Title: The impacts of copper, iron and manganese metal ions on the EPR assessment of beer oxidative stability.

Authors: David Jenkins, University of Nottingham, Sutton Bonington Campus, LE12 5RD, UK; **Sue James, Frieda Dehrmann and Katherine Smart** Anheuser-Busch InBev. Woking, GU21 6HT, UK; **and David Cook**¹, University of Nottingham, Sutton Bonington Campus, LE12 5RD, UK.

¹Corresponding author. E-mail: david.cook@nottingham.ac.uk

Jenkins, D., James, S., Dehrmann, F., Smart, K. and Cook, D.

Impact of metal ions on the EPR assessment of beer

Abstract

Beer flavour stability is a key quality parameter as brewers seek to maintain the quality of their product throughout the supply chain. The electron paramagnetic resonance (EPR) oxidative stability assay is one method that brewers are utilising to optimise their process with regards to flavour stability without the time requirements of stored aging and sensory testing of beer. There are still gaps in knowledge relating to the EPR measurement and the factors within the assay that affect the measured results. This investigation aimed to understand the influence that transition metal ions have on the measurement in four different beers (three lagers and one stout). The detrimental impact of copper and iron on the lag time (an indication of when staling may begin) of trial beers is demonstrated, whilst the influence of manganese is shown to differ between beers. The T450 value (an indication of how much staling may occur in a particular beer) is shown to increase with iron and manganese addition in most beers. However, copper reduces the T450 or maximum spin adduct concentration achieved and the potential reasons for this are discussed. Crucially for brewers it has been shown that as little as 10 ppb transition metal ion addition can make detectable difference to the measured oxidative stability.

Introduction

1 Brewers spend a significant amount of time and effort ensuring that their product meets
2 the desired flavour profile. However, during the time it takes for the product to reach the
3 consumer this flavour profile can change, losing positive attributes and developing aged
4 characteristics. Understanding this flavour instability is crucial to improving the customer
5 experience and realising potential savings in the supply chain.

6 Optimisation of the brewing process to limit flavour instability requires an effective
7 measurement against which improvements can be made. Many researchers and brewers
8 have adopted assays utilising EPR, also known as electron spin resonance (ESR), to
9 achieve this aim (4,17). EPR is not sensitive enough to detect short lived radicals
10 generated in beer and therefore the addition of a spin trap is used as part of the assay to
11 quench and stabilise radicals for subsequent measurement. A standard EPR method
12 usually involves artificially aging the beer at an elevated temperature, typically 60 °C, over
13 a short period of time (hours). Over this time the amount of spin adducts formed is
14 proportional to the observed EPR. This signal intensity is plotted against the time of the
15 assay, to generate a curve. There is often an absence of, or reduced, adduct formation
16 seen at the beginning of the assay and this time period is referred to as the lag time. This
17 lag time is suggested to be an indication of the endogenous antioxidant capacity of the
18 beer (32) and hence relates to the time before staling may start in a particular beer. The
19 intensity of the EPR signal at a given time (TXXX), such as a T120 (intensity after 120
20 minute of the assay) (33), is thought to be an indication of the oxidative stability of the
21 beer, which in turn is considered to predict the oxidative deterioration that may occur.
22 With these measures defined, a brewery may be able to use the EPR assay as a tool to
23 optimise their process to produce more flavour stable beer. Although a correlation with
24 sensory data has been suggested (34), this methodology has its limitations, most notably
25 only assessing the oxidative stability of beer and not the non-oxidative staling pathways
26 prevalent in beer and reviewed by Vanderhagen et al. (35). Instances where brewers note
27 a poor correlation of EPR data with subsequent sensorial assessment of aged product have

1 understandably led some to be sceptical that the technique would be suitable for
2 operational deployment.

3 Chapon and Chapon (6) discussed the importance of transition metal ions in the generation
4 of free radicals responsible for beer flavour deterioration, and the intricacies of the
5 reactions have been investigated by several researchers since this study (1,3,15,33).
6 Molecular triplet state oxygen, itself relatively unreactive, can be activated by reduced
7 transition metal ions, which function as electron donors, to superoxide anion ($\bullet\text{O}_2^-$) and
8 its protonated form, the hydroperoxyl radical ($\bullet\text{OOH}$). These species can be further
9 reduced under beer conditions to hydrogen peroxide (H_2O_2) which, in the presence of
10 reduced transition metals, can undergo decomposition to highly reactive hydroxyl radicals
11 ($\bullet\text{OH}$) via the Fenton reaction. Hydroxyl radicals are reactive towards virtually all beer
12 components, and are ultimately responsible for the generation of staling compounds (23).

13 Pohl (24) reviewed the determination of metals in beer, discussing studies which together
14 had measured iron in the range of 15 – 1006 ppb, copper 8 – 800 ppb and manganese 31
15 – 180 ppb. These wide ranges likely reflect differences in beer type, raw materials and
16 practices used in its production, whilst the method of detection may also influence the
17 results (26). A study of Polish beers measured iron in the range of 208-345 ppb, copper
18 at 72-114 ppb, and manganese at 70-165 ppb (26). Another study assessed beers
19 available in a Spanish convenience store and measured iron (62 – 494 ppb), copper (19-
20 68 ppb, with several below the detection limit of the method) and manganese (44-207
21 ppb) (5). A more recent study of 58 different commercial UK lagers (28) may indicate not
22 all breweries have achieved reduced metal ion contents, with the mean iron (461 ppb) and
23 copper (428 ppb) at the top or above these previously reported ranges, whilst the mean
24 concentration of manganese was comfortably within this range (100 ppb). However, there
25 is likely to be a trend in major breweries towards attaining lower concentrations as many
26 acknowledge the impact that metal ions may have on the quality of their products.
27 Changes in brewing practice may alter expected metal ion loadings, for example, the
28 renewed interest in dry hopping which has been suggested to increase the manganese

content of beer (27). In addition to their presence, the particular complexes formed and oxidation states of metal ions in beers may also determine their contribution to oxidative instability. Studies on the forms in which metal are present in beers are less prevalent, but it is suggested they can be found in their free form, bound to phenolic compounds or other organic species (26,31). Chelated metals may exhibit altered oxidation properties within the beer system (36). In this study transition metal ions were added to beers immediately prior to assessment for oxidative stability using the EPR assay. The impact of the transition metal ions on the assessment of oxidative stability and what this might mean for brewers is discussed.

Experimental

Beers

Beers were obtained from local supermarkets in the Nottingham (UK) area and were all within 6 weeks of production.

Metal Additions

Degassed deionised water was used to make stock solutions of the various metals which were added at the concentrations indicated. Ions were added in the form of FeCl₂ (Iron II), FeCl₃ (Iron III), CuCl₂ (Copper II) and MnCl₂ (Manganese II) (Sigma-Aldrich, USA). Additions were made immediately prior to commencement of the oxidative stability assay.

EPR Analysis with POBN As The Spin Trap

Prior to assaying samples were adjusted to 20 °C. α-(4-Pyridyl N-oxide)-N-tert-butyl nitrone (POBN) (Sigma-Aldrich, USA) was prepared as outlined in Kunz et al. (20). 168 mg POBN was dissolved in 1 ml of water and 50 µL of this stock was then added to 12 ml of beer sample. The EPR spectrum was recorded using an E-Scan Beer Analyser system (Bruker). The spectrum parameters were set-up to capture the central peak of the triplet peaks generated, with a centre field of 3475 G and a sweep width of 14 G. The microwave bridge had a power of 2.31 mW and frequency of 9.77. Receiver gain was 2000,

modulation frequency 86 kHz, modulation amplitude 1.49 G, modulation phase 0.85°, time constant 20.48 ms based on the settings of Kunz et al. (20). Samples were inserted into a heating block (60 °C) at staggered intervals. Assays were controlled by EPR Liquid and Beverage Analyser software (ELBA 2.0.2, Bruker) which took samples over at least 450 minutes. 18 scans were aggregated and the peak to peak height of the first derivative of the EPR spectra was recorded as the intensity value at a given time point. Samples were taken using an autosampler (Bruker, USA).

EPR Analysis With PBN As A The Spin Trap

452 mg N-tert-Butyl- α -phenylnitrone (PBN) was dissolved first in 500 μ L of ethanol (Fisher Scientific) and 500 μ L water added. 280 μ L of PBN solution was added to 7 ml beer sample. Samples were placed in a 60 °C heating block at staggered intervals. EPR spectra were recorded with a centre field of 3478 G and sweep width of 17 G. The microwave bridge had a power of 2.31 mW and frequency of 9.77. Receiver gain was 1261, modulation frequency 86 kHz, modulation amplitude 1.1 G, modulation phase 0.85°, time constant 20.48 ms. Scans were aggregated and the peak to peak height of the first derivative of the EPR spectra was recorded as the intensity value at a given time point. Assays were run for 150 minutes with 15 samples taken during this time. Samples were taken using an autosampler (Bruker, USA) and the running order was randomised.

Curve Analysis

A sigmoidal curve was fitted to the data using GraphPad Prism 6 (USA). From the resulting fitted curve, lag times were determined as the point at which the modelled curve reached an intensity equal to 12 % of the difference between the upper and lower limits of the curve. The T450 was the value of the curve at 450 minutes. The rate at the point of inflection was determined as the maximum rate (EPR Intensity / min) of the curve. In cases where the signal reduced, curves were fitted to the sample points prior to this reduction.

Metal Quantification

Nitric acid (trace metal grade, Fisher, UK) was added to beer samples to final concentration of 2%. Quantification of iron, copper and manganese was achieved using inductively coupled plasma mass spectrometry (ICP-MS) (Thermo-Fisher iCAP-Q) with a Flatopole collision cell (charged with helium gas) upstream of the analytical quadrupole. Internal standards were introduced to the sample stream via a T-piece with Sc ($50 \mu\text{g L}^{-1}$), Ge ($20 \mu\text{g L}^{-1}$) Rh ($10 \mu\text{g L}^{-1}$) and Ir ($5 \mu\text{g L}^{-1}$) included in the matrix of 2% HNO_3 ; External calibration standards for iron, copper and manganese were run at 0, 20, 40 and $100 \mu\text{g L}^{-1}$ (ppb). Samples were introduced via an autosampler (Cetac ASX-520) through a nebuliser. Sample processing was undertaken using Qtegra software (Thermo-Fisher Scientific).

Statistical Analysis of data

Means and standard deviations were calculated using Excel software, (Microsoft, USA).

Results and Discussion

Transition Metal Ion Content of Beer

The transition metal ion contents of four beers (one stout and three lager style) selected for this study were measured (Table 1). The iron content of the stout (Beer D) was highest (161 ppb), in keeping with previous studies of this beer style (29). The iron contents of beers A-C were low in comparison to the studies discussed previously ($25 - 37 \text{ ppb}$) as was copper ($33 - 57 \text{ ppb}$) and may represent best practice in the industry.

The Oxidative Stability of the Beers

Although efforts were made to select relatively fresh beers (within six weeks of production) from the trade, no knowledge of the supply chain from brewery to vendor was available. The oxidative stability of a beer, assessed using EPR, is a dynamic property altered by reactions in the beer which occur over time and are influenced by environment, particularly temperature. Therefore an unmeasured deterioration in the trial beers will have occurred prior to the commencement of this study. Variation may also occur between batches due

to production differences. Beers were assessed at the commencement of the trial with the knowledge that they would likely be in different states of oxidative stability and that this would provide variation within the experimental design. When utilising the oxidative stability assay, brewers target longer lag times and/or smaller TXXX for improved flavour stability. Using POBN as the spin trap, Beer A demonstrated the longest lag time (204 min), smallest rate (Max EPR Intensity / min) of radical production (2412) and a low T450 (5.8×10^5), indicating that it was the most oxidatively stable beer of the four at the time of the study. Beer D demonstrated an absence of any lag time and the highest T450 value (Figure 1), hence was the least flavour stable beer included in the study.

The Impact of Metal Ion Additions

Given the importance of transition metal ions in the promotion of oxidative instability in beer this study set out to investigate the impact of three transition metals (iron, copper and manganese) on the EPR oxidative stability. Individual metal salts were dosed into the beer in addition to the inherent metal ion contents (Table 1). Beer A, the most oxidatively stable beer at the time of the study, was spiked with 10, 20, 40, 80, 160 and 320 additional ppb of iron, copper and manganese. Beers B-C were spiked with 40, 80 and 160 ppb of the same metal ions.

Iron

The addition of iron directly to Beer A resulted in an earlier generation of spin adducts and higher overall production with greater iron concentrations (Figure 2). These curves translated into shortened lag times and raised T450 measurements, reducing the oxidative stability of the beer in agreement with previous studies (2,16,20,32). Examples of the curves generated with 160 ppb addition of iron to beers B-D can be found in Figures 3-5, whilst the measures derived from the curves generated at all concentrations trialled in these beers (0, 40, 80 and 160 ppb) are detailed in Table 1. The trend of increased iron being detrimental to oxidative stability markers is evident throughout the samples. However, whereas 40 ppb addition to Beer A increased the T450 by 37%, the same

addition to Beer C resulted in no change, and only 6 % increase at the highest iron addition (160 ppb). Beer C was the least oxidatively stable of the three lagers at the start of the trial and the reduced impact of additional iron may be an indication that further instability was harder to generate. Although Beer D had the highest initial T450 and by far the highest initial iron concentration (162 ppb) the addition of 40 ppb still produced a 12% increase in the T450 value. This is in keeping with theories of the pro-oxidation activity of iron previously explored.

It has been proposed that iron catalyses the initial reduction of oxygen leading to the formation of hydrogen peroxide, through a cycle between iron(II) and iron(III), whilst iron also catalyses the generation of the hydroxyl radical from hydrogen peroxide (23). For iron to have this catalytic effect it needs to be in the Fe(II) oxidative state. Filik and Giray (11) suggested that Fe(II) ions are predominant in fresh beer, but as the beer ages Fe(III) dominates. Although speciation of iron in the trial beers was not investigated, the effect of iron(III) addition to Beer A was trialled (data not included) resulting in similar results as iron(II), an observation also noted previously (23). When Fe(III) is formed, it can be reduced by a variety of compounds to return to Fe(II). Kunz et al (21) suggest this cycling in beer can be achieved through the reducing activities of intermediates in the Maillard reaction. In wine the recycling activity of polyphenols has been proposed as the key reaction mechanism in the cycling of iron (8).

Manganese

Manganese was the metal present in the highest concentration in the three lager beers trialled (Table 1). The addition of manganese ions had little impact on the rate (EPR Intensity / min), T450 or lag time when added to Beer A (Figure 6). In contrast Beers B and C both showed a reduction in lag time with increasing manganese addition (Beer D had no lag time prior to addition). Beers B-D also demonstrated increased T450, with 57%, 22% and 48 % increases respectively, at the highest manganese addition rate (160 ppb). This suggests that the action of manganese in beers B-D was more detrimental to oxidative stability than iron. Previous determination of the speciation of manganese in beer proposed

1 that it is likely to be present as Mn^{2+} (31), although a later study suggested that not all
2 manganese is in ionic form, but in fact a proportion may be bound, particularly in what
3 they suggest are premium beer brands (25). Phenolic compounds, amino acids or organic
4 acids were listed as potential ligands for manganese, which were not measured in this
5 study.

6 The oxidative stability measures of Beer A were relatively unaffected by the addition of
7 manganese. It is speculated that Beer A contained elevated levels of a ligand capable of
8 reducing the pro-oxidant activity of manganese, although this has not been proven here.

9 Manganese on its own in model wine has been shown to exert a relatively low oxidative
10 effect, but its' potency was greatly enhanced by the presence of copper and iron. It was
11 proposed that manganese may interact with intermediate complexes in iron and copper
12 oxidation, enhancing the catalytic effect (9). Therefore any lack of pro-oxidant activity
13 from manganese may also reflect the non-availability of active iron or copper ions in the
14 beer.

15 *Copper*

16 Copper is often postulated to have a similar pro-oxidant role to iron in contributing to
17 flavour instability (18,23), but its influence on the EPR assay has not been explored to the
18 same extent as iron in beer. The formation of spin adducts during the oxidative stability
19 assay when copper concentration is increased clearly differs from the other metals in this
20 study (Figure 7). The earlier formation of spin adducts is evident, resulting in the shortest
21 lag time for Beers A-C of all metals trialled (Table 1). T450 values however were
22 consistently reduced in beers as copper was increased, in strong contrast to the results of
23 iron. The maximum rate of adduct formation provided no clear trends when all beers are
24 considered. A correlation between copper and beer staling assessed by sensory panel has
25 previously been demonstrated (15). It has also been shown to have a dramatic effect on
26 thiobarbituric acid values, used to estimate staling substances, which were increased with
27 its addition (3). In addition to catalysing Fenton type reactions, the increase in oxidative

1 action of copper may be due to interactions with iron, since the copper ion has been
2 suggested to facilitate redox cycling of iron in wine systems (7).

3 It was not the intention at the outset of this work to compare EPR spin traps or
4 methodology, but the unusual result seen with copper required verification. POBN has
5 been suggested as a superior spin trap partly as it is not affected by the pH change during
6 the assay, unlike PBN (20). The trends seen with iron and copper are still seen when the
7 alternative spin trap PBN is used (Figures 8-9). However in this case the trend only became
8 apparent at 80 ppb additional copper, when the T150 is seen to be lower than the previous
9 40 ppb addition (Table 3). Similar early generation of spin adducts but reduced maximum
10 peaks have been generated when copper was added to wine (10). Although unusual,
11 certain beers can produce a signal which begins to reduce during the timeframe of the
12 assay (data not shown). When the assay is allowed to continue beyond the normal time
13 frame (up-to 39 hrs) the spin adduct life cycle is evident (Figure 10). Here the initial lag
14 time has been missed, with the second sample point at 180 min, but the formation of
15 adducts is clearly shown; a peak is reached and a more gradual reduction as the spin
16 adducts and traps break down. Manganese produced the highest peak intensity when 160
17 ppb of metal ions were added to beer, followed by iron, the control and copper
18 respectively. Copper again forms more spin adducts early in the assay compared to the
19 control but a smaller maximum peak is achieved. The EPR signal has been observed to
20 reach a plateau (19), with the authors suggesting this steady-state level is determined by
21 competition of the rates of formation and degradation of the spin adducts.

22 The smaller area under the curve when copper is added would suggest fewer spin adducts
23 are formed in the assay or that those adducts that are formed are less stable. Copper has
24 been shown to destabilise POBN spin adducts whereas iron did not, although this was not
25 in a beer system (13), and as the curves presented here are in theory determined by the
26 generation and degradation of spin adducts no clear evidence of copper interacting with
27 the adducts is provided.

Another potential scenario is that increased copper results in earlier radical formation, but less overall. To generate fewer radicals than the control suggests copper is interacting with component/s which are pro-oxidant in the beer system. In a similar way that Iron is thought to cycle between the iron(III) and iron(II) oxidation states, copper cycles between copper(II) and copper(I). It has been suggested that Maillard reaction products may reduce metal ions in beer (21) whilst ascorbate and glutathione have been highlighted as reductants in non-beer systems (30). In non-beer systems it has been shown that glutathione binds Cu(I) as a stable complex with no or greatly reduced reaction with hydrogen peroxide to form the hydroxyl radical (14). Whilst a scenario such as this may explain a reduction in copper pro-oxidative effect, it does not explain the reduction in radical production seen relative to the control.

Proteins have been shown to play a role in the oxidative stability of beer, as reviewed by Wu *et al.* (37). Of particular relevance to this study, proteins have the ability to bind to pro-oxidant metal ions and their subsequent precipitation has been suggested as a possible reason for an increased oxidative stability seen when beers were pasteurised (22). If increased copper resulted in greater protein precipitation this could potentially reduce the overall spin-adduct formation whilst also oxidising the sulphite compounds which are largely responsible for the initial lag time. However this hypothesis was not tested or proven in the present study.

Conclusions

Under experimental conditions, copper addition was shown to produce the greatest reduction in lag time whilst manganese typically resulted in the greatest increase in T450, with the exception of Beer A. Iron was consistently detrimental to both lag time and T450 measures. The influence of copper on the T450 value is currently unexplained and some potential reasons for this are discussed. Although only apparent at higher copper concentrations this observation may be helpful for brewers relying on EPR assays to consider when interpreting results. Crucially for brewers it has been shown that as little as 10 ppb transition metal ion addition can make a detectable difference to the oxidative

1 stability of relatively stable beers. Whilst the benefits of reducing the transition metal ion
2 content further was not directly demonstrated (due to the complexities of removing metal
3 ions innate to the beers), further gains may be achievable through reduction of transition
4 metal ions in beer beyond their current levels. Historically the focus has been on iron
5 content. However, with substantial reductions achieved, consideration must be given to
6 other transition metals, particularly copper and manganese which may be increased in
7 beer due to changing brewing practices.

8 The value of EPR spectroscopy to the brewing industry as a rapid prediction of flavour
9 stability, enabling proactive process changes, is clear. Changes in lag time and TXXX taken
10 from EPR assay curves have been shown to differ independently, confirming that in some
11 scenarios they offer different measures of a beer's oxidative stability. Previous publications
12 have alluded to the need to consider the relevance of curve changes on a case by case
13 basis (12), and the variety in changes demonstrated here reinforces this message.

14 **Acknowledgements**

15 David Jenkins gratefully acknowledges the financial support of this work from SABMiller
16 Plc. and the University of Nottingham through the Barry Axcell Fellowship in Brewing
17 Science. Thanks are also given to Anheuser-Busch InBev for permission to publish this work.

18 **References**

19
20 1. Andersen, M. L., H. Outtrup, and L. H. Skibsted. Potential Antioxidants in Beer
21 Assessed by ESR Spin Trapping. J. Agric. Food Chem. 48: 3106–3111. 2000.

22 2. Andersen, M. L., and L. H. Skibsted. Electron Spin Resonance Spin Trapping
23 Identification of Radicals Formed during Aerobic Forced Aging of Beer. J. Agric. Food Chem.
24 46: 1272–1275. 1998.

25 3. Bamforth, C. W., and R. Parsons. New Procedures to Improve the Flavor Stability of
26 Beer. J. Am. Soc. Brew. Chem. 43: 197–202. 1985.

4. Barr, D., B. Baker, S. Bosben, T. Bradshaw, E. Converse, A. Daar, R. Garner, C. Giarratano, S. Gonzalez, H. Hight, T. Kunz, D. Loyet, N. Penn, C. Rhodes, F. Saunders, L. Young, J. Zinanti, and R. Foster. Standard method for measurement of oxidative resistance of beer by electron paramagnetic resonance. *J. Am. Soc. Brew. Chem.* 66: 259–260. 2008.
5. Bellido-Milla, D., J. M. Moreno-Perez, and M. P. Hernández-Artiga. Differentiation and classification of beers with flame atomic spectrometry and molecular absorption spectrometry and sample preparation assisted by microwaves. *Spectrochim. Acta Part B At. Spectrosc.* 55: 855–864. 2000.
6. Chapon, L., and S. Chapon. Peroxidatic Step in Oxidation of Beers. *J. Am. Soc. Brew. Chem.* 37. 1979.
7. Danilewicz, J. C. Interaction of sulfur dioxide, polyphenols, and oxygen in a wine-model system: Central role of iron and copper. *Am. J. Enol. Vitic.* 58: 53–60. 2007.
8. Danilewicz, J. C. Reactions involving iron in mediating catechol oxidation in model wine. *Am. J. Enol. Vitic.* 64: 316–324. 2013.
9. Danilewicz, J. C. Chemistry of Manganese and Interaction with Iron and Copper in Wine. *Am. J. Enol. Vitic.: ajev.* 2016.16033. 2016.
10. Elias, R. J., M. L. Andersen, L. H. Skibsted, and A. L. Waterhouse. Key factors affecting radical formation in wine studied by spin trapping and EPR spectroscopy. *Am. J. Enol. Vitic.* 60: 471–476. 2009.
11. Filik, H., and D. Giray. Cloud point extraction for speciation of iron in beer samples by spectrophotometry. *Food Chem.* 130: 209–213. 2012.
12. Foster, R. I., E. J. Samp, H. Patino, and D. P. Barr. Electron Paramagnetic Resonance [EPR] profiling for potential flavor stability improvements in beer. *Tech. Q.-Master Brew. Assoc. Am.* 38: 247–250. 2001.
13. Gunther, M. R., P. M. Hanna, R. P. Mason, and M. S. Cohen. Hydroxyl Radical Formation from Cuprous Ion and Hydrogen Peroxide: A Spin-Trapping Study. *Arch. Biochem. Biophys.* 316: 515–522. 1995.

14. Hanna, P. M., and R. P. Mason. Direct evidence for inhibition of free radical formation from Cu(I) and hydrogen peroxide by glutathione and other potential ligands using the EPR spin-trapping technique. *Arch. Biochem. Biophys.* 295: 205–213. 1992.
15. Irwin, A. J., R. L. Barker, and P. Pipasts. The role of copper, oxygen, and polyphenols in beer flavour instability. *J. Am. Soc. Brew. Chem.* 49: 140–149. 1991.
16. Kaneda, H., Y. Kano, S. Koshino, and H. Ohya-Nishiguchi. Behavior and role of iron ions in beer deterioration. *J. Agric. Food Chem.* 40: 2102–2107. 1992.
17. Kaneda, H., Y. Kano, T. Osawa, N. Ramarathnam, S. Kawakishi, and K. Kamada. Detection of Free Radicals in Beer Oxidation. *J. Food Sci.* 53: 885–888. 1988.
18. Kaneda, H., N. Kobayashi, I. Takashio, I. Tamaki, and K. Shinotsuka. Beer staling mechanism. *MBAA TQ* 36: 41–47. 1999.
19. Kocherginsky, N. M., Y. Y. Kostetski, and A. I. Smirnov. Antioxidant Pool in Beer and Kinetics of EPR Spin-Trapping. *J. Agric. Food Chem.* 53: 6870–6876. 2005.
20. Kunz, T., C. Müller, and F.-J. Methner. EAP determination and beverage antioxidative Index (BAX) - Advantageous tools for evaluation of the oxidative flavour stability of beer and beverages (Part 1). *BrewingScience* 66: 12–22. 2013.
21. Kunz, T., A. Strähmel, N. Cortés, L. W. Kroh, and F.-J. Methner. Influence of intermediate maillard reaction products with enediol structure on the oxidative stability of beverages. *J. Am. Soc. Brew. Chem.* 71: 114–123. 2013.
22. Lund, M. N., S. Hoff, T. S. Berner, R. Lametsch, and M. L. Andersen. Effect of Pasteurization on the Protein Composition and Oxidative Stability of Beer during Storage. *J. Agric. Food Chem.* 60: 12362–12370. 2012.
23. Lund, M. N., A. C. Krämer, and M. L. Andersen. Antioxidative Mechanisms of Sulfite and Protein-Derived Thiols during Early Stages of Metal Induced Oxidative Reactions in Beer. *J. Agric. Food Chem.* 63: 8254–8261. 2015.
24. Pohl, P. Determination and fractionation of metals in beer: A review. *Food Addit. Contam. Part A* 25: 693–703. 2008.

25. Pohl, P., and B. Prusisz. Fractionation analysis of manganese and zinc in beers by means of two sorbent column system and flame atomic absorption spectrometry. *Talanta* 71: 1616–1623. 2007.
26. Pohl, P., and B. Prusisz. Chemical fractionation of Cu, Fe and Mn in canned Polish beers. *J. Food Compos. Anal.* 23: 86–94. 2010.
27. Porter, J. R., and C. W. Bamforth. NOTE: Manganese in Brewing Raw Materials, Disposition During the Brewing Process, and Impact on the Flavor Instability of Beer. *J. Am. Soc. Brew. Chem.* 2016.
28. Rodrigo, S., S. D. Young, M. I. Talaverano, and M. R. Broadley. The influence of style and origin on mineral composition of beers retailing in the UK. *Eur. Food Res. Technol.*: 1–9. 2016.
29. Sancho, D., C. A. Blanco, I. Caballero, and A. Pascual. Free iron in pale, dark and alcohol-free commercial lager beers. *J. Sci. Food Agric.* 91: 1142–1147. 2011.
30. Spasojević, I., M. Mojović, Z. Stević, S. D. Spasić, D. R. Jones, A. Morina, and M. B. Spasić. Bioavailability and catalytic properties of copper and iron for Fenton chemistry in human cerebrospinal fluid. *Redox Rep.* 15: 29–35. 2010.
31. Svendsen, R., and W. Lund. Speciation of Cu, Fe and Mn in beer using ion exchange separation and size-exclusion chromatography in combination with electrothermal atomic absorption spectrometry. *Analyst* 125: 1933–1937. 2000.
32. Uchida, M., and M. Ono. Improvement for Oxidative Flavor Stability of Beer -- Role of OH-Radical in Beer Oxidation. *J. Am. Soc. Brew. Chem.* 54. 1996.
33. Uchida, M., and M. Ono. Technological approach to improve beer flavor stability: Analysis of the effect of brewing processes on beer flavor stability by the electron spin resonance method. *J. Am. Soc. Brew. Chem.* 58: 8–13. 2000.
34. Uchida, M., S. Suga, and M. Ono. Improvement for oxidative flavor stability of beer - Rapid prediction method for beer flavor stability by electron spin resonance spectroscopy. *J. Am. Soc. Brew. Chem.* 54: 205–211. 1996.
35. Vanderhaegen, B., H. Neven, H. Verachtert, and G. Derdelinckx. The chemistry of beer aging – a critical review. *Food Chem.* 95: 357–381. 2006.

1 36. Wietstock, P. C., and T. H. Shellhammer. Chelating properties and hydroxyl-
2 scavenging activities of hop A- and iso- α -acids. J. Am. Soc. Brew. Chem. 69: 133–138.
3 2011.

4 37. Wu, M. J., P. J. Rogers, and F. M. Clarke. 125th Anniversary Review: The role of
5 proteins in beer redox stability. J. Inst. Brew. 118: 1–11. 2012.

6

1

2

3 **Figure 1. A comparison of the typical Electron Paramagnetic Resonance (EPR) assay**
 4 **curves generated from the four beers used in this trial (Beers A, B, C and D) with POBN**
 5 **as the spin trap. One example of triplicate runs for each Beer is shown. Measures derived**
 6 **from these curves can be found in Table 2.**

1

2 **Figure 2. The impact of Iron (II) addition to Beer A at 10, 20, 40, 80 and 160 ppb**
3 **concentrations with POBN as the spin trap. These curves represent examples chosen from**
4 **triplicate trials.**

1

2 **Figure 3. The impact of Iron (II), Copper (II) and Manganese (II) addition to Beer B at**
 3 **160 ppb with POBN as the spin trap. These curves represent examples chosen from**
 4 **triplicate trials. Measures from these curves can be found in Table 2.**

1

2

3 **Figure 4. The impact of Iron (II), Copper (II) and Manganese (II) addition to Beer C at**
 4 **160 ppb with POBN as the spin trap. These curves represent examples chosen from**
 5 **triplicate trials. Measures from these curves can be found in Table 2.**

6

1

2

3 **Figure 5. The impact of Iron (II), Copper (II) and Manganese (II) addition to Beer D at**
 4 **160 ppb with POBN as the spin trap. These curves represent examples chosen from**
 5 **triplicate trials. Measures from these curves can be found in Table 2.**

6

7

8

Figure 6. The impact of Manganese (II) addition to Beer A at 10, 20, 40, 80 and 160 ppb concentrations with POBN as the spin trap. These curves represent examples chosen from triplicate trials.

Figure 7. The impact of Copper (II) addition to Beer A at 10, 20, 40, 80 and 160 ppb concentrations with POBN as the spin trap. These curves represent examples chosen from triplicate trials.

Figure 8. The impact of Copper (II) addition to Beer A at 10, 20, 40, 80 and 160 ppb concentrations with PBN as the spin trap. These curves represent examples chosen from triplicate trials. Measures from these curves can be found in Table 3.

Figure 9. The impact of Iron (II) addition to Beer A at 10, 20, 40, 80 and 160 ppb concentrations with PBN as the spin trap. These curves represent examples chosen from triplicate trials. Measures from these curves can be found in Table 3.

1

2 **Figure 10. The EPR intensity measured with beer C using POBN as a spin trap over an**
3 **increased assay duration (39 hours). 160 ppb additional iron, copper and manganese**
4 **respectively. Example curves representative of triplicate runs.**

Table 1. Metal concentrations, pH, alcohol content and oxidative stability (lag time and T450) of beers used in this study. The standard deviations of triplicate measurements are shown.

Beer	A	B	C	D
Beer Style	Lager	Lager	Lager	Stout
Alc. % v/v	5.5±0.0	4.8±0.0	4.8±0.0	4.0±0.0
pH	4.2±0.0	4.1±0.0	4.1±0.0	3.9±0.0
Package	Can	Can	Can	Can
Fe (ppb)	26.2±0.9	25.0±0.7	37.1±1.3	161.6±6.6
Mn (ppb)	119.6±0.4	51.0±0.5	70.5±1.3	94.1±0.2
Cu (ppb)	57.7±1.1	33.1±0.5	35.7±1.6	14.1±0.1
Lag Time (min)	204±6	146±3	89±3	0±0
T450 x 10⁻⁵	5.75±0.42	5.29±0.34	9.67±0.58	11.85±0.58
Max Rate (EPR Intensity / min)	2412±212	3013±239	7238±373	7142±456

Table 2. Measures taken from the curve fitted to the results of the oxidative stability assay using POBN as the spin trap.

		Iron (II)			Copper (II)			Manganese (II)		
Beer	Metal Addition (ppb)	Lag Time (min)	Max Rate (EPR Intensity / min)	T450 x 10 ⁻⁵	Lag Time (min)	Max Rate (EPR Intensity / min)	T450 x 10 ⁻⁵	Lag Time (min)	Max Rate (EPR Intensity / min)	T450 x 10 ⁻⁵
A	0	204±6	2412±212	5.75±0.42						
	10	201±6	2466±203	6.39±0.57	167±6	3010±189	5.89±0.40	202±6	2578±163	5.81±0.52
	20	190±3	2898±198	6.91±0.63	145±9	3418±200	5.35±0.33	200±5	2642±189	5.79±0.43
	40	182±7	3229±251	7.89±0.41	130±10	3990±186	4.0±0.36	199±6	2618±050	5.92±0.37
	80	174±6	3877±260	8.79±0.41	99±8	3542±178	2.21±0.33	197±4	2774±186	6.01±0.43
	160	156±6	4265±230	9.77±0.43	76±7	3241±355	1.65±0.48	197±7	2752±180	6.21±0.47
B	0	146±3	3013±239	5.29±0.34						
	40	128±4	2365±455	5.54±0.35	129±4	3337±165	5.27±0.41	133±3	3450±305	6.91±0.42
	80	123±5	3691±363	5.85±0.40	116±4	3142±399	4.82±0.32	132±4	3218±389	7.46±0.36
	160	115±7	3619±421	6.31±0.41	108±6	3887±220	4.41±0.40	129±5	3763±400	8.30±0.37
C	0	89±3	7238±373	9.67±0.58						
	40	79±3	6410±351	9.67±0.33	64±5	8176±412	9.42±0.32	67±3	6516±256	10.73±0.32
	80	71±4	7361±394	9.94±0.45	58±2	7379±256	8.91±0.42	61±3	9970±401	11.12±0.12
	160	64±5	9297±220	10.33±0.35	48±3	7824±301	7.74±0.43	56±4	8557±324	11.81±0.25
D	0	N/A	7142±456	11.85±0.58						
	40	N/A	10848±564	12.87±0.47	N/A	7339±524	10.58±0.11	N/A	10661±666	13.33±0.89
	80	N/A	11420±389	13.60±0.83	N/A	8704±459	9.81±0.45	N/A	12086±327	14.05±0.48
	160	N/A	16869±587	15.32±0.33	N/A	7736±533	8.30±0.64	N/A	14603±721	17.50±0.14

Table 3. Measures taken from the curve fitted to the results of the oxidative stability assay using PBN as the spin trap.

		Copper (II)			Iron (III)		
Beer	Metal Addition (ppb)	Lag Time (min)	Max Rate (EPR Intensity / min)	T150 x 10 ⁻⁵	Lag Time (min)	Max Rate (EPR Intensity / min)	T150 x 10 ⁻⁵
A	0	86±5	1510±156	0.86±0.09			
	10	73±4	1452±200	1.25±0.10	78±2	1737.246	1.31±0.10
	20	67±3	1890±235	1.41±0.07	72±4	1777±356	1.67±0.15
	40	52±5	2028±346	1.90±0.10	66±5	2100±312	2.10±0.15
	80	50±6	1652±178	1.44±0.14	60±6	2681±286	2.75±0.17
	160	N/A	724±147	1.25±0.15	54±4	2880±167	3.14±0.18