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Possibilities to improve the antioxidative capacity of beer by optimized hopping regimes

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Different hopping regimes were evaluated to investigate the effect on the oxidative stability of wort and beer. Compared with a single hop dosage at the beginning of wort boil, it was possible to increase the concentration of α-acids in pitching wort and beer by applying incremental hop dosage, dry hopping or the use of a pre-isomerized hop product in combination with an α-acid extract, which concomitantly resulted in lower iron concentrations and an enhanced flavour stability as indicated by standard wort and beer analyses, atomic absorption spectroscopy, electron spin resonance spectroscopy and sensory analysis of fresh and force-aged beers. The functional principle of hop dosage variations is explained by saving of α-acids throughout the wort production process, which yields an increased formation and precipitation of pro-oxidative acting transition metal ions (e.g. Fe) in α-acid-complexes during the whirlpool rest and fermentation. Consequently, fewer reactive oxygen species are generated. Additional laboratory trials simulating wort cooling and beer storage in buffered model solutions proved that un-isomerized α-acids are strong iron chelators and confirmed the functional principle of the applied hopping regimes. Negative effects of higher α-acid contents on fermentation performance and depletion of the zinc concentration, which is an essential nutrient for yeast, could be excluded.

Keywords: hopping regimes; hop acids; chelate iron complexes; incremental hop dosage; electron spin resonance spectroscopy (ESR); oxidative beer stability

Introduction

A beer's freshness is the key to good drinkability. Compared with other beverages, beer is subject to complex reactions in the final package that can harm the product's character. Published literature *(1–6)* indicates that the stale flavours result from the formation of unsaturated, volatile carbonyl compounds, for example, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde, benzaldehyde, 2-furfural, hydroxymethylfurfual and *trans*-2-nonenal. Among the pathways thought to be involved in the formation of these carbonyls are the following: Strecker degradation of amino-acids; the melanoidin-mediated oxidation of higher alcohols; the autoxidation of unsaturated fatty acids; and the aldol condensation of short-chain aldehydes. Another possible pathway involving free-radical-promoted oxidation of amino acids has been suggested in recent studies *(7)*.

Based on findings from studies, using electron spin resonance (ESR) spectroscopy *(8–23)*, a beer deterioration mechanism was proposed where free radicals are formed from Fenton-type reactions during the aging of beer, which in turn can initiate a series of radical reactions that are responsible for the generation of aging components by oxidative processes. Atmospheric oxygen and transition metal ions are known to be two of the most detrimental substances when considering beer flavour stability, as they are producing highly reactive oxygen species (ROS, e.g. •OH). These reactions are primarily dependent on the availability of the reactants, catalysts, pH and temperature *(12,13,15,19,21,24)*. Beer should therefore be stored cool and the level of oxygen should be kept as low as practically possible during production and packaging. However, even though oxygen pick-up is mitigated, atmospheric oxygen can ingress through the crown cork's compound. The reactions can be suppressed by antioxidants that are present in the raw materials, added artificially or generated during fermentation, such as SO_2. Pro-oxidative substances may promote staling reactions by, for example, recycling Fe^{3+} to its lower valence state, Fe^{2+}, thus making it available again for the activation of oxygen and catalyses of radical generation by the Fenton reaction system *(15–17,21,24–26)*. Many attempts have been made to find a solution that would inhibit or delay the process of beer deterioration during storage. There are three possible fundamental approaches to solving the problem of beer flavour deterioration: a more sensorial approach where the stale flavours are masked by other substances, the reduction of the precursors for beer staling and the minimization of the rate of oxidation.

From a practical point of view, it is important to consider that all raw materials that are used for brewing (malt, hops, water, yeast, adjuncts) influence flavour stability and the staling potential of the resultant beer. Endogenous SO_2 is the strongest antioxidant in beer and is solely formed during fermentation by the yeast *(8,9,11,17,18,27–33)*. As for stale-related compounds, barley includes many precursors, enzymes, antioxidative and pro-oxidative substances that are involved in a multifaceted interplay during the production of malt and beer. The antioxidative activity of malt can result from the polyphenols and melanoidins. However, there is a debate about whether intermediates of the Maillard reaction and Maillard reaction products, such as melanoidins, have a pro- or antioxidative character *(3,25,34–39)*. Several authors *(35,37,39)* claim that reductones and melanoidins have high

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antioxidative capacities. On the other hand, according to Bravo *et al*. *(1,2)*, α-dicarbonyls, intermediates in the Maillard reaction, can markedly enhance the formation of specific aldehydes during beer storage, and blockage of α-dicarbonyls yielded a significantly lower accumulation of these aldehydes. Other authors *(25,36–38)* have discovered a positive correlation between the content of Maillard reaction products in special malts, caramel beers, stout and pro-oxidative acting reactions in wort and final beers. The colouring Maillard reaction products increased the level of radicals in the Fenton reaction assay, indicating that coloured malt or caramel colour is able to accelerate metal-catalysed oxidation of beer *(25,40)*. Kunz *et al*. *(25)* described a reaction mechanism of specific intermediate Maillard reaction products with reductone/endiol structure on the pro-oxidative process resulting in an acceleration of oxygen activation and the generation of ROS such as •OH radicals.

Several previous studies *(5,6,20,41–43)* have shown that adding hops during beer production can markedly enhance the flavour stability of beer or decrease oxidation indicators during beer storage. At present, hops are exclusively used in beer production because they contribute bitterness and aroma. The main bittering principles in hops are the hop α-acids that isomerize during wort boiling over an acyloin ring contraction, thereby forming the intensively bitter tasting *cis*- and *trans-iso*-α-acids. Recent studies from Haseleu *et al*. *(44)* and Krofta *et al*. *(45)* have demonstrated that certain degradation products from hop β-acids, which are formed during wort boiling, also contribute to beer bitterness.

Hops contain a number of antioxidative substances, whereby hop α- and β-acids contribute to the antioxidative potential to a large extent, as measurements using ESR spectroscopy have shown *(43,46)*. Hop α-acids have been demonstrated to have a high radical quenching ability, whereas *iso*-α-acids have a lesser effect. Hence, the isomerization of hop-α-acids to *iso*-α-acids was claimed to yield a lower antioxidant capacity. The investigations demonstrated that the main mechanism by which hop acids act as antioxidants is by chelating iron and by scavenging radicals *(42,43,46–51)*. However, newer studies *(26)* using a 2-deoxyribose oxidative degradation assay revealed that both hop α- and *iso*-α-acids are capable of suppressing radical formation by complexing iron, but are incapable of scavenging hydroxyl radicals. Other authors *(52–54)* have claimed that the oxidative degradation of *iso*-α-acids in beer plays a role in beer staling, but to a minor degree.

The role of malt and hop polyphenols is discussed controversially in the published literature *(20,43,46,48–50,55,56)*. Trials using ESR spectroscopy in combination with specific analytical methods for the determination of the antioxidative potential indicated that polyphenols possess a low antioxidant behaviour *(20,43,46,50,55,57)* However, when other assays are used, for example, scavenging of DPPH radicals, there is evidence that polyphenols show strong antioxidative properties *(14,42,43,46,48,49,57)*. Moreover, beer storage trials revealed that polyphenols markedly enhance beer flavour stability *(42,56)*. Aerts *et al*. *(58)* achieved an increase in the oxidative stability of beers by adding gallotannins during mashing and lautering. Recent studies *(59,60)*, using ESR spectroscopy could additionally verify the positive effects of gallotannins on the oxidative beer stability by adding them during mashing and wort boiling.

In the present study, different hopping regimes were tested to evaluate beer flavour stability using hop α-acids as an antioxidant. Oxidative stability of wort and beer were monitored using ESR spectroscopy and the fresh and stored (5 months at 25°C) beers were rated by sensory analyses. International bitter units (B.U.) and hop acid concentrations were evaluated by UV–vis spectroscopy and HPLC, respectively. Furthermore, the metallic ion concentration was determined by graphite furnace atomic adsorption spectroscopy (GF-AAS). The brewing trials were performed in the Technische Universität Berlin's 1.5 hL research pilot plant brewery.

Experimental procedures

Analytical methods

Beer analyses according to MEBAK *(61)*. The methods used were as follows: bitter units (2.22.1); colour (2.13.2); extract (2.10.3); alcohol (2.10.7); pH value (2.14); total nitrogen (2.8.1); magnesium sulphate precipitable nitrogen (2.8.3.1); foam stability (2.19); total polyphenols (2.17.1); anthocyanogens (2.17.2).

ESR methods used: determination of the EAP and T_{600}-value. The determination of the 'endogenous antioxidative potential' (EAP value) *(17,18)* is based on the detection of the radical generation during accelerated beer aging (60°C). Initially, the radical generation can be delayed or prevented by the endogenous antioxidative activity of beer. After the consumption of antioxidants, the ESR signal increases when spin-trap adducts, mainly hydroxyethyl radicals, are generated. The intersection of the two linear slopes, from this delay, gives a relative measure of the oxidative beer stability. The antioxidative potential (time, *x*-axis value) at the intersection is defined as the EAP value (Fig. 2). The T_{600}-value (*y*-axis value) is defined as the ESR signal intensity measured after 600 min of reaction time at a forced aging temperature of 60°C and estimates the amount of radicals that are generated (Fig. 1). The T_{600}-value is affected by metal ions, pH-value, polyphenols, proteins, intermediate Maillard reaction products, the ethanol concentration, etc.

Beer samples were degassed in an ultrasonic bath for 20 min (20°C). For each sample, 8.4 mg of α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN) was diluted in 50 µL of double-distilled water (3.5–3.6 mM POBN). For the preparation of ESR samples, 150 µL of ethanol and 12 mL beer sample were filled into 16 mL vials. As soon as the measurement was started, successively 50 µL POBN solution was added to each sample, which was shaken twice

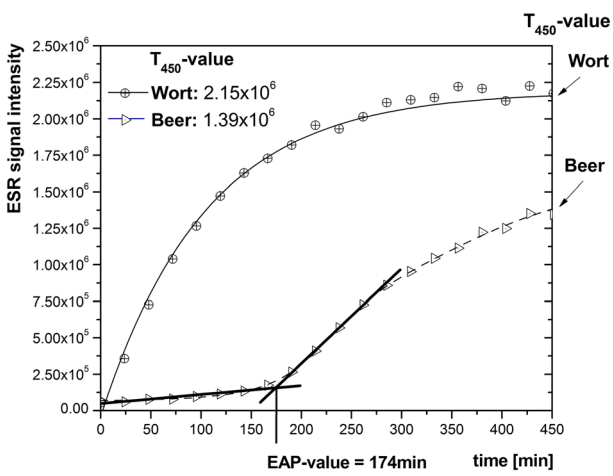

Figure 1. Evaluation of the ESR measurement *(17)*.

and placed in the heating block (60°C) of the autosampler. For the duration of the ESR measurement (10 h) the autosampler measured the concentration of spin trap adducts periodically (ranging from 30 to 70 min) *(17,18)*.

Instruments. ESR spectra were obtained with an X-band spectrometer (Bruker e-scan, Rheinstetten, Germany) with the following settings: centre field, 3475 G; attenuation, 0 dB; equivalent to 8.492 mW; sweep width, 14 G; receiver gain, 2.0×10^3: resolution; 512 modulation amplitude, 1.49 G; modulation frequency, 86 kHz; conversion time, 10 ms; time constant, 40 ms; scans, 30 for wort measurements and 25 for beer measurements.

Determination of iron and zinc concentrations using GF-AAS according to MEBAK *(62)*. Iron and zinc concentrations were determined using GF-AAS. After diluting the samples with HNO_3 (0.14 M), the autosampler was started and 20 µL of the sample was automatically mixed with 20 µL of a magnesium matrix modifier solution (0.015 mg/5 µL) and subsequently nebulized in the graphite-coated furnace.

SO_2 determination using CFA *(61,63)*. The determination of SO_2 was carried out by continuous flow analysis (CFA) under an optimized procedure using a Teflon® membrane *(63)*. SO_2 is released from beer at high temperatures as a gas and dialysed by a Teflon® membrane into a formaldehyde solution. *p*-Rosaniline is added and the molecule binds with the sulphur dioxide–formaldehyde complex at a temperature of 45°C, forming a red coloured complex, which can then be determined colorimetrically at 560 nm. The evaluation was performed with CFA software with consideration of peak heights and a calibration line.

Hop acids concentration by HPLC [based on ASBC Beer-23.B *(64)* and Donley *(65)*]. The ASBC method is based on solid phase extraction (SPE) of hydrophobic substances such as α-acids, *iso*-α-acids and β-acids. These hop acids are retained in the support material C_8 of the SPE cartridges, whereas polyphenols and hydrophilic substances are eluted from the packing material. The substances detained in the packing material are eluted with acidified methanol and subsequently determined using high-performance liquid chromatography (HPLC). The method was originally validated for *iso*-α-acids; however, in this study, this method was also applied to hop α- and β-acids. Chromatographic determination was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Böblingen, Germany) at a constant temperature of 40°C and a flow rate of 1.2 mL/min with a 5 µL injection volume. Two mobile phases were used. Mobile phase A was 100% methanol; mobile phase B was 55% methanol, 44% water and 1% phosphoric acid. The elution began isocratically with 50% of mobile phase B for the first 12 min followed by a gradient descent to 20% of mobile phase B over the next 3 min, which was then held for 10 min more. A Purosphere Star™ LC-18 5 µm C_{18} silica column was used for separation. Absorbance was measured at 270 and 314 nm. As reference for the *iso*-α-acids, an international calibration extract (ICS-I2) was used, and as reference for α- and β-acids, a standardized hop extract (ICE-2).

Sensory beer analyses according to DLG *(61)*. The beers were rated using the testing method of the German agriculture organization (DLG, Deutsche Landwirtschaftsgesellschaft e.V.). The taste panel consisted of 10–12 expert assessors who evaluated the beers' odour, taste, freshness, palate fullness and quality of bitterness on a scale from 1 to 5, where 5 represents the highest rating and 1 the lowest rating. A rating lower than 3 is considered as not suitable for selling. The single evaluations from two sessions were averaged and an overall grade was set. The panellists were also asked to describe the hop aroma qualitatively as variations were expected when using different hopping regimes.

Effect of hopping regimes on metal ion concentration and radical formation

All beers were produced from 100% Pilsner malt and adjusted to 11.5 °P original gravity. Wort boiling was carried out under atmospheric pressure (100°C) for 60 min and the worts were fermented for 5–7 days at 12°C using a bottom-fermenting yeast until the residual apparent extract was <3.5°P. The Institute's pilot plant consists of a two-roller mill (Künzel, Germany; gap 1.7 mm), a 1.5 hL mash kettle, a lauter tun and a 1.5 hL wort/whirlpool combi kettle with an external boiler system (Steinecker, Germany). Fermentations were conducted in 40 L cylindro-conical vessels with mantle cooling. After 7 days of maturation, the beers were membrane filtered (three-stage Donaldson-cartridge-filter) before carbonation and filling.

In a pre-trial, a brew where 100% of the hops [hop CO_2 extract: Magnum, 48.8% (w/w) α-acids; hop dosage calculated to 90 g/hL α-acids] were added at the beginning of boiling was compared with a brew where the hop dosage was divided and 20% of the total hop amount added at the beginning of boiling, 50% added after 30 min, and another 30% added after 50 min of boiling. The oxidative wort stability was determined by measuring the level of POBN spin trap-adducts (T_{600}-value) at certain time points during wort boiling using ESR spectroscopy.

For the main trial, brews with four different hop regimes were produced. In addition to a control brew (100% hops added at beginning of boiling, 90 g α-acids/hL), a second brew with an incremental hopping regime with 110% total hop addition (50% hops at the beginning of boil, 30% after 30 min and 30% after 50 min of boiling) was produced. Third, a brew with an addition of 90% hop CO_2 extract at the beginning of the boil was dry hopped with 100 g/hL of hop pellets [type 90, Hallertauer Perle, 7.9% (w/w) α-acids] added after fermentation was produced. Finally, a fourth 'pre-isomerized brew' with 50% hop CO_2 extract addition at the beginning of boil and a calculated mixture of pre-isomerized α-acid extract (2.75 g/hL pre-isomerized α-acids) in combination with a high α-acid extract (1.75 g/hL α-acids) added in the whirlpool was produced.

In all trials, the kettle hop additions were dosed directly into the boiling wort. For dry hopping, the whole pellets were added in a loose form into a 50 L keg and purged three times with CO_2 prior to addition to the green beer. Dry hopping was carried out for 7 days at 2°C and the hops were subsequently removed by filtration. A detailed description of the hopping regimes and the calculated hop acid concentrations is given in Fig. 2.

Iron precipitation trials in buffered model systems

In addition to the brewing trials, iron precipitation was modelled in acetate buffer solutions to simulate the reactions between hop acids and iron ions at beer- or wort-like conditions. First, two trials were conducted in which the process step of wort cooling to pitching temperature and the process step of beer filtration at 0°C were imitated. For simulating wort cooling, in each trial, 0.2 ppm Fe^{2+} ($FeSO_4$ x 7 H_2O) and 90 ppm of purified hop α-acids (89.2% w./w. purity) or hop *iso*-α-acids (90.5% w/w

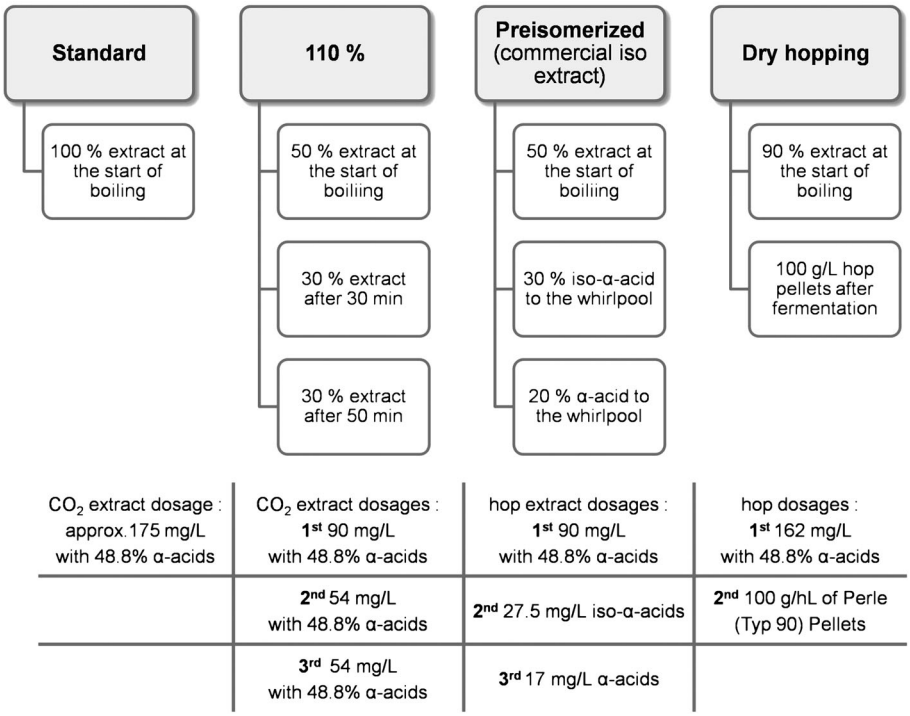

Figure 2. Different hop regimes of the brews.

purity) were added to an acetate buffer (20 mM, pH 5.3) and heated to 90°C for 5 min to allow the formation of potential hop acid–iron complexes. The sample solutions were subsequently cooled to 12°C in a temperature-controlled room and filtered (membrane filters, pore size 0.45 μm).

In a separate trial, the influence of the pH-drop during fermentation and the process step of filtration were simulated by adding 0.2 ppm Fe^{2+} and 90 ppm of hop α-acids or hop *iso*-α-acids, respectively, into a buffer–ethanol mixture [acetate buffer, 20 mM, pH 4.6; 5% (v/v) ethanol], and cooling the mixture subsequently to 0°C. The mixtures were incubated for 2 h and then filtered through membrane filters (pore size 0.45 μm). In both trials, the residual iron concentrations in the filtrates were determined by GF-AAS *(62)*.

To evaluate the effects of hop acid addition in greater detail, a second set of experiments was carried out. In these trials, the hop acid dosage into the model solution was decreased and different molar ratios between hop α-acids or hop *iso*-α-acids and Fe^{3+} were adjusted. Hop acids (50 μM) were added to an acetate buffer solution (20 mM, pH 4.3) and mixed with 50 μM Fe^{3+} ($FeCl_3 \times 6 H_2O$). Additionally, the molar ratio between Fe^{3+} and hop acids was altered to 2:1 (Fe^{3+}:hop acids, 50:25 μM) and 3:1 (Fe^{3+}:hop acids, 50:16.7 μM). The mixtures were incubated for 180 min at room temperature prior to filtering them with 0.45 μm membrane syringe filters. Subsequently, the residual iron concentrations in the filtrates were analysed, again using GF-AAS.

Zinc is an important nutrient for the yeast cells and affects the course of fermentation; a decrease in zinc would therefore have a negative effect on the fermentation performance. Therefore in a separate trial, the effect of hop acids on zinc precipitation was analysed by mixing 0.2 ppm zinc ($ZnSO_4 \times 7 H_2O$) with 60 ppm of hop α-acids or *iso*-α-acids, respectively. The same base (acetate buffer, 20 mM, pH 4.6) and the same procedure were used as for the iron precipitation models. After filtration, the zinc concentration in filtrates was analysed, again using GF-AAS.

Results and discussion

The described brewing trials were carried out in the pilot research brewery of the Technische Universität Berlin, with the analytical focus on the influences of the different hop regimes on the oxidative stability, bitterness, hop acid concentration and the overall flavour impressions in wort and beer.

In a pre-trial, a brew with 100% dosage of hop CO_2 extract at the beginning of boil was compared with a brew with an incremental hop dosage of 20% CO_2 extract added at the beginning of the boil, 50% at the middle of boil and 30% after 50 min boiling time. In both trials, the addition of hops yielded a drop in the ESR signal intensity, which indicates diminished radical formation and an improved resistance of the wort against oxidation (Fig. 3). These results confirm earlier investigations *(43)* and point to the strong antioxidative influence of hop ingredients on the wort. Throughout the course of wort boiling, the ESR signal intensity increased again until the end of boil. Dividing the hop dosage resulted in a drop in ESR signal intensity at every time point when hops were added, and the reduction of ESR signal intensity was dependent on the amount of hops dosed (Fig. 3). At the end of boil, the radical concentration (indicated by the ESR signal intensity) of the incremental brew was reduced by 28% as compared with the brew where 100% of the hops were added at the beginning of boil, which indicates a higher oxidative stability. However, the later hop dosage also yielded a 10% lower yield in bitterness units (43 BU/39 BU).

The literature indicates that α-acids possess strong antioxidative properties *(26,42,43,46–50)*. When un-isomerized α-acids were added later during the boil, they underwent a shorter isomerization time and a diminished conversion of α-acids to *iso*-α-acids. Consequently, when applying an incremental hop dosage, a higher concentration of antioxidative α-acids was present at the end of the wort boiling process. Both

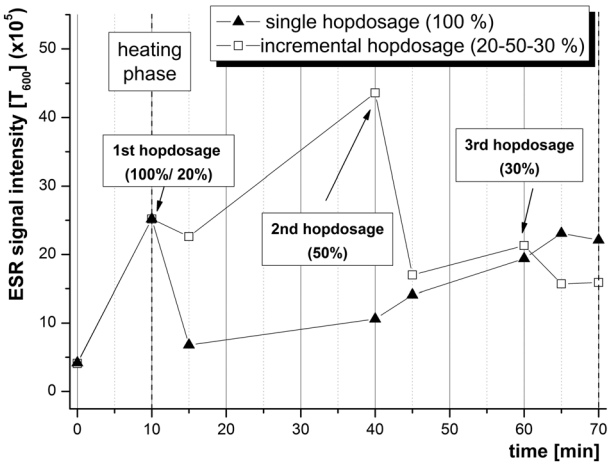

Figure 3. Wort boiling with single hop dosage vs incremental hop dosage (20/50/30%).

observations, the increased antioxidative effect and the diminished bitter yield, may be traced back to the enhanced presence of α-acids at the later stages of wort boiling, thus diminishing the radical generation.

As a consequence of the slightly lower bitter yield from the previous trial, 110% of hop CO_2 extract was used in a subsequent trial, with an incremental hop dosage of 50/30/30% using the same dosage time points as before (beginning of boil/30 min after boiling started/10 min before the end of boil). A beer with 100% of hops added at beginning of boil was produced as a control.

Additional brews were carried out and also compared with the control brew. The effects of a 50% hop CO_2 extract addition at the beginning of boil combined with a calculated mixture of pre-isomerized and high α-acid extract added during the whirlpool rest, and the effect of dry hopping, were investigated. The dry-hopped brew was produced with 90% CO_2 extract addition at the beginning of boiling and a dosage of 100 g/hL hop pellets type 90 during cold storage.

Table 1 depicts that all results of the standard wort analysis of the produced worts were similar in terms of colour, pH-values and nitrogen fractions, and close to the desired original gravity of 11.5 °P.

As demonstrated in Fig. 4, in analogy to the results of the pre-trial, with each hop addition during the incremental brew, the ESR signal intensity dropped, and at the end of boil showed a 35% lower radical generation as compared with the control brew. However, the bitter yield (indicated by bitter units) in the pitching wort was comparable, although the *iso*-α-acid concentration as well as the α-acid concentration as determined by HPLC varied slightly: less *iso*-α-acids but a higher concentration of α-acids were measured in the wort produced with the divided hop dosage (Table 1). Again, this fact can be explained by the overall shorter isomerization time when using second and third hop additions.

In the case of the dry-hopped brew, in logical order to the 90% hop addition at the beginning of boil, an approximately 10% lower bitter yield and hop acid concentration could be detected in the pitching wort of the latter. Accordingly, the 10% lower hop dosage in the dry-hopped brew is probably responsible for the lowest antioxidative potential of all of the pitching worts, as indicated by an increased radical generation (T_{600}-value: 1.56×10^6, Fig. 5).

The opposite result was seen in the mutual application of pre-isomerized and high α-acid extract during whirlpool rest combined with a 50% CO_2-extract dosage at the start of wort boil. This also led to a remarkable 38% higher *iso*-α-acid concentration and bitterness yield in the pitching wort (60 BU), as compared with the control. The use of a calculated mixture of pre-isomerized *iso*-α-acid and high α-acid extracts combined with 50% conventional hopping appeared to result in a better bitterness yield in comparison to hop dosage with 100% hop CO_2 extract at the beginning of wort boil. A possible explanation for the higher bitterness may lie in the dosage of the mixture during the whirlpool, resulting in a diminished precipitation of hop acids and better utilization during wort boiling. In this case, the total addition of 70% α-acids (50% at start of boiling in form of CO_2-extract + 20% during whirlpool rest in form of a purified α-acid extract) and 30% *iso*-α-acids (whirlpool rest) led to a 15% higher bitterness yield in comparison to the control. Apparently, the pre-isomerized *iso*-α-acids were completely preserved from precipitation during the whirlpool rest, whereas the added un-isomerized hop α-acids were lost owing to precipitation by approximately 20%.

Table 1. Results of standard wort analysis

Analyses	Control wort 100% single hop dosage	110% incremental hop dosage 50% – start 30% – 30 min 30% – 50 min	Dry hopping (90% of hops added)	Pre-isomerized α-acid (given in whirlpool)
Bitter units (EBC/MEBAK)	53	52	48	60
∑ *Iso*-α-acid (ppm)	34	27	28	47
∑ α–acid (ppm)	24	28	23	25
Original extract (%)	11.41	11.07	11.52	11.51
pH value	5.43	5.52	5.44	5. 52
Colour (EBC)	6.8	7.0	6.7	6.8
Total nitrogen (12 °P) (ppm)	1054	1067	1104	1069
$MgSO_4$ precipitable nitrogen (12 °P) (ppm)	268	274	272	272

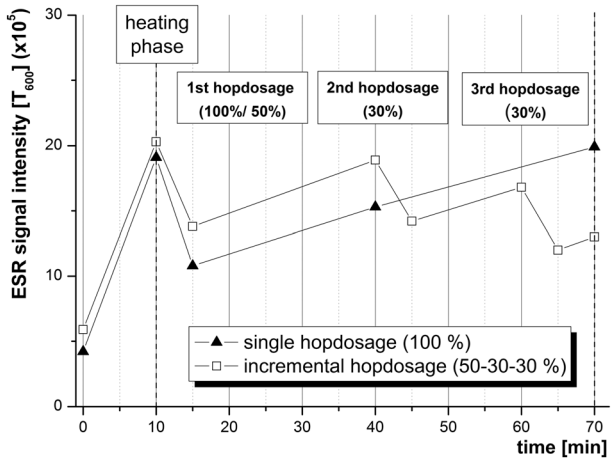

Figure 4. Wort boiling with single hop dosage vs incremental hop dosage (50/30/30%).

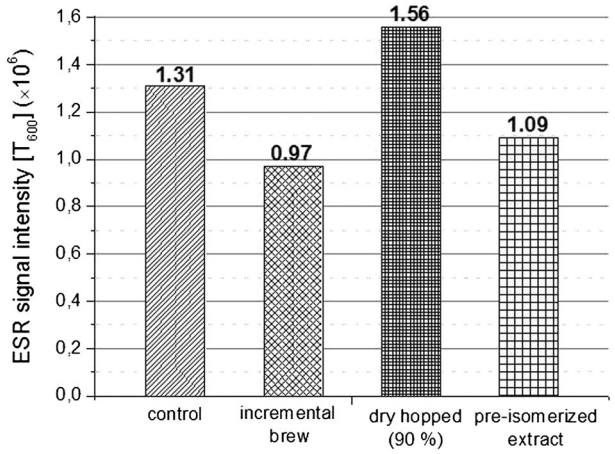

Figure 5. T_{600} values of pitching worts.

The detected influences of different hopping regimes affected the antioxidative potential of the worts. Despite the higher bitterness yield and small losses of α-acids by precipitation when using pre-isomerized hop acids, a comparable final α-acid content and an improved oxidative wort stability (T_{600}-value: 1.09×10^6, Fig. 5) could be detected in comparison to the control (1.31×10^6).

The fermented kieselguhr-filtered and bottled beers were analysed for standard parameters (extract, attenuation, alcohol, pH, colour, foam stability, nitrogen and polyphenols), for their hop acid concentration, and were analysed by ESR spectroscopy. The results of the standard beer analysis (Table 2) of the four brews did not vary significantly from each other. In terms of pH-values and nitrogen fractions, the results were also comparable. The wort analyses, showing a lower original gravity for the incremental brew, explain the lower alcohol concentration and real extract value in the subsequent beer. The head retention and the haze formation did not follow a particular pattern. The dry-hopped beer was higher in polyphenols and anthocyanogens than the other three brews, which can be explained by the insertion of additional phenolic material from hops during dry hopping. Hop pellets contain a higher fraction of polyphenols in contrast to CO_2 extract *(66,67)* and dosing hop pellets into beer thus yields an increase in polyphenols.

Among the values of the final beers, the SO_2 levels were quite similar. The dry-hopped beer and incremental hop dosage brew had a slightly higher value in the range of 1 ppm difference.

With regard to the influence of the hopping regimes on the bitterness yield and the antioxidative potential, the bitterness units and the concentration of hop α-acids has to be taken into account (Table 2). The incremental brew showed the same hop acid concentrations as the control, which is in accordance with the pitching worts. In contrast, the dry-hopped and pre-isomerized brews were characterized by higher bitter units of 28 and 30 BU, respectively, which can be directly linked to the

Table 2. Results of standard beer analysis

Analyses	Control beer, 100% single hop dosage	110% incremental hop dosage 50% – start 30% – 30 min 30% – 50 min	Dry hopping (100 g/hL)	Pre-isomerized α-acid (given in whirlpool)
Bitter units (EBC/MEBAK)	24	23	28	30
Σ *Iso*-α-acid (ppm)	24	24	33	28
Σ α-acid (ppm)	2.7	2.5	4.0	2.9
Original extract (GG%)	11.43	11.09	11.55	11.46
Attenuation (%)	75.4	75.3	75.4	75.6
Alcohol (vol%)	4.57	4.42	4.63	4.60
pH value	4.45	4.47	4.49	4.43
Colour (EBC)	5.4	5.4	5.8	5.9
Foam stability (NIBEM) (s)	24	23	28	30
$MgSO_4$ precipitable nitrogen (12 °P) (ppm)	294	278	289	284
Total nitrogen (12 °P) (ppm)	884	903	906	853
Polyphenols (ppm)	164	157	181	161
SO_2 (ppm)	2.4	2.9	3.2	2.2
EAP values (min)	87	112	98	87
Iron concentration (ppb)	69	43	24	26

higher *iso*-α-acid concentrations. In terms of the α-acid concentration, the dry-hopped brew, which had a dry-hopping regime after fermentation and prior to filtration of 100 g/hL of hop pellets, still retained 4 ppm of α-acids in the beer, whereas the remaining three brews had only 2.5–2.9 ppm left.

Transition metal ions such as Fe are known to be catalysts in the Fenton reaction system and responsible for an acceleration of oxygen activation by electron transfer promoting beer deterioration reactions *(15–17,21,24,25)*. Lowering the iron concentrations thus improved the oxidative stability, which was a major goal of the trials. In terms of iron concentration, the control brew showed a content of 69 ppb, while all other beers were characterized by lower iron concentrations (Table 2). The incremental brew and the pre-isomerized brew had an iron reduction of 38 and 62%, respectively, as compared with the control brew. The dry-hopped brew displayed a 65% reduction with a final iron concentration of 24 ppb.

Despite the similar SO_2 levels, brewing with individual hop additions affected the oxidative stability of the final beers significantly. The ESR results in Figs. 6 and 7 demonstrate that dry hopping after fermentation and the incremental hop dosage regimes markedly diminished the radical generation (T_{600}-value: 0.54×10^6) as compared with the control (T_{600}-value: 0.76×10^6), indicating an increased oxidative beer stability. Applying an incremental hop dosage with 110% of hops showed the highest EAP of all beers and displayed a 25% lower radical formation as indicated by the T_{600} values in comparison to the control brew.

As shown in Fig. 8, the differences in the oxidative stability became even more apparent after 5 months of storage at 25°C. The EAP-values and SO_2 contents of all brews were completely consumed (Fig. 7). However, the ESR signal intensity after 600 min of measurement (T_{600}) showed distinct differences. The incremental brew and the dry-hopped beer had an ~35% lower T_{600}-value, and the pre-isomerized brew had an almost 46% lower signal intensity as compared with the control brew.

The direct correlation between the detected lower iron concentrations in the brews with altered hop regimes and the similar influences on the radical generation (T_{600}-ESR-signal intensity) highly suggest a direct correlation between the ESR data and the iron content. In consideration of these facts, it becomes clear that the hopping regime is accountable for the final iron concentration, which in turn plays an important role in oxidative beer stability.

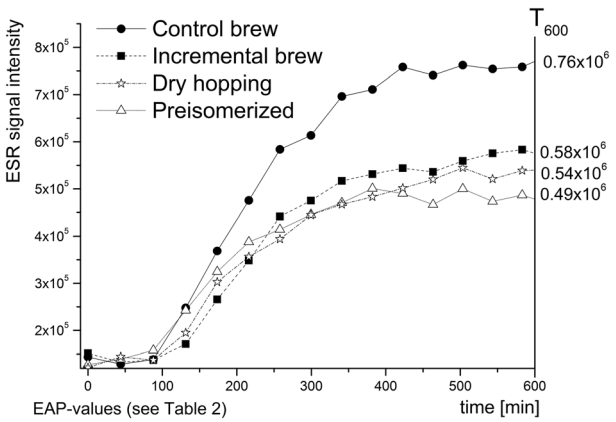

Figure 6. Results of the endogenous antioxidative potential (EAP) determination of freshly bottled beers.

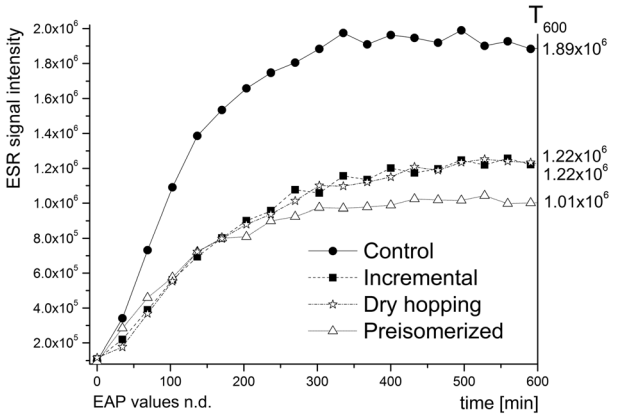

Figure 7. Results of the EAP determination after 5 months of storage (room temperature, bottled beer); T600- & EAP values (EAP-values not detectable).

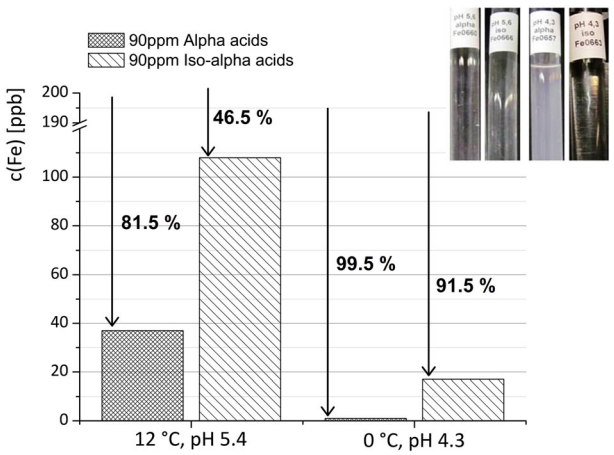

Figure 8. Iron precipitation by 90 ppm of hop acids.

Additionally, sensory analyses were used to investigate the described positive effects of the specific hopping regimes on the flavour in fresh beers and the flavour stability during storage. The results as depicted in Table 3 show that the goal of equal sensory bitterness and higher flavour stability was achieved for all of the modified brews.

In all sensory features (odour, taste, freshness, palate fullness, quality of bitterness), the ratings of all beers at the beginning of storage were in the typical range of pilsner-style beers (Table 3). Within this range, all modified brews with different hopping regimes showed slightly higher single ratings in comparison to the control brew, with the exception of the freshness rating of the dry hopped brew, which was ranked 0.3 lower. This lower rating might be derived from an oxygen influx during the process of dry hopping and would also explain the slightly higher ESR signal intensity as compared with the other brews with varied hopping regimes. In general, the altered hop regimes led to slightly improved sensory impressions in fresh beers, with a smoother hop aroma profile described by the panellists. Furthermore, the results of the sensory analyses after 5 months of storage at room temperature underline the previously demonstrated advantages of the different hop regimes on oxidative beer stability. The ratings had not changed immensely in terms of freshness, palate fullness and quality of

Table 3. Sensory analysis of bottled beers fresh vs stored

Sensory features	Control beer, 100% single hop dosage	110% incremental hop dosage 50% – start 30% – 30 min 30% – 50 min	Dry hopping (100 g/hL)	Pre-isomerized α-acid (given in whirlpool)
		Fresh		
Odour	4.1	4.5	4.5	4.3
Taste	4.2	4.3	4.5	4.2
Freshness	4.3	4.3	4.0	4.6
Palate fullness	4.4	4.4	4.6	4.6
Quality of bitterness	4.2	4.2	4.4	4.5
		After 5 months storage at room temperature		
Odour	3.3	4.2	4.3	3.9
Taste	3.5	4.2	4.2	3.9
Freshness	4.2	4.3	4.6	4.5
Palate fullness	4.2	4.5	4.7	4.5
Quality of bitterness	4.0	4.3	4.2	4.1

bitterness, but it should be noted that the attributes of odour and flavour were rated significantly lower in the control brew after storage.

Among the brews with a different hop regime, only the pre-isomerized brew deteriorated slightly in terms of odour and flavour. This rating stands in contrast to the ESR results of the fresh and aged beers, where the pre-isomerized brew showed one of the highest oxidative stabilities. This phenomenon may be linked to the commercial production process of pre-isomerized hop products.

Iron and zinc precipitation in buffered model systems

To get a better insight into the positive influence of hop acids, especially α-acids, on the oxidative beer stability and their reduction properties against pro-oxidative acting transition metal ions, a simulation of the iron precipitation in common pH ranges of the brewing process was carried out with acetate buffer solutions modelling wort or beer, respectively.

In a first experiment (Fig. 8), the reactions caused by hop acids during wort boiling and cooling (pH 5.4, 12°C) and cold beer storage [pH 4.5, 5% (v/v) ethanol, 0°C] on final iron levels were demonstrated by a trial in which 90 ppm hop α-acids or *iso*-α-acids was added to an acetate buffer, which already contained 200 ppb of Fe^{2+}. After incubation for 2 h at 0°C, to allow the formation of potential hop acid–iron complexes, the solutions were filtered using membrane filters (pore size 0.45 µm) to remove the generated hop acid–iron complexes.

After filtration, the wort model solution (pH 5.4) showed an iron reduction of 81.5% when α-acids were added and 46.5% reduction when *iso*-α-acids were added. In the lower pH range of the beer model solution (pH 4.3), almost all iron (>99.5%) precipitated when adding α-acids, and with addition of *iso*-α-acids the residual iron concentration was also diminished by 91.5%. These outcomes and the visible appearance of haze, as demonstrated in Fig. 8, give an impression of the different pH-dependent complex formation properties of hop acids. The results of the wort, as well as beer modelling precipitation trials (Fig. 8), demonstrate clearly that *iso*-α-acids have less effect on iron reduction in comparison to the α-acids in a wort and in a beer matrix. This outcome confirms earlier findings from Wietstock *et al. (26,43)*, in which they showed that α-acids are stronger chelators than *iso*-α-acids. Consequently, the isomerization during wort boiling minimizes the complex formation properties of the hop acids with iron ions. Furthermore, the results and visible haze formation in the beer model solution (pH 4.3) suggest a pH-dependency of the complex formation between iron ions and α- and *iso*-α acids. Taking the data together, there is a clear indication that lower temperature levels and lower pH values result in a markedly higher precipitation of iron.

To obtain more detailed information about the iron precipitation mechanism with hop acids, a second trial with a beer model solution was carried out (Fig. 9). In this trial, the molar ratio between Fe^{3+} and hop acids was chosen according to their molecular weight in ratios of 1:1, 2:1 and 3:1 with 50 µM Fe^{3+} each.

The 1:1 ratio resulted in a decrease of 53% Fe^{3+} using α-acids and 19% using *iso*-α-acids. A 2:1 ratio resulted in decreases of 31 and 7%, and a 3:1 ratio in 12 and 2%, respectively. Considering all of the data, the results showed clearly that even small concentrations of α-acids can be responsible for a significant decrease in pro-oxidative iron ions in the pH range of beer, whereas *iso*-α acids showed only slight complex formation properties In conclusion, in concurrent presence of α- and *iso*-α-acids in wort, during fermentation or in real beer samples, the already lower effect of *iso*-α-acids may be additionally inhibited by the faster reaction of α-acids in competitive situations.

The detailed scrutiny of the results gives additional information about the molar ratios between the hop acids and iron ions during complex formation, which are important to describe the reaction mechanism. The calculation reveals that at least 1.4–1.6 µM (~2 µM) α-acids are needed for the precipitation of 1 µM iron ions.

Zinc is an important metal ion that is necessary for yeast nutrition. To exclude the possibility that an alteration of the hopping regime also negatively affects the final zinc concentration in the wort, in addition to the experiments where the effect on iron was evaluated, the influences of the hop acids on the zinc content were analysed (Fig. 10). The same procedure as used for

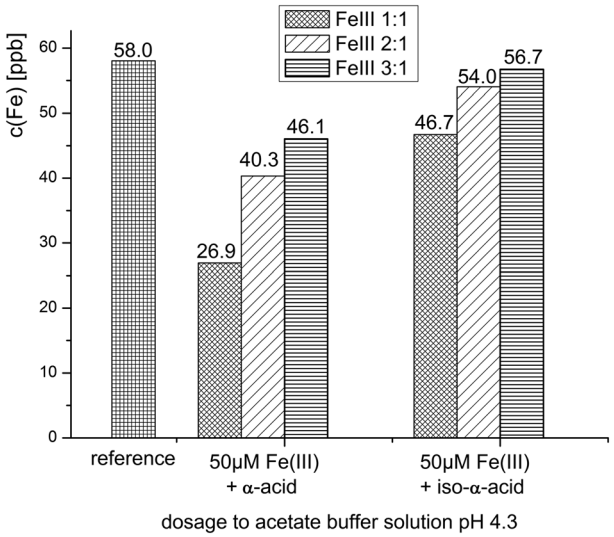

Figure 9. Iron precipitation by hop acids in molecular relation (1:1, 2:1, 3:1).

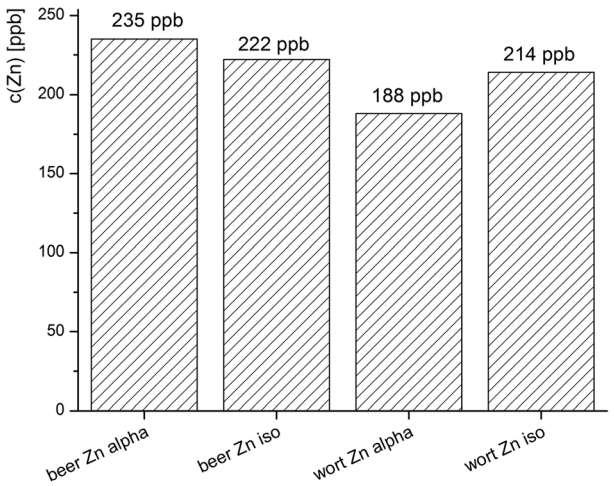

Figure 10. Zinc precipitation by 60 ppm of hop acids.

the iron trials was applied. In the trials where *iso*-α-acids were added, a reduction or effect on the zinc concentration could not be observed; the addition of α-acids also showed no effect at the pH range of beer and just a slight decrease of zinc (6%) at the higher pH range of wort (pH 5.3).

Conclusions

The results depicted in this study clearly demonstrate the positive influence of specific hop ingredients on the oxidative stability of wort and beer. In particular, the positive influence of hop acids as described in the literature *(6,20,24,26,41–43)* could be confirmed. It could be proven that mainly α-acids are responsible for the increase in oxidative beer stability and that the positive effect is presumably caused by the precipitation of pro-oxidative acting metallic ions, such as iron, in chelate complexes with α-acids during wort boiling, caused by the pH drop during fermentation, or by the process of dry hopping, thereby resulting in a decreased metal content in the final beer. As a consequence, a deceleration of oxidative processes caused by oxygen activation via electron transfer and the inhibition of very reactive hydroxyl radical generation initiated by the Fenton–Haber–Weiss reaction system is observable.

The functional principle of the incremental hop dosage is based on the saving of un-isomerized α-acids during wort boiling and whirlpool rest. With a later dosage of hops, the share of α-acids in comparison to *iso*-α-acids is higher; further, less hop acids are precipitated during boiling and therefore they are not lost with the hot trub in the whirlpool. Hence, the properties of α-acids to eliminate or reduce the iron content are accentuated and a high concentration of α-acids towards the end of boiling or in the whirlpool leads to augmented antioxidative properties. Under this aspect, the pH drop during fermentation plays an important role. In correspondence to the results of the precipitation trials, the pH drop leads to a significant precipitation of α-acids, which involves bound iron ions. The separation of these is caused by yeast crop or centrifugation after fermentation or during filtration. Recent studies *(51)* have also shown that the transition metal copper is involved in this kind of pH-dependent precipitation with α-acids, whereas magnesium and calcium are not involved in the complex formation *(51)*. Additionally, the decrease in the zinc concentration (zinc is fundamental to yeast nourishment) by precipitation with hop acids could be excluded in this work. The fact that magnesium, calcium and especially zinc are not affected is in the best interests of the brewer, since it does not lead to fermentation difficulties.

Although all sensory features (odour, taste, freshness, palate fullness, quality of bitterness) could be improved by the applied incremental hop dosage regimes, the unexpected deterioration in the pre-isomerized brew should be noted. The resulting question of why the pre-isomerized products combined with an α-acid enriched extract addition were responsible for an accelerated change in the sensory impression during storage, in comparison to the incremental application of pure CO_2-extract, remains open. Perhaps certain degradation products formed during the production process of pre-isomerized hop products are flavour-active and responsible for an accelerated change in the flavour impression during storage.

Taken together, the outcomes from this study show that applying an incremental hop dosage regime can be a useful alternative tool to increase the oxidative wort and beer stability, with a simultaneous enhancement of the hop aroma profile. These effects can justify the necessary surplus of approximately 10% hop application or the use of hop pellets for dry hopping.

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