Research article

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Complexation of transition metals by chelators added during mashing and impact on beer stability

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Beer inevitably changes due to an array of staling reactions. A major factor in beer ageing is the involvement of transition metals (iron, copper, manganese) in oxidative reactions. To tackle the flavour stability issue, metal chelation was investigated. Based on previous research, five primary chelators (tannic acid, gallic acid, EDTA, citric acid and phytic acid) were screened using experimental design for their capacity to reduce the content of wort transition metals. The chelating agents were added under varying conditions (mash out temperature, mash pH, grain bill, chelator concentration, addition time) during laboratory scale mashing to assess how they altered complexation and metal load. Fourteen alternative chelators (ferulic acid, tartaric acid, quercetin, chlorogenic acid and ten polyphenolic food extracts: green tea, pomegranate, grape seed, reishi, cinnamon, curcuma, milk thistle, ginkgo, grapefruit seed and raspberry) were also explored. Metal ions were analysed using inductively coupled plasma optical emission spectrometry and wort oxidative stability by electron spin resonance spectroscopy. Mash pH was the most decisive of all tested process variables: acidified mashing (pH 6 to 5) produced worts with more iron, manganese and zinc (230, 320 and 150%, respectively). Addition of effective chelators counteracted this undesirable effect for iron. Green tea extract, tannic acid and, particularly, pomegranate extract all resulted in lower wort iron. Conversely, addition of EDTA, caused iron, manganese and zinc to increase. Pomegranate extract (90% ellagic acid) was the best performing chelator and reduced radical generation in wort (80% reduction by 60 mg/L addition), making it a promising novel compound in the improvement of beer shelf life.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords: Oxidative beer stability; transition metals; chelate complexes; chelator efficacy; inductively coupled plasma optical emission spectrometry (ICP-OES); electron spin resonance (ESR) spectroscopy

Introduction

Problems with beer flavour stability can result in brand rejection and a decrease in sales *(1)*. Accordingly, it is paramount that beer retains its freshness until at least the best-before date. Unfortunately for brewers and consumers alike, this is not always the case: the issue of beer flavour (in)stability remains the unsolved Achilles' heel of the brewing industry. The problem is particularly apparent, now that beer is produced and retailed both nationally and internationally. Noticeable deterioration in flavour may start three months from packaging when stored at room temperature. Typical signs of beer ageing involve - but are not limited to - the characteristic decline in pleasant, crisp or 'fresh' bitterness *(2–4)* and the formation of undesirable stale flavours or off-flavours (often associated with aldehydes) *(5–7)*.

The complex nature of beer degradation *(8–10)* involves staling catalysts, of which transition metal ions (iron, copper, manganese) are the main concern *(11,12)*. Their presence originates primarily (96%) from malt *(13)*. These transition metal ions accelerate oxygen activation per electron transfer by the Fenton and Haber-Weiss reaction (Figure 1). The formed reactive oxygen species (ROS) - generated from ground state in-pack oxygen - trigger oxidative stress. ROS are aggressive pro-oxidative entities that chemically 'attack' any molecule in proximity (including desired flavour compounds).

Avoidance or removal of transition metal catalysts should slow the reaction rates, lowering the production of free radicals and other reactive species, and reducing the oxidative damage inflicted on wort and beer. This was already observed in work by Wietstock and colleagues, where mash hopping resulted in a more oxidative stable beer, with diminished aldehyde formation during ageing, through iron chelation by hop α-acids *(14–16)*.

Research *(19)*—conducted in beer and model wort solutions—showed several promising food-grade chelators for the removal of catalytic transition metals from the brewing process, such as tannic acid, gallic acid, and phytic acid. However, wort and beer matrices are more complex in their chemical make-up, requiring a fuller investigation of their chelation behaviour in those systems. The previous investigation *(19)* also showed that mash pH (5.4 ± 0.2) was superior to that of beer (4.3 ± 0.3) in terms of complex

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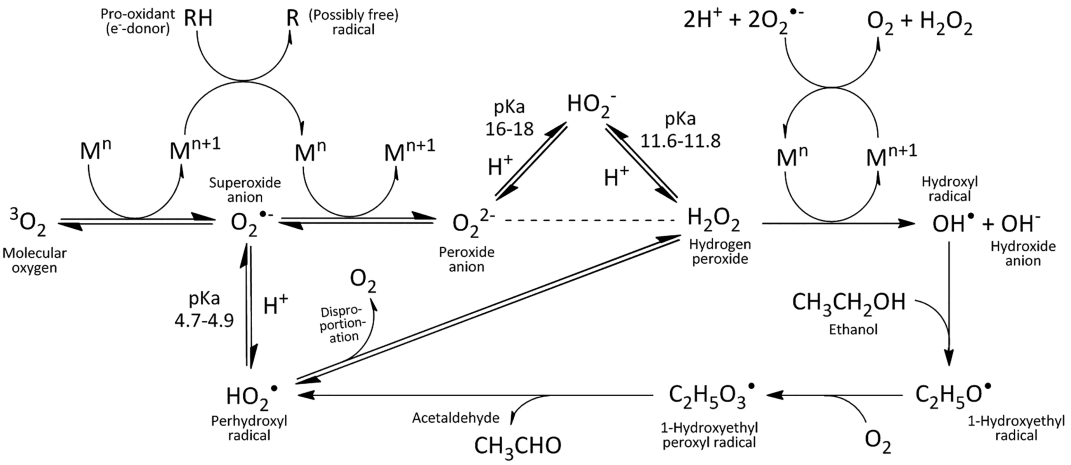

Figure 1. Fenton and Haber-Weiss reaction mechanism and the formation of reactive oxygen species, with M = transition metal (Fe, Cu or Mn); adapted with permission from *(17,18)*

formation. An additional advantage that chelation in mashing has over beer - apart from the higher pH - is the added protection provided by early catalyst removal, as opposed to late removal (e.g. during filtration). This is because transition metals actively promote staling throughout the whole brewing chain, not only during storage, and are especially reactive during high energy stages (mashing and boiling) *(20)*.

The production of wort is a complex and dynamic process, and it is uncertain if chelators have the same impact in a wort/mashing regime, as such a system is (in many ways) more complex than model solutions. Chelation and mineral load can be influenced by a myriad of unknown matrix effects (owing to the intricate makeup of wort as the active medium) and by the various process parameters (time of addition of the chelator, mash pH *(19,21)*, mashing temperature *(21,22)*, chelator concentration, grain composition *(21,23–25)*, mechanical stirring etc). All these factors will ultimately determine the final metal content of wort and beer.

In this study, five previously investigated 'primary chelators' (tannic acid, gallic acid, citric acid, EDTA and phytic acid) were assessed for their ability to diminish transition metals during the mashing stage of brewing (wort), as opposed to fermentation or post-fermentation (beer). To reduce the number of trials per chelator for the screening of their best working conditions, 'design of experiments' (DOE) software was used. Additionally, rapid screening was also conducted on fourteen ('alternative') chelators and experimental design was employed for the final optimisation of the overall best working chelating agent (out of the 19 chelators).

Chelation efficacy and metal load were assessed by inductively coupled plasma optical emission spectrometry (ICP-OES) *(26)*, and wort oxidative stability was quantified by electron spin resonance (ESR) spectroscopy *(27)*. Ideally, this would replicate the results with the model wort solution *(19)*. The ambition of this work was to identify food grade chelators - and their optimal working conditions during mashing - capable of forming filterable complexes with the transition metals iron, copper and manganese (but not with zinc), so that their effective removal (through filtration or lautering) resulted in heightened oxidative stability and increased shelf-life.

Materials and methods

Chemicals

Ferulic acid (≥ 99%), phytic acid sodium salt hydrate (≥ 90%) and quercetin dihydrate (≥ 98%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ethylenedinitrilo-tetraacetic acid disodium salt dihydrate (EDTA disodium salt or Titriplex® III; ≥ 99.0%), citric acid monohydrate (≥ 99.5%), tartaric acid (≥ 99.5%) and anhydrous gallic acid (≥ 98%) were obtained from Merck KGaA (Darmstadt, Germany). Chlorogenic acid (98%) was from Acros Organics (New Jersey, USA). N-tert-Butyl-α-(4-pyridyl-1-oxide) nitrone (POBN; > 98%) was from TCI Deutschland GmbH (Eschborn, Germany). Hydrochloric acid (34-37%) and concentrated nitric acid (67-70%) were from Th. Geyer GmbH & Co. KG (ChemSolute®, Renningen, Germany). Absolute ethanol (100%) and sodium hydroxide (≥ 98.5) were from VWR International S.A.S. (Fontenay-sous-Bois, France). Reference standards (1000 μg/mL) for iron, copper, manganese and zinc were from PerkinElmer LAS GmbH (Rodgau, Germany). Argon (99.999%) was from Air Liquide GmbH (Düsseldorf, Germany). Tannic acid (BrewTan B®; ≥ 98%) was from S.A. Ajinomoto OmniChem N.V. (Wetteren, Belgium). Other polyphenolic (food) extracts (Table 1) were from various sources: green tea (Fairvital B.V., Landgraaf, Netherlands), pomegranate (Healthtonics, Thornaby, United Kingdom), grape seed (PumpEffect BV, Maastricht, Netherlands), reishi (Kurkraft GmbH, Berlin, Germany), cinnamon & curcuma (Extrakt Manufaktur Hamburg GmbH, Hamburg, Germany), milk thistle (Alpha Zwo B.V., Eindhoven, Netherlands), Ginkgo biloba (Nuvi Health B.V., Heerlen, Netherlands), grapefruit seed (Bafoxx UG, Münster, Germany) and raspberry (GEN Nutrition UG, Aachen, Germany). All aqueous solutions were made with ultrapure water (by Sartopore® 2 MidiCap 0.2 μm filtration; Sartorius AG, Goettingen, Germany).

Screening by two-level full factorial design

A two-level full factorial screening design of experiments (DOE) was conducted for each of the five primary chelators: tannic acid, gallic acid, EDTA, citric acid and phytic acid. A screening DOE

Table 1. Polyphenolic (food) extracts

Extract	Active compound	Purity (%)
Green tea	Epigallocatechin gallate	50 (total polyphenol content of 98%)
Pomegranate	Ellagic acid	40, 90
Grape seed	Oligomeric proanthocyanidin	95
Reishi	Polysaccharides	35
Cinnamon	Polyphenols	30
Curcuma	Curcuminoid	95
Milk thistle	Silymarin	80
Ginkgo biloba	Flavonol glycosides	24
Grapefruit seed	Flavonoids	45
Raspberry	Raspberry ketone	100

determines whether the factors of choice (process parameters) have significant linear effect(s) on the responses of interest (level of metal ions) and if there are factor interactions. Accordingly, by varying two factor levels per experimental unit, the key determinants and their impacts can be identified.

Here, the factors were four independent variables: addition time of chelator, mash pH, mash out temperature and chelator concentration. The two factor levels (low and high) used for each variable are reported in Table 2. The reasoning behind the 'chelator concentration' levels (through preliminary trials) can be found in Supplementary Figure S1. The responses of interest were the four dependent (outcome) variables: concentration of iron, copper, manganese and zinc in lautered wort.

To estimate the pure error for the Lack-of-Fit test, all screening runs were conducted in duplicate, resulting in 32 runs (2×2^4) per design. The sequence of the experimental units was determined randomly for all DOEs. Randomisation protects against confounding (the distortion of associations, between the experimental factors and the outcomes, by extraneous factors), an important source of bias.

Rapid screening

For rapid screening, chelators were added to the mash at five or six concentrations, from 5 mg/L up to 100 or 200 mg/L for quercetin, ferulic, tartaric and chlorogenic acid and from 25 or 62.5 mg/L up to 1000 mg/L for the ten polyphenolic food extracts (green tea, pomegranate, grape seed, reishi, cinnamon, curcuma, milk thistle, ginkgo, grapefruit seed and raspberry).

The lautered worts were analysed for metal content and the data were plotted to check if fluctuations in metal load occurred. Mashing was performed with unadjusted pH (≈ 5.6), constant grain bill (50/50 Pilsner/Munich), mash out temperature of 78°C and chelator addition time of 0 minutes (onset of mashing).

Optimisation by rotatable full central composite design

A rotatable full central composite optimisation DOE was conducted with the most promising chelator, pomegranate extract (90% ellagic acid content). Like the screening DOE, the response surface (optimisation) DOE determines factor importance and interactions. It is, however, not limited to linearity, also allowing to check for quadratic or higher order trends. This helps determine the factor configuration leading to minimal metal load and maximal chelation of detrimental transition metal ions (through statistical modelling).

Table 2. Experimental parameters (factors and levels used) for the two-level full factorial design for testing the effect of chelator addition time, mash pH, mash out temperature and chelator concentration on the wort metal content (Fe, Cu, Mn, Zn)

	Factor level	
Experimental factor	Low (-1)	High (+1)
Addition time of chelator [min]	0	55
Mash pH [-]	5.0	6.0
Temperature (mash out) [°C]	72	80
Chelator concentration [μmol/L]	5.9	35.3

In this case, the selected factors were four independent variables: addition time of pomegranate extract, grain bill (Pilsner/Munich), mash pH and pomegranate extract concentration. The responses of interest were - as with the screening DOE - the four dependent (outcome) variables: concentration of iron, copper, manganese and zinc in lautered wort.

Each experimental unit was varied over five factor levels (Table 3): eight axial points (±α), sixteen factorial points (±1) and six centre points (0), resulting in 30 runs. The design is rotatable, with $\alpha = \pm 2$. The centre point run was performed in sextuplicate and was used to estimate pure error for the Lack-of-Fit test. Analogous to the screening DOE, the run sequence of the experimental units was determined randomly to prevent confounding bias.

Wort production

Laboratory scale sweet worts (400 mL) were prepared from mashing milled Pilsner and Munich malt Type I from Weyermann GmbH & Co. KG (Bamberg, Germany) and ultrapure water (1:4 w/w), using a congress Bender & Hobein mashing apparatus (Bruchsal, Germany) with stainless steel mashing cups. The water lost during mashing due to evaporation was replaced (by weight) after mashing, to ensure a grain/liquor-ratio of 1:4 w/w.

Adjustments of the natural mash pH (≈ 5.6) were made at the beginning of mashing either by addition of 2 M hydrochloric acid (acidification) or 1 M sodium hydroxide (basification). Unless otherwise stated, there was no wort boiling and mashing was with 50% Pilsner and 50% Munich malt with the following temperature program: mashing in at 63°C; rest for 30 min; heating up (1°C/min) to 72°C; rest for 15 min; heating up (1°C/min) to 78°C and rest for 1 min (mash out). Boiling was by simmering wort (150 mL) for 60

Table 3. Experimental parameters (factors and levels) for the rotatable full central composite design for testing the effect of pomegranate extract addition time, grain bill, mash pH and pomegranate extract concentration on wort metal content (Fe, Cu, Mn, Zn)

	Factor level				
Experimental factor	Axial point (-α)	Low (-1)	Centre point (0)	High (+1)	Axial point(+α)
Time of addition of pomegranate [min]	0	13.75	27.5	41.25	55
Grain bill (Pilsner/Munich) [%]	0	25	50	75	100
Mash pH	5	5.25	5.5	5.75	6
Pomegranate extract concentration [mg/L]	10	22.5	35	47.5	60

minutes in a 250 mL flask under reflux, followed by trub removal through simulated whirlpooling (swirling) and decantation (50 mL).

Lautering was simulated by filtering the mash through folded Whatman filter papers (Cytiva, Freiburg, Germany) and recirculating the first 100 mL of lautered wort through the filter paper (containing spent grains) for enhanced wort clarification. Sparged wort #1 and #2 were collected separately by consecutively pouring 100 mL of warm (78°C) ultrapure water (without pH adjustment) over the 'filter dry' spent grains.

Wort analyses (extract and pH measurement) were performed on samples at room temperature. For the determination of metal content and radical levels, the lautered wort samples were held frozen (-18°C) in metal free tubes (VWR International, Radnor, USA) and thawed in a 40°C water bath for 20 minutes before usage.

Table 4. Instrumental parameters for the ICP-OES analysis

Baffled Cyclonic Spray Chamber, GemCone Low-Flow Nebuliser, 2.0 mm Alumina Injector, Two-Dimensional Charge Coupled Device (CCD) Detector	
Parameter	Level
Radio Frequency (RF) power	1500 Watts
Nebuliser flow	0.4 L/min
Auxiliary flow	0.3 L/min
Plasma flow	8 L/min
Sample flow rate	1.00 mL/min
Equilibration time	15 sec
Torch position	0.00 mm
Carrier and purge gas	Gaseous argon
Shear gas	Air

Wort analysis

Extract and pH determination were on clear, undiluted wort samples according to MEBAK *(27)* (method 2.9.3 and 2.13, respectively) with an Anton Paar GmbH Alcolyzer Beer Analyzing System (Graz, Austria).

Determination of the metal content

Metal content in lautered wort was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) using an Avio 200 spectrometer (PerkinElmer, Rodgau, Germany) with the instrumental parameters in Table 4. The 20 mL wort samples were diluted to 90.9% with 1 mL ultrapure water and 1 mL nitric acid (end concentration: 3-3.2% HNO_3) and vortexed before each analysis.

For the determination of each metal ion, the following analytical emission lines were used: Fe, 238.204 nm; Cu, 327.398 nm; Mn, 257.612 nm; Zn, 206.200 nm. For quantification, four-point calibration curves ($R^2 > 0.99$) were constructed for each analyte in wort by standard addition. This was by spiking a 'blank' reference wort of equal extract with increasing levels of metal standards, achieving respective end concentrations, for iron, copper, manganese and zinc, of (in μg/L): 0, 0, 0, 0 (addition 0); 50, 50, 100, 150 (addition 1); 100, 100, 200, 300 (addition 2); 200, 200, 400, 600 (addition 3).

Determination of radical levels

Radical formation was measured in wort by electron spin resonance (ESR) spectroscopy using an X-band spectrometer (e-scan, Bruker BioSpin GmbH, Rheinstetten, Germany) with the following settings: centre field, 3475 G; attenuation, 3.0 dB; power, 8.920 mW; sweep width, 14 G; receiver gain, 3.99×10^3; resolution, 512; modulation amplitude, 1.47 G; modulation frequency, 86.00 kHz; conversion time, 10 ms; time constant, 40 ms; number of scans, 25.

Using the method described in MEBAK *(27)* by Kunz et al. *(28)*, 10 ml of sample was mixed with 700 μL absolute ethanol and 100 μL 0.82 M POBN in a glass vial (final sample concentrations: 6.5 v/v ethanol and 7.6 mM POBN). On adding the spin trap agent (POBN) to the sample, the vial was placed in the heating block at 60°C until the end of the analysis (ca. 10 hours). All vials were in duplicate and sampled by the autosampler, which periodically (every 30-70 minutes) measured the concentration of POBN spin adducts, formed by the emerging hydroxyl- and hydroxyethyl radicals.

The time dependent radical concentration data were graphically plotted using the statistical software package OriginPro (version 8.0891, OriginLab Corporation, Northampton, MA, USA). Final radical concentrations were reported as the radical intensities at minute 450 (T_{450}-value).

Data analysis

Two-level factorial experimental design (screening), response surface experimental design (optimisation) and multivariate data analysis were performed with Design-Expert® software (version 11, Stat-Ease, Inc., Minneapolis, MN, USA). The statistical significance (confidence level of 95% or $p \leq 0.05$) of the numerous factors and their interaction was determined with analysis of variance (ANOVA).

The removal of insignificant model terms was done with backward elimination (with $\alpha > 0.1$), by applying all blocks and forced terms to the model. This stepwise regression is used to algorithmically reduce the design to the minimum number of needed terms while giving all model terms a chance to be included. Non-significant terms were kept in the model when they were involved in interaction(s) with significant terms ('principle of hierarchy').

Results and discussion

The lautered first worts had an average specific gravity of 16.5 ± 0.2°P and were made with all-grain pale malts (Pilsner and Munich), because of the predominance of beers utilising this malt type *(29)*. Mixing Munich with Pilsner malt was because of its slightly higher roasting, making it extrude more iron, manganese and zinc during mashing. In our preliminary trials, the all-Munich wort contained 160-210% more iron than three diverse brands of all-Pilsner worts. This is a known effect, albeit not well-documented *(14,30)*.

Depending on the malts used, a standard 12°P gravity wort has levels of around 100-270 μg/L iron, 20-400 μg/L copper and 80-150 μg/L manganese with 100-5000 μg/L of the beneficial zinc *(31,32)*. Calcium and magnesium - two other beneficial brewing metals found in wort - were not screened in our trials. Neither appear to substantially chelate out of solution *(19)* and they are also present in wort at concentrations two orders of magnitude higher than the detrimental iron, copper and manganese ions (namely, 50-90 mg/L for Mg and 15-35 mg/L for Ca) *(31)*.

Mashing at pH 5.0 gave a slightly higher extract (16.6°P) compared to mashing at pH 6.0 (16.3°P), which is in accordance with the report of Taylor and MacWilliam *(33,34)*. This is likely to be due to the more efficient availability or activation of limit dextrinase *(35,36)*. This higher extract does not always indicate higher fermentability. The mash pH for maximised fermentability is around 5.5-5.6 *(37–39)*, since this is the pH optima at mashing temperatures (60-70°C) of α- and β-amylase, measured in wort at room temperature.

During the mashing process, the pH of the adjusted mash drifted towards a more natural wort pH: the pH 5.0 mash had an average pH of 5.07, while the pH 6.0 mashes had an average pH of 5.92. This is due to the release of various wort substances during mashing, such as amino nitrogen compounds and organic acids, which have buffering capability. The first and second wort sparges had average gravities of 10.5 ± 0.7°P and 5.7 ± 0.4°P. But here, the opposite effect could be seen with mash pH and extract: the first and second sparges of the spent grain mashed at pH 5.0 had lower extracts on average (10.3°P and 5.4°P) than the ones mashed at pH 6.0 (10.7°P and 5.9°P). This effect was particularly noticeable in the tannic acid sparges (Δ°P of 0.7 between different pHs).

For metal ion content (concentration in the sparged worts compared to the first worts), a progressive reduction could be seen. Zinc and manganese showed substantial displacement from the spent grains to the sparged worts. For zinc, the extent was mainly dependent on the mash pH. On average, over all the chelator trials, the first and second sparges of the pH 5 mashes had 121% and 82% of the Zn content of the first wort, respectively. In comparison, grains mashed at pH 6 contained only 86% and 56%.

The trials with phytic acid are reported in Figure 2. Diagrams for the other four chelators can be found in the supporting information (Figures S2-5), together with an overview of the average absolute metal values for every chelator (Table S1). As previously described, one screening DOE was conducted per chelator, resulting in a total of 20 models with one model for every metal ion (n = 4) for every chelator (n = 5). A summary of the data with correlation matrices is given in Table 5. It features the correlation coefficients between every experimental factor and metal ion. Strong linear dependencies, between variable and response, are coloured red for positive and blue for negative correlations. Some caution is needed when interpreting this data as some non-linear associations may be underestimated or overlooked (monotonic and non-monotonic relationships, respectively). The ANOVA results and fit statistics for every model can be found in the supplementary info (Table S2).

From Table 5 it can be observed that - for the five chelators - the time of addition does not have a high impact on the levels of iron, manganese and zinc found in the lautered wort. However, copper behaved differently as the time of addition did a to influence the concentration in wort slightly. But even with tannic acid, where this effect was particularly strong, late addition only led to a minor increase in copper of 20-30 μg/L (depending on the concentration of the added tannic acid). Further, the mash pH has a major effect on the levels of metal ions that leach into the lautered wort. For iron, manganese and zinc, a strong negative correlation exists with

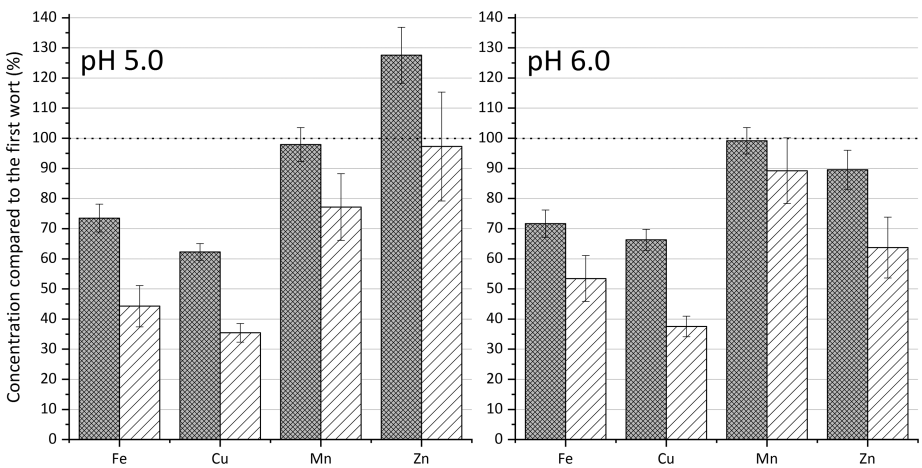

Figure 2. Decrease in metal ion concentration between the first wort and two consecutive sparges (first sparge: dense pattern; second sparge: sparse pattern), average at pH 5.0 (left, n = 16) and pH 6.0 (right, n = 16) of phytic acid ± standard deviation

Table 5. Pearson correlation coefficients (*r*) between experimental factors and metal ion concentrations in lautered wort

	Tannic acid				Gallic acid				EDTA				Citric acid				Phytic acid			
	[Fe]	[Cu]	[Mn]	[Zn]	[Fe]	[Cu]	[Mn]	[Zn]	[Fe]	[Cu]	[Mn]	[Zn]	[Fe]	[Cu]	[Mn]	[Zn]	[Fe]	[Cu]	[Mn]	[Zn]
Chelator addition time	0.00	0.74	-0.05	0.04	-0.05	0.32	-0.08	-0.08	-0.01	-0.29	-0.01	-0.28	-0.02	-0.12	-0.10	-0.06	-0.14	0.25	-0.10	-0.11
Mash pH	-0.59	0.49	-0.99	-0.87	-0.99	0.65	-0.99	-0.95	-0.66	0.55	-0.92	-0.05	-0.96	0.47	-0.98	-0.93	-0.96	0.70	-0.98	-0.92
Mash out temperature	-0.07	-0.17	-0.10	-0.35	-0.06	0.08	-0.07	-0.25	0.03	-0.10	-0.18	-0.13	0.08	0.04	-0.05	-0.15	-0.13	0.05	-0.10	-0.22
Chelator concentration	-0.66	0.08	0.05	0.16	0.01	0.10	0.01	0.04	0.69	0.01	0.27	0.93	-0.01	-0.02	-0.01	0.01	0.02	0.22	0.00	-0.05

Pearson's $r \in [-1,1]$ and coloured dark red at total positive linear correlation (1.00), white at no linear correlation (0.00) and dark blue at total negative linear correlation (-1.00)

a lower mash pH leading to a higher amount of Fe, Mn and Zn leaching out into the wort (see Supplementary Figure S6). A similar observation was reported by Holzmann and Piendl in 1977 *(21)*. This is likely to be due to most metal ions having an increased solubility in more acidic environments, as a higher pH promotes the hydroxide precipitation of metals. Additionally, the higher level of protons in an acidic mash compete with the metal cations for any binding sites within the wort matrix (such as endogenous polyphenols), thus increasing the number of free metal ions. Furthermore, the activity of antioxidants (e.g. chelators) is also often linked to their pH dependent solubility, as was seen in previous studies *(19, 40)*. Polyphenols, among other chelators, are more soluble and active at alkaline pH *(41)*. Again, copper is the exception. A lower pH leads to a slightly - but statistically significant - lower level of Cu (20-30 μg/L) which is independent of other factors (see Supplementary Figure S6). This could be attributed to the enhanced protein coagulation during mashing at pH 5 (as opposed to pH 6), as the isoelectric point of most wort proteins is around pH 5.2 or below *(37,42–44)*. Copper binds with the coagulating proteins, precipitates and is removed with the hot break during lautering *(45,46)*. With EDTA, there is the absence of the usual strong negative correlation of zinc level with the mash pH. It a that the chelation effect of EDTA outweighs the pH effect. Even at low concentrations of EDTA (5.9 μM), only a slight increase in zinc is observed when mashing at pH 5 compared to pH 6.

From the low correlation coefficients, the mash out temperature did not impact on the final metal content across the board. It is likely however, although the mash out temperature was found to be a significant factor in most models, that the difference between the low (72°C) and high (≤ 80°C) mash out temperature was too small to cause any tangible effect. There were weak correlations in concordance with the findings of Holzmann and Piendl *(21)*. Higher mashing temperature reduced the level of zinc and manganese, but for iron this was unnoticeable across 72-80°C.

Copper behaved atypically, especially with Cu-Citric acid and Cu-EDTA. As can be seen from the surface plots (Figure 3), a non-linear *and* non-monotonic relationship was found between the response factor (copper) and the two interacting variables (chelator addition time and mash out temperature), leading to the misleadingly low linear correlation coefficients (Table 5).

The correlation coefficients for chelator concentration (Table 5) show which chelator agents influence the metal ion composition of the lautered wort. Gallic, citric and phytic acids have a negligible impact, leastways at the concentration employed (5.9-35.3 μmol/L). EDTA enhanced the solubility of iron (Figure 4), copper, manganese and zinc, presumably by competing with endogenous chelators *(47,48)* and possibly stripping them away from other complexes consisting of 'soft' ligands (such as thiol groups). In agreement with Formanek and Bonte *(49)*, of the five screened chelators, tannic acid was the only chelator that successfully reduces the level of iron in wort after lautering (Figure 5). This iron chelating capability was also observed in model solutions *(19)*. The copper binding potential seen in the wort model solutions could, however, not be reproduced in the mash. Copper may

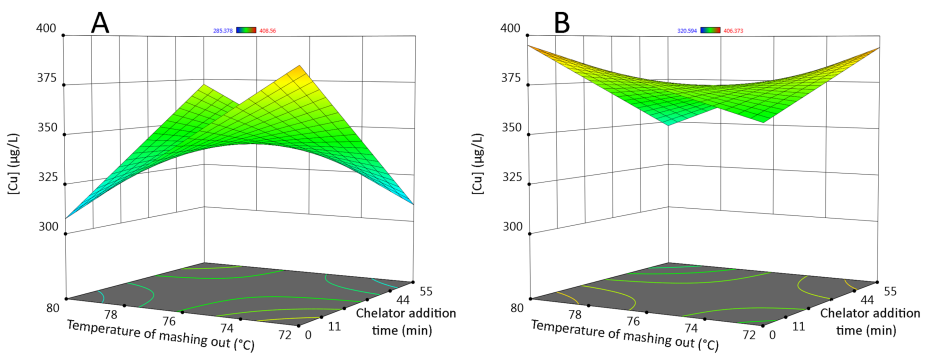

Figure 3. Response surface interaction model for the fitted value levels of copper concentration (μg/L) in lautered wort, after mashing at pH 5.6 with (A, left) 35.3 μM citric acid and (B, right) 35.3 μM EDTA, at varying levels of mash out temperature (°C) and chelator addition time (min) [Colour figure can be viewed at wileyonlinelibrary.com]

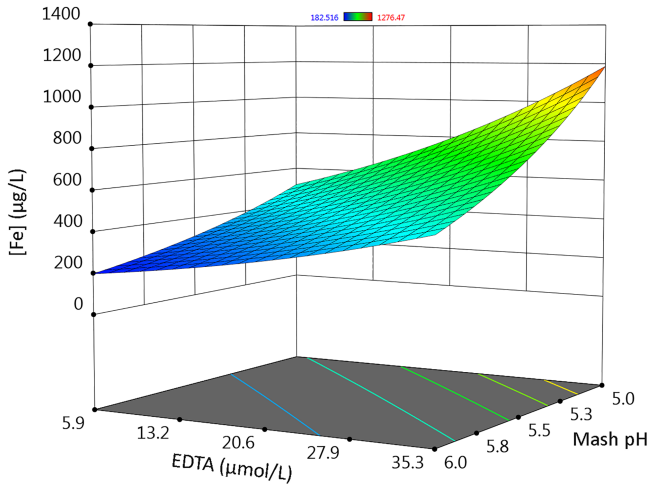

Figure 4. Response surface interaction model for the fitted value levels of iron concentration (µg/L) in lautered wort, after mashing with varying levels of EDTA (µmol/L) and mash pH, with chelator additions at the onset of mashing and mash out at 78°C [Colour figure can be viewed at wileyonlinelibrary.com]

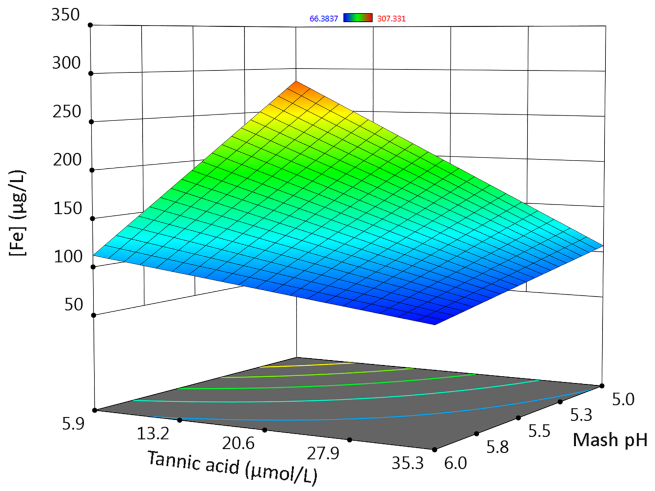

Figure 5. Response surface interaction model for the fitted value levels of iron concentration (µg/L) in lautered wort, after mashing with varying levels of tannic acid addition (µmol/L) and mash pH, with chelator additions at onset of mashing and mash out at 78°C [Colour figure can be viewed at wileyonlinelibrary.com]

already be too tightly bound to other matrix components, such as amino acids, peptides or proteins *(50)*.

In Figure 5, the large effect of mash pH on the iron content (as previously described) is clearly noticeable. Without the addition of tannic acid, reducing the mash pH from 6 to 5 causes a more than two-fold increase in iron content (from 140 to 310 µg/L, or 221%). On adding a low dosage of tannic acid (5.9 µM or 10.0 mg/L), a comparable (high) iron level was found in the wort at pH 5. However, this pH effect can largely be diminished (for iron) by applying a high dosage of tannic acid (35.3 µM or 60.0 mg/L) to the acidic mash. Unsurprisingly, the lowest Fe level (66 µg/L) was found with 35.3 µM of tannic acid *and* a mash pH of 6.0. With the same (high) concentration of tannic acid and a mash pH of 5.0, the iron content was 110 µg/L. The greater efficiency of removal by tannic acid at the higher pH is likely due to a more deprotonated state, which facilitates coordination interactions *(19,51)*.

The successful chelating capacity of some of the other chelators (gallic acid, phytic acid) in wort buffer solutions could not be reproduced during mashing. Indeed, even tannic acid was not capable of chelating out copper. This was not unexpected, as wort is a complex matrix and lautering is a crude form of filtration compared with microfiltration used in the previous study *(19)*. Even so, when comparing lautered wort with 0.45 µm microfiltered wort (data not shown), the difference (on average) was less than 3% for all metal ions and all chelators with the exception of phytic acid. In this case, a difference of 8-12% were seen for all metals and was similar to how phytic acid behaved at wort pH in the trials with the metal ion mix *(19)*.

What sets tannic acid apart from the other tested chelating agents is its capability (due to its large size and many hydroxyl groups) to form multiple crosslinks through coordination with metal ions *(19,52)*. Tannic acid contains a central glucose spine to which ten galloyl moieties are bound (deca-galloyl glucose), and each galloyl group has two adjacent hydroxyl groups that serve as binding sites for metal ions. At wort pH, bis- or tris-polyphenol complexes can be formed with iron (two or three tannic acid ligands octahedrally coordinated around one metal ion) *(53–55)*. Accordingly, tannic acid can form large (supramolecular) organic-metal aggregates, as there are more sites involved in metal-binding than just the galloyl groups *(56)*. Among other polyphenols, tannic acid is also known to strongly interact with proteins (particularly amino and hydroxyl groups) by hydrogen bonding, covalent bonding, ionic interactions and hydrophobic interactions *(57,58)*. These polyphenol-protein complexes also form strong chelates with metal ions (particularly iron and copper) *(11,59,60)*. Transition metal ions play an important role in polyphenol-protein association and precipitation *(61,62)*. Other factors that will influence the size and grade of polyphenol-protein complexation are the presence of oxidising agents (e.g. hydrogen peroxide), heightened temperatures and oxygen exposure, as these factors facilitate the formation of covalent bonds that irreversibly link polyphenols to proteins *(63,64)*.

For every chelator, a selection of unboiled wort samples (addition time, 0 min; mash out, 80°C; mash pH, 5.0 and 6.0; chelator concentration, 5.9 and 35.3 µM) were analysed by electron spin resonance (ESR) spectroscopy, which measures the radical concentration. Lower free radical intensity is a sign of lower oxidative stress and potentially reduced product degradation. Among other things, radical generation is influenced by transition metal content and the form in which the metal ions are present (free or bound) *(65)*. Among the ESR samples (data not shown), the only notable difference between 'high' and 'low' chelator addition was with EDTA. At both pH values, the area under the curve (radical intensity) was larger with the 35.3 µM addition. This can be explained by the observation that the addition of EDTA (a strong chelating agent) caused more (transition) metals to leach out into the lautered wort. Further, EDTA metal complexes are known to behave pro-oxidatively at molar ratios of EDTA and iron below 1, spurring free radical generation *(66,67)*.

In summary, from the primary five chelators screened by DOE, only tannic acid was of interest in enhancing beer flavour stability. This is not a new finding *(68)*. Thus, further examination was required, and additional (rapid) screening was performed for other chelators that - like tannic acid - perform well during mashing. Four chelators (ferulic acid, tartaric acid, quercetin and chlorogenic

acid) - also employed in the previous study - were screened, together with ten polyphenolic food extracts (curcuma, cinnamon, raspberry, grapefruit seed, ginkgo, grape seed, green tea, reishi, pomegranate and milk thistle). Many of these fourteen chelators are similar to tannic acid in that they possess multiple hydroxyl and/or carboxylate groups, capable of chelation *(69)*.

In the range of 0-200 mg/L, ferulic, tartaric and chlorogenic acids did not reduce the concentration of iron, copper or manganese. Indeed, at high concentration they led to slightly higher levels of Fe and Mn, which is likely to reflect the slight decrease in pH. Quercetin, however, did cause a reduction in iron with 3 μg/L at 50.0 mg/L (147.8 μM) and 39 μg/L at 200 mg/L (591.2 μM) (Figure 6). However, this was far from the efficacy found with tannic acid.

The ten polyphenolic food extracts were tested at a broader range (0-1000 mg/L). Most (curcuma, cinnamon, raspberry, grapefruit seed, ginkgo, grape seed, reishi and milk thistle) had no impact on the transition metal content (Figure 6). Green tea and pomegranate extract however, were successful in the chelation and depletion of iron (Figure 7). What distinguishes the green tea and pomegranate extracts from the other food extracts is that they are rich in phenolics that, like tannic acid, possess multiple catechol or galloyl groups. The green tea extract contains 50% epigallocatechin gallate and the pomegranate extract, 40% ellagic acid. Both molecular structures- and their possible binding sites- are shown in the supporting information (Figures S7-8).

As the pomegranate extract performed exceptionally well as a chelating agent, the evaluation was performed with an extract containing 90% ellagic acid (rather than 40%). The results (Figure 8) are compared with a 60 mg/L addition of tannic acid. It can be concluded that the pomegranate extract with the 90% ellagic acid is the best chelator of iron. No significant effects were seen with other metal ions, albeit the wort with the addition of tannic acid added was slightly higher in copper. An advantage of using ellagic acid is that it is naturally found in some beers (0-1.5 mg/L) *(70,71)*.

Accordingly, an optimisation experimental design was performed with pomegranate extract (90% ellagic acid). An optimisation DOE requires the most runs per factor, but it also gives the

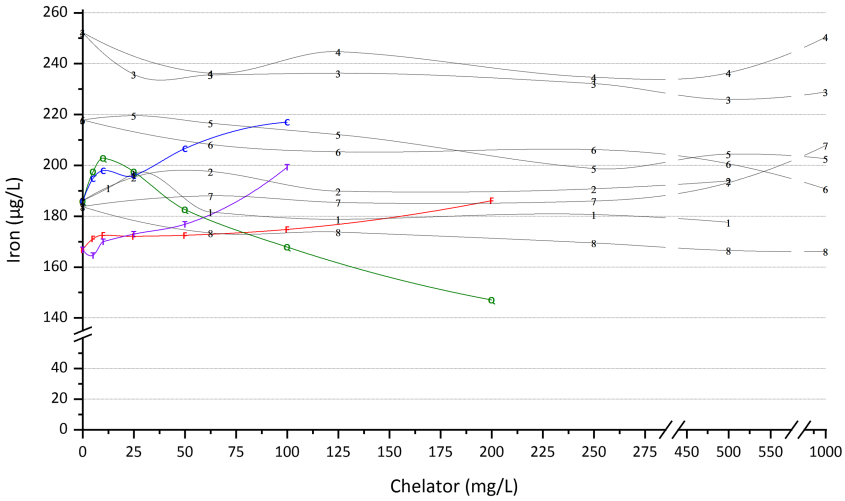

Figure 6. Levels of iron (μg/L) with increasing concentrations of chelators (mg/L), with ferulic acid (F) in red, tartaric acid (T) in purple, quercetin (Q) in green, chlorogenic acid (C) in blue and eight polyphenolic food extracts in grey (curcuma, 1; cinnamon, 2; raspberry, 3; grapefruit seed, 4; ginkgo, 5; grape seed, 6; reishi, 7; milk thistle, 8) [Colour figure can be viewed at wileyonlinelibrary.com]

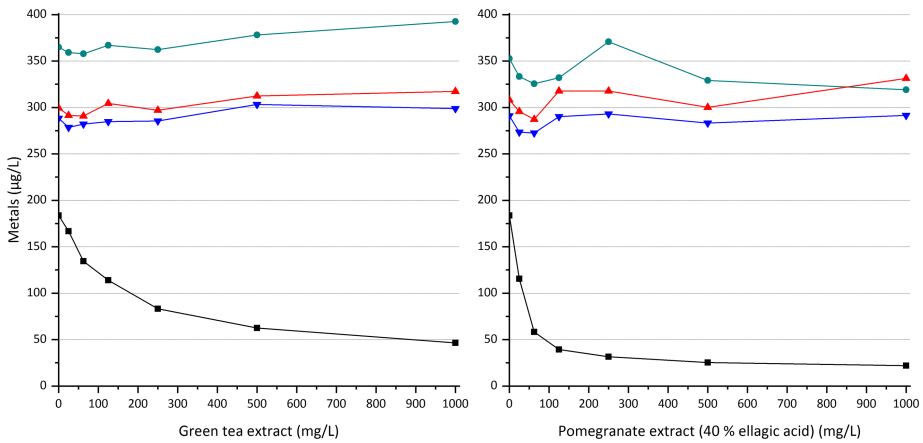

Figure 7. Levels of iron, copper, manganese and zinc (μg/L) with increasing concentrations of green tea (left) and pomegranate extract (right) (mg/L: 0, 25, 62.5, 125, 250, 500 and 1000), with iron in black (■), copper in green (●), manganese in red (▲) and zinc in blue (▼) [Colour figure can be viewed at wileyonlinelibrary.com]

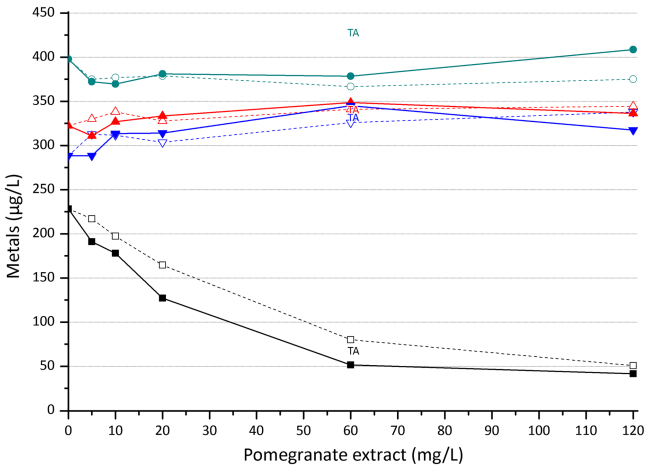

Figure 8. Levels of iron, copper, manganese and zinc (µg/L) with increasing concentrations of pomegranate extract (mg/L: 0, 5, 10, 20, 60 and 120), with iron in black (■), copper in green (●), manganese in red (▲) and zinc in blue (▼). The dotted line is of pomegranate extract (40% ellagic acid) and the solid line with 90% ellagic acid. Tannic acid (TA) addition (60 mg/L) is included for comparison. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 6. ANOVA and fit statistics for the response surface models (optimisation)

	Pomegranate extract (90% ellagic acid)			
	Iron	Copper	Manganese	Zinc
Model	Reduced Cubic	Reduced Cubic	Reduced Quartic	Reduced Cubic
Transformation	None	None	None	None
R^2	0.99	0.71	0.99	0.94
Adjusted R^2	0.99	0.55	0.98	0.91
Predicted R^2	0.97	- 0.58	NA	0.84
Adequate precision	50.3	8.1	38.5	21.6
Model F-value	171	2.6	85.9	29.1
Lack of fit F-value	1.32	0.76	0.12	0.54

most information of any design. As with the screening DOE, this approach determines the important factors, fits a quadratic polynomial model to the response to model second-order effects (curvature) and can be used to find the factor settings that lead to a maximised/optimised response, which (in this case) is the point of minimal transition metal content. The ANOVA results and statistics of the resulting models can be found in Table 6. A summary of the pomegranate trials by correlation matrix is in Table 7.

In agreement with the chelators tested in the screening trials (Table 5), the time of addition had little impact. The grain bill- which was kept at a constant 50/50 ratio in the screening design- had a low negative correlation with iron, manganese and zinc, with higher ratios of Munich malt aligned with higher levels of Fe, Mn and Zn in the wort (see Figure 9). It is assumed that this results from the more acidic mash from using more highly kilned malts containing higher levels of acidic melanoidins, facilitating metal release. However, since the pH difference between an all-Pilsner wort and an all-Munich wort is

Table 7. Pearson correlation coefficients (r) between experimental factors and metal ion concentrations in lautered wort

	Pomegranate extract (90% ellagic acid)			
	[Fe]	[Cu]	[Mn]	[Zn]
Chelator addition time	-0.06	-0.01	-0.01	0.02
Grain bill (Pilsner/Munich)	-0.19	0.05	-0.12	-0.12
Mash pH	-0.56	-0.38	-0.95	-0.89
Chelator concentration	-0.62	0.07	0.09	0.17

Pearson's $r \in [-1,1]$ and coloured dark red at total positive linear correlation (1.00), white at no linear correlation (0.00) and dark blue at total negative linear correlation (-1.00)

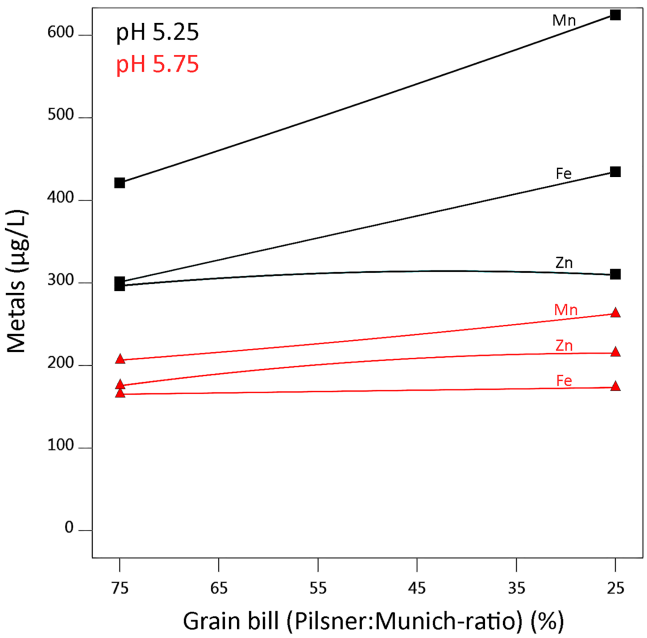

Figure 9. Levels of iron, manganese and zinc (µg/L) at increasing ratio of Munich malt (left to right) and two pH levels (pH 5.25, black; pH 5.75, red) [Colour figure can be viewed at wileyonlinelibrary.com]

0.20, this is only part of the explanation. It is likely that an additional mechanism is at play, such as the loss of metal binding capacity of malt solids by roasting *(24,30)*- with copper, again, being the exception.

In Figure 9 it is also notable that, in terms of mash pH, similar effects were seen as with the other chelators for iron, manganese and zinc. With respect to the chelator concentration, the correlations were almost identical to those of tannic acid, suggesting that

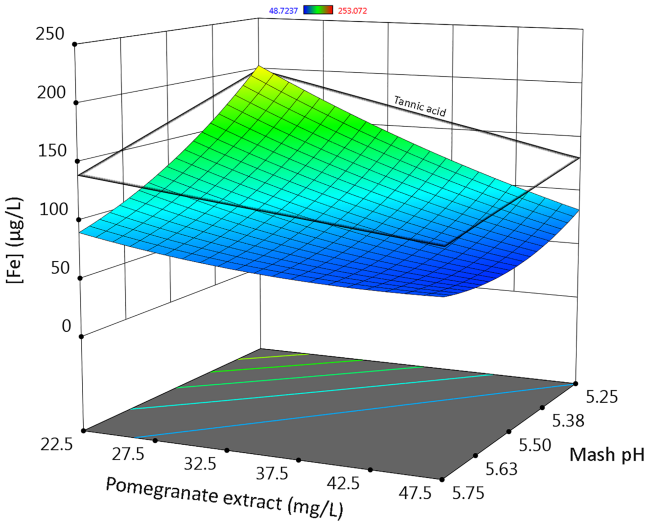

Figure 10. Response surface interaction model for the fitted value levels of iron concentration (µg/L) in lautered wort, after mashing with varying levels of pomegranate extract addition (mg/L) and mash pH, with a 50/50 Pilsner/Munich grain bill, chelator additions after 27.5 min mashing and mash out at 78°C. The responses with tannic acid are included for reference. [Colour figure can be viewed at wileyonlinelibrary.com]

- like tannic acid - the pomegranate extract effectively reduces the amount of iron during mashing. The three dimensional plot of iron fluctuation, with pomegranate extract added at varying concentrations and different mash pH, is presented in Figure 10. The iron level with additions of tannic acid are included for reference. In all, pomegranate extract is more effective at diminishing iron than tannic acid at the given concentrations.

As can be seen from the correlation matrices (Tables 5 and 7), mash pH and (if the chelators are effective) chelator concentration are the two main contributing factors to the metal content of wort. This derivation is seen in the ANOVA results of all models (namely, the F- and p-values of the experimental factors). Table 8 and 9, respectively, feature a summary of these outcomes for the iron-tannic acid model and the iron-pomegranate extract model. In both models, the chelator concentration term leads the total model contribution, with mash pH second. Together with iron-EDTA, and presumably with iron-green tea extract (which was not investigated by DOE), these are the only instances where this occurs. In all other models, mash pH is the main contributor to the model (with Cu-tannic acid, Cu-EDTA and Cu-citric acid being exceptions).

Electron spin resonance (ESR) spectroscopy was employed to further investigate the effects of pomegranate extract on mashing. A selection of the pomegranate worts were boiled and subsequently measured on ESR (Figure 11). The data are from a single experiment with technical replicates per sample. The ESR assay allows for a deeper understanding of the antioxidative mechanism of pomegranate as ICP-OES does not differentiate between free and bound (transition) metal ions within the lautered wort, and there are important oxidative differences between free and bound metals. Even among (bound) metal-complexes, there can be great

Table 8. ANOVA results of the Fe-tannic acid model terms

Factor	Contribution (%)	F-value	p-value
[Tannic acid]	44.2	1423	< 0.0001
Mash pH	34.0	1095	< 0.0001
Mash pH * [Tannic acid]	18.9	606	< 0.0001
Mash pH * Addition time	1.3	42.5	< 0.0001
Temperature mash out	0.5	14.5	0.0009
[Tannic acid] * Addition time	0.3	7.9	0.0098
Mash pH * Temperature mash out	0.2	5.0	0.0358
Addition time	0.0	0.1	0.8128

Table 9. ANOVA results of the Fe-pomegranate extract model terms

Factor	Contribution (%)	F-value	p-value
[Pomegr. extr.]	38.4	867	< 0.0001
Mash pH	30.7	694	< 0.0001
Mash pH * [Pomegr. extr.]	10.8	245	< 0.0001
Mash pH^2	6.8	154	< 0.0001
Grain bill	3.7	84.4	< 0.0001
Mash pH * Grain bill	3.3	73.9	< 0.0001
[Pomegr. extr.]^2	2.2	50.4	< 0.0001
[Pomegr. extr.] * Grain bill	1.4	31.8	< 0.0001
Mash pH * [Pomegr. extr.] * Grain bill	0.8	18.4	0.0006
Mash pH * Addition time	0.5	10.3	0.0055
Addition time	0.4	8.9	0.0086
[Pomegr. extr.] * Addition time	0.2	3.9	0.0667
Addition time^2	0.1	3.1	0.0952

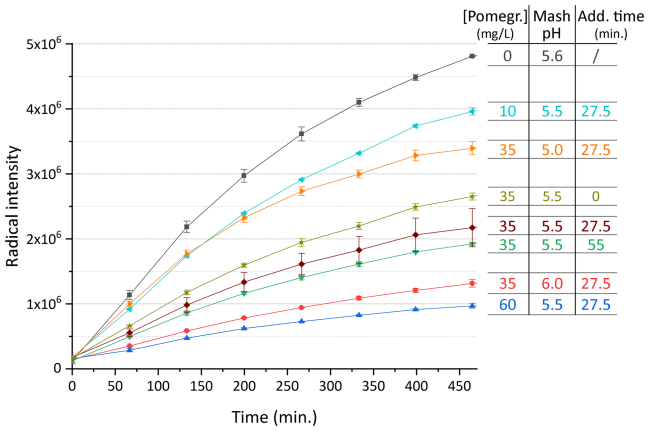

Figure 11. ESR measurement (T450-values) of boiled worts (grain bill, 50/50 Pilnser/Munich; mash out temperature, 78°C) with various levels of pomegranate extract (mg/L), mash pH and chelator addition time (min) [Colour figure can be viewed at wileyonlinelibrary.com]

disparity, with some complexes being antioxidative and others prooxidative. ESR spectroscopy can help resolve this.

The reference wort (no pomegranate extract added) shows the highest radical intensity (T_{450}-value) of all samples, indicating the antioxidative capabilities of pomegranate. The wort with the highest amount of pomegranate extract added (60 mg/L) shows the lowest radical intensity or the highest oxidative stability. Apart from the established iron chelation - which is the main antioxidative effect - further antioxidative properties, such as radical quenching *(72–74)*, may contribute to the effectiveness of pomegranate extract in lowering the radical generation. For the worts that had the same concentration of pomegranate extract added, pH was a significant factor, with a lower mash pH leading to a higher generation of radicals. This effect cannot be solely explained by the greater iron content of the pH 5 mash since it is only 12 μg/L higher than that of the pH 6 mash. An additional determinant is the pH dependent speciation of some metal ions (particularly Fe) and the pH dependent coordination number of some organic ligands (e.g. ellagic acid), all of which will change the coordination chemistry of the metal complexes that will ultimately affect the reactivity of the Fenton and Haber-Weiss mechanism *(75,76)*.

Later addition times of the extract appear to be more beneficial than an early addition time. From an oxidative standpoint this is surprising, since with the early addition of the chelating agent, the endogenous wort antioxidants would be better preserved (as they are already protected at the onset of mashing from oxidising early), resulting in a lower radical concentration. This is may be due to the earlier additions having a slightly higher (5-17 μg/L) iron content than the late addition, and ESR measurements are very sensitive to iron. Jenkins et al. *(12)* reported that as little as 10 μg/L transition metal ion can make a detectable difference to the oxidative stability. This effect was also seen by Maxminer *(32)*, where a control beer (with no tannic acid added) had a lower ESR area under the curve than two other beers (that *did* have tannic acid additions) since the control beer was ca. 20 μg/L lower in Fe than the two other samples. Other authors *(16,77)* have also reported the accelerative ability of iron to activate oxygen by electron transfer with an excellent correlation ($R^2 = 0.99$) between ESR area and Fe content.

Conclusions

The results show that pomegranate extract (high in ellagic acid) is an excellent chelator in lowering the prooxidative iron content of wort during mashing. Its antioxidative capabilities were reaffirmed in boiled wort, where it reduced radical generation during prolonged heating (ESR analysis). Tannic acid and, to a lesser degree, green tea extract share iron chelating properties.

With regard to mashing conditions, it is noteworthy that acidic (5.2 ± 0.2) mashes will result in significantly higher levels of iron, manganese and zinc in the wort (and possibly the beer), as compared to mashing at more alkaline pH (5.6 ± 0.2). This effect can, to a great extent, be nullified for the detrimental iron by sufficient (early) addition of pomegranate extract or tannic acid. Manganese content, however, was not readily diminished and its final concentration remained pH dependent. Copper largely remained unaffected. Apart from less metal leaching out with non-acidified mashing, a more natural mash pH (≈ 5.6) provides an environment that is better suited to chelation, since the chelating agents are less protonated. Further, mashing at a pH of 5.5-5.6 produces a more fermentable wort, since it is the optimum working range for both α- and β-amylase.

Mash out temperature and chelator addition time were not as significant a parameter as chelator concentration or mash pH in terms of influencing metal content. This does not, however, imply that these factors have no impact on oxidative stability. As seen with the chelator addition time, samples with late mash addition of pomegranate extract appeared to show lower radical generation, compared to earlier additions (although this may be due to minor differences in iron content). It is also likely that a higher mash out temperature (although it causes slightly more transition metals to drop out of solution) will result in a more oxidised wort due to higher thermal load.

Pomegranate extract containing ellagic acid has exciting potential as a flavour (and colloidal) stability enhancer, not only by adding it during mashing but also during wort boiling, beer maturation and filtration. Further investigation is needed and a follow-up study will explore whether beer shelf-life can be increased by applying these findings in (pilot scale) brewing trials. Similarly, further work is required on the influence of chelators on *de novo* aldehyde formation and whether or not, by decreasing the generation of reactive oxygen species, their formation can be slowed down substantially.

Author contributions

Tuur Mertens – conceptualisation of the research plan, project administration, conceived and performed experiments, created the models and conducted formal analysis, writing (original draft).
Thomas Kunz - conceptualisation of the research plan and interpretation of the data, writing (review & editing).
Philip Wietstock - conceptualisation of the research plan and interpretation of the data, writing (review & editing).
Frank-Jürgen Methner - acquisition of funding, supervisor.

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Conflict of interest

The authors declare there are no conflicts of interest.

References

1. Stephenson WH, Bamforth CW. 2002. The impact of lightstruck and stale character in beers on their perceived quality: a consumer study. *J Inst Brew* 108:406–9. https://doi.org/10.1002/j.2050-0416.2002.tb00568.x
2. King BM, Duineveld CA. 1999. Changes in bitterness as beer ages naturally. *Food Qual Prefer* 10:315–24. https://doi.org/10.1016/S0950-3293(98)00040-8
3. Cooman L, Aerts G, Overmeire H, Keukeleire D. 2000. Alterations of the profiles of iso-α-acids during beer ageing, marked instability of trans-iso-α-acids and implications for beer bitterness consistency in relation to tetrahydroiso-α-acids. *J Inst Brew* 106:169–78. https://doi.org/10.1002/j.2050-0416.2000.tb00054.x
4. Jaskula B, Syryn E, Goiris K, De Rouck G, Van Opstaele F, De Clippeleer J, Aerts G, De Cooman L. 2007. Hopping technology in relation to beer bitterness consistency and flavor stability. *J Am Soc Brew Chem* 65:38–46. https://doi.org/10.1094/ASBCJ-2007-0112-03
5. Baert JJ, De Clippeleer J, Hughes PS, De Cooman L, Aerts G. 2012. On the origin of free and bound staling aldehydes in beer. *J Agric Food Chem* 60:11449–72. https://doi.org/10.1021/jf303670z
6. Baert JJ, De Clippeleer J, Bustillo Trueba P, Jaskula-Goiris B, De Rouck G, Aerts G, De Cooman L. 2018. Exploring aldehyde release in beer by 4-vinylpyridine and the effect of cysteine addition on the beer's pool of bound aldehydes. *J Am Soc Brew Chem* 76:257–71. https://doi.org/10.1080/03610470.2018.1518639
7. Malfliet S, Opstaele F, Clippeleer J, Syryn E, Goiris K, Cooman L, Aerts G. 2008. Flavour instability of pale lager beers: determination of analytical markers in relation to sensory ageing. *J Inst Brew* 114:180–92. https://doi.org/10.1002/j.2050-0416.2008.tb00324.x
8. Vanderhaegen B, Neven H, Verachtert H, Derdelinckx G. 2006. The chemistry of beer aging – a critical review. *Food Chem* 95:357–81. https://doi.org/10.1016/j.foodchem.2005.01.006
9. Bamforth CW. 1999. The science and understanding of the flavour stability of beer: a critical assessment. *Brauwelt Int* 17:98–110.
10. Takashio M, Shinotsuka K. 1998. Preventive production of beer against oxidation-recent advances in brewing technology. *Food Sci Technol Int Tokyo* 4:169–77. https://doi.org/10.3136/fsti9596t9798.4.169
11. Kaneda H, Kano Y, Koshino S, Ohya-Nishiguchi H. 1992. Behavior and role of iron ions in beer deterioration. *J Agric Food Chem* 40:2102–7. https://doi.org/10.1021/jf00023a013
12. Jenkins D, James S, Dehrmann F, Smart K, Cook D. 2018. Impacts of copper, iron, and manganese metal ions on the epr assessment of beer oxidative stability. *J Am Soc Brew Chem* 76:50–7. https://doi.org/10.1080/03610470.2017.1402585
13. Wietstock PC, Kunz T, Waterkamp H, Methner F-J. 2015. Uptake and release of Ca, Cu, Fe, Mg, and Zn during beer production. *J Am Soc Brew Chem* 73:179–84. https://doi.org/10.1094/ASBCJ-2015-0402-01
14. Wietstock PC, Kunz T, Methner F-J. 2016. Influence of hopping technology on oxidative stability and staling-related carbonyls in pale lager beer. *BrewSci* 69:73–84.
15. Wietstock PC, Shellhammer TH. 2011. Chelating properties and hydroxyl-scavenging activities of hop α- and iso-α-acids. *J Am Soc Brew Chem* 69:133–8. https://doi.org/10.1094/ASBCJ-2011-0718-01
16. Kunz T, Frenzel J, Wietstock PC, Methner F-J. 2014. Possibilities to improve the antioxidative capacity of beer by optimized hopping regimes. *J Inst Brew* 120:415-25. https://doi.org/10.1002/jib.162
17. Kaneda H, Kobayashi N, Takashio M, Tamaki T, Shinotsuka K. 1999. Beer staling mechanism. *Tech Q Master Brew Assoc Am* 36:41–7.
18. Andersen ML, Skibsted LH. 1998. Electron spin resonance spin trapping identification of radicals formed during aerobic forced aging of beer. *J Agric Food Chem* 46:1272–5. https://doi.org/10.1021/jf9708608
19. Mertens T, Kunz T, Methner FJ. 2020. Assessment of chelators in wort and beer model solutions. *BrewSci* 73:58–67. https://doi.org/10.23763/BrSc20-01mertens
20. Zazo JA, Pliego G, Blasco S, Casas JA, Rodriguez JJ. 2011. Intensification of the Fenton process by increasing the temperature. *Ind Eng Chem Res* 50:866–70. https://doi.org/10.1021/ie101963k
21. Holzmann A, Piendl A. 1977. Malt modification and mashing conditions as factors influencing the minerals of wort. *J Am Soc Brew Chem* 35:1–8. https://doi.org/10.1094/ASBCJ-35-0001
22. Jacobsen T, Lie S. 1977. Chelators and metal buffering in brewing. *J Inst Brew* 83:208–12. https://doi.org/10.1002/j.2050-0416.1977.tb03796.x
23. Hopulele T, Piendl A. 1973. Effect of barley variety, environment, and malting technology on the mineral substances of malt. *Proceedings Annu Meet - Am Soc Brew Chem* 31:75–83. https://doi.org/10.1080/00960845.1973.12006006
24. Pagenstecher M, Maia C, Andersen ML. 2021. Retention of iron and copper during mashing of roasted malts. *J Am Soc Brew Chem* 79:138–144. https://doi.org/10.1080/03610470.2020.1795609
25. Charalambous G, Bruckner KJ. 1977. Analysis of metallic ions in brewing materials, wort, beer and wine by inductively coupled argon plasma-atomic emission spectroscopy. *Tech Q Master Brew Assoc Am* 14:197–208.
26. Petrich M, Paulsen O, Mertens T, Grothusheitkamp D, Kunz T, Cahoon E. 2019. Direct analysis of trace elements in beer and wort by ICP-OES. *European Winter Conference on Plasma Spectrochemistry*. Pau. Book of Abstracts, p 354.
27. MEBAK®. 2012. *Brewing analysis methods: wort, beer and beer-based beverages*. 4th ed. Self-published by MEBAK, Freising Weihenstephan, Germany.
28. Kunz T, Methner F-J, Hüttermann J, Kappl R. Patent: WO 2007/028635 Al, 2006. Method for determining the endogenous antioxidative potential of beverages by means of ESR spectroscopy. Technische Universität Berlin & Universität des Saarlandes.
29. Boulton C. 2013. *Encyclopedia of Brewing*, p 462. John Wiley & Sons, Ltd, Oxford, UK. https://doi.org/10.1002/9781118598115
30. Hoff S, Lund MN, Petersen MA, Jespersen BM, Andersen ML. 2012. Influence of malt roasting on the oxidative stability of sweet wort. *J Agric Food Chem* 60:5652–9. https://doi.org/10.1021/jf300749r
31. Gibson BR. 2011. 125th anniversary review: improvement of higher gravity brewery fermentation via wort enrichment and supplementation. *J Inst Brew* 117:268–84. https://doi.org/10.1002/j.2050-0416.2011.tb00472.x
32. Maxminer JP. 2016. *Assessing the flavour stability of lager-style beers*. PhD Thesis. University of Nottingham, Nottingham, UK.
33. Taylor JRN. 1992. Mashing with malted grain sorghum. *J Am Soc Brew Chem* 50:13–18. https://doi.org/10.1094/ASBJ-50-0013
34. MacWilliam IC. 1975. pH in malting and brewing - a review. *J Inst Brew* 81:65–70. https://doi.org/10.1002/j.2050-0416.1975.tb03663.x
35. Stenholm K, Home S. 1999. A new approach to limit dextrinase and its role in mashing. *J Inst Brew* 105:205–10. https://doi.org/10.1002/j.2050-0416.1999.tb00020.x
36. De Rouck G, Jaskula B, De Causmaecker B, Malfliet S, Van Opstaele F, De Clippeleer J, De Brabanter J, De Cooman L, Aerts G. 2013. The influence of very thick and fast mashing conditions on wort composition. *J Am Soc Brew Chem* 71:1–14. https://doi.org/10.1094/ASBCJ-2013-0113-01
37. Kunze W, Pratt S, Hans M. 2004. *Technology Brewing and Malting*. 3rd ed. VLB Berlin, Berlin, Germany.
38. Bamforth CW. 2001. pH in brewing: an overview. *Tech Q Master Brew Assoc Am* 38:1–9.
39. Briggs DE, Boulton CA, Brookes PA, Stevens R. 2004. *Brewing: Science and Practice*. Woodhead Publishing Limited, Cambridge, England. https://doi.org/10.1533/9781855739062
40. Wietstock PC, Kunz T, Pereira F, Methner F-J. 2016. Metal chelation behavior of hop acids in buffered model systems. *BrewSci* 69:56–63.
41. Jeantet R, Croguennec T, Schuck P, Brulé G. 2016. *Handbook of Food Science and Technology 1: Food Alteration and Food Quality*. John Wiley & Sons, Inc., Hoboken, NJ, USA. https://doi.org/10.1002/9781119268659
42. Miedaner H. 1986. Wort boiling today - old and new aspects. *J Inst Brew* 92:330–5. https://doi.org/10.1002/j.2050-0416.1986.tb04419.x
43. Dadic M, Van Gheluwe G. 1972. Experiments with a whirlpool tank. *Brewers Digest* 47:120–6.
44. Lewis MJ, Wahnon NN. 1984. Precipitation of protein during mashing: evaluation of the role of calcium, phosphate, and mash pH. *J Am Soc Brew Chem* 42:159–63. https://doi.org/10.1094/ASBCJ-42-0159
45. Gorinstein S. 1974. Metal protein complexes in ethanol media. *J Food Sci* 39:953–6. https://doi.org/10.1111/j.1365-2621.1974.tb07285.x

46. Zufall C, Tyrell T. 2008. The influence of heavy metal ions on beer flavour stability. *J Inst Brew* 114:134–42. https://doi.org/10.1002/j.2050-0416.2008.tb00318.x
47. McDonald M, Mila I, Scalbert A. 1996. Precipitation of metal ions by plant polyphenols: optimal conditions and origin of precipitation. *J Agric Food Chem* 44:599–606. https://doi.org/10.1021/jf950459q
48. Andjelkovic M, Van Camp J, De Meulenaer B, De Paemelaere G, Socaciu C, Verloo M, Verhe R. 2006. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chem* 98:23–31. https://doi.org/10.1016/j.foodchem.2005.05.044
49. Formanek JA, Bonte P. 2017. Use of tannic acid in the brewing industry for colloidal and organoleptic stability. *Tech Q Master Brew Assoc Am* 54:11–6. https://doi.org/10.1094/TQ-54-1-0112-01
50. Irwin AJ, Barker RL, Pipasts P, St S. 1991. The role of copper, oxygen, and polyphenols in beer flavor instability. *J Am Soc Brew Chem* 49:140–9. https://doi.org/10.1094/ASBJ-49-0140
51. Rahim MA, Lin G, Tomanin PP, Ju Y, Barlow A, Björnmalm M, Caruso F. 2020. Self-assembly of a metal–phenolic sorbent for broad-spectrum metal sequestration. *ACS Appl Mater Interfaces* 12:3746–54. https://doi.org/10.1021/acsami.9b19097
52. Yang L, Han L, Ren J, Wei H, Jia L. 2015. Coating process and stability of metal-polyphenol film. *Colloids Surfaces A Physicochem Eng Asp* 484:197–205. https://doi.org/10.1016/j.colsurfa.2015.07.061
53. Perron NR, Brumaghim JL. 2009. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochem Biophys* 53:75–100. https://doi.org/10.1007/s12013-009-9043-x
54. Rahim MA, Ejima H, Cho KL, Kempe K, Müllner M, Best JP, Caruso F. 2014. Coordination-driven multistep assembly of metal–polyphenol films and capsules. *Chem Mater* 26:1645–53. https://doi.org/10.1021/cm403903m
55. Ejima H, Richardson JJ, Liang K, Best JP, van Koeverden MP, Such GK, Cui J, Caruso F. 2013. One-step assembly of coordination complexes for versatile film and particle engineering. *Science* 341:154–7. https://doi.org/10.1126/science.1237265
56. Khokhar S, Owusu Apenten RK. 2003. Iron binding characteristics of phenolic compounds: some tentative structure–activity relations. *Food Chem* 81:133–40. https://doi.org/10.1016/S0308-8146(02)00394-1
57. Zhang X, Do MD, Casey P, Sulistio A, Qiao GG, Lundin L, Lillford P, Kosaraju S. 2010. Chemical cross-linking gelatin with natural phenolic compounds as studied by high-resolution NMR spectroscopy. *Biomacromolecules* 11:1125–32. https://doi.org/10.1021/bm1001284
58. Matheis G, Whitaker JR. 1984. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J Food Biochem* 8:137–62. https://doi.org/10.1111/j.1745-4514.1984.tb00322.x
59. Gramshaw JW. 1970. Beer polyphenols and the chemical basis of haze formation, part II: changes in polyphenols during the brewing and storage of beer - the composition of hazes. *Tech Q Master Brew Assoc Am* 7:122–33.
60. Aron PM. 2011. *The effect of hopping technology on lager beer flavor and flavor stability and the impact of polyphenols on lager beer flavor and physical stability*. PhD Thesis. Oregon State University, Corvallis, Oregon, USA.
61. Ho C-T, Lee CY, Huang M-T. 1992. *Phenolic Compounds in Food and their Effects on Health I: Analysis, Occurrence, and Chemistry*. American Chemical Society, Washington, DC, USA. https://doi.org/10.1021/bk-1992-0506
62. Liu C, McClements DJ, Li M, Xiong L, Sun Q. 2019. Development of self-healing double-network hydrogels: enhancement of the strength of wheat gluten hydrogels by in situ metal–catechol coordination. *J Agric Food Chem* 67:6508–16. https://doi.org/10.1021/acs.jafc.9b01649
63. Brudzynski K, Maldonado-Alvarez L. 2015. Polyphenol-protein complexes and their consequences for the redox activity, structure and function of honey. A current view and new hypothesis – a review. *Polish J Food Nutr Sci* 65:71–80. https://doi.org/10.1515/pjfns-2015-0030
64. Strauss G, Gibson SM. 2004. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. *Food Hydrocoll* 18:81–9. https://doi.org/10.1016/S0268-005X(03)00045-6
65. Frederiksen AM, Festersen RM, Andersen ML. 2008. Oxidative reactions during early stages of beer brewing studied by electron spin resonance and spin trapping. *J Agric Food Chem* 56:8514–20. https://doi.org/10.1021/jf801666e
66. Aisen P, Cohen G, Kang JO. 1990. Iron Toxicosis. In: *International Review of Experimental Pathology: Transition Metal Toxicity*. Academic Press Inc, San Diego, California, USA. p 1–46. https://doi.org/10.1016/B978-0-12-364931-7.50006-9
67. Aust SD, Morehouse LA, Thomas CE. 1985. Role of metals in oxygen radical reactions. *J Free Radic Biol Med* 1:3–25. https://doi.org/10.1016/0748-5514(85)90025-X
68. Withouck H, Boeykens A, Jaskula B, Goiris K, De Rouck G, Hugelier C, Aerts G. 2009. Upstream beer stabilisation during wort boiling by addition of gallotannins and/or PVPP. *BrewSci* 63:14–22.
69. Gülçin İ, Huyut Z, Elmastaş M, Aboul-Enein HY. 2010. Radical scavenging and antioxidant activity of tannic acid. *Arab J Chem* 3:43–53. https://doi.org/10.1016/j.arabjc.2009.12.008
70. Pontes Guimarães B, Eduardo Pereira Neves L, Gonçalves Guimarães M, Ferreira Ghesti G. 2020. Evaluation of maturation congeners in beer aged with Brazilian woods. *J Brew Distill* 9:1–7. https://doi.org/10.5897/JBD2019.0053
71. Ullucci PA, Thomas D, Acworth IN. 2016. Application note 1020: chalconoids and bitter acids in beer by HPLC with UV and electrochemical detection. Chelmsford, MA; Chelmsford, MA;
72. Priyadarsini KI, Khopde SM, Kumar SS, Mohan H. 2002. Free radical studies of ellagic acid, a natural phenolic antioxidant. *J Agric Food Chem* 50:2200–6. https://doi.org/10.1021/jf011275g
73. Evtyugin DD, Magina S, Evtuguin D V. 2020. Recent advances in the production and applications of ellagic acid and its derivatives. A Review. *Molecules* 25:2745. https://doi.org/10.3390/molecules25122745
74. Żymańczyk-Duda E, Szmigiel-Merena B, Brzezińska-Rodak M, Klimek-Ochab M. 2018. Natural antioxidants–properties and possible applications. *J Appl Biotechnol Bioeng* 5:251–8. https://doi.org/10.15406/jabb.2018.05.00146
75. Salgado P, Melin V, Contreras D, Moreno Y, Mansilla HD. 2013. Fenton reaction driven by iron ligands. *J Chil Chem Soc* 58:2096–101. https://doi.org/10.4067/S0717-97072013000400043
76. Salgado P, Melin V, Albornoz M, Mansilla H, Vidal G, Contreras D. 2018. Effects of pH and substituted 1,2-dihydroxybenzenes on the reaction pathway of Fenton-like systems. *Appl Catal B Environ* 226:93–102. https://doi.org/10.1016/j.apcatb.2017.12.035
77. Ogane O, Yokoyama F, Hirano T. 2000. Quantification of beer freshness, based on the original freshness scale. *Tech Q Master Brew Assoc Am* 37:69–72.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

