the dominant effect of the SO_2 concentration and the concentration in this model, similar to concentrations found in commercial lager, might have been defined too low to have a significant influence.

6.3.1.2.3 **dx matrix**

The dx matrix has been reported to indicate the presence of retardant antioxidant classes (Foster, 2009). A shallower slope (higher dx value) of the EPR curve indicates a reduced rate of free radical formation that suggests a higher retardant antioxidant concentration.

In the present model, the dx matrix resulted in a very complex model ($R^2 = 0.66$) with a large number of single factors and two factor interactions being significant. Apart from the α-extract addition, all single factors had a significant influence. Furthermore four two-factor interactions were detected (TIPO – Age, TIPO – SO_2, TIPO – iso-α-extract, Age – SO_2). The effect of the single factor and two-factor interactions was not clear, as erratically increasing and decreasing dx values were detected in the two factor interactions.

Figure 6.8 and Figure 6.9 show the two-factor interaction of TIPO and iso-α-extract for fresh and 60 days aged samples.

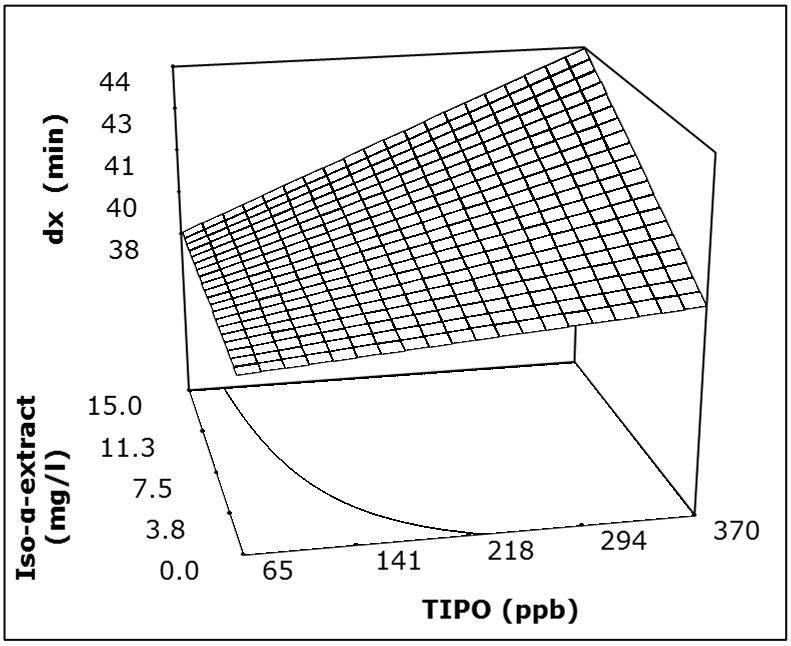


Figure 6.8: 3D-plot of two factor interaction of TIPO (ppb) and iso-α-extract (mg/l) for fresh samples of modelled EPR dx data. Actual factors: SO_2 = 7 mg/l, Fe = 75 ppb, α-extract = 2 mg/l (addition), Model R^2 = 0.67

In the fresh samples (Figure 6.8), an increase in the TIPO concentration generated higher dx values. With increasing iso-α-extract concentration the dx value also increased. For the aged samples (Figure 6.9), the trend changes; increasing the TIPO concentration decreases the value of dx.

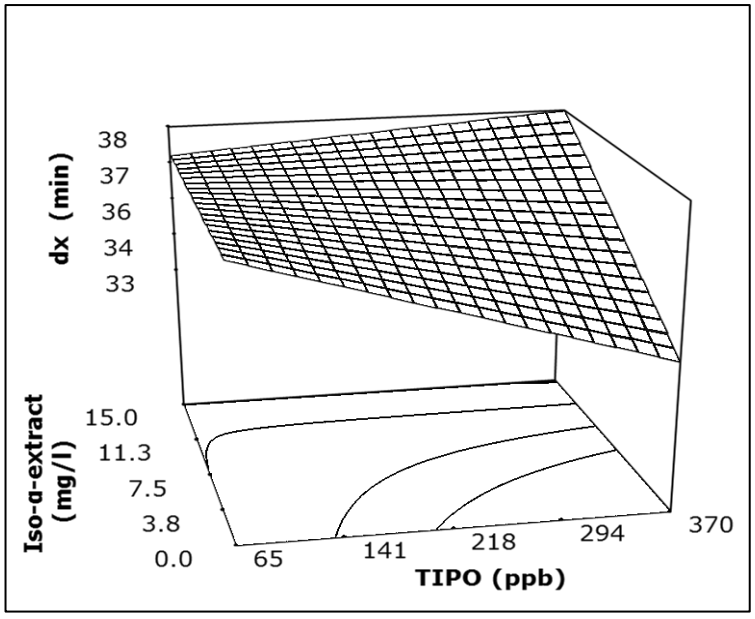


Figure 6.9: 3D-plot of two factor interaction of TIPO (ppb) and iso-α-extract (mg/l) for 60 days aged (30 ˚C) samples of modelled EPR dx data. Actual factors: SO_2 = 7 mg/l, Fe = 75 ppb, α-extract = 2 mg/l (addition), Model R^2 = 0.67

In the given design space, the determination of the dx-value did not illustrate any effect of retardant antioxidant classes. The model might be too complex to clearly delineate the expected relatively small effects of retardant antioxidant classes.

6.3.1.3 *Thiobarbituric acid index for fresh and aged beer samples (Model A)*

The TBI has traditionally been used as a reliable, uncomplicated and inexpensive indicator to evaluate the brewhouse operations in reference to heat impact and the resulting formation of Maillard reaction products. During the TBI assay a large number of the Maillard reaction products and other aldehydes are measured and TBI values can be used to quantify the extent of beer staling. Lower TBI values will indicate a lower impact on flavour stability.

The modelling for the TBI was not successful for Model A. The model probability itself was very good ($p < 0.0001$) but the coefficient of determination ($R^2 = 0.25$) was

very low and therefore indicates an insufficient modelling for TBI. Figure 6.10 shows the significant single factor interaction of days aged ($p < 0.0001$) on the TBI value.

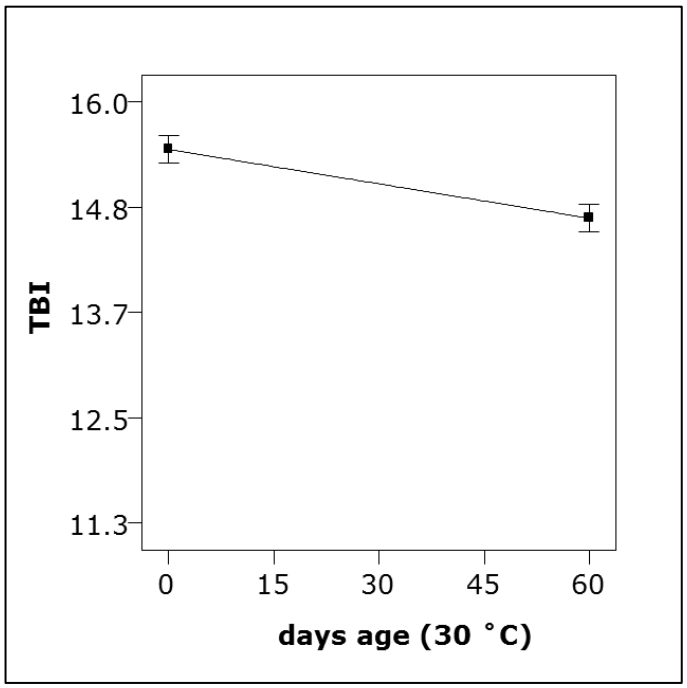


Figure 6.10: Significant single factor days aged (30 ˚C) for TBI, Actual factors: SO_2 = 7 mg/l, TIPO = 65 ppb, Fe = 75 ppb, iso-α-extract = 7.5 mg/l, α-extract = 2 mg/l, Model R^2 = 0.25. Model predicts linear relationship over the 60 days of ageing.

TBI is a common tool to indicate heat stress during brew house operations. The effect of dicarbonyl compounds and Strecker aldehydes on TBI values has been reported (Fegredo et al., 2009) but could not be verified in this study, as the TBI value decreased during storage (Figure 6.10) but the concentration of Strecker aldehydes increased during storage (Figure 6.11). The wavelength used for the TBI determination (448 nm or 530 nm) directly influences the results. Thus, experiments at 530 nm have been reported (Li et al., 2015) to show the maximum absorption for the reaction products of thiobarbituric acid with the aldehydes. However the more traditionally and in this study used wavelength of 448 nm, as the publication referring to 530 nm was after the

experiment was started. The traditional TBI was not able to indicate changes in the concentration of the aldehydes related to staling in the given design space.

6.3.1.4 *GC-MS SPME measurement of staling aldehydes in fresh and aged beer samples (Model A)*

The concentration of aldehydes associated with typical staling off-flavours (e.g. papery, bready, or sherry characters) generally increase during storage and they play a very important role in flavour change during storage. They can be formed during storage as a result of Strecker degradation or Maillard reactions, as well as be released during storage from the bisulfite bound form (Baert et al., 2015).

The measurements and data analysis of the benzaldehyde concentration of the samples for Model A did not result in a significant model. An additional assessment of the GC results (R^2 > 0.95 for the calibration curve) and the modelling approach did not highlight any abnormalities. At this point it can only be speculated why the modelling approach failed and the benzaldehyde measurements did not model against the design factors. In any case, benzaldehyde concentrations did not approach the flavour threshold of 500 ppb (concentration < 15 ppb) in any of the beers.

In Figure 6.11, the effect of the SO_2 concentration on the concentration of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal during the forced ageing period is shown.

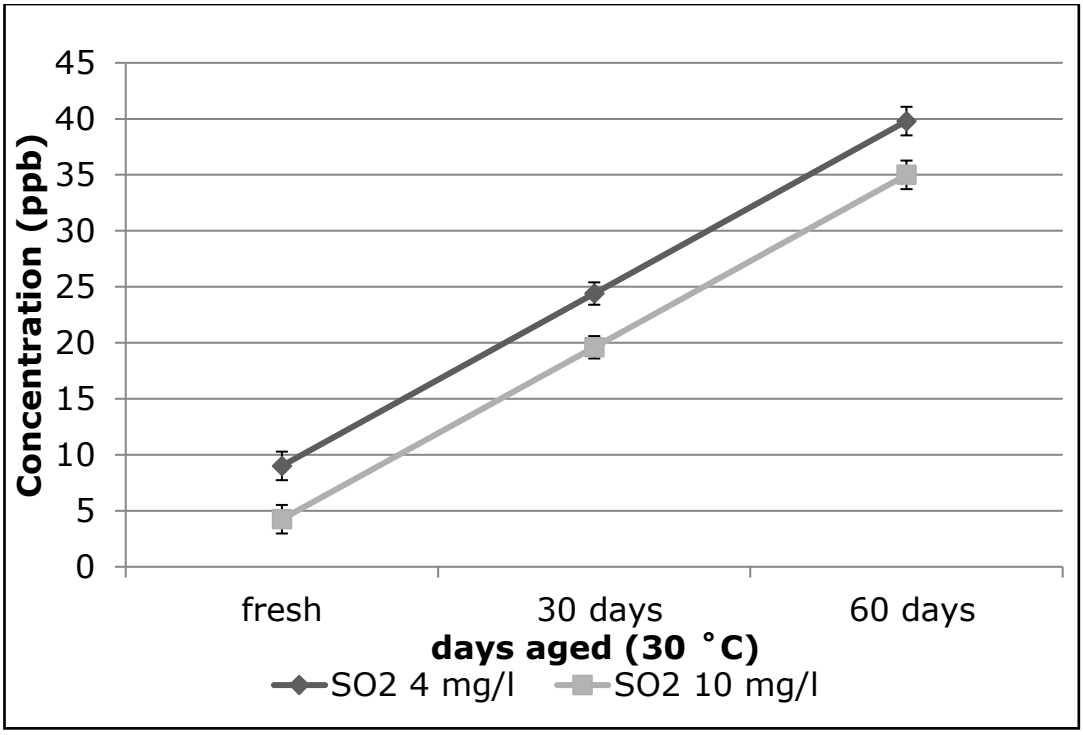


Figure 6.11: Effect of SO_2 on concentration of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal during forced ageing period. Actual factors: TIPO = 65 ppb, Fe = 75 ppb, iso-α-extract = 7.5 mg/l, α-extract = 2 mg/l, Model R^2 = 0.92

The concentrations of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal were combined into a single response, indicative of 'Strecker aldehydes'. In the model (R^2 = 0.93), only the SO_2 concentration (p< 00001) and sample age (p< 0.0001) had a significant effect on Strecker aldehyde concentrations. Figure 6.11 shows the model data for the pooled concentration of these three aldehydes with respect to the SO_2 concentration of the model over the ageing period. For all three sample points, the concentration of the three aldehydes increased for all samples. The 10 mg/l SO_2 concentration samples had a ca. 5 ppb lower concentration of 'Strecker aldehydes' compared to the 4 mg/l SO_2 concentration samples for all time points. 2-methylpropanal, 2-methylbutanal and 3-methylbutanal have been described as not being significant with respect to flavour degradation but are viewed as suitable markers for beer oxidation (Narziss et al., 1999). For this experiment, the increased TIPO

concentration did not result in an increase in the formation of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal. This could be related to the strong antioxidant properties of SO_2 (immediate reaction of SO_2 with formed H_2O_2 from increased TIPO during pasteurisation), which limits the effect of the increased TIPO concentration. Furthermore the limited O_2 permeation into the samples through the crown cap during the forced ageing procedure might have also influenced the aldehyde formation. The relatively small difference between in aldehyde concentrations between the two SO_2 concentrations (4 mg/l and 10 mg/l, respectively) could be related to the limited formation of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal during storage (Suda et al., 2007). Only ca. 15 % of the 2-methylpropanal, 2-methylbutanal and 3-methylbutanal present in beer are produced during storage by the Strecker degradation. The rest has already been formed during the boiling of the wort. As the same base beer batch was used for the experiments, the low increases during storage for the entire model supports this assumption.

Figure 6.12 displays the significant effect of the SO_2 concentration on the concentration of phenyl acetaldehyde during forced ageing period over the design space.

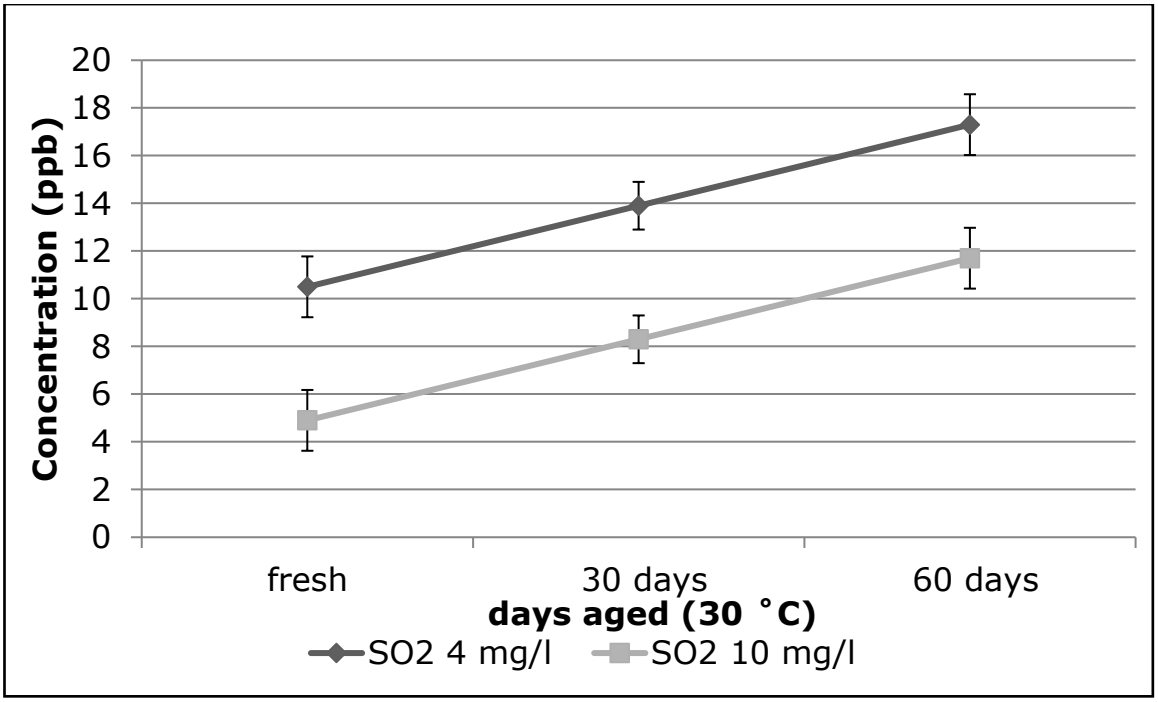


Figure 6.12: Effect of SO_2 on concentration of phenyl acetaldehyde during forced ageing period. Actual factors: TIPO = 65 ppb, Fe = 75 ppb, iso-α-extract = 7.5 mg/l, α-extract = 2 mg/l, Model R^2 = 0.45

The model showed significant effects of the SO_2 concentration ($p < 0.0001$) and the forced ageing ($p < 0.0001$) on the phenyl acetaldehyde concentration. Over the entire storage trial, the concentration of the samples with 4 mg/l SO_2 was significantly higher compared to the samples with 10 mg/l SO_2 (over 60% after 60 days) (Figure 6.12).

Compared to the other Strecker aldehydes (2-methylpropanal, 2-methylbutanal and 3-methylbutanal, Figure 6.11), the difference of the phenyl acetaldehyde concentration between the two SO_2 concentrations was more significant. This could be related to an increased formation during storage compared to the other Strecker aldehydes (30 % formed during storage) (Suda et al., 2007).

A 3D-plot presents the significant two factor interaction of SO_2 and days aged for the furfural concentration of modelled data in Figure 6.13.

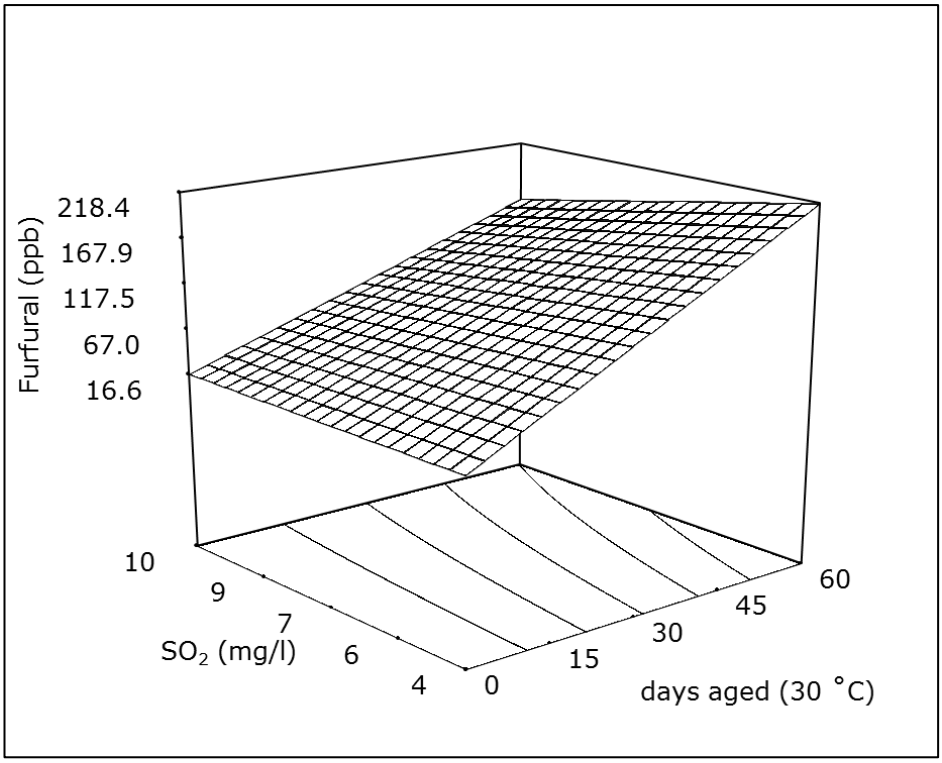


Figure 6.13: 3D-plot of two factor interaction of SO_2 and days aged for furfural concentration of modelled data. Actual factors: TIPO = 65 ppb, Fe = 75 ppb, iso-α-extract (mg/l) = 7 mg/l, α-extract = 2 mg/l (addition), Model R^2 = 0.89

For the furfural concentration, the model revealed SO_2 concentration ($p < 0.0001$) and days aged ($p < 0.0001$) as significant factors. Furthermore the two factors were involved in a significant two-factor interaction ($p < 0.0005$) shown in Figure 6.13. In the fresh samples, the SO_2 concentration had no effect on the furfural concentration. For all samples, the furfural concentration was ca. 16.6 ppb. During the forced ageing period, the difference in SO_2 concentration resulted in a concentration difference of up to 65 ppb for furfural. The almost identical concentration of furfural for all fresh samples could be directly related to the use of the same base beer and a lower chemical affinity to SO_2 compared to the smaller Strecker aldehydes, as the SO_2 concentration had no effect in the fresh samples. The increased formation furfural during storage for the samples with a lower SO_2 concentration and therefore lower concentration of

antioxidants, might be associated with an increased formation of ROS resulting in an increased furfural formation (Shimizu et al., 2001).

Figure 6.14 displays the 3D-plot of the significant two factor interaction of SO_2 and the storage period for (E)-2-nonenal concentration of modelled data.

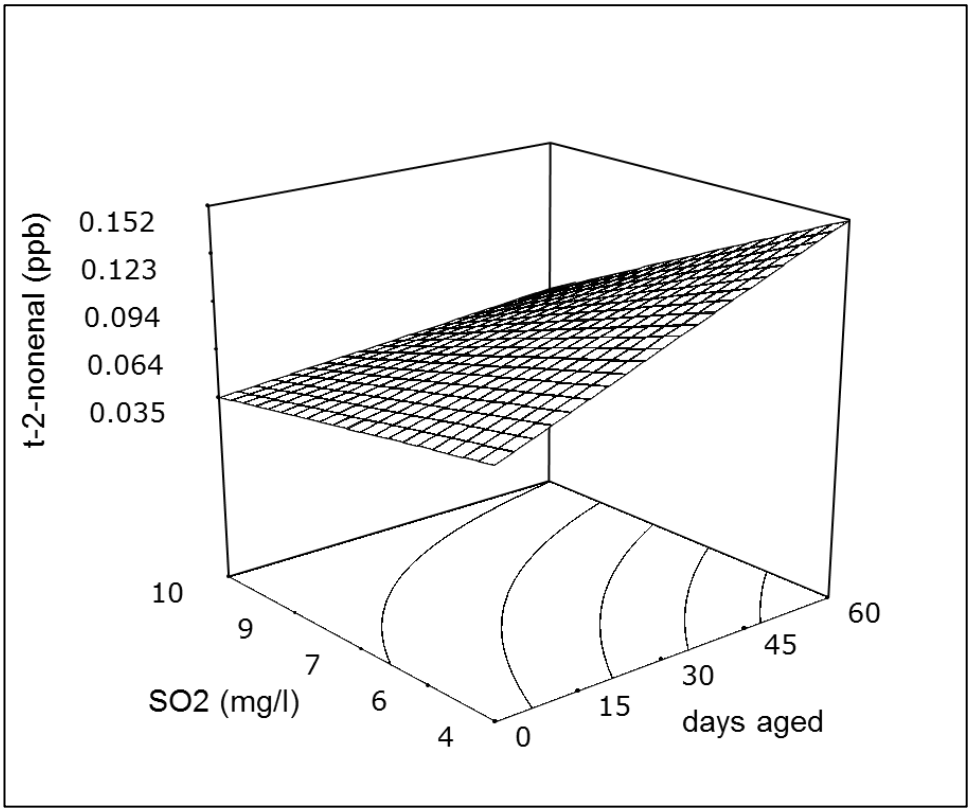


Figure 6.14: 3D-plot of two factor interaction of SO_2 and days aged for (E)-2-nonenal concentration of modelled data. Actual factors: TIPO = 65 ppb, Fe = 75 ppb, iso-α-extract (mg/l) = 7 mg/l, α-extract = 2 mg/l (addition), Model R^2 = 0.51

The model for the (E)-2-nonenal concentration displayed a significant effect with SO_2 concentration ($p < 0.0001$) and days aged ($p < 0.0001$). Additionally, the two factors were involved in a significant two-factor interaction ($p < 0.0014$) shown in Figure 6.14. For the fresh samples, only a very small difference in the concentration of t-2-noneal (0.035 ppb to 0.034 ppb, respectively) could be detected for increasing SO2 concentrations (4 mg/l and 10 mg/l, respectively). During the storage trial, a very large

increase (400 %) of the (E)-2-nonenal concentration for the samples with a low SO_2 concentration occurred, whereas the (E)-2-nonenal concentration for samples with high SO_2 concentrations only marginally increased. This indicates that in the samples with low SO_2 concentrations, (E)-2-nonenal was released from the non-volatile bisulfite adduct form during storage (Barker et al., 1983). Due to the chemical structure of (E)-2-nonenal, the SO_2 could be bound on to the carbonyl function and on to the double bound. The decreasing SO_2 concentration during storage and the much lower SO_2 concentration remaining in the samples with an initially lower SO_2 concentration could explain the difference in (E)-2-nonenal concentration after 60 days of storage. Nevertheless it has been reported, that binding SO_2 to the double bound will form an irreversible bond (Dufour et al., 1999) and that the amount of reversible bisulfite addition to (E)-2-nonenal will be minimal (Lermusieau et al., 1999). Therefore increased concentrations of (E)-2-nonenal could probably be linked to auto-oxidation or enzymatic oxidation earlier in the production process (Vanderhaegen et al., 2006). For this particular experiment, a difference in auto-oxidation or enzymatic oxidation earlier in the process can be excluded, as the base beer for all samples was produced on production scale until conditioning. Consequently the fraction of reversible bisulfite adducts might have been increased in this experiment.

The TIPO level was not a significant factor in the modulation of the (E)-2-nonenal concentration in the model. This is in line with previously reported results, which show that the oxygen level in the bottled sample does not influence the (E)-2-nonenal concentration (Noel et al., 1999, Lermusieau et al., 1999).

6.3.1.5 *Sensory evaluation of fresh and aged beer samples (Model A)*

The sensory evaluation of fresh and aged beer samples play a very important role in the evaluation of flavour stability. The organoleptic evaluation is often used as a borderline for the determination of flavour stability and shelf life.

The data analysis revealed a significant model ($P < 0.0001$) but a relatively low predictive power. ($R^2 = 0.42$). The ageing of the sample ($p < 0.0001$) and the SO2 concentration ($p = 0.0046$) had a significant influence on the sensory evaluation and are presented in Figure 6.15.

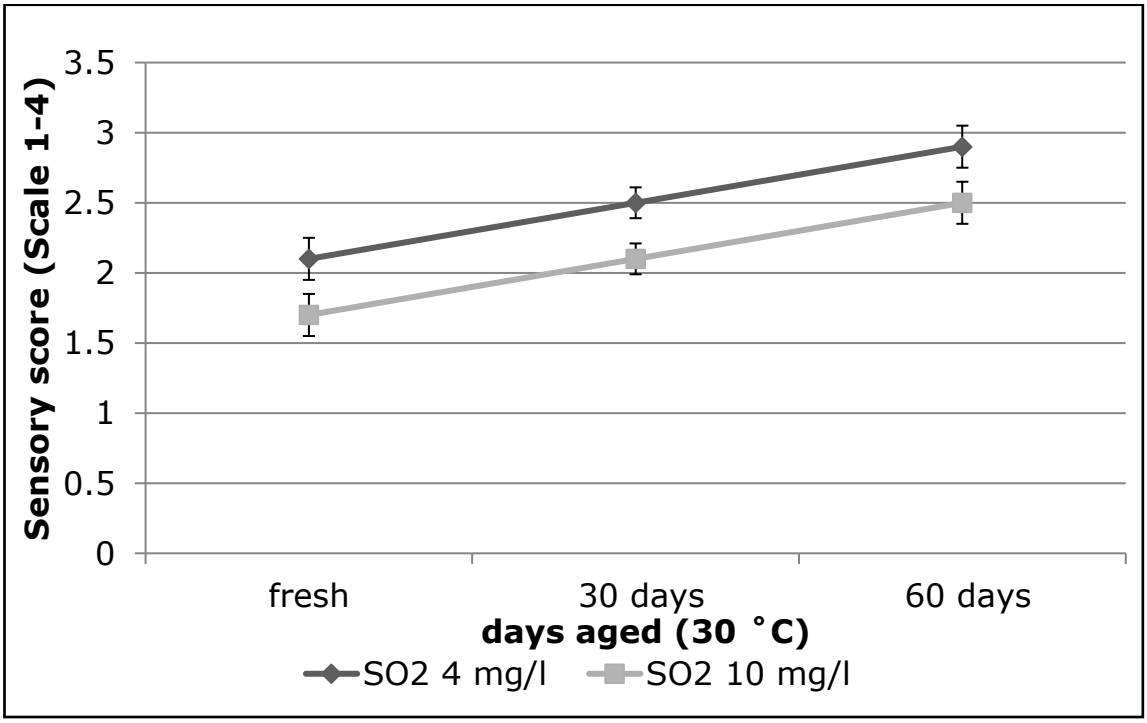


Figure 6.15: Effect of SO_2 on sensory scores during forced ageing period. Actual factors: TIPO = 65 ppb, Fe = 75 ppb, iso-α-extract = 7.5 mg/l, α-extract = 2 mg/l, Model $R^2 = 0.42$

A difference between the low (4 mg/l) and high (10 mg/l) SO_2 concentration of 0.4 sensory score points could be observed for each time point. For both SO_2 concentrations, the sensory score increased by 0.4 sensory score points for each time point during the entire ageing procedure resulting in a total increase of 0.8 score points

from the fresh to the 60 days aged sample. Increasing the SO_2 concentration resulted in significantly better sensory scores. Nevertheless for the high SO_2 concentration samples (addition of SO_2 into the beer sample), the tasters increasingly commented on unpleasant sulphur flavours during the tasting sessions. In the design space no significant effect of TIPO or Fe concentration could be observed for the sensory evaluation.

The coefficient of determination for the sensory model (R^2 = 0.42) was relatively small. Due to the nature of sensory evaluations, the deviation was high despite utilizing trained tasters. The expert sensory panel (see Section 5.1.9) might have been a better option for the sensory evaluation but due to the large number of samples, only a simplified sensory evaluation was feasible. A direct correlation (R^2 > 0.3) of the sensory scores with the EPR data was not possible for the results obtained in the model. The Staling aldehyde measurements (Figure 6.12 and Figure 6.13) and the sensory scores (6.15) showed very similar trends.

6.3.1.6 *Summary results for Model A*

For the majority of the ageing parameters that were analysed, the SO_2 concentration had a significant effect and played an important role in limiting the effects of the forced ageing. Increased concentrations of SO_2 resulted in a reduced radical formation due to the antioxidant properties of SO_2. The suppression of the formation of radicals, the formation of α-hydroxy-sulfonate and a slower release from bound form during storage resulted in lower staling aldehyde concentrations at higher SO_2 concentrations. The lower aldehyde concentrations also resulted in positive sensory scores, regardless of the detectable sulphur flavours. Apart from a strong influence on the radical formation, increased Fe concentrations did not influence any of the other ageing parameters. Since

EPR detected radical formation is mostly dependent on increased oxygen levels, the forced ageing procedure which was used might have reduced the influence of Fe, as the permeation of oxygen through the crown cap was limited in the 60 days ageing time. On the other hand, the total Fe concentration might not be directly influencing the flavour degradation during storage as relatively low Fe levels could be sufficient for the Fenton reaction due to the catalytic action or Fe and the long storage periods.

The increased TIPO concentrations appear to play a minor role, as only the EPR metrics were significantly impacted by TIPO concentrations. This could be related to a direct reaction of SO_2 with a reduced form of the ground state oxygen formed during pasteurization. Lower TIPO levels will lead to higher remaining SO_2 concentrations after bottling and as SO_2 was the main factor in the model, reduced TIPO concentrations should increase flavour stability.

Both hop acid additions had only minor or insignificant effects. The addition of iso-α-extract caused a significant but small reduction in the EPR area values. This showed the antioxidant potential but in relation to the influence of SO_2 and Fe, the effects were minor. Over the entire design space, the addition of α-acids did not significantly influence, positively or negatively, any of the ageing parameters.

6.3.2 Results for Model B

A summary of the significant factors ($p < 0.05$) and their impacts (+/-) on the measured flavour stability indices, the significant two factor interactions ($p < 0.05$) and model statistics for Model B are presented in Table 6.5. The structure of Model B was very similar to Model A, apart from exchanging the hop acids with glutathione and (+)-catechin. Glutathione was introduced into the model to examine the effects of protein thiol groups on flavour stability (Lund and Andersen, 2014). As one of the

quantitatively largest polyphenols present in beer, the addition of (+)-catechin was used to investigate the potential effects due to antioxidant or metal-chelating activities which have long been associated with polyphenols (Aron and Shellhammer, 2010).

Table 6.5 Summary table of significant factors and their impacts on the flavour stability indices, two factor interactions and model statistics for Model B (x = no significant interaction, p > 0.05)

Flavour stability indices	Factors						Two factor interaction		Model statistics	
	Age	TIPO	SO_2	Fe	Cat.	GHS	Factors interacting	p-value	Model R^2	Model sign. (p-value)
SO_2	< 0.0001 (-)	0.0075 (-)	< 0.0001 (+)	x	x	x	Age – SO_2	< 0.0001	0.95	< 0.0001
EPR Area	< 0.0001 (+)	x	< 0.0001 (-)	< 0.0001 (+)	0.0021 (+)	x	x	x	0.92	< 0.0001
EPR x0 lag time	< 0.0001 (-)	x	< 0.0001 (+)	< 0.0001 (-)	x	x	x	x	0.90	< 0.0001
EPR dx	< 0.0001 (-)	x	< 0.0001 (+)	< 0.0001 (-)	x	x	Age – SO_2	0.0024	0.65	< 0.0001
TBI	< 0.0001 (-)	x	x	x	x	< 0.0001 (-)	Age - GHS	0.0077	0.54	< 0.0001
2-MP, 2-MB, 3-MB	< 0.0001 (+)	x	0.0007 (-)	x	x	0.05	Age - GHS	0.025	0.87	< 0.0001
Benzaldehyde	x	x	x	x	x	x	x	x	x	x
Phenyl acetaldehyde	< 0.0001 (+)	x	< 0.0001 (-)	x	x	x	x	x	0.45	< 0.0001
Furfural	< 0.0001 (+)	x	x	x	x	< 0.0001 (-)x	Age – GHS	0.0026	0.67	< 0.0001
(E)-2-nonenal	< 0.0001 (+)	x	0.0001 (-)	x	x	x	Age – SO_2	0.05	0.45	< 0.0001
Sensory	< 0.0001 (+)	x	0.008 (-)	x	x	0.017 (+)	Age – SO_2	0.006	0.53	< 0.0001

6.3.2.1 *Model SO_2 concentration in comparison to analysed concentration of SO_2 for Model B*

In Figure 6.16, the significant effect of TIPO on analysed SO_2 concentration (mg/l) during ageing in displayed.

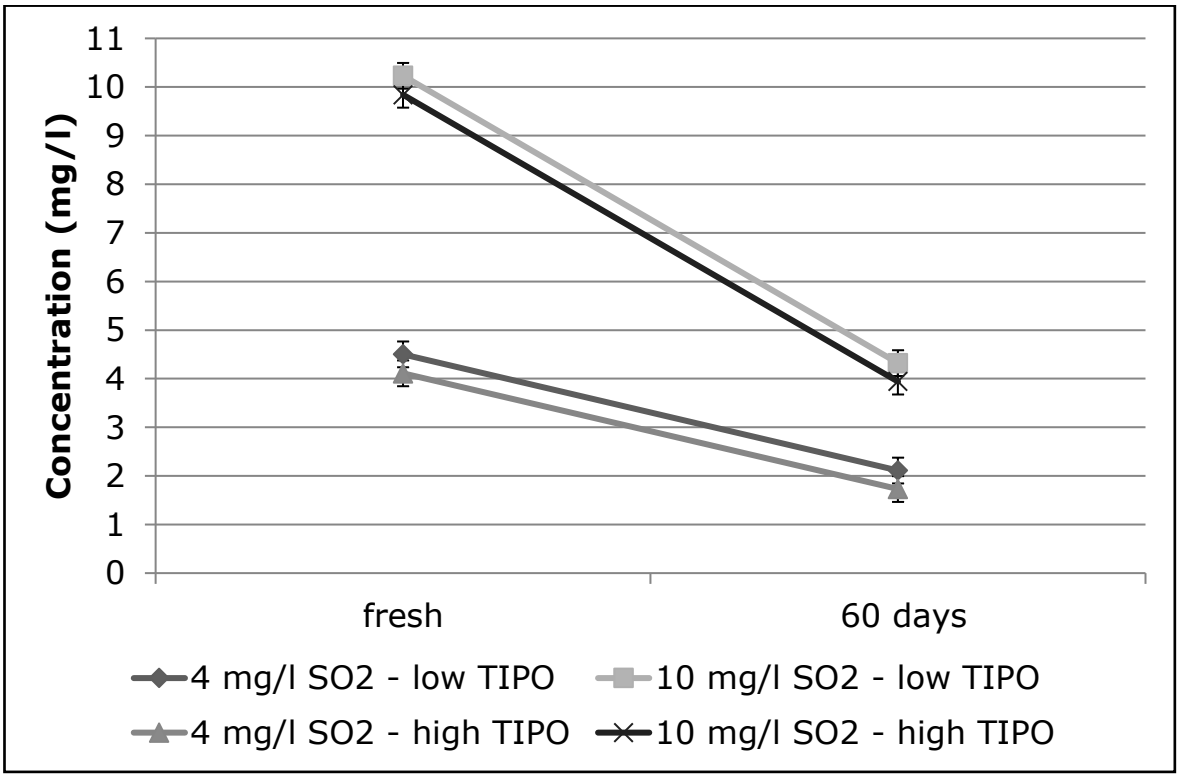


Figure 6.16: Effect of TIPO on analysed SO_2 concentration (mg/l) during ageing. Errorbars represent LSD. Modelled data, Model R^2 = 0.95

For model B, an increase in TIPO concentration from 65 ppb to 370 ppb resulted in a ca. 0.5 mg/l lower SO_2 concentration in the fresh and the forced aged sample (Figure 6.16). None of the other factors influenced the SO_2 concentration either in the fresh or the aged samples.

A 3D plot of the significant two factor interaction of the storage time and model SO_2 concentration on analysed SO_2 concentration (mg/l) is displayed in Figure 6.17.

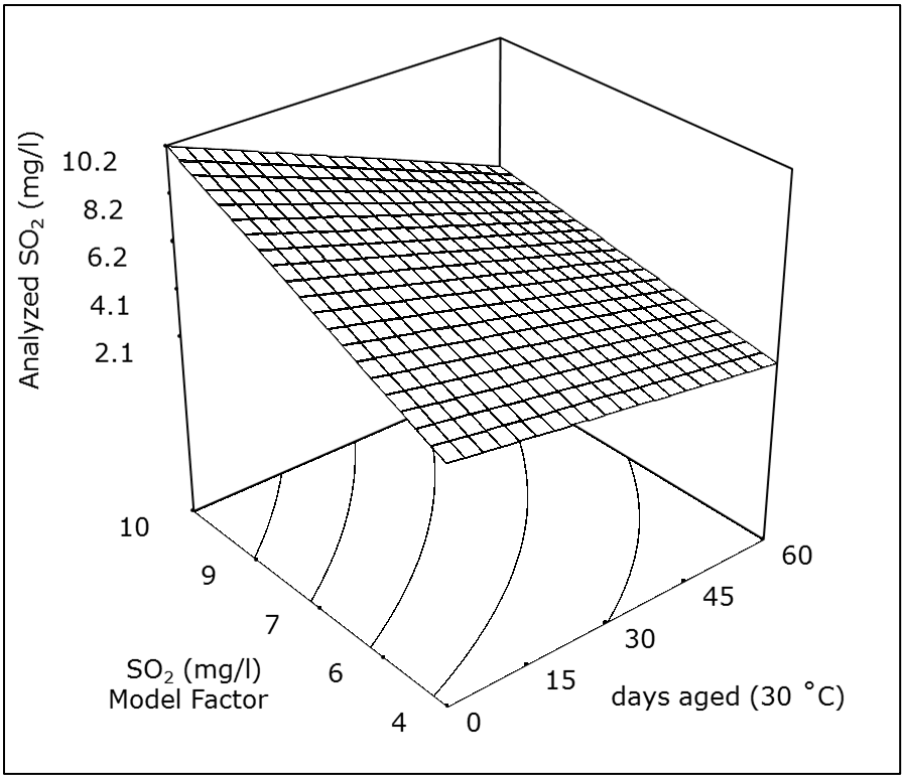


Figure 6.17 3D plot of two factor interaction of days aged and SO_2 on analysed SO_2 concentration (mg/l). Actual factors: TIPO = 65 ppb, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), glutathione = 20 mg/l (addition). Modelled data, Model R^2 = 0.95

In the model a significant two factor interaction ($p < 0.0001$) between ageing and the SO_2 concentration as model factor (4 mg/l and 10 mg/l) could be detected (Figure 6.17) for the analysed SO_2 concentration over the entire design space. The two factor interaction of SO_2 and days aged did show a constant decrease of the SO_2 concentration over the ageing period in the entire design space. The decrease in the design space correlates with data reported by Ilett and Simpson (1995).

6.3.2.2 *EPR spectroscopy measurements of beer oxidative potential for Model B*

The different EPR matrixes: area under the curve, x0-lag time and dx based on the Boltzmann fitting will be discussed for Model B.

6.3.2.2.1 **Area under the curve**

In Figure 6.18, a comparison of the area under the curve values for lowest and highest values of significant single factors SO_2, Fe and (+)-catechin for Model B in the fresh sample are displayed. The effect of single factors set to the lowest and the highest value are presented in the model on the EPR area value in the fresh sample.

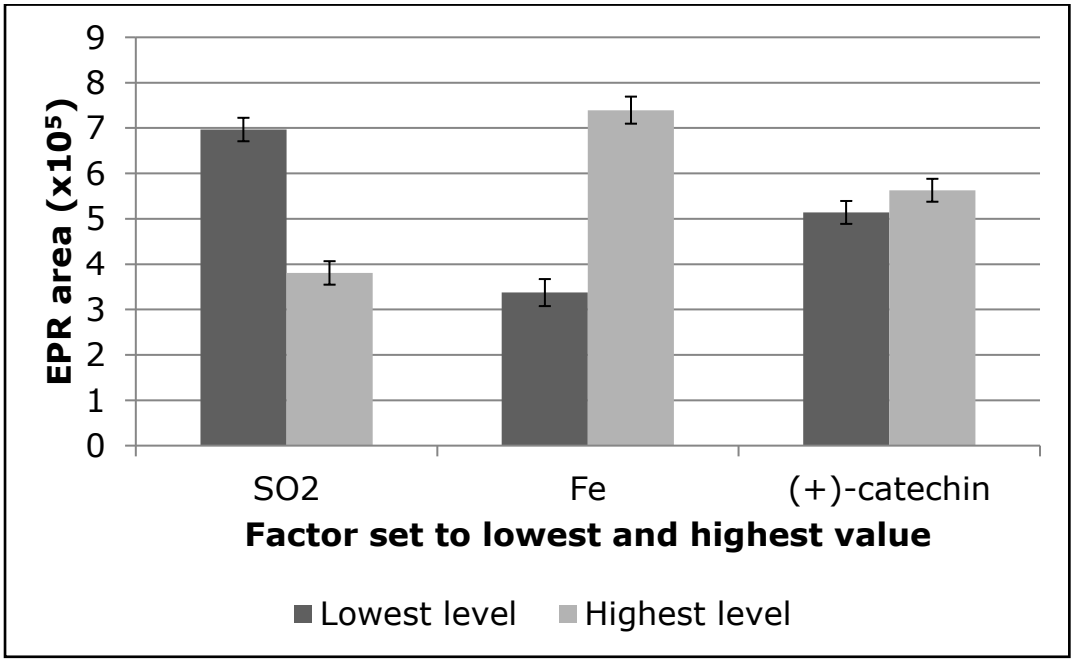


Figure 6.18: Comparison of area under the curve values for lowest and highest values of significant single factors SO_2, Fe and (+)-catechin for Model B in the fresh sample (TIPO = low, SO2 = 7 mg/l, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), glutathione = 20 mg/l (addition) if factor not set for lowest and highest value). Errorbars represent LSD. Modelled data, Model R^2 = 0.92

A significant impact of SO_2, Fe and (+)-catechin concentrations could be detected. The increased SO_2 concentration (4 mg/l to 10 mg/l) approximately halved the EPR area value (- 45 %) (Figure 6.18). The increased Fe concentration (30 mg/l to 120 mg/l) more than doubled the EPR area value (+ 119 %), whereas the addition of

50 mg/l compared to no addition of (+)-catechin had only a small increasing effect (+ 9 %).

The effects of SO_2 (Andersen et al., 2000) and Fe concentration (Zufall and Tyrell, 2008) identified in our experiments on the model B solutions, correlate well with these previous studies. The increased EPR area (5.14 $x10^5$ to 5.62 $x10^5$) that follows (+)-catechin addition is difficult to rationalise. The antioxidant properties of (+)-catechin in beer have been documented (Woodman, 2009, Vinson et al., 2003) but in this experiment, the increased EPR area suggests a pro-oxidant effect for (+)-catechin. As the increase in EPR area was small (5.14 $x10^5$ to 5.62 $x10^5$) in comparison to the other significant factors (Figure 6.18), the pro-oxidant impact of the addition of (+)-catechin should not be stated but no anti-oxidative properties could be detected in the model based on the EPR area matrix.

Glutathione has been reported to improve flavour stability, based on an antioxidant potential (Lund and Andersen, 2011). The EPR area matrix, as a strong indicator for oxidative processes, was not significantly influenced by the addition of glutathione up to an addition 40 mg/l. This might be related to the dominating antioxidant properties of SO_2 in the model solution in comparison to glutathione (Lund and Andersen, 2014).

No two factor interaction between any of the design factors was identified in the model for the EPR area.

6.3.2.2.2 **x0-lag time**

A comparison of x0-lag time values for lowest and highest values of significant single factors SO_2, and Fe for Model B in the fresh sample are shown in Figure 6.19.

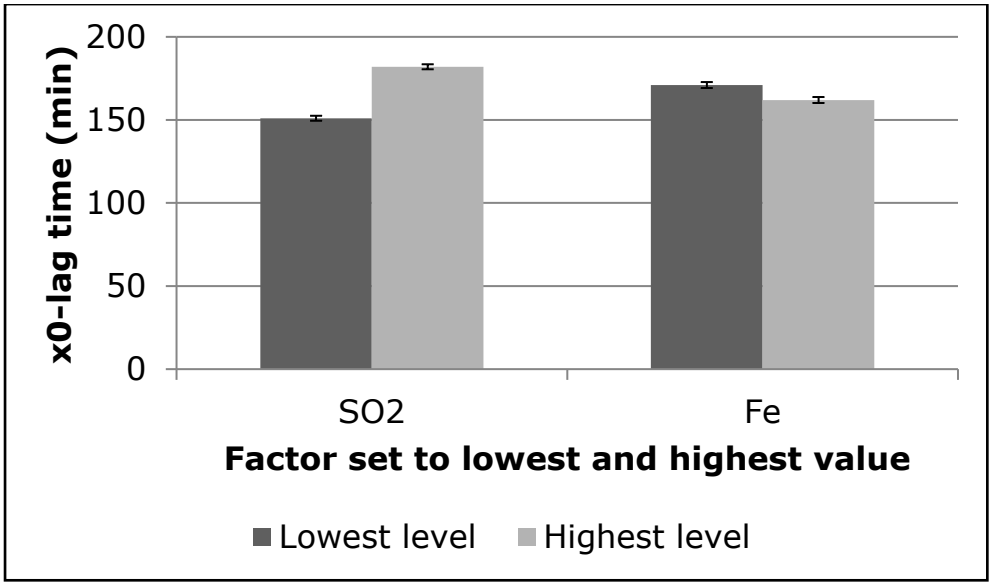


Figure 6.19: Comparison of x0-lag time values for lowest and highest values of significant single factors SO_2, and Fe for Model B in the fresh sample (TIPO = low, SO2 = 7 mg/l, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), glutathione = 20 mg/l (addition) if factor not set for lowest and highest value). Errorbars represent LSD. Modelled data, Model R^2 = 0.90

The effect of the SO_2 and Fe concentration on the x0-lag time in the fresh beer are presented in Figure 6.19. The maximum SO_2 concentration (10 mg/l) resulted in an increased lag-time (+ 31 min) compared to the low SO_2 concentration (4 mg/l). Increasing the Fe concentration (30 ppb up to 120 ppb) reduced the lag time value significantly (- 9 min) but the effect was much lower compared to the impact of SO_2. Ageing the samples over 60 days (30 ˚C) resulted in an average decrease in lag time of 19 min over the entire design space.

For the lag time values for Model B, the SO_2 concentration was the dominant factor. In the fresh samples increasing the concentration of SO_2 up to 10 mg/l prolonged the lag time and the decreasing concentration of SO_2 to 4 mg/l during storage resulted in shorter lag times. Interestingly the increased Fe concentration (up to 120 mg/l) reduced

the lag time values (9 min). This could be related to the increase in the rate of formation of ROS caused by the higher Fe concentration.

The addition of glutathione or (+)-catechin had no effect on the lag time, despite their antioxidant properties and regardless of using the x0-lag time matrix as an indicator of retardant antioxidant classes (Foster, 2009).

6.3.2.2.3 **dx-value**

Figure 6.20 displays the 3D-plot for the significant two factor interaction of SO_2 (mg/l) and storage period at 30 ˚C for samples of modelled EPR dx data.

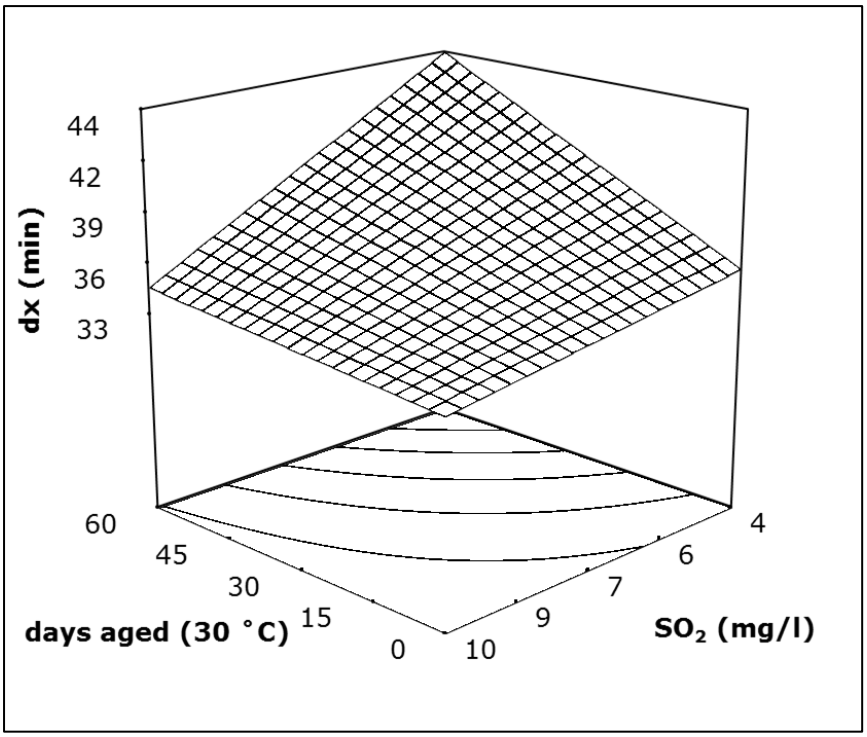


Figure 6.20: 3D-plot of two factor interaction of SO2 (mg/l) and days aged (30 ˚C) for samples of modelled EPR dx data. Actual factors: TIPO = low, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), glutathione = 20 mg/l (addition) Model R^2 = 0.65

For the dx-value, a significant two factor interaction between the concentration of SO_2 and the storage time could be identified (p = 0.0024). An increase from 33 min to 44 min for the low SO_2 concentration (4 mg/l) for the storage trial is shown in Figure

6.20. For the high SO_2 concentration (10 mg/l), only a small increase of the dx value of ca 3 min could be detected.

As a higher dx-value represents a shallower slope of the EPR curve, a reversed result for the dx-value would have been expected. The increased SO_2 concentration suppresses the radical formation and results in a shallower EPR curve. In this experiment, higher SO_2 concentrations resulted in a lower dx-value and therefore in steeper EPR curves. In the given design space, the context of increasing dx-values could not be established. The addition of glutathione or (+)-catechin did not significantly affect the dx-value.

6.3.2.3 *Thiobarbituric acid index for fresh and aged beer samples (Model B)*

The modelling of TBI resulted in a significant two factor interaction ($p = 0.0077$) between the added glutathione and the days the samples were aged (Figure 6.21). For the fresh samples, an increased glutathione addition resulted in a lower TBI value over the entire design space. Incomprehensibly, during the storage trial the TBI value decreased over the entire design space. A further decrease could be detected for increasing glutathione additions. In general a difference between TBI values of 1 to 2 cannot be considered as very high, but a decrease of TBI over time could not be explained, as an increased carbonyl compound concentration occurring during ageing should result in higher TBI values (Fegredo et al., 2009).

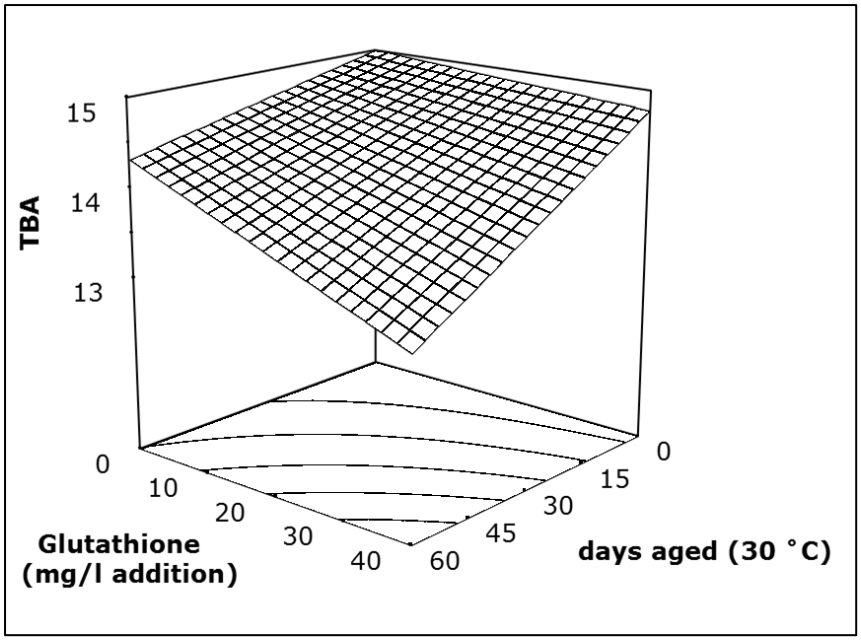


Figure 6.21 3D plot of two factor interaction of days aged and added glutathione on TBI. Actual factors: TIPO = 65 ppb, SO_2 = 7 mg/l, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition). Modelled data, Model R^2 = 0.54

Thus, in our experiments the TBI measurements at 448 nm did not further the understanding of changes occurring during beer storage.

6.3.2.4 *GC-MS SPME measurement of staling aldehydes in fresh and aged beer samples (Model B)*

The measurements and data analysis of the benzaldehyde concentration of the samples for Model B did not result in a significant model. An additional assessment of the GC results ($R^2 > 0.95$ for the calibration curve) and the modelling approach did not result in a valid explanation for the failure of the modelling. At this point it can only be speculated why the modelling approach failed and the benzaldehyde measurements did not correlate significantly with any of the model factors. For all samples, the measured concentration was never near the flavour threshold of 500 ppb (concentration < 15 ppb).

To reduce the complexity of the results for the aldehyde measurements, the concentration of the three Strecker aldehydes 2-methylpropanal, 2-methylbutanal and 3-methylbutanal were combined. In Figure 6.22, the effect of the SO_2 concentration on the concentration of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal during forced ageing period are displayed.

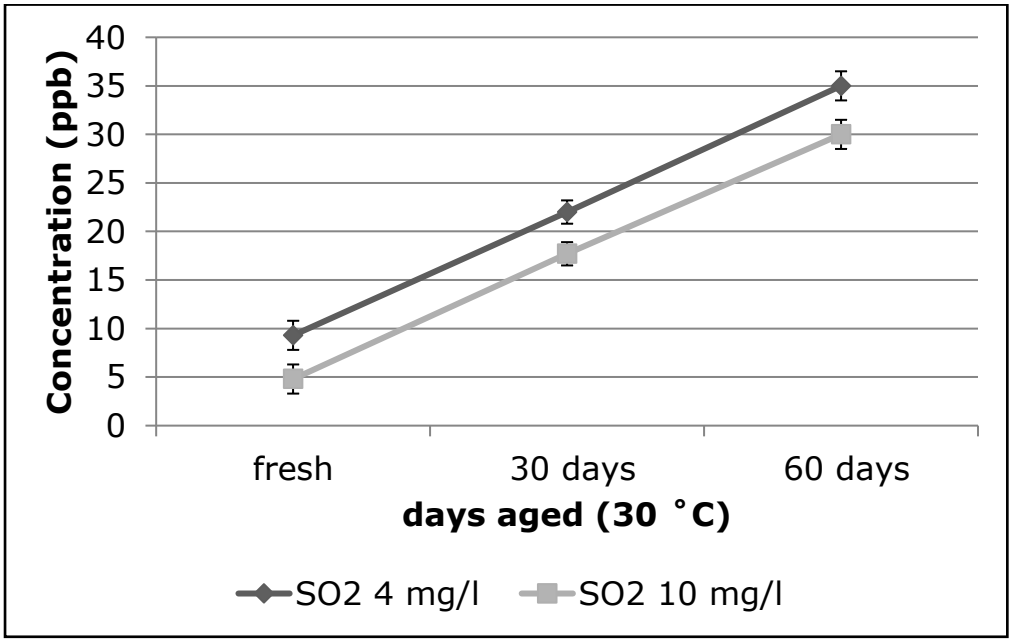


Figure 6.22: Effect of SO_2 on concentration of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal during forced ageing period. Actual factors: TIPO = 65 ppb, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), glutathione = 20 mg/l (addition), Model R^2 = 0.87, error bars represent LSD

An increased SO_2 concentration ($p < 0.0001$) had a significant effect on the combined aldehyde concentration (Figure 6.22). During the storage trial, the concentration of the aldehydes increased for both SO_2 concentrations (4 mg/l and 10 mg/l). For the lower SO_2 concentration (4 mg/l), an increase of ca. 390 % for the aldehyde concentration was detected. The aldehyde concentration for the higher SO_2 concentration (10 mg/l) increased by ca. 600 % over the 60 days of storage. The increase in 2-methylpropanal, 2-methylbutanal and 3-methylbutanal concentration can be directly correlated to the

decrease in SO_2 concentration (Figure 6.13) and the release of the aldehydes from the bisulfite adducts.

A 3D-plot of the significant two factor interaction of glutathione (mg/l addition) and storage period (30 ˚C) on concentration of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal is shown in Figure 6.23.

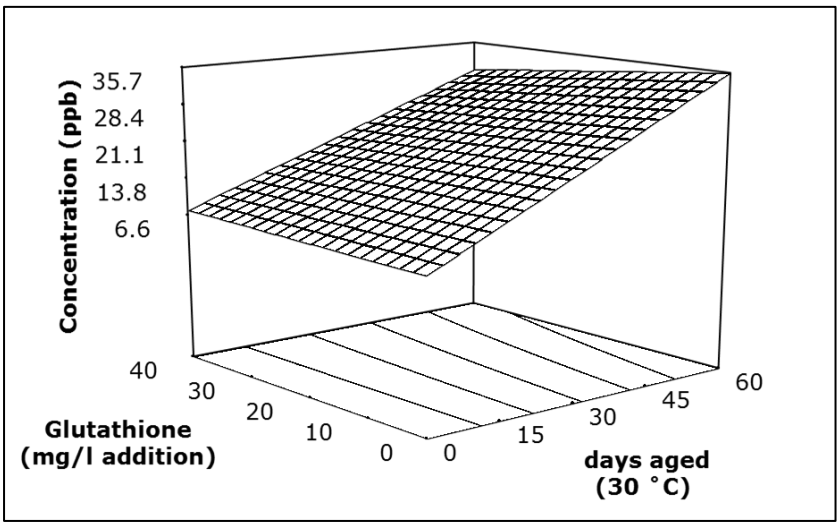


Figure 6.23: 3D-plot of two factor interaction of glutathione (mg/l addition) and days aged (30 ˚C) on concentration of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal. TIPO = 65 ppb, SO_2 = 7 mg/l, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), Model R^2 = 0.87

The influence of the addition of glutathione (p = 0.05) on the total 2-methylpropanal, 2-methylbutanal and 3-methylbutanal concentration is shown in Figure 6.23. In the fresh samples, no correlation between the aldehyde and the glutathione concentrations were resolved. However, over the storage trial the influence of the glutathione appears to increase, resulting in a reduced aldehyde concentration of ca. 5 ppb for a 40 mg/l addition of glutathione. Compared to the SO_2 concentration, the impact of the glutathione addition appears to be secondary and could be related to the binding of the aldehydes with the thiol group of the glutathione.

The effect of an increased SO_2 concentration ($p < 0.0001$) on the phenyl acetaldehyde concentration during the forced ageing period is shown in Figure 6.24.

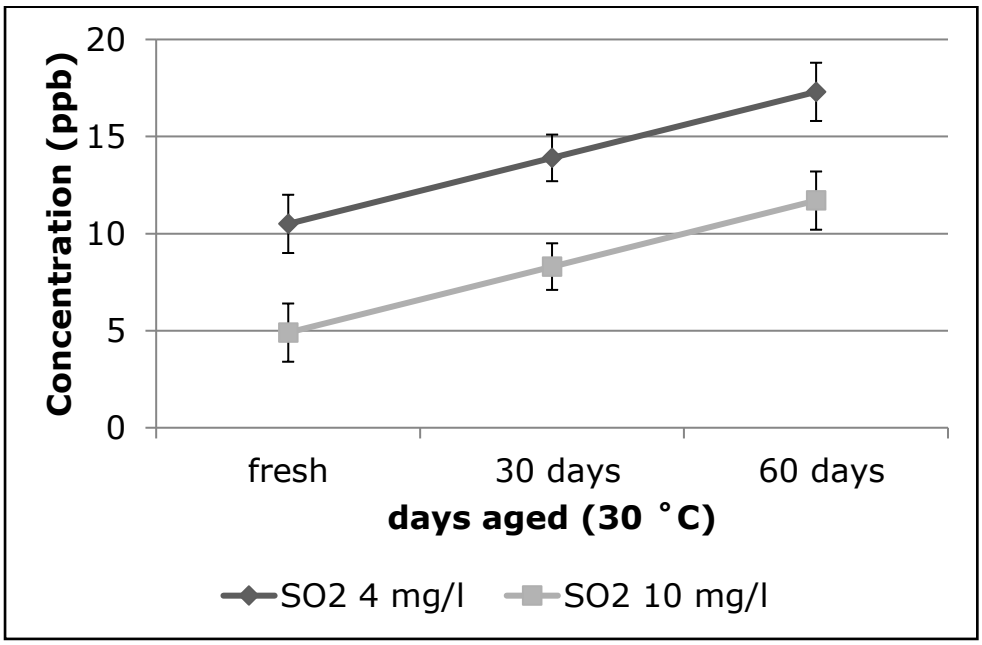


Figure 6.24: Effect of SO_2 on concentration of phenyl acetaldehyde during forced ageing period. Actual factors: TIPO = 65 ppb, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), glutathione = 20 mg/l (addition), Model R^2 = 0.45, error bars represent LSD

During the entire storage trial of 60 days, for the higher SO_2 concentration (10 mg/l), only half the phenyl acetaldehyde concentration compared to 4 mg/l SO_2 concentration was detected.

The significant two factor interaction of glutathione (mg/l addition) and days aged (30 ˚C) on concentration of furfural is displayed in a 3 D plot in Figure 6.25.

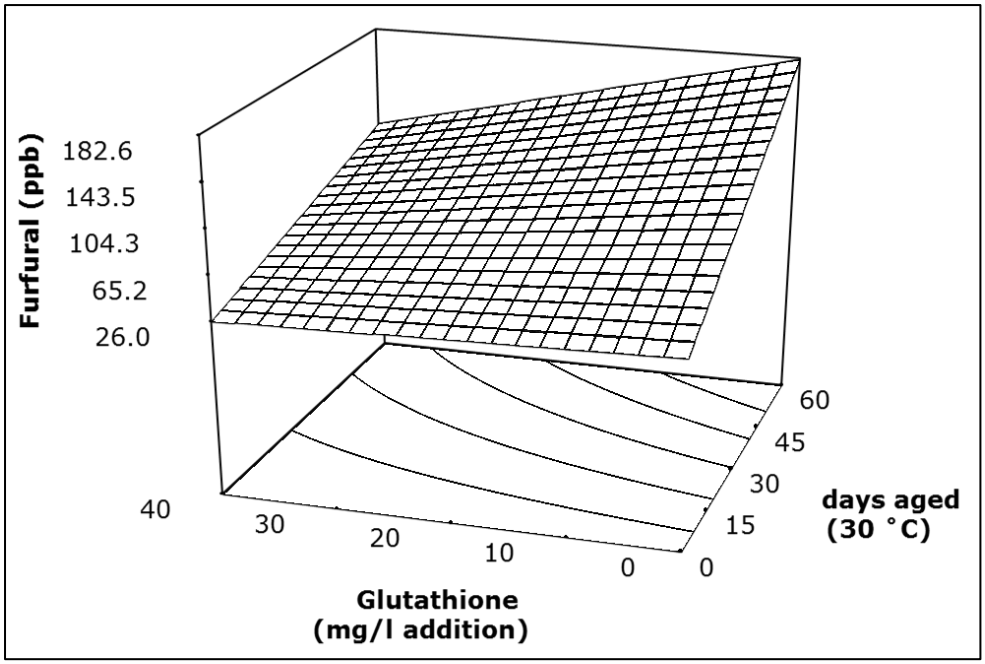


Figure 6.25: 3D-plot of two factor interaction of glutathione (mg/l addition) and days aged (30 ˚C) on concentration of furfural. TIPO = 65 ppb, SO_2 = 7 mg/l, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), Model R^2 = 0.67

The glutathione addition ($p < 0.0001$) was the only factor that significantly influences the concentration of furfural during the storage trial. For fresh samples, the glutathione addition appears to have no effect on the concentration of furfural (ca. 30 ppb furfural for all samples, Figure 6.25). After 60 days of storage, a glutathione addition of 40 mg/l resulted in a 50 % lower furfural concentration compared to no glutathione addition (93 ppb to 180 ppb). The positive effect of glutathione on the furfural concentration might be related to numerous factors. SO_2 is known to have a stronger preference to bind to smaller aldehydes (Dufour et al., 1999) and therefore the influence of SO_2 could be limited for the larger aldehyde furfural. In the model system, the glutathione concentration might have more influence on the ROS formation later in the storage trial (Lund and Andersen, 2014) and therefore the furfural concentration

was reduced ascribable to the antioxidant properties of glutathione (Shimizu et al., 2001).

Figure 6.26 displays the 3D-plot showing the significant two factor interaction of SO_2 (mg/l) and days aged (30 ˚C) on concentration of (E)-2-nonenal in the model.

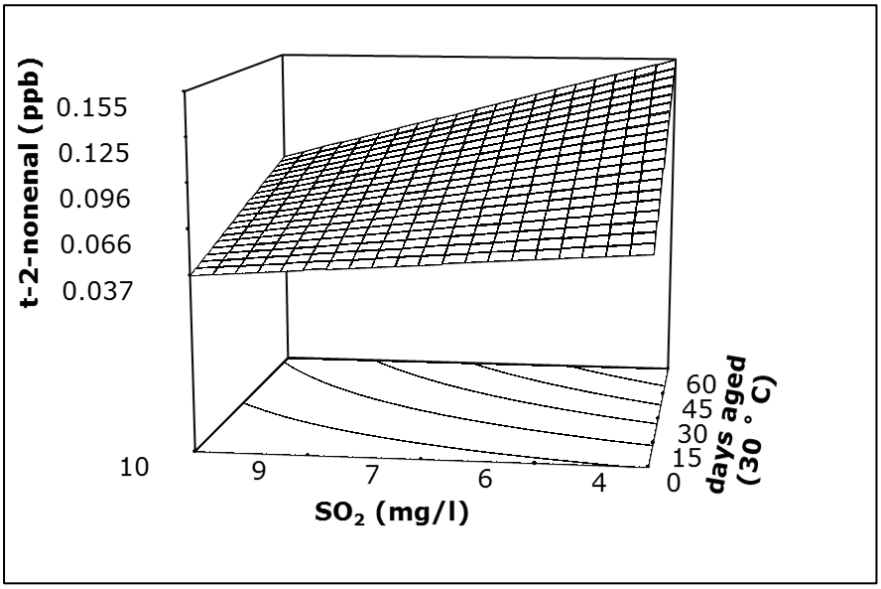


Figure 6.26: 3D-plot of two factor interaction of SO_2 (mg/l) and days aged (30 ˚C) on concentration of (E)-2-nonenal. TIPO = 65 ppb, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), Glutathione = 20 mg/l (addition), Model R^2 = 0.45

The (E)-2-nonenal concentration (Figure 6.26) was significantly influenced by the SO_2 concentration ($p < 0.0001$). In the fresh samples, the detected (E)-2-nonenal levels were very similar for all samples. During the storage trial, increasing SO_2 concentration resulted in lower (E)-2-nonenal concentrations over the entire design space. This was very similar to the findings for Model A and has been discussed previously (6.3.1.4).

For the entire set of aldehydes measured, no significant effects of TIPO or (+)-catechin could be detected. Previously, the positive influence of the (+)-catechin addition on the aldehyde concentration has been reported (Walters et al., 1997b), but in

combination with the other additions, the (+)-catechin concentration appears to play a secondary role.

6.3.2.5 *Sensory evaluation of fresh and aged beer samples (Model A)*

The 3D-plot of the significant two factor interaction of SO_2 (mg/l) and storage period (30 ˚C) on sensory scores (Scale 1 to 4) is displayed in Figure 6.27.

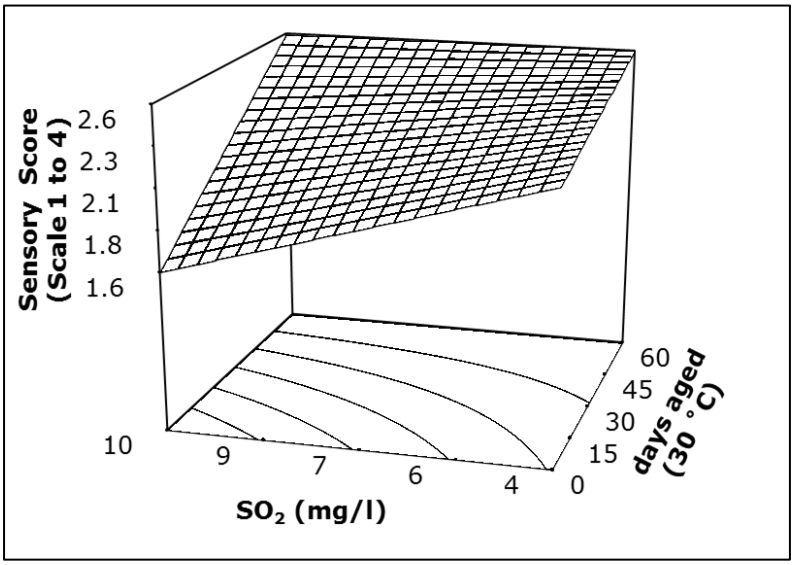


Figure 6.27: 3D-plot of two factor interaction of SO_2 (mg/l) and days aged (30 ˚C) on sensory scores. TIPO = 65 ppb, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), Glutathione = 20 mg/l (addition), Model R^2 = 0.53

For the sensory evaluation, a two factor interaction (p = 0.006) between SO_2 concentration and the storage time was determined (Figure 6.27). In the fresh samples, increasing the SO_2 concentration (4 mg/l to 10 mg/l) resulted in a difference of up to 0.6 in the sensory scores. In the 60 days aged samples, the SO_2 concentration had no influence on the sensory scores (2.6 for all samples). In correlation with the staling aldehyde concentrations and the EPR data, the trend was difficult to explain.

Increasing aldehyde concentrations and intensified oxidation should result in increased sensory scores for the samples with low SO_2 concentrations.

Figure 6.28 displays the single factor interaction of glutathione on the sensory scores in fresh samples.

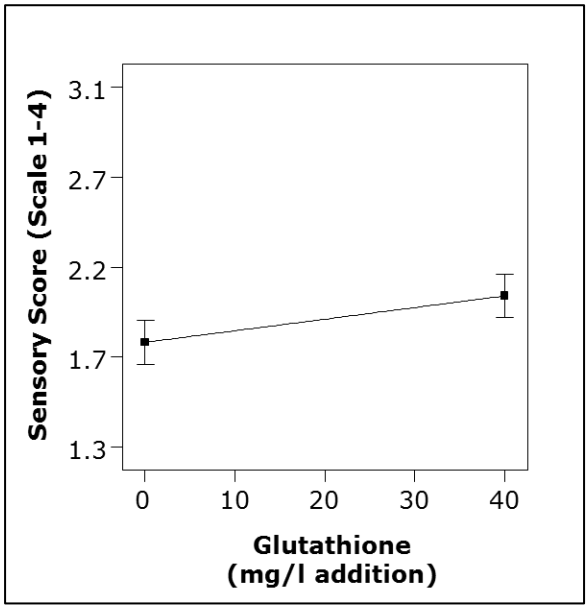


Figure 6.28: Single factor interaction of glutathione on the sensory scores in fresh samples. Actual factors: TIPO = 65 ppb, SO_2 = 7 mg/l, Fe = 75 ppb, (+)-catechin = 25 mg/l (addition), Model R^2 = 0.53, error bars represent LSD. Model predicts linear relationship of the sensory evaluation with the glutathione addition from 0 mg/l 40 mg/l.

The increased glutathione addition had a significant impact on the sensory evaluation ($p = 0.117$). In the fresh samples, the addition of 40 mg/l glutathione resulted in a 0.2 points higher sensory score on a scale of 1 to 4. Furthermore the tasters added comments on a bitter aftertaste for the samples with the glutathione added. Based on the sensory evaluation, the glutathione addition appears to be a questionable method to improve the flavour stability. Furthermore, none of the other factors had a significant impact on the sensory scores.

6.3.2.6 *Summary Model B*

Model B showed strong similarity to Model A. Again the SO_2 concentration appears to be the principal factor in effecting the different flavour stability indices. The radical formation detected by EPR measurements showed the strong antioxidant properties of SO_2 and an increased SO_2 concentration resulted in reduced staling aldehyde concentrations. The data from the sensory evaluation were not easy to interpret, as for the aged samples, the sensory scores appeared to be relatively unaffected.

Increased TIPO concentrations (from 65 ppb to 370 ppb) appeared to have little effect across the entire design space.

Despite its previously reported antioxidant properties (Walters et al., 1997a), (+)-catechin did not appear to reduce the formation of radical species or positively impact any of the flavour stability indices for model B.

Our data showed that glutathione reduced the release and the formation of Strecker aldehydes and furfural. The lower concentrations of the aldehydes with increasing glutathione additions suggests that they were being bound to either the thiol or amino groups present in glutathione. Furthermore, the antioxidant behaviour of the glutathione still present after the SO_2 concentration was reduced over storage, may have also affected the formation of furfural.

As with model A, the EPR metrics did not correlate with, or help to predict the sensory staling data.

6.4 Conclusion

The main focus of attention of the studies reported in this chapter was to incorporate and vary a large number of factors reported to influence beer flavour stability in a single experiment. Despite the large number of factors included and the complexity of the experiment, the modelling approach was capable of identifying the relative significance of, and the interactions between, the main factors in determining the flavour stability metrics of this commercial lager across the given design space.

6.4.1 The impact of So2 concentration

Based on the model results, an effective management of the SO_2 concentration will be one of the most important factors to improve flavour stability. Increased concentrations will reduce radical formation, staling aldehyde concentrations and improve sensory scores. The management of the concentration will be very important to avoid negative effects, including sulphur off-flavours by addition or negative influences on taste and production process by restrained fermentations.

6.4.2 The impact of TIPO

Overall the impact of the TIPO concentration on the different flavour stability indices appeared to be less pronounced when compared to the significant effects on flavour stability described previously in the literature (Bamforth et al., 1993). However TIPO is not the only source of oxygen that may influence flavour stability and throughout the entire brewing process, oxygen uptake during processing and transfers also has the potential to influence flavour stability by producing staling precursors. Reducing oxygen uptake across all stages of processing remains an important goal and will result

in a reduced formation of ROS and increased levels of antioxidants present in the finished product.

6.4.3 The impact of Fe concentration

The Fe concentration directly influences the radical formation measured using EPR spectroscopy of the lager beer samples, but had no effect on any other flavour stability index. Limiting Fe levels will lead to a reduction in the rate of formation of radical species and their total concentration. For the concentrations of Fe used in the model, no direct positive or negative effect could be determined from the formation of aldehydes or from sensory scores, which was perhaps surprising bearing in mind the prominence of Fe in the models of the measured EPR data.

6.4.4 The impact of glutathione concentration

The strong effect of the glutathione addition on Strecker aldehyde and furfural concentration is indicative of the redox potential of glutathione and points towards the possible binding of aldehydes to a protein-thiol group or imine formation (Baert et al., 2015). Further investigation will be necessary to fully understand the formation of aldehydes in the model solutions and their release in relation to protein thiol group concentration. Modulating the concentrations of protein thiol group containing compounds present in a commercial beer could be very difficult, without any negative impact on the general beer quality. Furthermore the addition of glutathione resulted in a bitter aftertaste, and therefore the direct addition in levels positively effecting flavour stability can be problematic.

6.4.5 The impact of hop acids and (+) catechin

The addition of hop acids and (+) catechin had only limited to no effect across the entire design space. The added amounts were representative for a commercial lager but in the given design space had a low potential to improve flavour stability.

6.4.6 Correlation between oxidative stability measures and sensory evaluation

The correlation between the EPR spectroscopic results and sensory scores must be examined further. A different sensory scoring system that is better aligned towards typical ‘oxidised’ stale flavours may be more appropriate. Nevertheless the radical formation detected by EPR spectroscopy, is not the only parameter influencing flavour degradation and therefore a direct correlation may not be possible. For future work, a different approach for the sensory evaluation and a prolonged storage trial at a lower temperature should be used to further explore the interaction between the different factors.

7 Conclusion

Beer flavour stability has become increasingly important to the brewing industry. As multinational brewers export their products into distant markets, products are exposed to longer and more challenging storage conditions, including elevated temperatures and extended distribution distances. Controlling these storage conditions is almost impossible, as after the beer leaves the brewery gate, the brewer has almost no influence regarding the conditions during transport and distribution. Furthermore customer expectations concerning the quality of the final product have increased and a highly competitive beer market leaves little margin for error. Despite being the subject of a large number of studies, many questions remain unanswered concerning the chemical reactions involved in flavour degradation and the best methods to use for the prediction of beer flavour stability. This study has attempted to elucidate the influence of modifications in the brewhouse, filtration and in bright beer on beer flavour stability, with the focus on using and further developing electron paramagnetic resonance (EPR) spectroscopy in addition to more traditional flavour stability indices to gain a further insight into flavour stability.

In Chapters 2 and 3 the development of experimental methods to evaluate flavour stability has been described. This included the determination of one of the major antioxidants present in beer, sulphur dioxide (SO_2), via distillation. Despite being recognised as one of the major antioxidants in beer for decades, one of the most common methods for its determination shows significant methodological differences between the standard methods of analysis reported by the EBC and MEBAK. Our combination and development of the two methods resulted in a robust technique to determine the SO_2 levels of beer. With the use of a beer with a known SO_2 level to

evaluate the method on a daily basis, the reliability of the technique could be improved. We also established a solid phase micro extraction (SPME)-GC-MS method with on fibre derivatization as a reliable detection method for aldehydes related to off flavours perceived in aged beer. As carbonyl compounds play a very important role in the perception of stale flavours in aged beer samples, a reliable method for their detection is indispensable. As those compounds are extremely volatile and are only present in concentrations in the ppb to ppt range, their reliable determination requires special care. This required accurate calibration against external standards ($R^2 < 0.98$), 'just in time' sample preparation (or chilled storage of prepared samples) and regular equipment maintenance. The technique of on fibre derivatization in combination with SPME is capable of reliably measuring staling aldehydes in beer with standard deviations of below 20 % between technical replicates. Nevertheless further development will be required to enable a detailed understanding of pathways of free and bound staling aldehydes. We also measured oxidative stability by electron paramagnetic resonance (EPR) spectroscopy. As a major focus of this work was the evaluation and development of the EPR spectroscopy as a technique to evaluate and predict flavour stability of lager style beers, the technique was subject to extensive assessment. Despite the use of EPR to measure the oxidative stability of beer and wort samples for over two decades, this method of analysis is far from being routine. The application of different data work-up routines and the use of various units like lag time, T_{150} or area under the curve to appraise the oxidative stability measured, illustrates the attempts to link the EPR values to other flavour stability indices and sensory evaluation. Furthermore the accuracy of the results can be affected by the quality and the type of the spin trap used. However, EPR spectroscopy has significant

potential as a research tool to help to further understand the mechanisms that impact the flavour stability of beer.

In Chapter 4 the effects of three different filtration systems, a large scale Kieselguhr frame filter (400 hl/h), a membrane filtration system (8 hl/h) and a membrane filter combined with a centrifuge (20 hl/h), on the oxidative stability measured by EPR were evaluated. Three different lager style beer samples were analysed pre- and post-filtration. The results illustrate how EPR spectroscopy is sensitive to metal ion pick-up from traditional filter media and oxidative stability measures were evaluated versus oxygen pick up across filtration and the sulphur dioxide content of beer samples. The large scale brewing environment required reasonable compromises regarding the trial setup. Nevertheless the results obtained showed clear differences in the performance of the filtration systems. A better performance of both membrane cross-flow filtration systems regarding the oxidative stability compared to the standard Kieselguhr filter could be detected. A constant Fe pickup of 30-40 ppm from pre to post filtration was observed for the Kieselguhr filter systems. Only a limited effect of oxygen pick during filtration on the EPR measurements was observed. A further development was an issue with water supply in one of the breweries related to rusty supply tanks. Overall EPR spectroscopy shows great potential for evaluating the effects of varying process technology, such as the example here regarding beer filtration.

In Chapter 5 remarkable improvements of the oxidative stability of cooled wort samples produced on a pilot scale could be achieved by the addition of gallotannins during mashing (30 % reduced EPR area) as well as for an addition at the end of boil (70 % reduced EPR) in comparison to the control brew. The EPR measurements showed strong evidence for the radical scavenging and metal chelating properties of gallotannins, regardless of the brewhouse addition point. The more traditional

thiobarbituric acid index (TBI) displayed the positive effect of gallotannin addition on reducing the formation of aldehydes used to evaluate the brewhouse performance and overall the wort produced using gallotannin additions complied with all requirements to result in an increased flavour stability in the finished beer in comparison to the control brew. However, the improvements for both addition points in the wort did not clearly transfer to the finished pilot scale beer samples. Oxidative stability measures via EPR spectroscopy as well as TBI, aldehyde formation and sensory results did not result in a conclusive improvement in the finished beer. To further verify the effects in finished beer, the pilot scale trials were repeated on full scale production with focus on the evaluation of the finished beer. Even after 9 months of storage at 20 ˚C none of the indices used, namely EPR, TBI, aldehyde measurement and sensory evaluation, were able to display any significant differences regarding the flavour stability of the beers produced with a gallotannin addition in the brewhouse as compared with the control brews.

In Chapter 6 a factorial experimental design was adopted to probe the interactions between seven factors known to impact on beer flavour stability. A commercial lager beer was brewed, where chemical additions were made to the bright beer prior to bottling, to vary factors as follows: total in pack oxygen (40ppb to 400ppb), SO_2 (3mg/l to 10mg/l), total iron (Fe; 30ppb to 120 ppb), iso-α-acid content (5 IBU to 15 IBU) and α-acid (addition of up to 4 mg/l), (+)-catechin (addition of up to 50mg/l) and glutathione (addition of up to 40mg/l). The aim of this study was to integrate and vary a large number of factors reported to influence flavour stability into a single experiment. Using a factorial design approach revealed significant interactions between single factors but also gave the possibility to evaluate the significance of interactions between the different factors. The oxidative stability, staling aldehyde

formation, SO_2 degradation, TBI and sensory evaluation were measured to determine the effects of the different factors on a commercial lager across the given design space. Despite being reported as one of the main factors influencing flavour stability, the impacts of increased TIPO levels in the present experiment were rather limited. In contrast, a significant impact regarding the oxidative stability could be observed for increasing Fe concentration, highlighting the significance of pro-oxidative effects of transition metals. The impact of the added hop acids were very limited. Apart from the EPR measurements, none of the other stability indices were affected significantly. The similar observation could be made for the addition of catechin. Apart from increasing the EPR area values, no significant effect on the evaluate flavour stability indices could be observed. A strong effect of increasing glutathione concentrations on reducing concentrations of Strecker aldehydes and furfural was detected. This was most likely related to the redox potential associated with glutathione. Unfortunately the sensory scores were affected negatively. Increased SO_2 concentrations had the largest impact across the entire design space, resulting in reduced radical formation, staling aldehyde concentrations and improved sensory scores. The correlation between the EPR results and sensory scores must be further examined, because it was not evident in the present study. The sensory evaluation might need to be adapted to focus solely on stale characters related to beer oxidative stability (e.g. ‘leathery’ ‘papery’ ‘oxidised’) in order to see a better correlation with predictions based on EPR spectroscopy. Overall the use of experimental design gave further insights into the effects of single factors and especially in the significant interactions between the factors.

7.1 Effect of sulphur dioxide

The strong antioxidant activity of the endogenous sulphite as well as the positive impact on beer flavour stability are widely recognized (Andersen et al., 2000). Based on the results presented for all trials, an effective management of the SO_2 concentration will be one of the most important factors to improve flavour stability. Increased concentrations will reduce radical formation, staling aldehyde concentrations and improve sensory scores. However, there are limits (both legal and sensory) on the amounts of SO_2 a beer may contain. Increasing the concentration of SO_2 present in beer above a certain point risks sensory impairment due to sulfury off-notes. Thus direct addition into the finished beer can lead to unpleasant off-flavours. In contrast, technological interventions to increase the SO_2 concentration, such as the use of yeast with a low viability or low wort aeration rates can lead to the deterioration of the flavour profile of the fresh beer. This would have a contrary effect on flavour stability. Therefore a sophisticated technological management and a balanced addition of SO_2 are strong possible measures to improve beer flavour stability. Current evidence suggests that an SO_2 concentration of around 8 mg/L in fresh beer may be a 'sweet spot' to aim for with lager beers. Certainly, the management of the SO_2 content in the finished beer and the degradation rate during storage deserves close attention in any attempt to improve flavour stability.

7.2 Use of EPR spectroscopy to predict flavour stability

The results presented in this work demonstrate the wide range of application of EPR spectroscopy for the characterization of stability and antioxidant properties of beer and wort samples. We have demonstrated that EPR spectroscopy is sensitive to differences

in the oxidative stability of beer and wort samples obtained by the adjustment of a range of technological parameters. Furthermore, on the basis of the work presented in this thesis it can be concluded that EPR spectroscopy did not allow reliable prediction of the speed of sensory flavour deterioration in a given beer. The need for an appropriate control sample for the standardisation of EPR measurements has to be considered.

7.3 Final remarks

Based on the results and conclusions, the following headlines can be summarised:

- SO_2 showed a strong antioxidant effect and significantly influenced the formation and release of staling aldehydes during storage
- Increasing amounts of transition metal ions (FE, Cu, Mn) reduced the anti-oxidant potential of beer
- A negative effect of increased oxygen uptake could be observed but was not as pronounced as expected, especially during the bottling process
- Hop compounds (iso-α-acids and α-acids) overall had only minor effects on flavour stability
- Glutathione, as a molecule containing a protein thiol, increased the antioxidant potential but had negative effect on the sensory evaluation
- Increasing polyphenol concentrations had neither a significant positive or negative effect on beer flavour stability

Beer flavour stability depends on a range of chemical pathways and control parameters and has to be described as a multifactorial problem. When approaching the problem of flavour degradation during storage, the large complexity of the brewing process and the possible effect of any intended or unintended alteration in the process on the

flavour stability of the packaged beer must be considered. Furthermore, the special characteristics of the natural product beer need to be considered. This includes, but is not limited to, the impact of varying raw material composition, water quality or yeast performance. For every technological experiment, only the effects of varied parameters can be identified. The results are accordingly only valid for this test and opposing effects of other parameters not intentionally varied in the experiment cannot be excluded. Within a brewery or an experiment, the quality of the samples is almost equally dependent on intentionally altered parameters and random variables in the brewing process. Therefore, the results reflect only the impact of the varied parameters in the given experimental setup or test series but allow no widely applicable conclusions about the significance of the studied relationship for the entire brewing process or for operations in different breweries. The results are therefore only valid for the actual experimental setup or examined brewery trial. Nevertheless the transfer of the findings to practice in other breweries or experimental setups needs to be considered. However, in the case of the multifactorial design experiments (with the addition of different compounds in a later stage of the brewing process) the question arises as to whether the effects of the addition of a substance may be subtly different to the effects achieved by regulating concentrations of the same components through process control. Therefore further such experiments through process control are needed to establish the broader applicability of the present results. Nonetheless our experiment represents a significant step forwards in that it worked at scale with a commercial product and investigated the impacts of varying multiple factors across an experimental design space.

7.4 Future work

To gain further insight into the formation, degradation and release of staling aldehydes during beer ageing, a further improvement in reproducibility and sensitivity of the detection of those aldehydes will be required. The use of tandem MS in combination with the SPME and on fibre derivatization could be an excellent tool to fulfil those requirements for beer. It has already been established for the evaluation of carbonyl compounds with increased sensitivity in different food applications (Schmarr et al., 2008). Using this technique in the matrix beer, should be very useful to further evaluate aldehyde formation and release during storage.

To further develop the predictive power of EPR spectroscopy regarding beer flavour stability, a further evaluation of the effects of different spin traps used to measure the radical formation during forced beer ageing would be of interest. The most common spin trap for oxidative stability measurements in beer, PBN, directly influences the pH and therefore the radical formation during the forced ageing test. POBN does not affect the pH during the oxidative stability measurement but influences the oxidation state of Fe. As one of the major objectives of EPR spectroscopy utilizing a spin trap is limiting or even eliminating the effect of the spin trap on the measured sample, further development will be required to exploit the full potential of EPR spectroscopy for the evaluation and prediction of beer flavour stability.

The use of alternative spin traps or varying the microwave frequency to examine the effects beyond the hydroxyl ethyl radical formation during forced beer ageing should be capable of revealing other radical induced reactions pathways and make EPR spectroscopy an even more powerful tool in the hunt for increased flavour stability.

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