Impacts of Copper, Iron, and Manganese Metal Ions on the EPR Assessment of Beer Oxidative Stability

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ABSTRACT

Beer flavor 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 utilizing to optimize their process with regard to flavor stability without the time requirements of stored aging and sensory testing of beer. There are still gaps in our 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 staling) is demonstrated, while 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 a 10 ppb transition metal ion addition can make a detectable difference to the measured oxidative stability.

Introduction

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

Optimization of the brewing process to limit flavor instability requires an effective measurement against which improvements can be made. Many researchers and brewers have adopted assays utilizing electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR), to achieve this aim.\textsuperscript{[1,2]} EPR is not sensitive enough to detect short-lived radicals generated in beer, and therefore the addition of a spin trap is used as part of the assay to quench and stabilize radicals for subsequent measurement. A standard EPR method usually involves artificially aging the beer at an elevated temperature, typically 60°C, over a short period of time (hr). Over this time the amount of spin adducts formed is proportional to the observed EPR signal. This intensity signal is plotted against the time of the assay, to generate a curve. There is often an absence of, or reduced, adduct formation seen at the beginning of the assay, and this time period is referred to as the lag time. This lag time is suggested to be an indication of the endogenous antioxidant capacity of the beer\textsuperscript{[3]} and, hence, relates to the time before staling may start in a particular beer. The intensity of the EPR signal at a given time (TXXX), such as a T120 (intensity after 120 min of the assay),\textsuperscript{[4]} is thought to be an indication of the oxidative stability of the beer, which, in turn, is considered to predict the oxidative deterioration that may occur. With these measures defined, a brewery may be able to use the EPR assay as a tool to optimize their process to produce more flavor stable beer. Although a correlation with sensory data has been suggested,\textsuperscript{[3]} this methodology has its limitations, most notably only assessing the oxidative stability of beer and not the non-oxidative staling pathways prevalent in beer and reviewed by Vanderhaegen et al.\textsuperscript{[6]}

Instances where brewers note a poor correlation of EPR data with subsequent sensorial assessment of aged product have understandably led some to be skeptical that the technique would be suitable for operational deployment.

Chapon and Chapon\textsuperscript{[7]} discussed the importance of transition metal ions in the generation of free radicals responsible for beer flavor deterioration, and the intricacies of the reactions have been investigated by several researchers since this study.\textsuperscript{[8–10]} Molecular triplet state oxygen, itself relatively unreactive, can be activated by reduced transition metal ions, which function as electron donors, to superoxide anion (\(\cdot O_2^-\)) and its protonated form, the hydroperoxyl radical (\(\cdot OOH\)). These species can be further reduced under beer conditions to hydrogen peroxide (H\(_2\)O\(_2\)), which, in the presence of reduced transition metals, can undergo decomposition to highly reactive hydroxyl radicals (\(\cdot OH\)) via the Fenton reaction. Hydroxyl radicals are reactive toward virtually all beer components, and are ultimately responsible for the generation of staling compounds.\textsuperscript{[11]}

Pohl\textsuperscript{[12]} reviewed the determination of metals in beer, discussing studies that measured iron in the range of 15–1006

KEYWORDS

Beer; EPR spectroscopy; ESR; flavor stability; metal ions
ppb, copper 8–800 ppb and manganese 31–180 ppb. These wide ranges likely reflect differences in beer type, raw materials, and practices used in its production, while the method of detection may also influence the results. A study of Polish beers measured iron in the range 208–345 ppb, copper at 72–114 ppb, and manganese at 70–165 ppb. Another study assessed beers available in a Spanish convenience store and measured iron (62–494 ppb), copper (19–68 ppb, with several below the detection limit of the method), and manganese (44–207 ppb). A more recent study of 58 different commercial U.K. lagers may indicate not all breweries have achieved reduced metal ion contents, with the mean iron (461 ppb) and copper (428 ppb) at the top or above these previously reported ranges, while the mean concentration of manganese was comfortably within this range (100 ppb). However, there is likely to be a trend in major breweries toward attaining lower concentrations, as many acknowledge the impact that metal ions may have on the quality of their products. Changes in brewing practice may alter expected metal ion loadings, for example, the renewed interest in dry hopping that has been suggested to increase the manganese content of beer. 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. Chelated metals may exhibit altered oxidation properties within the beer system. 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 (U.K.) area and were all within 6 weeks of production.

Metal additions

Degassed deionized water was used to make stock solutions of the various metals that 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, U.S.A.). 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. The α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN) (Sigma-Aldrich, USA) was prepared as outlined in Kunz et al. The 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 Analyzer system (Bruker Billerica, MA, U.S.A.). The spectrum parameters were set-up to capture the central peak of the triplet peaks generated, with a center 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. Samples were inserted into a heating block (60°C) at staggered intervals. Assays were controlled by EPR Liquid and Beverage Analyzer software (ELBA 2.0.2, Bruker), which took samples over at least 450 min. Eighteen 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, Billerica, MA, U.S.A.).

EPR analysis with POBN as the spin trap

The 452 mg N-tert-Butyl-α-phenyl-nitrone (PBN) was dissolved first in 500 μL of ethanol (Fisher Scientific) and 500 μL of water added. The 280 μL 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 center 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 min with 15 samples taken during this time. Samples were taken using an autosampler (Bruker, Billerica, MA, U.S.A.) and the running order was randomized.

Curve analysis

A sigmoidal curve was fitted to the data using GraphPad Prism 6 (La Jolla, CA, U.S.A.). 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 min. 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 Scientific, Loughborough, U.K.) was added to beer samples to a final concentration of 2%. Quantification of iron, copper, and manganese was achieved using inductively coupled plasma–mass spectrometry (ICP–MS) (Thermo-Fisher iCAP-Q, Waltham, MA, U.S.A.) 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 μg L⁻¹), Ge (20 μg L⁻¹), Rh (10 μg L⁻¹), and Ir (5 μg L⁻¹) included in the matrix of 2% HNO₃; External calibration standards for iron, copper, and manganese were run at 0, 20, 40, and 100 μg L⁻¹ (ppb). Samples were introduced via an autosampler (Cetac ASX-520; Thermo Scientific, Waltham, MA, U.S.A.) through a
nebulizer. Sample processing was undertaken using Qtegra software (Thermo-Fisher Scientific).

**Statistical analysis of data**

Means and standard deviations were calculated using Excel software, (Microsoft, Redmond, WA, U.S.A).

**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. The iron contents of beers A–C were low in comparison to the studies discussed previously (25–37 ppb) as was copper (33–57 ppb) and may represent best practice in the industry.

**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 that 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 utilizing the oxidative stability assay, brewers target longer lag times and/or smaller T450 for improved flavor 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) and, hence, was the least flavor stable beer included in the study.

**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. Examples of the curves generated with 160 ppb addition of iron to beers B–D can be found in Figures 3–5, while
the measures derived from the curves generated at all concentrations trialed in these beers (0, 40, 80, and 160 ppb) are detailed in Table 2. 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 accordance with theories of the pro-oxidation activity of iron previously explored.

It has been proposed that iron catalyzes the initial reduction of oxygen leading to the formation of hydrogen peroxide, through a cycle between iron(II) and iron(III), while iron also catalyzes the generation of the hydroxyl radical from hydrogen peroxide. For iron to have this catalytic effect, it needs to be in the Fe(II) oxidative state. Filik and Giray 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 trialed (data not included) resulting in similar results as iron(II), an observation also noted previously. Filik and Giray 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 trialed (data not included) resulting in similar results as iron(II), an observation also noted previously. When Fe(III) is formed, it can be reduced by a variety of compounds to return to Fe(II). Kunz et al 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.

Manganese

Manganese was the metal present in the highest concentration in the three lager beers trialed (Table 1). The addition of manganese ions had a minimal 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 that it is likely to be present as Mn$^{2+}$. However, a later study suggested that not
all manganese is in ionic form, but, in fact, a proportion may be bound, particularly in what they suggest are premium beer brands. \[26\] Phenolic compounds, amino acids, or organic acids were listed as potential ligands for manganese, which were not measured in this study.

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

Manganese on its own in model wine has been shown to exert a relatively low oxidative effect, but its potency was greatly enhanced by the presence of copper and iron. It was proposed that manganese may interact with intermediate complexes in iron and copper oxidation, enhancing the catalytic effect. \[27\] Therefore, any lack of pro-oxidant activity from manganese may also reflect the non-availability of active iron or copper ions in the beer.

**Copper**

Copper is often postulated to have a similar pro-oxidant role to iron in contributing to flavor instability, \[11,28\] but its influence on the EPR assay has not been explored to the same extent as iron in beer. The formation of spin adducts during the oxidative stability assay when copper concentration is increased clearly differs from the other metals in this study (Figure 7). The earlier formation of spin adducts is evident, resulting in the shortest lag time for Beers A–C of all metals trialed (Table 1). T450 values, however, were consistently reduced in beers as copper was increased, in strong contrast to the results of iron. The maximum rate of adduct formation provided no clear trends when all beers were considered. A correlation between copper and beer staling assessed by sensory panel has previously been demonstrated. \[10\] It has also been shown to have a dramatic effect on thiobarbituric acid values, used to estimate staling substances, which were increased with its addition. \[9\] In addition to catalyzing Fenton-type reactions, the increase in oxidative action of copper may be due to interactions with iron, since the copper ion has been suggested to facilitate redox cycling of iron in wine systems. \[29\]

It was not the intention at the outset of this work to compare EPR spin traps or methodology, but the unusual result seen with copper required verification. POBN has been suggested as a superior spin trap, partly as it does not result in a pH change during the assay, unlike PBN. \[19\] The trends seen with iron and copper are still seen when the alternative spin trap PBN is used (Figures 8 and 9). However, in this case the trend only became apparent at 80 ppb additional copper, when the T150 is seen to be lower than the previous 40 ppb addition (Table 3). Similar early generation of spin adducts but reduced maximum peaks have been generated when copper was added to wine. \[30\] Although unusual, certain beers can produce a signal that begins to reduce during the timeframe of the assay (data not shown). When the assay is allowed to continue beyond the normal time frame (up-to 39 hr) the spin adduct life cycle is evident (Figure 10). Here, the initial lag time has been missed, with the second sample point at 180 min. However, the formation of adducts is clearly shown; a peak is reached and a more gradual reduction as the spin adducts and the traps break down. Manganese produced the highest peak intensity when 160 ppb of metal ions was added to beer, followed by iron, the
control, and copper, respectively. Copper again forms more spin adducts early in the assay compared with the control but a smaller maximum peak is achieved. The EPR signal has been observed to reach a plateau,\cite{33} with the authors suggesting this steady-state level is determined by competition of the rates of formation and degradation of the spin adducts.

The smaller area under the curve, when copper is added would suggest fewer spin adducts are formed in the assay or that those adducts that are formed are less stable. Copper has been shown to destabilize POBN spin adducts whereas iron did not, although this was not in a beer system.\cite{32} Because the curves presented here are, in theory, determined by the generation and degradation of spin adducts, no clear evidence of copper interacting with the adducts is provided.

Another potential scenario is that increased copper results in earlier radical formation, but less overall. Generating fewer radicals than the control suggests copper is interacting with component/s that are pro-oxidant in the beer system. In a similar way, iron is thought to cycle between the iron(III) and iron(II) oxidation states and copper cycles between copper(II) and copper(I). It has been suggested that Maillard reaction products may reduce metal ions in beer\cite{24} while ascorbate and glutathione have been highlighted as reductants in non-beer systems.\cite{33} 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.\cite{34} Although 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.\cite{35} 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 pasteurized.\cite{36} If increased copper

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

| Beer | Metal addition (ppb) | Lag time (min) | Max rate (EPR intensity/min) | $T_{450} \times 10^{-5}$ | Lag time (min) | Max rate (EPR intensity/min) | $T_{450} \times 10^{-5}$ | Lag time (min) | Max rate (EPR intensity/min) | $T_{450} \times 10^{-5}$ |
|------|-----------------------|----------------|----------------------------|--------------------------|----------------|----------------------------|--------------------------|----------------|----------------------------|--------------------------|
| A    | 0                     | 204 ± 6        | 2412 ± 212                 | 5.75 ± 0.42              | 167 ± 6       | 3010 ± 189                 | 5.89 ± 0.40              | 202 ± 6       | 2578 ± 163                 | 5.81 ± 0.52              |
|      | 10                    | 201 ± 6        | 2466 ± 203                 | 6.39 ± 0.57              | 145 ± 9       | 3418 ± 200                 | 5.35 ± 0.33              | 200 ± 5       | 2624 ± 189                 | 5.79 ± 0.43              |
|      | 20                    | 190 ± 3        | 2898 ± 198                 | 6.91 ± 0.63              | 99 ± 8        | 3542 ± 178                 | 2.21 ± 0.33              | 197 ± 4       | 2774 ± 186                 | 6.01 ± 0.43              |
|      | 40                    | 182 ± 7        | 3229 ± 251                 | 7.89 ± 0.41              | 76 ± 7        | 3241 ± 355                 | 1.65 ± 0.48              | 197 ± 7       | 2752 ± 180                 | 6.21 ± 0.47              |
|      | 80                    | 174 ± 6        | 3877 ± 260                 | 8.79 ± 0.41              | 76 ± 7        | 3241 ± 355                 | 1.65 ± 0.48              | 197 ± 7       | 2752 ± 180                 | 6.21 ± 0.47              |
|      | 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              |

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

| Beer | Metal addition (ppb) | Lag time (min) | Max rate (EPR intensity/min) | $T_{150} \times 10^{-5}$ | Lag time (min) | Max rate (EPR intensity/min) | $T_{150} \times 10^{-5}$ |
|------|-----------------------|----------------|----------------------------|--------------------------|----------------|----------------------------|--------------------------|
| A    | 0                     | 86 ± 5         | 1510 ± 156                 | 0.86 ± 0.09              | 78 ± 2        | 1737 ± 246                 | 1.31 ± 0.10              |
|      | 10                    | 73 ± 4         | 1452 ± 200                 | 1.25 ± 0.10              | 72 ± 4        | 1777 ± 356                 | 1.67 ± 0.15              |
|      | 20                    | 67 ± 3         | 1890 ± 235                 | 1.41 ± 0.07              | 66 ± 5        | 2100 ± 312                 | 2.10 ± 0.15              |
|      | 40                    | 52 ± 5         | 2028 ± 346                 | 1.90 ± 0.10              | 60 ± 6        | 2681 ± 286                 | 2.75 ± 0.17              |
|      | 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              |
resulted in greater protein precipitation, this could potentially reduce the overall spin-adduct formation while also oxidizing the sulfite 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 while 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 stability of relatively stable beers. Although the benefits of reducing the transition metal ion content further were not directly demonstrated (due to the complexities of removing metal ions innate to the beers), further gains may be achievable through reduction of transition metal ions in beer beyond their current levels. Historically, the focus has been on iron content. However, with substantial reductions achieved, consideration must be given to other transition metals, particularly copper and manganese, which may be increased in beer due to changing brewing practices.

The value of EPR spectroscopy to the brewing industry as a rapid prediction of flavor stability, enabling proactive process changes, is clear. Changes in lag time and TXXX taken from EPR assay curves have been shown to differ independently, confirming that in some scenarios, they offer different measures of a beer’s oxidative stability. Previous publications have alluded to the need to consider the relevance of curve changes on a case by case basis[32] and the variety in changes demonstrated here reinforces this message.

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