T. Mertens, T. Kunz, G. De Rouck, B. Gibson, G. Aerts and L. De Cooman (†)

Effects of Mash Chelator Addition on Transition Metal Content and Oxidative Stability of Brewer's Wort

Oxidative stability in brewing refers to the ability of wort and beer to resist degradation by free radicals and reactive oxygen species. It is a critical quality aspect in beer production, as it affects both the shelf life and overall excellence of the final product. The catalytic role of iron, copper and manganese in radical-associated staling is widely acknowledged. In this context, the present study investigates the effectiveness of polyphenolic chelators (tannic acid, pomegranate and green tea extract) in enhancing oxidative wort stability by sequestering transition metals during mashing. The results, obtained from 12 comparable brews from two distinct pilot breweries, show that incorporating either pomegranate extract or tannic acid during mashing effectively lowers the levels of transition metals, specifically iron, in the brewhouse. Early mash addition of pomegranate extract (90 % ellagic acid) demonstrates the highest efficacy, with an almost 90 % decrease in iron levels and a nearly 80 % reduction in radical concentration, as measured in the final wort by ICP-OES and ESR spectroscopy, respectively. While the investigated chelators do not facilitate the removal of copper or manganese, their levels naturally decline during the brewing process. Chelator addition yields an average reduction of 40 – 60 % in total post-boil aldehydes. Furthermore, strong correlations are identified between iron levels, polyphenols and wort aldehydes after boiling, whereas only weak to moderate correlations with copper and manganese. Aldehydes levels, however, are greatly influenced by thermal stress throughout the brewing process. The findings suggest that natural chelators have the potential to enhance beer flavour stability by diminishing radical formation during brewing and lowering the amount of transition metals and aldehydes in the final product. However, further research is needed to fully understand the implications of these findings on beer stability, given the intricacy of staling.

Descriptors: oxidative stability; transition metals; chelators; pilot scale brewing; radical generation; staling aldehydes

1 Introduction

Beer is the oldest alcoholic beverage that is universally consumed. In recent times, however, the beer industry has become increasingly competitive and, for a business to attain or keep a leading position in the current market, delivering a product that exhibits both high quality and an extended shelf life is essential. Consumers expect beer to taste similar to prior batches they have enjoyed and consistent throughout storage. Accomplishing that often proves challenging. Despite being primarily made from only four ingredients (water, malt, hop, yeast), beer is incredibly complex; and the mechanisms involved in beer staling present an even greater level of complexity.

https://doi.org/10.23763/BrSc23-06mertens

Authors

Tuur Mertens, Thomas Kunz, Brian Gibson, Technische Universität Berlin, Institute of Food Technology and Food Chemistry, Chair of Brewing and Beverage Technology, Berlin, Germany; Gert De Rouck, Guido Aerts, Luc De Cooman (†), Katholieke Universiteit Leuven, Faculty of Engineering Technology, Department of Microbial and Molecular Systems, Laboratory of Enzyme, Fermentation and Brewing Technology, Ghent, Belgium; corresponding author: mertens.tuur@gmail.com

To maintain the flavour of a beer over time – or, in other words, increase its flavour stability – it is crucial to prevent the beer from (oxidative) ageing. Common industry practices include the control of dissolved oxygen levels (throughout brewing and particularly packaging), the limit of heat load and the use of healthy yeast with high oxygen scavenging activity, antioxidants that quench free radicals and chelators that remove oxidation catalysing transition metal ions (iron, copper, manganese) [1].

The deleterious effects of metals – particularly iron – on wort and beer oxidative stability are well documented [1–11] and are also seen in other beverages, such as wine [12–14], coffee [15] and spirits [16]. Through the activation of molecular oxygen by reduced transition metal ions, reactive oxygen species (ROS) can be generated, such as superoxide anions ($O_2^{\bullet -}$), perhydroxyl radicals ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$). These highly reactive entities play a crucial role in the formation of many stale flavour characteristics by oxidising various components present in wort and beer (fatty acids, hop compounds, amino acids, thiols, polyphenols, etc.).

In addition to reducing the number of metal ions potentially ending up in the beer, effectively removing Fe, Cu and Mn during brewing should minimise the formation of ROS and mitigate oxidative

damage throughout the process. By decreasing radical formation – especially during the high-temperature stages of mashing and boiling – *de novo* formation of wort aldehydes is anticipated to be partially impeded since Strecker aldehydes can be directly generated through radical attack of their parent amino acid [17, 18]. A reduction in bound-state aldehydes would also be expected, consequently [19].

The focus of this work is to achieve wort transition metal removal through the employment of polyphenolic chelators (tannic acid, pomegranate and green tea extract) during mashing. By sequestering transition metals early, we aim to positively impact wort oxidative stability, and thus, beer flavour stability. This assertion is substantiated by studies demonstrating a positive relationship between heightened oxidative stability in wort and improved quality, as well as extended shelf life, of the resultant beers under comparable conditions [20–32].

Some researchers, however, have expressed scepticism regarding this claim. Arguments on the insignificance of wort oxidation include the inherent capacity of the brewing process to strongly negate the content of transition metals (without the help of processing aids) during wort separation [33, 34] and fermentation [2, 35, 36], the oxygen ingress during brewing being low (especially for large vessels) [18, 21, 37], the aldehydes evaporating during wort boiling [4, 38] and the ability of yeast to scavenge any of the undesirable compounds that may reside after boiling [38–40]. It is important to note though that a wort with high oxidative stability does not inherently ensure good flavour stability, as a beer's shelf life is ultimately determined by its weakest component. In the absence of adequate control over downstream factors, such as total package oxygen, optimisation of upstream processes will have little impact.

This study seeks to address a specific facet within the complex mechanism of staling by investigating the impact of chelator addition (at different mashing stages) on metal ion concentration, prooxidative radical generation and aldehyde levels in wort. The results of which augment the current, albeit limited, understanding of the effects of external chelators in brewing and the influence of transition metal concentrations on wort aldehyde content. The authors hope that this work may serve as a cornerstone for future research on the topic of metal chelation in brewing – an area in the field of beer flavour stability still underexplored – and to put another tool in the brewer's arsenal to combat oxidative ageing.

2 Materials and methods

2.1 Chemicals and consumables

Calcium chloride dihydrate (≥ 99.0 %), glacial acetic acid (≥ 99.0 %), ethanol absolute (≥ 99.2 %), 2-thiobarbituric acid (≥ 99.0 %), disodium hydrogen phosphate dihydrate (≥ 99.5 %), potassium dihydrogen phosphate (≥ 99.5 %), D-fructose (≥ 99.0 %), ninhydrin (≥ 99.0 %), potassium iodate (≥ 99.7 %), glycine (≥ 99.7 %), carboxymethylcellulose (CMC; low viscosity), ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA, Titriplex® III; ≥ 99.0 %), n-butanol (≥ 99.9), isooctane (≥ 99.8 %), iron(II) sulphate heptahydrate (≥ 99.0 %), ammonium iron(III) citrate (≥ 16.5 % Fe), ammonia solution (≥ 25.0 %), bovine serum albumin (BSA, albumin fraction V; ≥ 98.0 %), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL; ≥ 97.0 %), O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine-hydrochloride (PFBHA; ≥ 99.0 %), *trans*-2-nonenal (≥ 97.0 %), phenylacetaldehyde (≥ 90.0 %), furfural (≥ 99.0 %), hexanal (≥ 98.0 %), 3-methylbutanal (≥ 97.0 %), 2-methylbutanal (≥ 95.0 %), 2-methylpropanal (≥ 99.0 %) and deuterated benzaldehyde (benzaldehyde-d_6; 98 atom %D) were purchased from Merck KGaA (Darmstadt, Germany). Deuterated 2-methylbutanal (2-methylbutanal-d_{10}; 100 atom %D) was synthesised upon ordering from MercaChem (Nijmegen, the Netherlands). MN-polyamide CC 6 was from Macherey-Nagel™ GmbH & Co. KG (Düren, Germany). Bradford's protein assay dye reagent concentrate (0.01 % Coomassie® Brilliant blue G-250) was made by Bio-Rad Laboratories GmbH (Munich, Germany). Concentrated nitric acid (≥ 65.0 %) was obtained from Th. Geyer GmbH & Co. KG (ChemSolute®, Renningen, Germany). Hydrochloric acid (≥ 32 %) and sodium hydroxide (≥ 98.5 %) were acquired from VWR International (Radnor, USA). Glycerine (≥ 98.0 %) and hydrogen

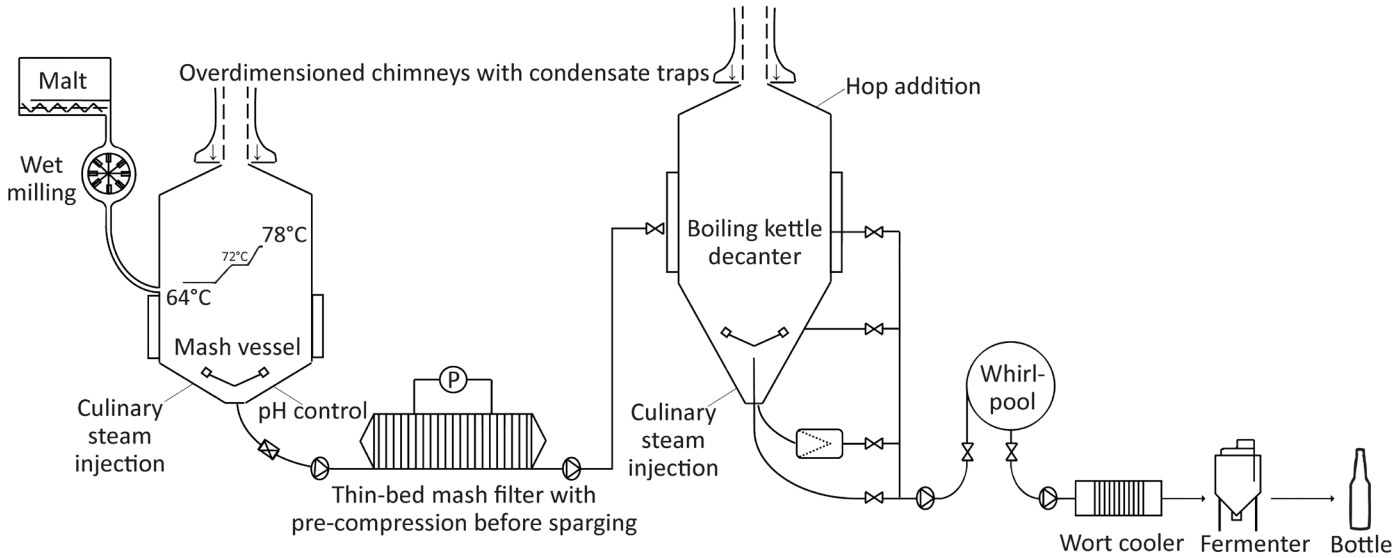

Fig. 1 Process outline of the 5-hL pilot brewery of KU Leuven, Technology Campus Ghent

peroxide (ROTIPURAN®; ≥ 30 %) were procured from Carl Roth GmbH (Karlsruhe, Germany). Methional (≥ 95.0 %) was attained from Acros Organics (New Jersey, USA). N-tert-Butyl-α-(4-pyridyl-1-oxide)-nitrone (POBN; ≥ 98.0 %) was bought from TCI Deutschland GmbH (Eschborn, Germany). Reference standards (1000 µg/mL) for iron, copper, manganese and zinc were acquired from Perkin Elmer LAS GmbH (Rodgau, Germany). Argon (Alphagaz™ 1; ≥ 99.999 %), nitrogen gas (≥ 99,999 %), helium (Alphagaz™ 2; ≥ 99.9999 %) and methane (≥ 99,995 %) were from Air Liquide GmbH (Düsseldorf, Germany). Tannic acid (BrewTan B®; ≥ 98.0 %) was supplied by S.A. Ajinomoto OmniChem N.V. (Wetteren, Belgium), pomegranate extract (ellagic acid, ≥ 90.0 %) by PureBulk Inc. (Oregon, United States) and green tea extract (epigallocatechin gallate, ≥ 50.0 %) by Fairvital B.V. (Landgraaf, The Netherlands). All aqueous solutions were made with ultrapure water, either through Milli-Q® purification (Merck Millipore, Darmstadt, Germany) or via Sartopore® 2 MidiCap 0.2 µm filtration (Sartorius AG, Goettingen, Germany). For the KU Leuven brewing trials, pilsner malt was sourced from Holland Malt (Lieshout, The Netherlands) and Munich (15 MD) malt from Mouterij Dingemans (Stabroek, Belgium). Hop extract (IsoHop®; 20 % iso-α-acids) was provided by Barth-Haas (Paddock Wood, England). For the TU Berlin brewing trials, both pilsner and Munich (type II) malt were sourced from Weyermann Malting Company (Bamberg, Germany). Hop extract (30 % iso-α-acids) was provided by Hopsteiner (Mainburg, Germany).

2.2 Wort samples

2.2.1 Pilot scale production at the Katholieke Universiteit Leuven

Eight batches of wort were produced at the pilot brewery of KU Leuven, Technology Campus Ghent (Fig. 1), under analogous, atmospheric conditions and with identical materials and equipment. All batches were brewed on a 5-hL scale by mixing 88 kg of kilned malt (50:50 Pilsner:Munich) with 1.94 hL of mash water at a rate of 2.2 L/kg (malt/liquor ratio of 1:2.2 w/w) during fine milling with a wet disc mill (disc gap, #19; Hydromill®, Meura SA, Péruwelz, Belgium). Mashing-in occurred at 64 °C for 30 min, followed by an intermediate temperature rest at 72 °C for 15 min (ΔT_{rise} = 1.5 °C/min) and mashing-off at 78 °C for 1 min.

The mashing liquor was pre-heated reverse osmosis water, enriched with 81 mg/L of Ca^{2+} ions, but without acid addition (unadjusted mash pH of 5.6 at the onset of boiling, measured at 20 °C). For the non-reference brews, chelator addition occurred either at the onset of mashing (mashing-in) or at the end of mashing (mashing-off), as summarised in table 1. Mash filtration was performed with a membrane-assisted thin-bed filter (Meura 2001, Meura SA). The resulting filter cake was sparged with 2 hL of water (sparging rate, 2.3 L/kg; first running, 25 °P; last running, 3 °P; collected wort, 16-17 °P). The sweet wort was adjusted to 13.4 °P during boiling. Additionally, isomerised hop extract was added, aiming for a final bitterness of 29 mg/L iso-α-acids (presumed yield, 50 %), and 10 µL/L of zinc. After the 60 min boil, the hot wort was pumped into an open whirlpool (filling, 6 min; rest, 20 min; emptying, 20 min), where trub was removed. The clarified wort was subsequently cooled to 20 °C. Wort samples were collected in metal-free tubes (VWR International) at the onset of mashing, end of mashing, mash filtration, onset of boiling, end of boiling and end of cooling (before wort aeration) and stored at – 20 °C until analysis.

2.2.2 Pilot scale production at the Technische Universität Berlin

Likewise, four batches of wort were produced at the pilot brewery of TU Berlin, Department of Brewing and Beverage Technology. All batches were brewed on a 2-hL scale by mixing 24 kg of kilned malt (50:50 Pilsner:Munich) with 80 L of mash water (malt/liquor-ratio of 1:3.3 w/w) after milling with a two-roll mill (grinding gap, 1.2 mm; Künzel Maschinenbau GmbH, Mainleus, Germany). Mashing-in occurred at 62 °C for 5 min, followed by a temperature rest at 66 °C for 30 min, one at 72 °C for 20 min (ΔT_{rise} = 0.5 °C/min) and mashing-off at 78 °C for 10 min. The mashing liquor was pre-heated reverse osmosis water, enriched with 68 mg/L of Ca^{2+} ions, but without acid addition (unadjusted mash pH of 5.4 at the onset of boiling, measured at 20 °C). For the non-reference brews, chelator addition occurred solely at the onset of mashing (mashing-in), as summarised in table 1. Mash filtration was performed by lauter tun. The spent grain was sparged with 40 L of water (first running, 16.5 °P; collected wort, 11.3 °P). The sweet wort was adjusted to 12.0 °P during boiling and isomerised hop extract was added, aiming for a final bitterness of 30 mg/L iso-α-acids (presumed yield, 85 %). After the 60 min boil, the hot wort was pumped into an open whirlpool (filling, 10 min; rest, 20 min; emptying, 10 min), where trub was removed. The clarified wort was subsequently cooled to 12 °C. Wort samples were collected in metal-free tubes at the onset of mashing, end of mashing, onset of boiling, end of boiling and end of cooling (before wort aeration) and stored at – 18 °C until analysis.

2.3 Standard wort analyses

Density, extract and pH were evaluated using a DMA 5000 Alcolyzer beer analysing system (Anton Paar GmbH, Graz, Austria) on clear, undiluted samples, according to MEBAK (method 2.9.6.3 and 2.13,

Table 1 Chelator mash addition protocol

	Brew	Chelator	Addition time	Concentration ($mg_{chel.}/kg_{malt}$)
KU Leuven	Ref_1 (△) Ref_2 (□)	–	–	0
	PG_e (▲) GT_e (▲) TA_e (▲)	Pomegranate extract Green tea extract Tannic acid	Onset of mashing (“early”)	0.17
	PG_l (■) GT_l (■) TA_l (■)	Pomegranate extract Green tea extract Tannic acid	End of mashing (“late”)	
TU Berlin	Ref_e (◇)	–	–	0
	PG_e (◆) GT_e (◆) TA_e (◆)	Pomegranate extract Green tea extract Tannic acid	Onset of mashing (“early”)	0.20

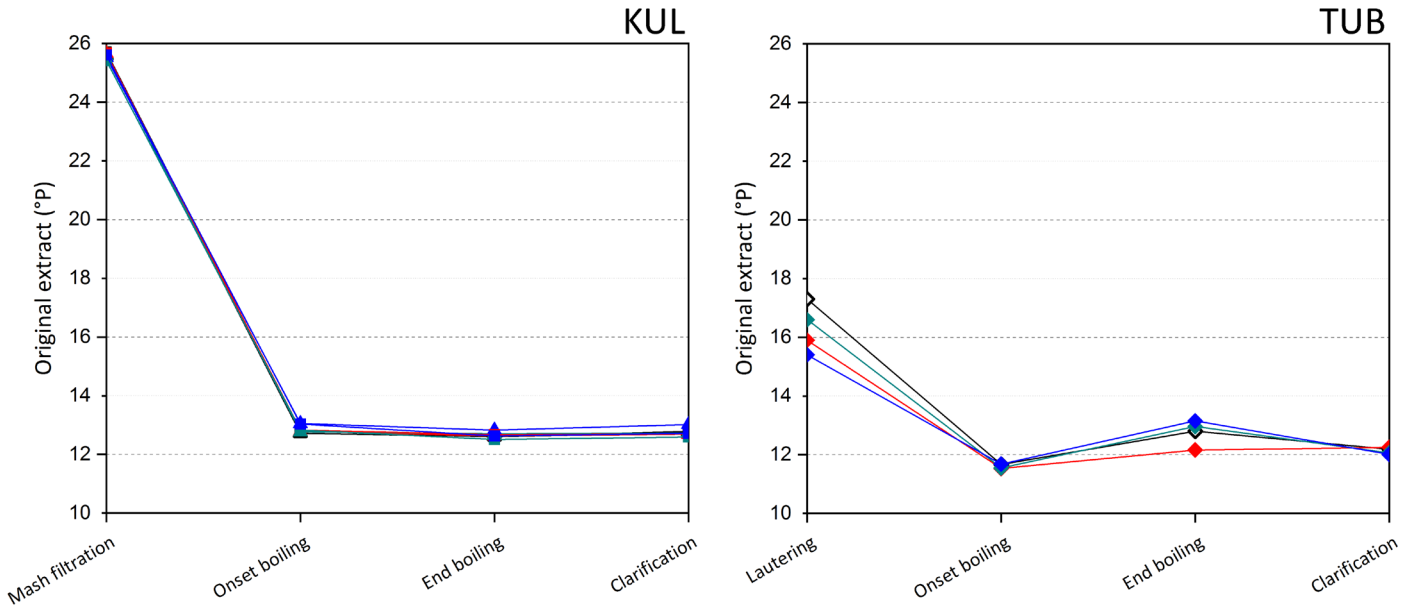

Fig. 2 **Original extracts of the KUL and TUB worts. Reference brews (no chelator addition) are in black with hollow symbols (△, □, ◇), pomegranate extract brews are in red (early addition, ▲ & ◆; late addition, ■), green tea extract brews are in green (early addition, ▲ & ◆; late addition, ■) and tannic acid brews are in blue (early addition, ▲ & ◆; late addition, ■)**

respectively) [41]. Thiobarbituric acid index (TBI) and soluble protein content were determined using ultraviolet-visible spectrophotometry (Varian Cary 100 UV-Vis spectrophotometer, Agilent Technologies, California, USA), according to MEBAK (method 2.4) [41] and the Bradford protein assay [42]. Anthocyanogens and bitterness were quantified by continuous flow analysis (continuous flow analyser, type San+; Skalar Analytical B.V., Breda, The Netherlands), according to MEBAK (method 2.16.2 and 2.17.1, respectively) [41]. Free amino nitrogen (FAN) and total polyphenols were analysed according to MEBAK (method 2.6.4.1 and 2.16.1, respectively) [41], using UV-Vis spectrophotometry for the KU Leuven samples and continuous flow analysis for the TU Berlin samples.

2.4 Determination of the metal content

Quantification of metal ions (iron, copper, manganese and zinc) in wort was performed by inductively coupled plasma-optical emission spectroscopy (ICP-OES), for which an Avio 200 spectrometer (PerkinElmer, Rodgau, Germany) was employed, in accordance with a preceding study [6]. Preliminary sample preparation consisted of centrifugation of the wort.

2.5 Determination of radical levels

Radical formation was measured in the wort samples under force-ageing conditions (60 °C) through electron spin resonance (ESR) spectroscopy by using an X-band spectrometer (e-scan, Bruker BioSpin GmbH, Rheinstetten, Germany), in accordance with a preceding study [6] and MEBAK (method 2.15.3) [41]. Final radical concentrations were reported as the radical intensities at minute 450 (T_{450}-value).

2.6 Determination of (free) aldehydes

Aldehydes (methional, *trans*-2-nonenal, phenylacetaldehyde, furfural, hexanal, 3-methylbutanal, 2-methylbutanal and 2-methylpropanal) were quantified by capillary gas chromatography-mass spectrometry (CGC-MS), based on the method of Baert et al. [43, 44]. The setup employs headspace-solid phase microextraction (HS-SPME), using a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre (StableFlex; Supelco, Bellefonte, USA) and on-fibre PFBHA derivatisation, coupled with a Thermo Scientific™ FOCUS GC gas chromatograph (Thermo Fisher Scientific Inc., Waltham, USA) and a Thermo Scientific™ ISQ™ EC Single Quadrupole mass spectrometer. Standards for external calibration were prepared from stock solutions of eight ethanol-dissolved aldehyde markers.

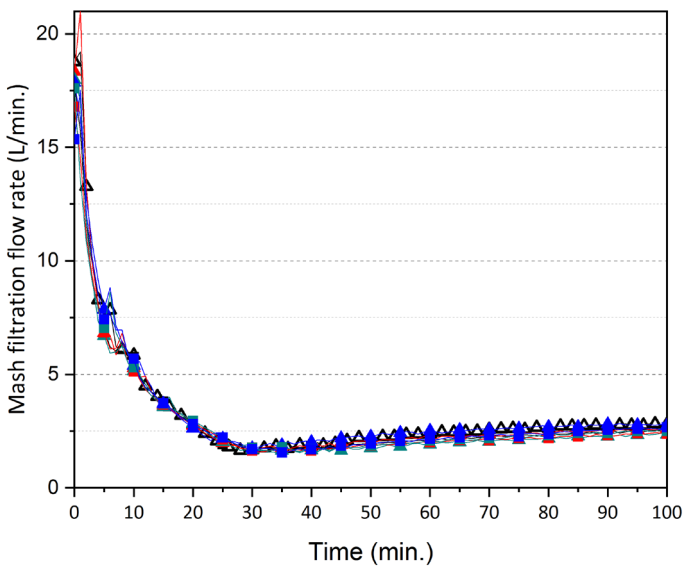

Fig. 3 **Mash filtration flow rates of the KUL worts. Reference brews (no chelator addition) are in black with hollow symbols (△, □), pomegranate extract brews are in red (early addition, ▲; late addition, ■), green tea extract brews are in green (early addition, ▲; late addition, ■) and tannic acid brews are in blue (early addition, ▲; late addition, ■)**

2.7 Data analysis

Mean values and standard deviations (SDs; graphically represented through error bars, where applicable) are determined via two technical replicates unless noted otherwise. Scientific graphing and data analysis were conducted using OriginPro (version 9.6.5.169, OriginLab Corporation, Northampton, MA, USA) and Microsoft Excel for Office 365 (version 16.0.12527.22286, Redmond, WA, USA).

3 Results and discussion

3.1 Chelator effects on brewing-relevant parameters

The eight batches of wort produced at the KU Leuven pilot brewery, henceforth referred to as the 'KUL worts', were very similar in terms of extract (SDs of 0.1 °P for every mashing stage). It verifies suitable batch reproducibility and the inconsequentiality of chelator addition on starch conversion. The same applied to the four batches of wort produced at the TU Berlin pilot brewery, henceforth referred to as the 'TUB worts'.

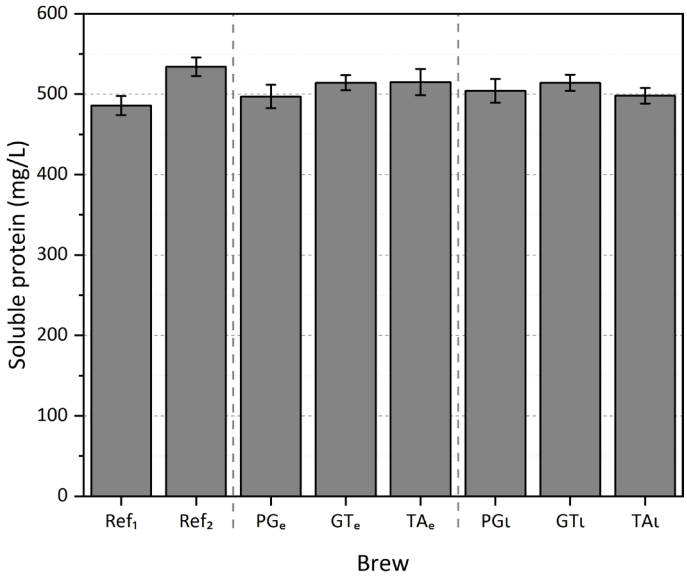

Fig. 4 Soluble protein contents of the clarified KUL worts. Calculated from 2 x 2 biological/technical replicates

The graphed data are shown in figure 2 and are also summarised in table 5 (see page 68). The latter serves as a comprehensive compilation of all the analyses and results of the study, allowing a comparison of averages between both breweries and providing insight into parameter fluctuations during brewing. In addition, the inclusion of standard deviations alongside the means provides information on the impact of chelator additions on each variable. A large standard deviation indicates a noticeable effect, whereas a small SD suggests a negligible impact.

Extract yields are important, as considerable variations in the extract can lead to different concentrations of staling-related wort compounds that are extracted from the malt (such as polyphenols, transition metals, aldehydes, etc.). Similarly, no consequential deviations were found for the KUL wort mash filtration times (Fig. 3). It is not unthinkable that an effect would have been observed if a more oxidised wort or a lauter tun was used, since tannic acid – at the concentrations employed – has been shown to positively influence lautering performance when added to the brewing liquor [45, 46]. It does this i.a. by preventing top-dough or 'teig' formation (a layer of oxidised cross-linked proteins that hinders wort separation) through coagulation and flocculation of thiol-containing proteins [47].

The ability to precipitate sensitive proteins makes tannic acid a valuable physicochemical stabilisation agent during wort boiling [48]. Given that pomegranate and green tea extract also contain polyphenols with protein-binding capabilities [49–51], it is reasonable to assume that these chelators may have similar potential for

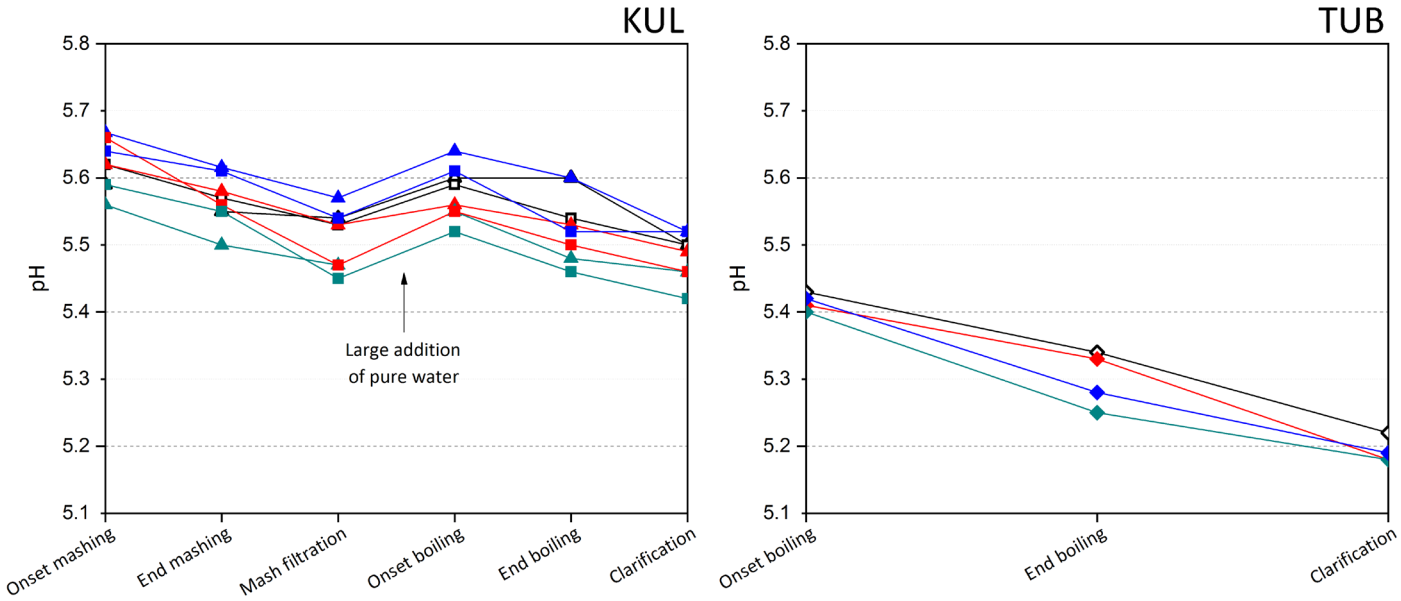

Fig. 5 pH of the KUL and TUB worts. Reference brews (no chelator addition) are in black with hollow symbols (△, □, ◇), pomegranate extract brews are in red (early addition, ▲ & ◆; late addition, ■), green tea extract brews are in green (early addition, ▲ & ◆; late addition, ■) and tannic acid brews are in blue (early addition, ▲ & ◆; late addition, ■)

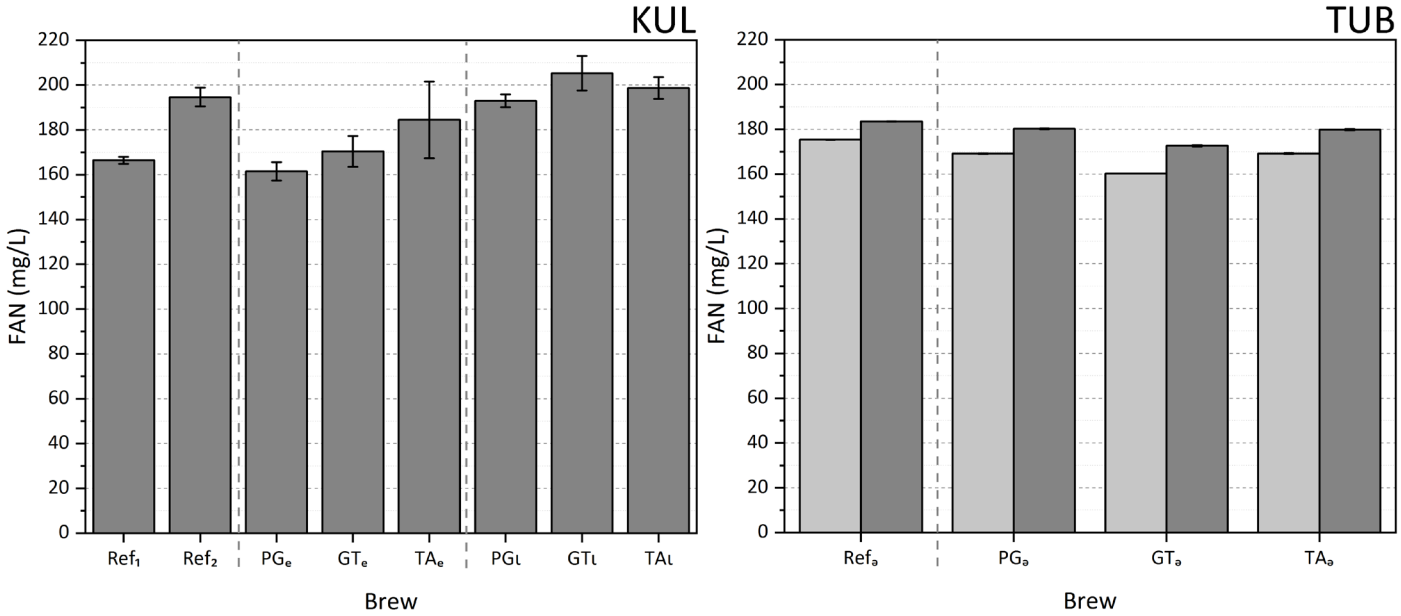

Fig. 6 FAN contents of the KUL and TUB worts, measured at the onset of boiling (light grey) and end of cooling (grey)

beer stabilisation. However, as yet, no studies have explored their effectiveness in this regard. Although this study found no measurable differences in soluble protein content among the clarified KUL worts (Fig. 4), pronounced differences in protein concentration would likely have been observed if the chelators were added to the boiling kettle, due to the lower protein content of filtered wort.

At the concentrations employed (75 and 60 $mg_{extract}/L_{mash\ liquor}$ for the KUL and TUB worts, respectively), the chelators did not affect mash pH. The acidities of the KUL and TUB worts (measured at 20 °C) did, however, show natural pH fluctuations throughout the brewing process. As seen in figure 5, wort pH gradually declines during mashing mainly due to the liberation of (organic and inorganic) malt phosphates [52]. Further pH decrease takes place during boiling by i.a. addition of hop bitter acids and production of Maillard reaction products (MRPs) [53].

Negligible inter-batch pH variations were detected for both breweries, with SDs ∈ [0.01–0.05] and ΔpH_{maxima} ∈ [0.03–0.14], depending on the mashing stage. The larger inter-brewery variation (ΔpH_{means} ∈ [0.16–0.29], depending on the mashing stage) seen is likely due to the utilisation of more strongly roasted Munich malt in the TU Berlin trials (25 EBC, as opposed to the KU Leuven's 15 EBC Munich malt), leading to more acidic melanoidins being released in the wort.

Only minor deviations were seen in the free amino nitrogen contents of the KUL and TUB worts (Fig. 6), with both breweries achieving

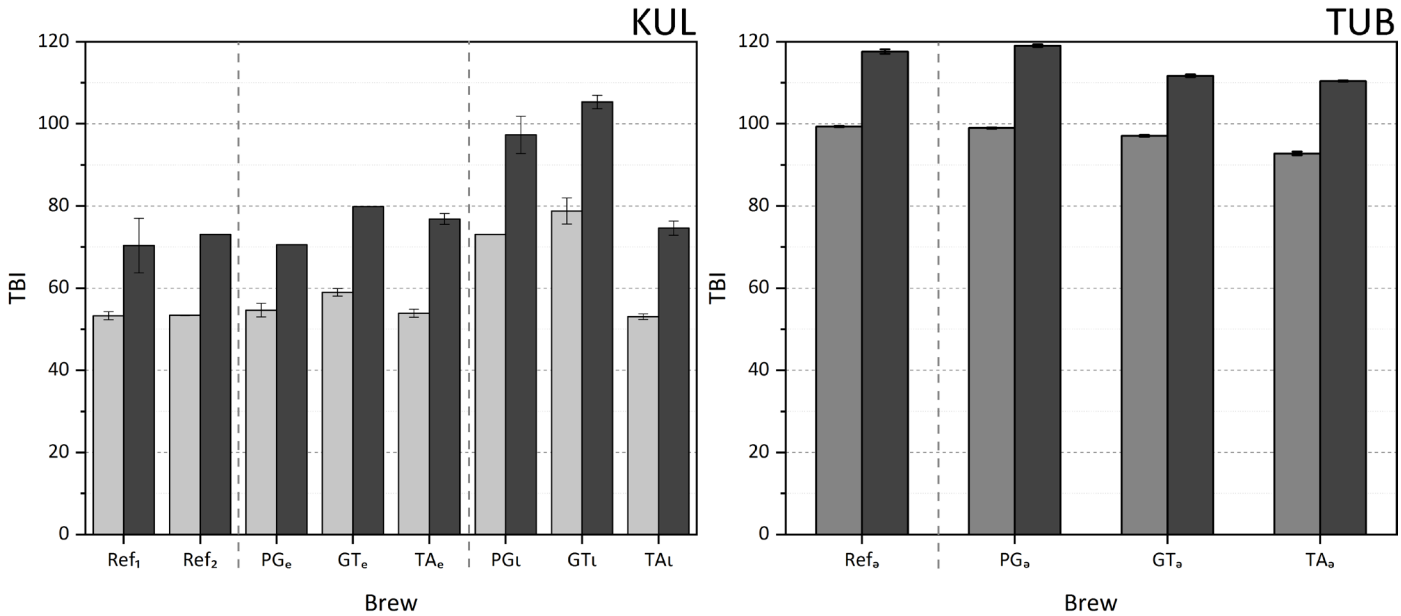

Fig. 7 TBI values of the KUL and TUB worts, measured at mash filtration (light grey), the onset of boiling (grey) and end of boiling (dark grey). All values were normalised to 12 % w/w extract

FAN levels of ca. 180 ± 13 mg/L after clarification. This is relevant because significant differences in amino acid content can lead to a high disparity in aldehydes, due to amino acids being important ageing precursors [54–56].

The levels of total polyphenols in the clarified KUL and TUB worts were all very similar (Table 5), with an average of 241 ± 15 mg/L for both breweries. As with the FAN content, the significance is that polyphenols can also influence staling by i.a. increasing the reducing activity and decreasing the formation of carbonyls [57]. The utilisation of isomerised (CO_2) hop extract in this study was motivated by the aim to exclude the potential influence of hop-derived polyphenols as a confounding factor.

These results suggest that the additions of the polyphenolic extracts (pomegranate, green tea, tannic acid) during mashing did not lead to a measurable increase in the polyphenol content of the clarified worts. The excess polyphenols seem to remain in the brewhouse (during mash filtration and clarification, adsorbed to wort proteins and trub) and, thus, do not carry over into fermentation. To substantiate this claim, however, a more elaborate examination is needed. Tannic acid, for example, has been known to impart (di)gallic acid residues to finished beer when added in the brewhouse [46, 58].

A significant discrepancy was observed in the thiobarbituric acid index – a value used for gauging cumulative thermal stress in malt, wort and beer, brought about by heat exposure during kilning, mashing, boiling, etc. – for two of the KUL brews (Fig. 7). Both brews PGI and GTI (late additions of pomegranate and green tea extract, respectively) had higher TBIs compared to the other KUL brews, before and after boiling. Worts and beers with higher TBIs generally have heightened staling-related benchmarks, such as increased aldehyde levels, colour and radical concentrations, as will be discussed in greater detail later on.

As stated, the elevated heat stress in brews PGI and GTI appeared to originate prior to wort boiling. One potential explanation is the possible presence of Maillard intermediates in the pomegranate and green tea extracts, carrying over into the filtered/boiled wort when added late to the mash but not with early mash addition, due to cross-linking and co-precipitation of the MRPs with wort proteins during mashing [59, 60]. The elevated TBI values observed in the TUB worts, e.g. when comparing the 'end of boiling' data points from both breweries, can be attributed to the use of the more intensely roasted Munich malt, as previously mentioned.

The worts with higher TBI values, PGI and GTI, were visibly darker than the others. This observation is in line with the evident correlation that exists between the thermal loads of malt, wort and beer and the corresponding increase in their colour, due to greater production of coloured Maillard reaction products [61–63]. It is important to note that similar associations have been established between these factors and prooxidative radical generation [8, 62, 64–66]. This is attributed to specific MRPs, such as reductone intermediates, which can rapidly reduce oxidised metal ions, promoting e.g. catalytic iron redox cycling ($Fe^{3+} \leftrightarrows Fe^{2+}$) [67, 68].

3.2 Chelator effects on metal ion, free radical and aldehyde content in wort

Transition metal (Fe, Cu, Mn, Zn) concentrations naturally fluctuate throughout the brewing process (Table 5). Copper concentrations decrease every subsequent step, whereas manganese is less readily retained in the brewhouse but ultimately declines during whirlpooling. Overall, metal levels decline during mashing, wort boiling and/or at the whirlpool step as a result of metal retention with spent grain, hot break and/or trub [1, 8, 35].

In the present work, however, the primary objective was to coercively reduce wort transition metal levels further. This was successfully achieved for iron in both pilot breweries by the addition of the external chelators, as shown in figure 8, which replicates

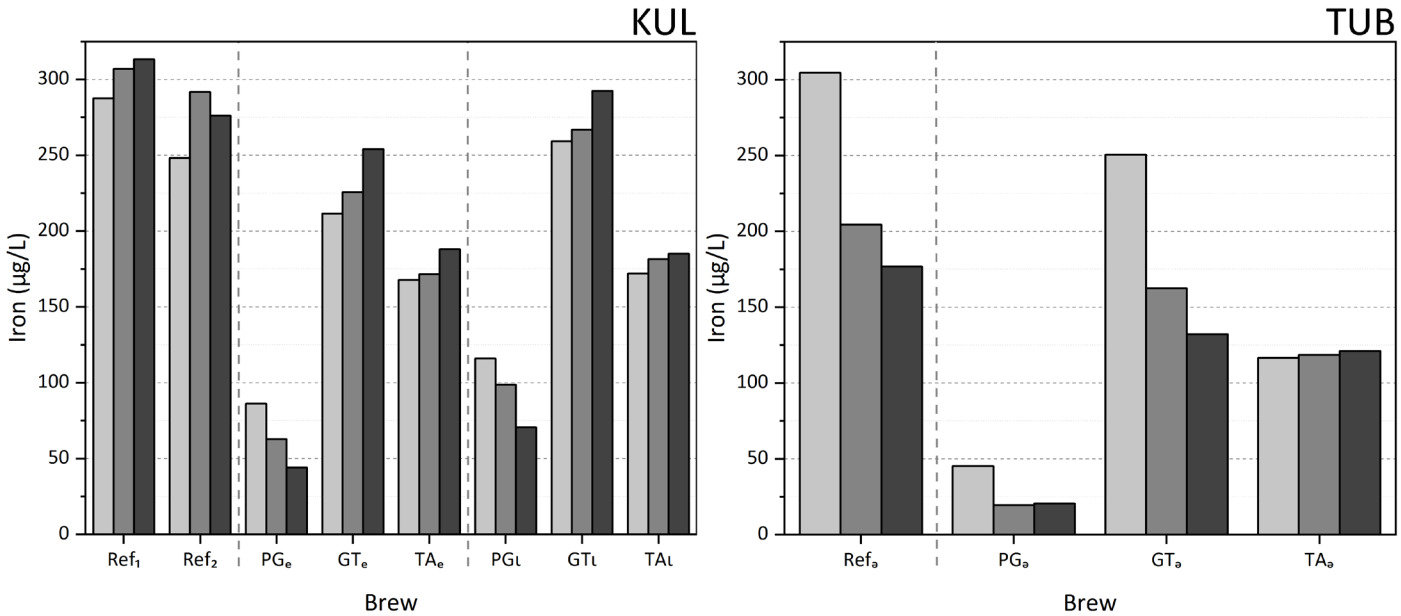

Fig. 8 **Iron concentrations of the KUL and TUB worts during mashing, measured at the onset of boiling (light grey), end of boiling (grey) and end of cooling (dark grey). All values were calculated from four technical replicates, with an average relative standard deviation below 2 %, and normalised to 12 % w/w extract**

Table 2 **Transition metal concentrations of the clarified KUL and TUB worts. All values were calculated from four technical replicates, with an average relative standard deviation below 2 %, and not subjected to normalisation for w/w extract**

	Brew	Chelator	Addition time	[Fe] (µg/L)	[Cu] (µg/L)	[Mn] (µg/L)	Sum (µg/L)
KU Leuven	Ref_1 (△)	–	–	339.3	91.3	95.9	526.4
	Ref_2 (□)			298.1	76.9	95.1	470.0
	PG_e (▲)	Pomegranate extract	Onset of mashing ("early")	**47.4**	89.1	100.1	236.5
	GT_e (▲)	Green tea extract		274.4	91.7	98.1	464.1
	TA_e (▲)	Tannic acid		204.5	91.0	114.6	410.1
	PG_l (■)	Pomegranate extract	End of mashing ("late")	**76.0**	89.7	88.4	254.0
	GT_l (■)	Green tea extract		312.6	90.7	94.0	497.3
	TA_l (■)	Tannic acid		200.0	94.0	96.5	390.5
TU Berlin	$Ref_\ominus$ (◇)	–	–	180.1	39.1	68.7	287.9
	$PG_\ominus$ (◆)	Pomegranate extract	Onset of mashing ("early")	**21.1**	26.0	71.2	118.3
	$GT_\ominus$ (◆)	Green tea extract		133.3	24.4	68.5	226.2
	$TA_\ominus$ (◆)	Tannic acid		121.5	21.3	73.6	216.4

the findings of a previous lab-scale study [6]. Similarly, ellagic acid and tannic acid – despite their ability to chelate copper in wort-pH buffer solutions [69, 70] – appeared unable to influence the final wort concentrations of copper and manganese. Their low standard deviations, observed in table 5, indicate little variation among the brews and provide further confirmation of the chelators' inefficacy in removing Cu and Mn ions.

Copper ions likely have stronger binding affinities for amino acids and proteins, which are abundant in wort, than for endo- or exogenous polyphenols. According to Jacobsen et al., certain amino acids can form complexes with copper at wort pH that may compete with potent chelating agents such as EDTA and EGTA [71]. This assertion is supported by brewing studies that have found heightened concentrations of copper bound to proteinaceous matter [2, 72–74]. In contrast, divalent manganese ions tend to form unstable complexes with organic ligands and are therefore easily separated from their binding partners [75–78]. This behaviour is confirmed by studies that have shown minimal losses of Mn during the brewing process [2, 7, 9].

A slight discrepancy with the preceding lab-scale study [6] was the better performance of the early (pomegranate) extract addition towards iron removal at pilot scale. This effect explains the lower ESR signal intensities observed after the 450-minute assay (represented by T_{450}-values) with the early additions in this study (Fig. 9).

The KUL reference brews exhibited an average, unnormalised iron concentration of 320 ± 30 µg/L in the clarified worts, which was higher than any of the 'chelator brews'. In terms of iron removal, compared to the reference brews, both the early and late additions of pomegranate extract were the most effective (– 85 % and – 76 %, respectively), followed by tannic acid (– 36 % and – 38 %) and green tea extract (– 14 % and – 2 %). These results were reaffirmed

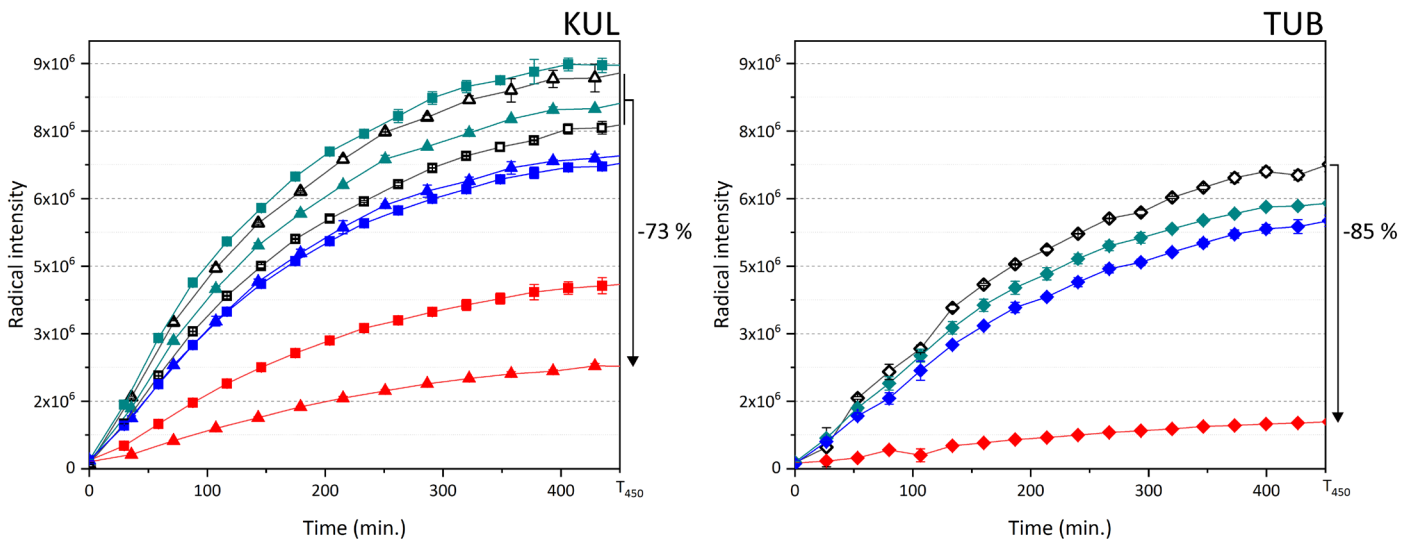

Fig. 9 **ESR results of the clarified KUL and TUB worts. Reference brews (no chelator addition) are in black with hollow symbols (△, □, ◇), pomegranate extract brews are in red (early addition, ▲ & ◆; late addition, ■), green tea extract brews are in green (early addition, ▲ & ◆; late addition, ■) and tannic acid brews are in blue (early addition, ▲ & ◆; late addition, ■)**

Table 3 **Concentrations of eight distinct aldehydes, as measured in the respective KUL worts. All values were normalised to 12 % w/w extract, are means ± standard deviation of eight brews and include both reference and chelator brews**

	Aldehyde content (µg/L)		
	Mash filtration	End of boiling	Change over boiling (%)
Furfural	73.1 ± 6.1	92.9 ± 7.5	+28 ± 10
2-Methylpropanal	567.6 ± 137.4	109.0 ± 102.4	-80 ± 20
2-Methylbutanal	440.8 ± 85.2	66.4 ± 66.5	-84 ± 18
3-Methylbutanal	683.2 ± 158.2	108.7 ± 109.3	-83 ± 19
Methional	397.1 ± 53.2	207.5 ± 22.8	-47 ± 5
Phenylacetaldehyde	87.8 ± 11.5	78.4 ± 17.1	-11 ± 10
Hexanal	37.7 ± 4.6	3.0 ± 3.9	-91 ± 12
Trans-2-nonenal	0.1 ± 0.0	0.0 ± 0.0	-79 ± 20

in the TUB trials (– 88 %; – 33 %; – 26 %), meaning that the findings are brewery independent and irrespective of mash filtration or lautering. It should be noted that the TUB worts had smaller iron contents at mashing-in (Table 5), resulting in their lower final iron concentrations. Comparatively, however, larger quantities of iron were removed from the KUL brews.

Concentrations of iron, copper and manganese in the clarified worts can be found in table 2. Apart from the declarations already made, it is noticeable that the metal levels were lower in the TU Berlin trials, even though a more roasted Munich malt was used, which typically bestows excess iron and manganese to the wort [6, 33]. While there was, indeed, a slightly higher concentration of the chelators used, this would not account for the differences seen in the reference brews. The observed effect is likely caused by differences in barley cultivar [79], malt roasting conditions [80], as well as the use of high extract brewing with fine milling at KU Leuven, as opposed to e.g. the method of wort separation (lautering vs. mash filtration) – although these factors are often interrelated.

Due to the variations in the total transition metal content among the different brews, different radical formation rates are anticipated in the worts. Figure 9 depicts the results of the electron spin resonance (ESR) assay for the clarified worts. Compared to the reference brews, the early additions of pomegranate extract resulted in a ca. 80 % reduction in T_{450}-values, indicating a significant decline in radical generation. Incorporations of tannic acid also led to reduced T_{450}-values, seemingly independent of addition time. The radical reduction efficacies of green tea extract were observed to be minimal, as they also did not demonstrate very effective iron removal.

While the low metal content of pomegranate brews certainly contributes to their low T_{450}-values, it is plausible that an additional antioxidative effect is at play. Notably, the additions of tannic acid and green tea in the TUB trials show a lower amount of total transition metals (216 and 226 µg/l, respectively), compared to the pomegranate additions in the KUL trials (237 and 254 µg/L, early and late); yet, the latter two worts have lower T_{450}-values.

Ellagic acid, for example, is also likely to engage in free radical scavenging [81, 82]. It is questionable, however, whether the low residual concentrations would account for the impact observed in figure 9. In reality, there is a strong correlation between the T_{450}-values and the wort Fe content (*r* of 0.95; R^2 of 0.90). The coefficients of correlation (*r*) and determination (R^2) are significantly lower with Cu (0.39; 0.15), Mn (0.33; 0.11) and even the sum of transition

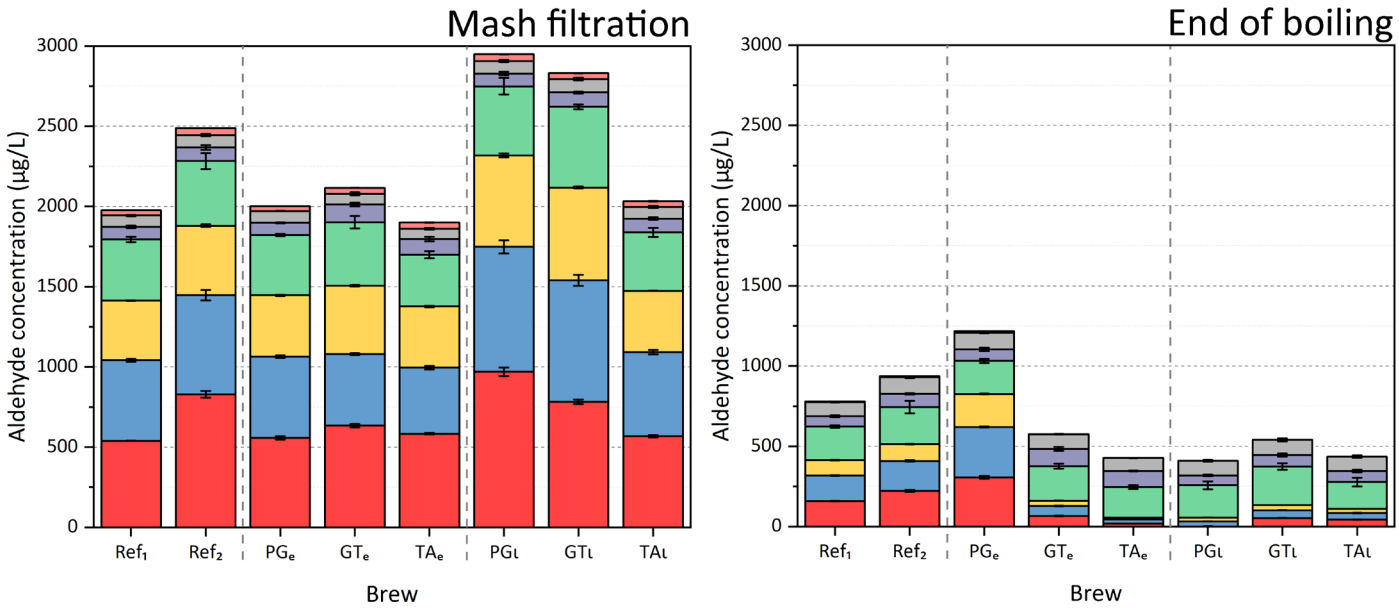

Fig. 10 **Aldehyde concentrations of the KUL worts, measured at mash filtration and the end of boiling. All values were normalised to 12 % w/w extract and calculated from three technical replicates. From bottom to top: 3-methylbutanal (red), 2-methylpropanal (blue), 2-methylbutanal (yellow), methional (green), phenylacetaldehyde (purple), furfural (grey), hexanal (pink), *trans*-2-nonenal (indistinguishable)**

Table 4 Notable coefficients of correlation (*r*) and determination (R^2) between seven selected variables and total wort aldehyde concentrations at two brewing stages (pre- and post-boil), with inclusion of brew PG_e. Correlations excluding the brew PG_e (as an outlier in terms of post-boil aldehydes) are provided and discussed in the text

	Mash filtration		End of boiling	
	r	R^2	*r*	R^2
TBI	0.86	0.74	– 0.53	0.28
Furfural	0.82	0.67	0.76	0.57
FAN*	0.64	0.41	– 0.57	0.32
Iron	0.39	0.15	– 0.07	0.00
Copper	– 0.21	0.04	– 0.26	0.07
Manganese	0.09	0.01	0.02	0.00
Total polyphenols*	0.00	0.00	– 0.48	0.23

*Clarified wort data

metals (0.88; 0.78). Accordingly, the three worts with the lowest amount of iron (Table 2, bolded) display the lowest T_{450}-values.

It is reasonable to anticipate distinctive behaviour in beer, considering how radical formation, and hence ESR values, are highly dependent on various matrix factors [1], including extract, pH, levels of MRPs and reductones, presence of transition metals, complexing agents (e.g. hop acids), radical-quenchers (e.g. sulphite, ethanol), and the mutual concentration ratios of all pertinent compounds. Indeed, copper and manganese have been shown to affect beer lag times (i.e. the duration required for antioxidants to be depleted during ESR analysis) [5, 7].

Table 3 depicts the normalised aldehydes contents, before and after wort boiling. The data show that despite the added heat stress – and consequent furfural increase – over the course of boiling, there is a notable decrease in the overall aldehyde levels. This trend has been observed in several other studies [53, 83–86], and is attributed to volatilisation, particularly for the aldehydes with boiling points below 100 °C (2-methylpropanal, 2- and 3-methylbutanal). It is apparent that the amount of aldehydes lost through evaporation during boiling surpasses the amount that is generated through release or *de novo* formation (e.g. via Strecker degradation of amino acids, the Maillard reaction or oxidative processes) [87].

The total aldehyde contents of each brew are visually represented in figure 10 through stacked column graphs for both brewing stages. The highest aldehyde levels were observed at mash filtration; particularly in brews PG_I and GT_I, which correlate with the elevated TBIs and colour intensities of these two worts (Fig. 7). The latter observation strengthens the existing understanding that aldehydes are closely linked to heat load, as recognised by numerous researchers [8, 20, 88–93] and evident from table 4. In addition to demonstrating the high predictive capability of TBI and furfural (both heat load indicators) for pre-boil total aldehyde levels, the coefficients in 4 also reveal a moderate association with iron in this regard.

It is worth noting that the correlation between TBI and furfural prior to wort boiling (*r* of 0.69) attenuates after boiling (*r* of – 0.09). Likewise, the correlation between TBI and total aldehydes also inverts and weakens post-boiling. Furfural, however, does not exhibit this behaviour and seems to be the more reliable predictor for post-boil wort aldehydes in this study. Nevertheless, it should be emphasised that the thiobarbituric acid index has been proposed by De Schutter et al. as the superior method for determining various types of heat load on wort [94, 95]. This is because, in addition to the volatile 5-hydroxymethylfurfural, the TBI also accounts for non-volatile precursors of volatile ageing compounds (such as Amadori rearrangement products and α-dicarbonyl compounds). It is therefore less susceptible to the shortcomings of volatile heat load indicators such as furfural, where information about the applied thermal load on wort is lost through evaporation.

As illustrated in figure 10, the brew (PG_e) with the least amount of transition metals and the lowest T_{450}-value remarkably demonstrated the highest aldehyde levels after boiling, exceeding even the boiled reference worts. This unexpected outcome can be solely attributed to an aberration during the boiling process, which impeded the evaporation of aldehydes. The elevated presence in brew PG_e of aldehydes in the higher volatility range (2-methylpropanal, 2- and 3-methylbutanal) bolsters this hypothesis. It is plausible that the brewing process for PG_e involved a relatively subdued boiling state (simmering, rather than a rolling boil), leading to inefficient volatilisation [96, 97]. A comparison of the brewing log boiling times revealed that brew PG_e spent a considerably shorter duration at temperatures of ≥ 100 °C (22 min), in stark contrast to the other brews (58 ± 7 min).

Consequently, including outlier brew PG_e in the calculations of the coefficients presented in 4 introduces potential inaccuracies. The exclusion of brew PG_e causes minimal changes in the 'mash filtration' coefficients ($\Delta_{abs} \leq 0.1$) but considerably alters the 'end of boiling' coefficients for certain variables. Omitting the outlier reveals positive correlations between the levels of free aldehydes detected in the boiled wort and the quantities of iron (*r* of 0.83; R^2 of 0.69) and manganese (0.45; 0.20), while a strong negative correlation with total polyphenols (– 0.81; 0.66) emerges. The recalculated coefficients align with the expected prooxidative influence of iron and manganese as transition metal catalysts and the antioxidative effects of polyphenols, as discussed previously. It is worth noting that a weak negative correlation between wort aldehydes and copper (– 0.32; 0.10) persists, which contradicts the prevailing understanding but is in line with recent observations made by other researchers [5, 33, 74].

Each chelator addition led to a lower level of total aldehydes in the boiled worts when compared to the reference brews. On average, there was a reduction of – 39 % for green tea extract, – 55 % for tannic acid and – 58 % for pomegranate extract (excluding brew PG_e). Although these results are promising, further investigation is needed to determine the impact of the depleted iron levels during the brewing process, and the subsequent lower T_{450}-values and aldehyde levels of the final worts, on the final beer quality and flavour stability. A follow-up study will analyse the fresh and force-aged beer samples resulting from these worts to address these queries. The potential benefits of these specified conditions have been speculated and are supported by the widespread use of tannic acid-based products (such as gallotannins) in the brewing industry.

Table 5 **Summary of results obtained from the analyses of worts produced at the KU Leuven and TU Berlin pilot breweries. The reported values are means ± standard deviation of either eight (KUL) or four (TUB) brews and include both reference and chelator brews**

	KU Leuven worts (n = 8)						TU Berlin worts (n = 4)				
Stage	**Onset mashing**	**End mashing**	**Mash filtration**	**Onset boiling**	**End boiling**	**End cooling**	**Onset mashing**	**End mashing**	**Onset boiling**	**End boiling**	**End cooling**
Analysis											
Extract (°P)			25.6 ± 0.1	12.9 ± 0.1	12.7 ± 0.1	12.7 ± 0.1		16.3 ± 0.8	11.6 ± 0.1	12.8 ± 0.4	12.1 ± 0.1
pH	5.6 ± 0.0	5.6 ± 0.0	5.5 ± 0.0	5.6 ± 0.0	5.5 ± 0.1	5.5 ± 0.0			5.4 ± 0.0	5.3 ± 0.0	5.2 ± 0.0
Flow rate of initial 100 min (L/min)			3.1 ± 0.2								
TBI*			59.9 ± 10.2		81.0 ± 13.1				97.0 ± 3.0	114.7 ± 4.3	
FAN (mg/L)						184.3 ± 16.3			168.5 ± 6.2		179.1 ± 4.6
Soluble protein (mg/L)						507.8 ± 14.8					
Total polyphenols (mg/L)						236.6 ± 10.8					255.9 ± 15.1
Anthocyanogens (mg/L)											49.4 ± 11.3
Iron* (µg/L)	486.3 ± 95.0	290.4 ± 60.9	380.0 ± 130.6	193.5 ± 70.8	200.7 ± 88.8	202.9 ± 101.0	236.8 ± 173.5	180.3 ± 114.1	179.2 ± 119.3	126.2 ± 79.3	112.7 ± 66.0
Copper* (µg/L)	326.1 ± 38.2	288.2 ± 11.3	277.7 ± 5.6	190.3 ± 10.6	134.5 ± 28.3	82.7 ± 4.8	234.7 ± 9.8	191.6 ± 32.5	106.4 ± 15.3	28.8 ± 4.6	27.3 ± 7.6
Manganese* (µg/L)	631.4 ± 85.2	242.4 ± 10.4	133.2 ± 9.1	145.7 ± 6.5	168.8 ± 25.0	90.6 ± 6.7	854.4 ± 117.2	376.8 ± 36.7	220.9 ± 10.3	135.0 ± 34.3	69.6 ± 2.7
Zinc* (µg/L)	1093.6 ± 162.7	430.3 ± 38.8	152.4 ± 17.8	183.7 ± 10.3	395.2 ± 103.3	251.5 ± 58.0	970.1 ± 143.9	504.3 ± 50.3	202.5 ± 11.8	93.2 ± 30.0	19.8 ± 3.2
T_{450}-value (x 10^6)						6.7 ± 2.4					4.8 ± 2.6
Bitterness (IBU)											23.7 ± 1.0

*Data normalised to 12 % w/w extract

The application of gallotannins (either pre- and post-wort boil, during maturation or before filtering/centrifugation) has been shown to enhance beer stability [45, 47, 98, 99]. Nevertheless, recent brewing research, including the present study, has shown that ellagic acid [6], and in particular punicalagin (a glycoside of ellagic acid) [69, 100], outperform gallotannins in terms of iron chelation and overall antioxidative capabilities. Punicalagin, like tannic acid, is a high molecular weight polyphenol with a large number (16) of phenolic hydroxyl groups per molecule and has the potential to be an effective stabilising and antioxidative agent. Further scientific exploration, however, is required to determine its full potential in brewing.

4 Conclusion

Incorporation of chelators during mashing influences the transition metal content of wort and does not adversely affect any of the pertinent brewing parameters (extract, pH, filterability, soluble protein, free amino acid content, total polyphenols, heat load). Among the polyphenolic substances investigated, pomegranate extract (90 % ellagic acid) and tannic acid are particularly effective in lowering iron levels in the wort throughout the brewing process. The addition of pomegranate extract during the early stage of mashing leads to an approximate 90 % reduction in the iron content of the final wort and a nearly 80 % reduction in prooxidative radical generation. None of the examined chelators significantly affect concentrations of copper and manganese. However, the levels of these metals naturally decline during brewing through wort separation (filtration and clarification).

On average, the chelator additions result in a 40 – 60 % reduction in total post-boil aldehydes compared to the reference brews, depending on the chelator used. However, only the pomegranate extract brews exhibit significantly higher oxidative wort stabilities, as evidenced by the diminished radical generation in ESR spectroscopy analysis (indicated by low T_{450}-values), which strongly correlate with wort iron content. Our investigation also identifies strong correlations between iron levels, polyphenols and total wort aldehydes after boiling, while only weak to moderate correlations are seen with copper and manganese. These associations, however, are not evident before boiling. It must furthermore be noted that aldehydes display a strong dependence on heat load throughout the brewing process.

Wort oxidative stability is important, as it can affect the quality and shelf life of the final beer product. In this regard, the use of pomegranate extract in brewing demonstrates great potential,

particularly for high-iron beers such as stouts and other dark ales. However, staling is complex; and the relationship between wort oxidative stability and beer flavour stability is not necessarily direct. Further investigation is warranted to determine the impact of these findings on oxidative beer stability and beer flavour stability. Subsequent studies will evaluate the fresh and aged characteristics of the resulting beers.

Acknowledgements

This article is dedicated to the loving memory of prof. Luc De Cooman. The author would also like to express gratitude to colleagues from the Laboratory of Enzyme, Fermentation and Brewing Technology (Katholieke Universiteit Leuven) and the Chair of Brewing and Beverage Technology (Technische Universität Berlin) for their valuable contributions to several of the experiments performed in this study.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 722166.

The author(s) would like to declare that a patent application was filed on the application of punicalagin/ellagic acid to improve the oxidative and colloidal stability of beverages (esp. beer).

5 References

1. Mertens, T.; Kunz, T. and Gibson, B.R.: Transition metals in brewing and their role in wort and beer oxidative stability: a review, Journal of the Institute of Brewing, **128** (2022), no. 3, pp. 77-95.
2. Chrisfield, B.J.; Hopfer, H. and Elias, R.J.: Impact of Copper Fungicide Use in Hop Production on the Total Metal Content and Stability of Wort and Dry-Hopped Beer, Beverages, **6** (2020), no. 3, pp. 1-17.
3. Vanderhaegen, B.; Neven, H.; Verachtert, H. and Derdelinckx, G.: The chemistry of beer aging – a critical review, Food Chemistry, **95** (2006), no. 3, pp. 357-381.
4. Bamforth, C.W.; Muller, R.E. and Walker, M.D.: Oxygen and Oxygen Radicals in Malting and Brewing: A Review, Journal of the American Society of Brewing Chemists, **51** (1993), no. 3, pp. 79-88.
5. Jenkins, D.; James, S.; Dehrmann, F.; Smart, K. and Cook, D.: Impacts of Copper, Iron, and Manganese Metal Ions on the EPR Assessment of Beer Oxidative Stability, Journal of the American Society of Brewing Chemists, **76** (2018), no. 1, pp. 50-57.
6. Mertens, T.; Kunz, T.; Wietstock, P.C. and Methner, F.: Complexation of transition metals by chelators added during mashing and impact on beer stability, Journal of the Institute of Brewing, **127** (2021), no. 4, pp. 345-357.
7. Zufall, C. and Tyrell, T.: The Influence of Heavy Metal Ions on Beer Flavour Stability, Journal of the Institute of Brewing, **114** (2008), no. 2, pp. 134-142.
8. Maia, C.; Cunha, S.; Debyser, W. and Cook, D.: Impacts of Adjunct Incorporation on Flavor Stability Metrics at Early Stages of Beer Production, Journal of the American Society of Brewing Chemists, **81** (2023), no. 1, pp. 54-65.
9. Porter, J.R. and Bamforth, C.W.: Manganese in Brewing Raw Materials, Disposition during the Brewing Process, and Impact on the Flavor Instability of Beer, Journal of the American Society of Brewing Chemists, **74** (2016), no. 2, pp. 87-90.
10. Ferreira, I.M.; Carvalho, D.O. and Guido, L.F.: Impact of storage conditions on the oxidative stability of beer, European Food Research and Technology, **249** (2023), no. 1, pp. 149-156.
11. Irwin, A.J.; Barker, R.L. and Pipasts, P.: The Role of Copper, Oxygen, and Polyphenols in Beer Flavor Instability, Journal of the American Society of Brewing Chemists, **49** (1991), no. 3, pp. 140-149.
12. Comuzzo, P. and Zironi, R.: Biotechnological Strategies for Controlling Wine Oxidation, Food Engineering Reviews, **5** (2013), no. 4, pp. 217-229.
13. Kreitman, G.Y.; Danilewicz, J.C.; Jeffery, D.W. and Elias, R.J.: Reaction Mechanisms of Metals with Hydrogen Sulfide and Thiols in Model Wine. Part 2: Iron- and Copper-Catalyzed Oxidation, Journal of Agricultural and Food Chemistry, **64** (2016), no. 20, pp. 4105-4113.
14. Danilewicz, J.C.: Review of Reaction Mechanisms of Oxygen and Proposed Intermediate Reduction Products in Wine: Central Role of Iron and Copper, American Journal of Enology and Viticulture, **54** (2003), no. 2, pp. 73-85.
15. Yeretzian, C.; Pascual, E.C. and Goodman, B.A.: Probing Free Radical Processes during Storage of Extracts from Whole Roasted Coffee Beans: Impact of O2 Exposure during Extraction and Storage, Journal of Agricultural and Food Chemistry, **61** (2013), no. 13, pp. 3301-3305.
16. Wang, L.; Chen, S. and Xu, Y.: Distilled beverage aging: A review on aroma characteristics, maturation mechanisms, and artificial aging techniques, Comprehensive Reviews in Food Science and Food Safety, **22** (2023), no. 1, pp. 502-534.
17. Wietstock, P.C. and Methner, F.-J.: Formation of aldehydes by direct oxidative degradation of amino acids via hydroxyl and ethoxy radical attack in buffered model solutions, BrewingScience, **66** (2013), no. 7/8, pp. 104-113.
18. Wietstock, P.C.; Kunz, T. and Methner, F.-J.: Relevance of Oxygen for the Formation of Strecker Aldehydes during Beer Production and Storage, Journal of Agricultural and Food Chemistry, **64** (2016), no. 42, pp. 8035-8044.
19. Lehnhardt, F.; Nobis, A.; Skornia, A.; Becker, T. and Gastl, M.: A Comprehensive Evaluation of Flavor Instability of Beer (Part 1): Influence of Release of Bound State Aldehydes, Foods, **10** (2021), no. 10, p. 2432.
20. Hashimoto, N.: Melanoidin-mediated oxidation: A greater involvement in flavour staling, Technical Report of Kirin Brewery Co. Research Laboratory, (1988), no. 31, pp. 19-32.
21. Meilgaard, M.: Effects on Flavour of Innovations in Brewery Equipment and Processing: A Review, Journal of the Institute of Brewing, **107** (2001), no. 5, pp. 271-286.
22. Takashio, M. and Shinotsuka, K.: Preventive Production of Beer against Oxidation-Recent Advances in Brewing Technology., Food Science and Technology International, Tokyo, **4** (1998), no. 3, pp. 169-177.
23. Narziss, L.; Back, W.; Miedaner, H. and Lustig, S.: Untersuchungen zur Beeinflussung der Geschmacksstabilität durch Variation technologischer Parameter bei der Bierherstellung, Monatsschrift für Brauwissenschaft, **52** (1999), no. 11/12, pp. 192-206.
24. Wietstock, P.C.; Kunz, T.; Shellhammer, T.H.; Schön, T. and Methner, F.-J.: Behaviour of Antioxidants Derived from Hops During Wort Boiling, Journal of the Institute of Brewing, **116** (2010), no. 2, pp. 157-166.
25. Lustig, S.; Miedaner, H.; Narziß, L. and Back, W.: Untersuchungen flüchtiger Aromastoffe bei der Bieralterung mittels multidimensionaler Gaschromatographie, Proceedings of the 24th Congress of the

European Brewery Convention, 1993, pp. 445-452.

26. Ohtsu, K.; Hashimoto, N.; Innoue, K. and Miyaki, S.: Flavor Stability of Packaged Beer in Relation to the Oxidation of Wort, Brewers Digest, **61** (1986), no. 6, pp. 18-23.
27. Uchida, M. and Ono, M.: Technological Approach to Improve Beer Flavor Stability: Adjustments of Wort Aeration in Modern Fermentation Systems Using the Electron Spin Resonance Method, Journal of the American Society of Brewing Chemists, **58** (2000), no. 1, pp. 30-37.
28. Uchida, M. and Ono, M.: Technological Approach to Improve Beer Flavor Stability: Analysis of the Effect of Brewing Processes on Beer Flavor Stability by the Electron Spin Resonance Method, Journal of the American Society of Brewing Chemists, **58** (2000), no. 1, pp. 8-13.
29. Narziss, L.; Reicheneder, E. and Lustig, S.: Oxygen optimization in wort preparation – new findings in pilot-plant and commercial-scale tests, BRAUWELT International, **7** (1989), no. 3, pp. 238-250.
30. Taylor, D.G.; Bamber, P.; Brown, J.W. and Murray, J.P.: Uses of Nitrogen in Brewing, Master Brewers Association of the Americas Technical Quarterly, **29** (1992), pp. 137-142.
31. Burkert, J.; Wittek, D.; Geiger, E. and Wabner, D.: Why aerate wort? A critical investigation of processes during wort aeration, Brauwelt International, **22** (2004), no. 1, pp. 40-43.
32. Singruen, E.: Oxidation Turbidities and their Causes, Modern Brewer, **23** (1940), no. 1, p. 31.
33. Pagenstecher, M.; Maia, C. and Andersen, M.L.: Retention of Iron and Copper during Mashing of Roasted Malts, Journal of the American Society of Brewing Chemists, **79** (2021), no. 2, pp. 138-144.
34. Frederiksen, A.M.; Festersen, R.M. and Andersen, M.L.: Oxidative Reactions during Early Stages of Beer Brewing Studied by Electron Spin Resonance and Spin Trapping, Journal of Agricultural and Food Chemistry, **56** (2008), no. 18, pp. 8514-8520.
35. Wietstock, P.C.; Kunz, T.; Waterkamp, H. and Methner, F.-J.: Uptake and Release of Ca, Cu, Fe, Mg, and Zn during Beer Production, Journal of the American Society of Brewing Chemists, **73** (2015), no. 2, pp. 179-184.
36. Mochaba, F.; O'Connor-Cox, E.S.C. and Axcell, B.C.: Effects of Yeast Quality on the Accumulation and Release of Metals Causing Beer Instability, Journal of the American Society of Brewing Chemists, **54** (1996), no. 3, pp. 164-171.
37. Bamforth, C.W.: Making Sense of flavor Change in Beer, Master Brewers Association of the Americas Technical Quarterly, **37** (2000), no. 2, pp. 165-171.
38. Bamforth, C.W.: The science and understanding of the flavour stability of beer: a critical assessment, Brauwelt International, **17** (1999), no. 2, pp. 98-110.
39. Bamforth, C.W.: 125th Anniversary Review: The Non-Biological Instability of Beer, Journal of the Institute of Brewing, **117** (2011), no. 4, pp. 488-497.
40. Bamforth, C.W.: A Critical Control Point Analysis for Flavor Stability of Beer, Master Brewers Association of the Americas Technical Quarterly, **41** (2004), no. 2, pp. 97-103.
41. MEBAK: Wort, Beer, Beer-based Beverages: Collection of Brewing Analysis Methods of the Mitteleuropäische Brautechnische Analysenkommission, new ed., Self-published by MEBAK (Freising-Weihenstephan, Germany), 2013.
42. Bradford, M.M.: A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, Analytical Biochemistry, **72** (1976), no. 1/2, pp. 248-254.
43. Baert, J.J.; De Clippeleer, J.; De Cooman, L. and Aerts, G.: Exploring the Binding Behavior of Beer Staling Aldehydes in Model Systems, Journal of the American Society of Brewing Chemists, **73** (2015), no. 1, pp. 100-108.
44. Baert, J.J.; De Clippeleer, J.; Jaskula-Goiris, B.; Van Opstaele, F.; De Rouck, G.; Aerts, G. and De Cooman, L.: Further Elucidation of Beer Flavor Instability: The Potential Role of Cysteine-Bound Aldehydes, Journal of the American Society of Brewing Chemists, **73** (2015), no. 3, pp. 243-252.
45. Aerts, G.; De Cooman, L.; De Pril, I.; De Rouck, G.; Jaskula-Goiris, B.; De Buck, A.; De Pauw, C. and van Waesberghe, J.: Improved brewhouse performance and beer stability by addition of a minimal, but effective concentration of gallotannins to the brewing and sparging liquor, Proceedings of the 29th Congress of the European Brewery Convention, Fachverlag Hans Carl, Dublin, Ireland, 2003, pp. 1-13.
46. Aerts, G.; De Cooman, L.; De Rouck, G.; Pénzes, Z.; De Buck, A.; Mussche, R. and van Waesberghe, J.: Evaluation of the addition of gallotannins to the brewing liquor for the improvement of the flavor stability of beer, Master Brewers Association of the Americas Technical Quarterly, **41** (2004), no. 3, pp. 298-304.
47. Mussche, R.A. and De Pauw, C.: Total stabilisation of beer in a single operation, Journal of the Institute of Brewing, **105** (1999), no. 6, pp. 386-391.
48. Withouck, H.; Boeykens, A.; Jaskula, B.; Goiris, K.; De Rouck, G.; Hugelier, C. and Aerts, G.: Upstream Beer Stabilisation during Wort Boiling by Addition of Gallotannins and/or PVPP, BrewingScience – Monatsschrift für Brauwissenschaft, **63** (2010), no. 1/2, pp. 14-22.
49. Kulkarni, A.P.; Mahal, H.S.; Kapoor, S. and Aradhya, S.M.: In Vitro Studies on the Binding, Antioxidant, and Cytotoxic Actions of Punicalagin, Journal of Agricultural and Food Chemistry, **55** (2007), no. 4, pp. 1491-1500.
50. Almajano, M.P.; Delgado, M.E. and Gordon, M.H.: Changes in the antioxidant properties of protein solutions in the presence of epigallocatechin gallate, Food Chemistry, **101** (2007), no. 1, pp. 126-130.
51. Oudane, B.; Boudemagh, D.; Bounekhel, M.; Sobhi, W.; Vidal, M. and Broussy, S.: Isolation, characterization, antioxidant activity, and protein-precipitating capacity of the hydrolyzable tannin punicalagin from pomegranate yellow peel (Punica granatum), Journal of Molecular Structure, **1156** (2018), pp. 390-396.
52. Stewart, G.G.; Russell, I. and Anstruther, A.: Handbook of Brewing, 3rd ed., CRC Press, 2017.
53. Willaert, R.G. and Baron, G. V.: Wort Boiling Today – Boiling Systems with Low Thermal Stress in Combination with Volatile Stripping, Cerevisia, **26** (2001), no. 4, pp. 217-230.
54. Nobis, A.; Lehnhardt, F.; Gebauer, M.; Becker, T. and Gastl, M.: The Influence of Proteolytic Malt Modification on the Aging Potential of Final Wort, Foods, **10** (2021), no. 10, p. 2320.
55. Ferreira, I.M. and Guido, L.F.: Impact of Wort Amino Acids on Beer Flavour: A Review, Fermentation, **4** (2018), no. 2, p. 23.
56. Jaskula-Goiris, B.; De Causmaecker, B.; De Rouck, G.; De Cooman, L. and Aerts, G.: Detailed multivariate modeling of beer staling in commercial pale lagers, BrewingScience – Monatsschrift für Brauwissenschaft, **64** (2011), no. 11/12, pp. 119-139.
57. Mikyška, A.; Hrabák, M.; Hašková, D. and Šrogl, J.: The Role of Malt and Hop Polyphenols in Beer Quality, Flavour and Haze Stability, Journal of the Institute of Brewing, **108** (2002), no. 1, pp. 78-85.
58. Dadic, M. and Belleau, G.: Determination of Tannic Acid in Beer by Thin-Layer Chromatography, Journal of the American Society of Brewing Chemists, **36** (1978), no. 4, pp. 161-167.

59. Böhm, W.; Stegmann, R.; Gulbis, O. and Henle, T.: Amino acids and glycation compounds in hot trub formed during wort boiling, European Food Research and Technology, **249** (2023), no. 1, pp. 119-131.
60. Hellwig, M.; Witte, S. and Henle, T.: Free and Protein-Bound Maillard Reaction Products in Beer: Method Development and a Survey of Different Beer Types, Journal of Agricultural and Food Chemistry, **64** (2016), no. 38, pp. 7234-7243.
61. Yin, X.S.: A Look at Three Key Malt-Derived Mechanisms of Beer Staling and Practices of Mitigation, Master Brewers Association of the Americas Technical Quarterly, **59** (2022), no. 2, pp. 39-48.
62. Kunz, T.; Müller, C.; Mato-Gonzales, D. and Methner, F.-J.: The influence of unmalted barley on the oxidative stability of wort and beer, Journal of the Institute of Brewing, **118** (2012), no. 1, pp. 32-39.
63. Bößendörfer, G.; Birkenstock, B. and Thalacker, R.: A contribution to taste stability of beer, BRAUWELT International, **20** (2002), no. 2, pp. 80-84.
64. Cortés, N.; Kunz, T.; Suárez, A.F.; Hughes, P. and Methner, F.-J.: Development and Correlation between the Organic Radical Concentration in Different Malt Types and Oxidative Beer Stability, Journal of the American Society of Brewing Chemists, **68** (2010), no. 2, pp. 107-113.
65. Furukawa Suárez, A.; Kunz, T.; Cortés Rodríguez, N.; MacKinlay, J.; Hughes, P. and Methner, F.-J.: Impact of colour adjustment on flavour stability of pale lager beers with a range of distinct colouring agents, Food Chemistry, **125** (2011), no. 3, pp. 850-859.
66. Kunz, T.; Woest, H.; Lee, E.J.; Müller, C. and Methner, F.-J.: Improvement of the oxidative wort and beer stability by increased unmalted barley proportion, BrewingScience – Monatsschrift für Brauwissenschaft, **64** (2011), no. 7/8, pp. 75-82.
67. Kunz, T.; Strähmel, A.; Cortés, N.; Kroh, L.W. and Methner, F.-J.: Influence of Intermediate Maillard Reaction Products with Enediol Structure on the Oxidative Stability of Beverages, Journal of the American Society of Brewing Chemists, **71** (2013), no. 3, pp. 114-123.
68. Kunz, T.; Müller, C. and Methner, F.-J.: EAP Determination and Beverage Antioxidative IndeX (BAX) – Advantageous Tools for Evaluation of the Oxidative Flavour Stability of Beer and Beverages, BrewingScience – Monatsschrift für Brauwissenschaft, **65** (2012), no. 1/2, pp. 12-22.
69. Kunz, T.; Mertens, T.; Hahne, K.; Kuhn, F.; Bergmann, T. and Gibson, B.: Punicalagin – a novel brewing stabilizing agent for beer shelf-life enhancement, 15th International Trends in Brewing Symposium, Ghent (Belgium), 2023.
70. Mertens, T.; Kunz, T. and Methner, F.-J.: Assessment of Chelators in Wort and Beer Model Solutions, BrewingScience, 73 (2020), no. 5/6, pp. 58-67.
71. Jacobsen, T.; Wathne, B. and Lie, S.: Chelators and metal buffering in fermentation, Journal of the Institute of Brewing, **83** (1977), no. 3, pp. 139-143.
72. Gorinstein, S.: Metal Protein Complexes in Ethanol Media, Journal of Food Science, **39** (1974), no. 5, pp. 953-956.
73. Jacobsen, T. and Lie, S.: Metal binding in wort – an evaluation of practical stability constants, Proceedings of the 17th European Brewery Convention Congress, Nürnberg, Germany, 1979, pp. 117-129.
74. Pagenstecher, M.; Bolat, I.; Bjerrum, M.J. and Andersen, M.L.: Copper Binding in Sweet Worts Made from Specialty Malts, Journal of Agricultural and Food Chemistry, **69** (2021), no. 23, pp. 6613-6622.
75. Irving, H. and Williams, R.J.P.: The stability of transition-metal complexes, Journal of the Chemical Society (Resumed), **8** (1953), pp. 3192-3210.
76. Miličević, A.; Branica, G. and Raos, N.: Irving-Williams Order in the Framework of Connectivity Index $^3\chi v$ Enables Simultaneous Prediction of Stability Constants of Bivalent Transition Metal Complexes, Molecules, **16** (2011), no. 2, pp. 1103-1112.
77. Frausto da Silva, J.J.R. and Williams, R.J.P.: The kinetics of Mn(II) complexes, The Biological Chemistry of the Elements: The Inorganic Chemistry of Life, 2nd ed., Oxford University Press, New York (USA), 2001, p. 561.
78. Oñate-jaén, A.; Bellido-milla, D. and Hernández-artiga, M.P.: Spectrophotometric methods to differentiate beers and evaluate beer ageing, Food Chemistry, **97** (2006), no. 2, pp. 361-369.
79. Ma, J.F.; Higashitani, A.; Sato, K. and Takeda, K.: Genotypic variation in Fe concentration of barley grain, Soil Science and Plant Nutrition, **50** (2004), no. 7, pp. 1115-1117.
80. Kunz, T.; Kanzler, C.; Fröhlich, H.; Gastl, M. and Methner, F.-J.: Special malt production – Selective steering of the roasting process to reduce prooxidative effects of roasted malt, World Brewing Congress, Denver, Colorado (USA), 2016.
81. Galano, A.; Francisco Marquez, M. and Pérez-González, A.: Ellagic Acid: An Unusually Versatile Protector against Oxidative Stress, Chemical Research in Toxicology, **27** (2014), no. 5, pp. 904-918.
82. Dalvi, L.T.; Moreira, D.C.; Andrade, R.; Ginani, J.; Alonso, A. and Hermes-Lima, M.: Ellagic acid inhibits iron-mediated free radical formation, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, **173** (2017), pp. 910-917.
83. Ditrych, M.; Filipowska, W.; De Rouck, G.; Jaskula-Goiris, B.; Aerts, G.; De Cooman, L. and Andersen, M.L.: Investigating the evolution of free staling aldehydes throughout the wort production process, BrewingScience, **72** (2019), no. 1/2, pp. 10-17.
84. Bustillo Trueba, P.; Jaskula-Goiris, B.; Ditrych, M.; Filipowska, W.; De Brabanter, J.; De Rouck, G.; Aerts, G.; De Cooman, L. and De Clippeleer, J.: Monitoring the evolution of free and cysteinylated aldehydes from malt to fresh and forced aged beer, Food Research International, **140** (2021), no. 4, p. 110049.
85. Yamashita, H.; Kühbeck, F.; Hohrein, A.; Herrmann, M.; Back, W. and Krottenthaler, M.: Fractionated boiling technology: wort boiling of different lauter fractions, Monatsschrift für Brauwissenschaft, **59** (2006), no. 7/8, pp. 130-147.
86. Kretschmer, H.; Miedaner, H.; Narziss, L. and Back, W.: Der Einfluss des Sauerstoffgehaltes beim Keimen, Schwelken und Darren auf die Qualitaet von Malz und Bier, Proceedings of the 25th Congress of the European Brewery Convention, Brussels (Belgium), 1995, pp. 507-513.
87. Nobis, A.; Kwasnicki, M.; Lehnhardt, F.; Hellwig, M.; Henle, T.; Becker, T. and Gastl, M.: A comprehensive evaluation of flavor instability of beer (Part 2): The influence of De Novo formation of aging aldehydes, Foods, **10** (2021), no. 11, pp. 1-15.
88. Ogane, O.; Imai, T.; Ogawa, Y. and Ohkochi, M.: Influence of Wort Boiling and Wort Clarification Conditions on Aging-Relevant Carbonyl Compounds in Beer, Master Brewers Association of the Americas Technical Quarterly, **43** (2006), no. 2, pp. 121-126.
89. Suda, T.; Yasuda, Y.; Imai, T. and Ogawa, Y.: Mechanisms for the development of Strecker aldehydes during beer aging, 31st Congress of the European Brewery Convention, Fachverlag Hans Carl (Nürnberg, Germany), Venice (Italy), 2007, pp. 1-7.
90. Jaskula-Goiris, B.; De Causmaecker, B.; De Rouck, G.; Aerts, G.; Paternoster, A.; Braet, J. and De Cooman, L.: Influence of transport and storage conditions on beer quality and flavour stability, Journal of the Institute of Brewing, **125** (2019), no. 1, pp. 60-68.

91. Gastl, M.; Spieleder, E.; Hermann, M.; Thiele, F.; Burberg, F.; Kogin, A.; Ikeda, H.; Back, W. and Narziss, L.: The influence of malt quality and malting technology on the flavour stability of beer, Monatsschrift fur Brauwissenschaft, **59** (2006), no. 9/10, pp. 163-175.
92. Malfliet, S.; Opstaele, F.; Clippeleer, J.; Syryn, E.; Goiris, K.; Cooman, L. and Aerts, G.: Flavour Instability of Pale Lager Beers: Determination of Analytical Markers in Relation to Sensory Ageing, Journal of the Institute of Brewing, **114** (2008), no. 2, pp. 180-192.
93. Baert, J.J.; De Clippeleer, J.; Hughes, P.S.; De Cooman, L. and Aerts, G.: On the Origin of Free and Bound Staling Aldehydes in Beer, Journal of Agricultural and Food Chemistry, **60** (2012), no. 46, pp. 11449-11472.
94. De Schutter, D.P.; Saison, D.; Delvaux, F.; Derdelinckx, G.; Rock, J.-M.; Neven, H. and Delvaux, F.R.: Release and Evaporation of Volatiles during Boiling of Unhopped Wort, Journal of Agricultural and Food Chemistry, **56** (2008), no. 13, pp. 5172-5180.
95. Miedaner, H.; Narziß, L. and Schneider, F.P.: Influence of Evaporation on Wort and Beer Quality, EBC Symposium Wort Boiling and Clarification, Strasbourg (France), 1991, pp. 37-44.
96. Hudson, J.R. and Birtwistle, S.E.: Wort-boiling In Relation To Beer Quality, Journal of the Institute of Brewing, **72** (1966), no. 1, pp. 46-50.
97. Bamforth, C.W.: Dimethyl Sulfide – Significance, Origins, and Control, Journal of the American Society of Brewing Chemists, **72** (2014), no. 3, pp. 165-168.
98. Formanek, J.A. and Bonte, P.: Use of Tannic Acid in the Brewing Industry for Colloidal and Organoleptic Stability, Master Brewers Association of the Americas Technical Quarterly, **54** (2017), no. 1, pp. 11-16.
99. Reinhardt, C.; Kunz, T. and Methner, F.-J.: Gallotannins - A useful tool to improve colloidal and oxidative Beer Stability, Master Brewers Association of the Americas Annual Conference, Austin, Texas (USA), 2013.
100. Burrer, F. and Kunz, T.: Alternative stabilization agents for cross-flow membrane filtration, 15th International Trends in Brewing Symposium, Ghent (Belgium), 2023.

Received 23 May 2023, accepted 7 June 2023