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Assessment of Chelators in Wort and Beer Model Solutions

Long-term storage of beer often results in flavour deterioration and quality reduction. Prolonging beer freshness would benefit both brewers and consumers worldwide. Oxygen has always been a major focus in regard to oxidation. Less naturally is the key involvement of catalytic transition metals (Fe, Cu, Mn) in oxidative beer ageing. Physicochemically removing these entities through chelation – rendering them incapable of forming reactive oxygen species – could greatly benefit flavour stability. This study aims to explore nine chelating compounds (EDTA, citric acid, tartaric acid, quercetin, chlorogenic acid, ferulic acid, gallic acid, phytic acid, and tannic acid) for their capacity to form complexes with seven metal ions (Fe^{II}, Fe^{III}, Cu^{II}, Mn^{II}, Ca^{II}, Zn^{II}, Mg^{II}) and to examine whether the complexes can effectively be removed by filtration. Chelators and metal ions were mixed and incubated in two distinct acetate buffer solutions, one with the pH and ethanol content of wort (5.60; 0.0 vol%) and one with that of finished beer (4.30; 5.0 vol%). Measurements were conducted by UV-Vis and ICP-OES spectroscopy, to respectively assess complex formation (through absorbance changes) and filterability (through metal level reductions). Ideally, chelators deplete iron, copper, and manganese, without affecting any metals vital for brewing (Ca, Zn, Mg). The findings suggest tannic acid to be the most promising chelator in this aspect, followed by quercetin, gallic acid, chlorogenic acid, and ferulic acid. EDTA, citric acid and tartaric acid did not form filterable complexes with any of the metal ions. Phytic acid chelated out zinc; among others when introduced to a mix of metal ions. Compared to beer pH, wort pH proved far superior overall in terms of transition metal removal by complex formation.

Descriptors: chelation, transition metals, flavour stability, oxidation, UV/Vis, ICP-OES

1 Introduction

Beer is one of the most all-time beloved beverages around the globe [1 chap. 1]; and in several aspects, it is also an exceedingly complex drink. Beer's intricate nature brings forth a broad range of different styles and an almost inexhaustible range of possibilities. This complexity, however, comes with the downside of (fresh) beer being unstable, as it is not in a state of chemical equilibrium.

The poor (flavour) stability causes beer to change and turn unpalatable rather fast; especially considering the detrimental effects of transport and unrefrigerated storage. And even though beer will technically not expire and become unsafe to drink, its lacking stability is a modern brewer's biggest headache. The flavour changes that occur over time are almost always unpleasant and unintended. Adverse storage conditions with time can also cause haze defects and lack of foam in the beer. These phenomena are all very off-putting for the consumer. Given that buyers are becoming more aware and increasingly demanding of beer quality and flavour [2] – along with an ever-expanding global beer distribution chain and a continuous rise in craft beer popularity – it's safe to say that the issue of beer flavour stability has never been more relevant than today.

A big culprit in beer staling is oxygen, as oxidation is the main cause of beer turning 'aged' [3, 4]. That is why breweries go to great lengths avoiding any unnecessary oxygen pickup during brewing and why good bottling practices are so important. Big improvements with total package oxygen (TPO) were already seen over recent years – bottle fillers now allow for a TPO of 50 ppb or less [5]. But even though it continues to improve, complete avoidance of oxygen has its practical limits and is downright impossible.

But there is more to oxidation than oxygen alone. Due to spin restrictions, molecular (ground state) oxygen is rather inert [6]. Unfortunately, transition metals (iron, copper, and manganese) are – through electron exchange – able to undermine this energy barrier and cause oxygen to go out of its latent state. Resulting derivatives are called 'reactive oxygen species' (ROS), of which O_2^{-}, $OOH^{\bullet}$, H_2O_2, and $OH^{\bullet}$ – generated by the Fenton and Haber-Weiss reaction mechanism [7] – are chief examples. These ROS are highly reactive entities and cause substantial oxidative damage by attacking compounds directly, whilst also inducing a cascade of additional oxidizing reactions, resulting in the formation of flavour-active, volatile substances (such as aldehydes), which are generally – depending on their concentrations – responsible

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for atypical tastes and odours. Thus, when it comes to preventing oxidation, one school of thought is to shift the focus more towards debilitating or removing these catalytic transition metals, instead of solely restricting oxygen.

1.1 Chelation

The detrimental effects of transition metals should not be made light of; without them, the vast majority of the "auto-oxidative" reactions in beer would cease to happen [8]. Despite this, content of transition metals is seldom monitored and/or controlled.

The bulk of metals, found in wort and beer, unavoidably enter through the grain at mashing [9]. Despite being added at significantly lower quantities, also hops are a relatively substantial source of manganese in beer (and especially so with dry hopping) [10]. Apart from the unwanted catalytic metals, there is also a leaching-out of desirable metal ions for brewing – such as calcium, magnesium, and zinc. The most feasible way of strictly removing the undesirable metal ions whilst brewing would have to be physicochemical, through means of chelation (the act of binding metal ions to other molecules or chelators). Only limited research has been conducted on the captivation of stale inducing metal ions in brewing, albeit with some very positive outcomes. Wietstock, Kunz and Methner showed that addition of a strong chelator – ethylenediaminetetraacetic acid (EDTA) – to beer actively reduces the amount of Strecker aldehydes formed during its storage [4] (note that fresh beer is low in aldehydes). Analogous anti-oxidative effects through iron complexation are seen with hop acids, albeit weaker [11–15]. Amino acids and melanoidins have been known to chelate copper ions [16], which is also the reason why the 'hot break' (which largely consists out of coagulated protein) is rich in metals [17]. Certain polyphenols, like prenylflavonoids and pro-anthocyanidins, should also act as anti-oxidants by similar metal-confining mechanisms, yet some debate still exists [18, 19].

The existing amount of controversy can largely be attributed to the fact that complexes may act as double-edged swords; behaving oxidation retardant under certain conditions, but accelerative under others. A certain crossline exists on which every chelator balances: the agent being reducing enough for it to serve protectively, by reducing ROS; and it being overly reducing, causing the metal ions to be converted back to their reduced states [20]. The cross-over phenomenon – whether a ligand acts pro- or anti-oxidative – is influenced by factors like chelator concentration and metal-to-chelator ratio [21, 22]. In this aspect, high chelator concentrations are usually preferred.

To add to the complexity of it all, the pH and the way the complex is chemically structured will play a role too [13]. If the chelating agent succeeds in shielding off the central metal ion from its environment, it prevents the electrons from taking part in the whole electron exchanging interplay, which greatly reduces the system's redox potential. However, if the complexed transition metal is still oxygen accessible, the ligands surrounding the metal ion will often get degraded through site-specific oxidation [23]. This is problematic, as these ligands are usually biomolecules which we wish to keep intact. On the other hand, by introducing a strong chelator, transition metal ions can be detached from these vulnerable bio-compounds, thus enhancing flavour stability. For chelation strength, the rule of thumb is: the more donor atoms a given chelator has, the stronger and more stable it will bind with metal ions [24].

Because of the obscure nature that complexes have on oxidative stability, we tried to focus more in this study on actual removal of catalytic transition metals. In practice, this meant screening for chelators that would form complexes with them, large enough so that they could effectively be filtered out. However, an important aspect of the study was to also monitor the beneficial metal ions (Ca, Mg, Zn). As mentioned earlier, we would prefer that these metals remain available for the workings of the brewing process.

2 Materials and method

2.1 Chemicals

Ferulic acid (99.0 %), phytic acid sodium salt hydrate (90 %), tannic acid (99.0 %), quercetin dihydrate (98.0 %), iron(II) sulphate heptahydrate (99.0 %) and calcium(II) acetate hydrate (99.0 %) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ethylenedinitrilo-tetraacetic acid disodium salt dihydrate (Titriplex® III; 99.0 %), citric acid monohydrate (99.5 %), tartaric acid (99.5 %), anhydrous gallic acid (98.0 %), iron(III) chloride hexahydrate (99.0 %), and manganese(II) sulphate monohydrate (99.0 %) were obtained from Merck KGaA (Darmstadt, Germany). Chlorogenic acid (98.0 %) was acquired from Acros Organics (New Jersey, USA). Magnesium(II) acetate tetrahydrate (99.5 %) was bought from Honeywell Riedel-de Haën AG (Seelze, Germany). Zinc(II) acetate dihydrate (99.5 %) was acquired from AppliChem GmbH (Darmstadt, Germany). Copper(II) acetate monohydrate (99.0 %), dimethyl sulfoxide (99.5 %), and glacial acetic acid (100 %) was attained from Carl Roth GmbH (Karlsruhe, Germany). Hydrochloric acid (37 %), ethanol absolute (100 %) and sodium hydroxide came from VWR International S.A.S. (Fontenay-sous-Bois, France). Sodium acetate (99.0 %) was obtained from Chemsolute (Renningen, Germany). All aqueous solutions were made

Table 1 Instrumental parameters for the ICP-OES analysis

Parameter	Baffled Cyclonic Spray Chamber, Meinhard K1 Nebulizer, 2.0 mm Alumina Injector, Two-Dimensional Charge Coupled Device (CCD) Detector
Radio Frequency (RF) Power	1500 Watts
Nebulizer Flow	0.35 L/min
Auxiliary Flow	0.2 L/min
Plasma Flow	11 L/min
Sample Flow Rate	1.00 mL/min
Equilibration Time	20 sec
Torch Position	– 3.00 mm
Carrier and purge gas	Gaseous argon
Shear gas	Air

Table 2 Emission lines used for the quantification of Fe, Cu, Mn, Zn, Ca, and Mg by ICP-OES

Element	Emission line used [nm]
Fe	238.204
Cu	327.398
Mn	257.612
Zn	213.857
Ca	317.933
Mg	279.553

with ultrapure water (by Sartopore® 2 MidiCap 0.2 µm filtration; Sartorius AG, Goettingen, Germany).

2.2 Preparation of reagent

Buffer 1 ('wort' buffer; pH 5.60, 0.0 vol% ethanol) and buffer 2 ('beer' buffer; pH 4.30, 5.0 vol% ethanol) were both 0.1 M sodium acetate buffers. Where necessary, minor pH adjustments were made with either hydrochloric acid (HCl) or sodium hydroxide (NaOH).

All metal ion solutions (Fe^{II}, Fe^{III}, Cu^{II}, Mn^{II}, Ca^{II}, Zn^{II}, Mg^{II}) and chelator solutions (EDTA, citric acid, tartaric acid, quercetin, chlorogenic acid, ferulic acid, gallic acid, phytic acid, and tannic acid) had concentrations of 500 µmol/L – allowing for a final concentration of 250 µmol/L, of both metal ion and chelator, when mixed in equal volumes. All solutions were aqueous, with exception of quercetin (dissolved in dimethyl sulfoxide) and ferulic acid (80:20 water:ethanol).

The 'metal ion mix' contained six metal ions (Fe^{II}, Cu^{II}, Mn^{II}, Mg^{II}, Zn^{II}, and Ca^{II}) with concentrations of 250 µmol/L (half that of the sole metal ion solutions). Blending equal volumes of metal ion mix and chelator solution yield final concentrations of 125 µmol/L for each metal ion and 250 µmol/L for the chelating agent.

2.3 Spectrophotometric determination of complex formation

The stabilization of any charge-transfer complex accompanies the transferring of electrical charges between electron-donor and -acceptor (e.g. metal ions and ligands). This electron distribution change gives rise to the creation of charge-transfer bands – which are optical absorption bands – that are detectable through ultraviolet–visible (UV/Vis) spectroscopy. So technically, complex formation can be monitored through spectrophotometry: a shift in absorbance (spectrum change) should indicate that chelation occurred.

Spectra of metal ion complexes, as well as each individual metal ion and chelator, were examined with a Lambda 25 UV/Vis spectrophotometer (PerkinElmer, Waltham, USA) and a 10 mm high precision cell made of special optical glass (Hellma Analytics, Jena, Germany), in a wavelength range of 200 – 800 nm (full spectrum).

2.4 Determination of complex filterability and metal concentration

The filterability of metal complexes was tested by pushing each metal-chelator mixture through a microfilter (0.2 µm) and analysing the filtrate for residual metal with inductively coupled plasma-optical emission spectroscopy (ICP-OES). For the latter, an Avio 200 spectrometer (PerkinElmer, Rodgau, Germany) was employed with following instrumental parameters (Table 1).

The analytical wavelengths used for the determination of each metal ion are stated in table 2. A seven-point matrix-matched calibration curve, ranging from 0 to 300 µmol/L for each individual metal ion, was used to quantify the sample concentrations ($r^2 > 0.99$).

2.5 Main methodology

In order to expand upon the current knowledge of metal chelation in wort and beer, we tested nine food-grade chelators (EDTA, citric acid, tartaric acid, quercetin, chlorogenic acid, ferulic acid, gallic

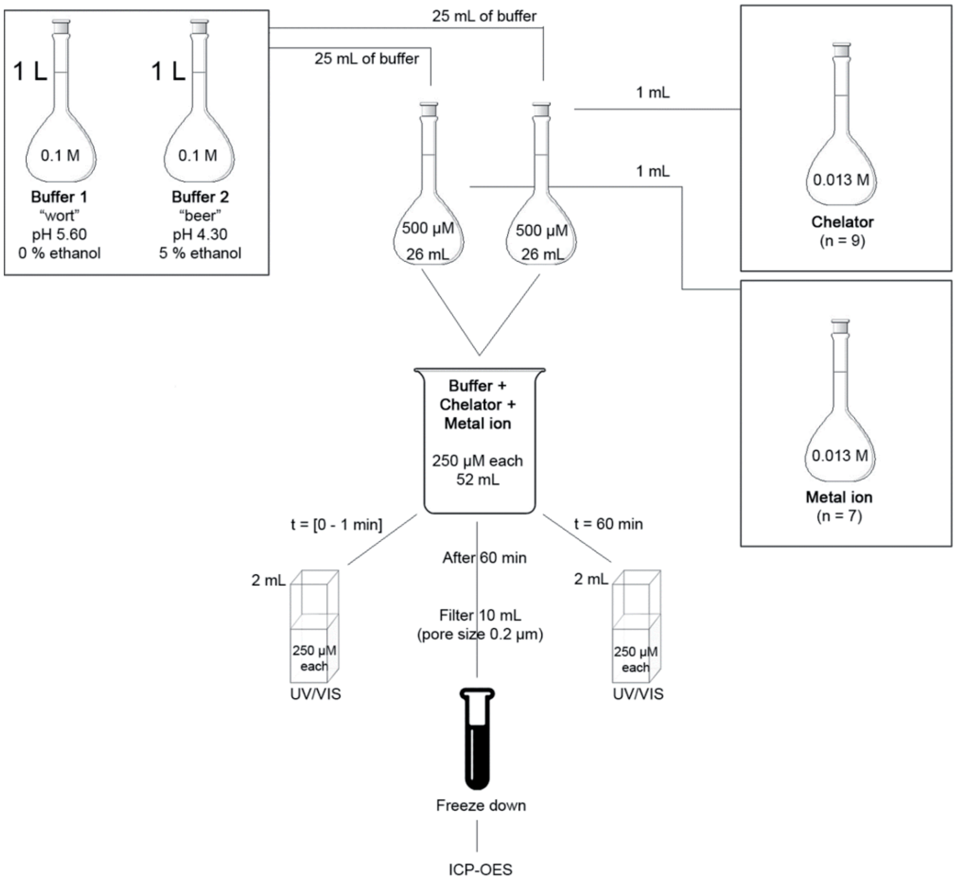

Fig. 1 Experimental setup for the measurement of complex formation and the filterability

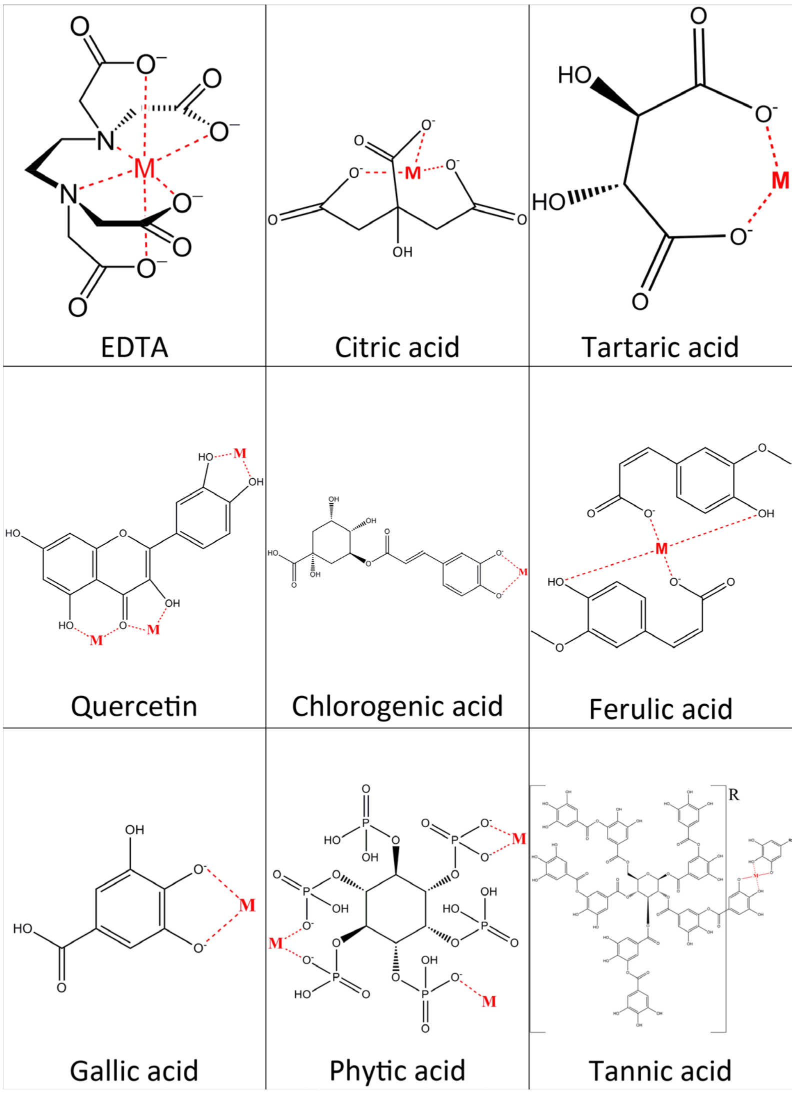

Fig. 2 Chemical structures and possible chelation mechanisms of all tested chelators

acid, phytic acid, and tannic acid) on their capacity to form filterable complexes with seven metal ions (Fe^{II}, Fe^{III}, Cu^{II}, Mn^{II}, Ca^{II}, Mg^{II}, and Zn^{II}) in both ‘wort’ and ‘beer’ buffer solutions (buffer 1 and buffer 2, respectively). Likewise, each chelator was also subjected to a mix of these metals to check the binding affinities – chelators bind metals in order of decreasing complex-stability constants – and performance consistency – whether similar efficiencies to sole metal ions are seen.

As depicted in figure 1, both metal and chelator solution were transferred simultaneously into a beaker and mixed. Molar concentrations were 250 µmol/L for each, entailing that chelator:metal-ratio was always 1:1. The only exception being the trials with metal ion mix, which had final molar concentrations of 125 µmol/L for each of the six different metal ions and 250 µmol/L for the chelator. All samples were carried out in duplicate; except for the blanks (no chelator added), which were conducted in triplicate.

The chelator-metal mixtures were analysed by spectrophotometer: once at the beginning (within 1 minute from mixing) and once at the ‘end’ (60 minutes from mixing). Separate absorbance spectra of the (blank) buffer solutions, chelators, and metal ions were also collected.

After that, the chelator-metal mixtures were filtered through a syringe filter with 0.2 µm cellulose acetate membrane (VWR International, Radnor, USA) and the filtrates analysed for residual metal ions by ICP-OES. All syringes (Henke-Sass Wolf, Tuttlingen, Germany) and tubes (VWR International, Radnor, USA) were single-use and metal-free.

3 Results & Discussion

In order to find compounds with the potential of slowing down radical formation in beer and brewing, a multitude of chelators was screened on two key features: Whether the chelator forms complexes with the metal ion(s) in question and, if so, whether these complexes are large enough to be effectively filtered out. Figure 2 displays the chemical structures and possible chelation mechanisms of all tested chelators. A quick and easy experimental setup allowed for the numerous combinations to be explored.

Due to the high variability and extreme complexity of beer and wort matrices, acetate buffer solutions were used. Using buffers negate any unwanted interferences coming from endogenous beer/wort components, such as naturally present chelators, metal ions, colour, etc.

Some metal-chelator combinations produced distinct colour formations, visible to the naked eye (Table 3); meaning that complex formation indisputably occurred.

UV/Vis spectrophotometry was used to more accurately detect whether complexes were formed between metal and chelator. The findings can be found in table 4 (see page 62). The table only addresses buffer 1 (‘wort’), but the spectrum changes generally

Table 3 Combinations leading to visibly coloured complex formations

	Quercetin		Chlorogenic acid		Gallic acid		Tannic acid	
	pH 4.30	pH 5.60	pH 4.30	pH 5.60	pH 4.30	pH 5.60	pH 4.30	pH 5.60
Fe(II)	–	Green	–	Dark Green	–	Dark purple	Purple	Dark purple
Fe(III)	Green	Dark green	Green	Dark green	Dark purple	Purple	Dark purple	Purple
Cu(II)	–	–	–	White (cloudy)	–	–	–	White (cloudy)

Table 4 **Complex formation – study findings and percentage of bound metal ion (as calculated by Visual MINTEQ software) in ‘wort’ buffer 1 (pH 5.60)**

Cation	Chelator									
	No chelator*	EDTA	Citric acid	Tartaric acid	Quercetin	Chloro-genic acid	Ferulic acid	Gallic acid	Phytic acid	Tannic acid
Fe(II)	(49.2)	99.8 (0.1)	56.5 (21.4)	3.3 (47.5)	56.6 (21.2)	0.0 (49.1)	–	28.8 (35.0)	5.1 (45.6)	99.8 (0.1)
Fe(III)	(100)[a]	100 (0.0)	90.4 (9.6)[b]	0.5 (99.5)[c]	100 (0.0)	83.7 (16.3)[d]	8.6 (91.4)[e]	100 (0.0)	–	99.4 (0.6)[f]
Ca(II)	(37.3)	91.6 (3.2)	21.9 (29.1)	2.1 (36.5)	0.0 (37.0)	–	–	63.3 (13.7)	0.0 (36.2)	–
Zn(II)	(62.1)	100 (0.0)	58.1 (26.0)	5.1 (58.9)	0.0 (61.8)	–	0.0 (62.1)	81.3 (11.6)	97.5 (1.5)	0.1 (62.1)
Mg(II)	(41.7)	47.3 (22.0)	21.2 (32.8)	0.7 (41.3)	0.0 (41.4)	–	–	28.5 (29.8)	0.0 (40.6)	–
Mn(II)	(49.3)	99.7 (0.1)	27.0 (35.9)	5.8 (46.3)	–	–	–	0.0 (49.2)	98.8 (0.6)	0.1 (49.2)
Cu(II)	(93.1)[g]	100 (0.0)	79.8 (18.8)[h]	3.3 (90.0)[i]	–	0.0 (93.1)[j]	10.3 (83.5)[k]	95.8 (3.9)[l]	0.0 (92.7)[m]	–

Grey cells imply substantial UV/Vis absorbance changes were seen (indicating complex formation occurred).

The primary value indicates the percentage of chelator-bound metal. The secondary value (in brackets) indicates the percentage of metal bound to other species present (acetate, hydroxide). Even though these latter complexes are weak (log k < 2; specified if otherwise), the metal ions are technically not considered free.

Vacant cells (–) are due to lacking component information.

* In the ‘no chelator’ column, the buffer’s acetate operates as the active chelator.

$[Fe(acetate)_2]^+$ (log K 7.6), $[Fe(acetate)_3]^0$ (log K 9.6) and $[Fe(OH)_2]^+$ (log K -5.8): [a] 11.9 %, 68.2 %, 19.9 %; [b] 1.1 %, 6.6 %, 1.9 %; [c] 11.9 %, 0.6 %, 19.8 %; [d] 1.9 %, 11.1 %, 3.2 %; [e] 10.9 %, 62.3 %, 18.2 %; [f] 0.1 %, 0.4 %, 0.1 %

$[Cu(acetate)_3]^-$ (log K 3.9), $[Cu(acetate)]^+$ (log K 2.2) and $[Cu(acetate)_2]^0$ (log K 3.4): [g] 11.0 %, 43.9 %, 38.2 %; [h] 2.2 %, 8.9 %, 7.7 %; [i] 10.6 %, 42.5 %, 36.9 %; [j] 11.0 %, 43.9 %, 38.2 %; [k] 9.8 %, 39.4 %, 34.3 %; [l] 0.5 %, 1.8 %, 1.6 %; [m] 10.8 %, 44.3 %, 37.6 %

Table 5 **Residual metal content (µmol/L) of metal-chelator mixtures after 60 minutes reaction time at room temperature and filtration with a 0.2 µm filter**

Metal ion added (250 µmol/L)	pH	Chelator added (250 µmol/L)									
		No chelator	**EDTA**	**Citric acid**	**Tartaric acid**	**Quercetin**	**Chlorogenic acid**	**Ferulic acid**	**Gallic acid**	**Phytic acid**	**Tannic acid**
Fe(II)	4.30	267.5	259.5	253.9	249.4	239.5	164.6	264.4	236.8	223.1	220.8
	5.60	175.6	243.2	224.6	245.5	114.9	238.6	159.1	251.4	245.6	10.6
Fe(III)	4.30	241.1	232.6	236.5	224.6	186.6	213.0	229.9	213.6	215.2	182.2
	5.60	149.5	168.7	207.1	194.7	5.7	14.6	132.1	40.9	211.0	4.8
Ca(II)	4.30	244.2	218.0	231.4	231.8	247.7	262.2	264.5	216.2	308.5	218.5
	5.60	237.4	200.6	204.5	208.7	237.8	232.9	246.7	197.9	262.9	195.2
Zn(II)	4.30	270.3	267.5	276.2	276.2	235.3	237.8	234.5	264.2	131.9	269.4
	5.60	226.9	212.9	228.2	228.4	229.2	211.9	224.4	222.5	67.3	215.3
Mg(II)	4.30	233.4	214.1	221.3	219.7	223.9	231.6	217.6	212.6	288.4	216.8
	5.60	213.2	240.4	202.0	203.5	211.6	204.7	204.8	197.7	241.6	206.1
Mn(II)	4.30	268.5	238.7	251.6	251.0	244.8	238.3	277.7	238.2	329.9	240.0
	5.60	241.3	207.5	196.8	201.4	233.0	221.8	232.5	186.2	232.2	227.1
Cu(II)	4.30	251.8	229.8	239.7	236.6	225.0	225.8	215.9	229.1	214.4	219.2
	5.60	239.1	198.7	190.6	225.3	233.3	238.1	245.4	143.7	219.8	4.1

The larger the decrease in metal ion concentration after filtration, the more intensely shaded the cell.

did not differ greatly between the two buffers. The represented values were calculated by using chemical equilibrium modelling software (Visual MINTEQ version 3.1). The data depict the (predicted) percentages of metal ion bound to any given chelator in buffer 1 (pH 5.60). Predictive calculations could not be made for all metal-chelator combinations, because of lacking stability constants published in literature for some.

According to the results depicted in table 4, all investigated iron species easily form complexes with all tested chelators (cells highlighted in grey). Even though the calculated values for tartaric acid with iron predict only very low percentages of tartrate-bound Fe, we do observe a clear spectrum change. The opposite effect is also seen: high calculated ratios of bound metal, yet no noted absorbance changes. Thus, at least for this setup, it seems dif-

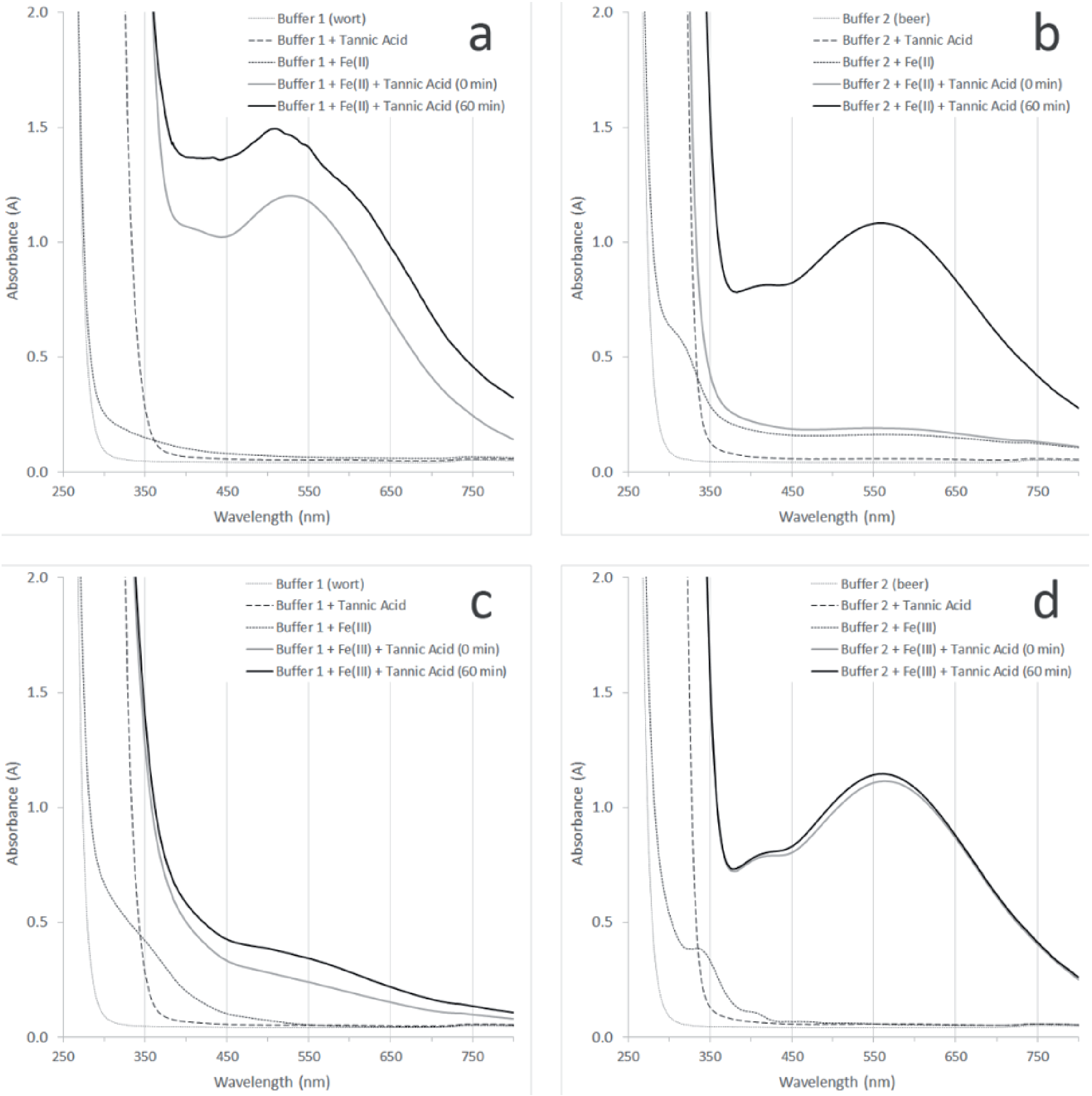

Fig. 3 Absorbance spectra of tannic acid with Fe(II) in 'wort' buffer 1 (a) & 'beer' buffer 2 (b); and Fe(III) in 'wort' buffer 1 (c) & 'beer' buffer 2 (d)

ficult to draw a hard line between complex formation and a clear change in absorbance.

Additionally, and unfortunately, it appeared that in many cases the chelator-metal complexes were small enough to allow for filter penetration, given that often no substantial metal decrease could be noticed after filtration. Nevertheless, as said, even unfilterable/small complexes could have anti-oxidative properties, as the transition metals could be bound in such a way that they no longer behave pro-oxidatively. This potential should not be dismissed. For this study, it was decided not to focus on this aspect. However, follow-up studies will further investigate the chelation behaviour of some of these chelators in wort during mashing; and electron spin resonance (ESR) spectroscopy will be employed to look deeper into their anti/pro-oxidative effects.

We could deduct, however, that decreases in residual metal after filtration (Table 5: shaded cells) are always accompanied with big changes in UV/Vis absorbance (e.g. Fig. 3a and 3c). This only goes one way, because – like already explained – some formed complexes will be small enough to permeate the microfilter. Thus, an obvious absorbance change is no guarantee that a decrease of the particular metal after filtration will take place (e.g. Fig. 3b and 3d).

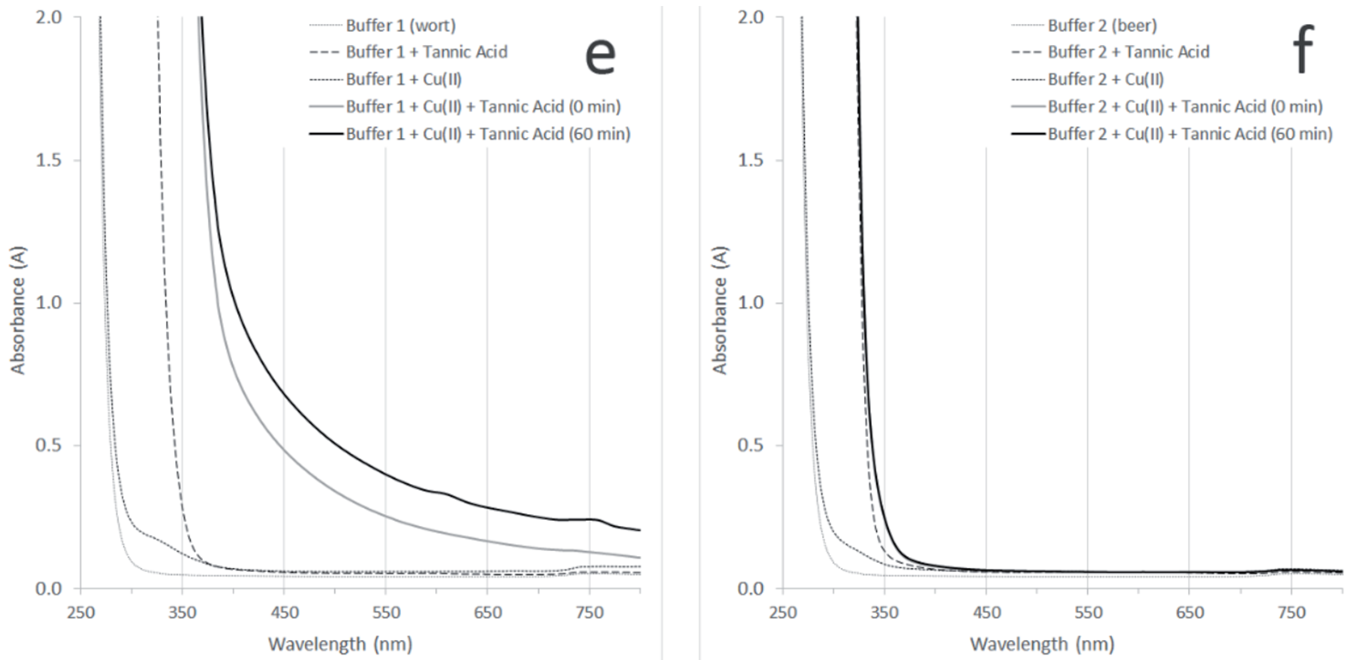

Fig. 4 **Absorbance spectra of tannic acid with Cu(II) in ‘wort’ buffer 1 (e) & ‘beer’ buffer 2 (f)**

Table 6 **Residual metal content (in µmol/L) of metals-chelator mixtures after 60 minutes reaction time at room temperature and filtration with a 0.2 µm filter**

Metal ion added (125 µmol/L)		pH	Chelator added (250 µmol/L)									
			No chelator	**EDTA**	**Citric acid**	**Tartaric acid**	**Quercetin**	**Chlorogenic acid**	**Ferulic acid**	**Gallic acid**	**Phytic acid**	**Tannic acid**
Mix	Fe(II)	4.30	116.6	118.1	119.0	115.0	95.5	104.2	93.2	114.3	42.6	99.8
	Ca(II)		121.6	128.9	129.0	126.1	106.8	158.8	105.4	126.4	121.7	133.6
	Zn(II)		114.8	116.0	114.6	115.3	107.5	121.8	103.6	112.2	80.0	120.0
	Mg(II)		121.2	119.2	122.8	121.4	109.7	141.2	106.6	124.9	125.8	125.2
	Mn(II)		116.3	117.5	116.9	114.8	104.5	141.9	102.1	116.8	100.8	122.5
	Cu(II)		117.3	117.8	117.7	108.9	109.5	119.8	122.6	105.6	93.5	127.6
Mix	Fe(II)	5.60	89.0	122.5	138.9	137.8	45.8	84.7	71.1	110.6	15.1	15.9
	Ca(II)		91.7	95.7	110.7	97.1	82.9	83.0	90.2	100.9	61.2	97.2
	Zn(II)		126.0	122.1	122.9	124.3	122.5	119.9	124.2	125.7	17.2	92.6
	Mg(II)		127.6	125.3	123.5	128.1	118.9	122.7	135.0	143.3	136.3	155.4
	Mn(II)		129.3	124.9	124.3	127.9	124.5	123.7	129.7	135.3	68.0	133.8
	Cu(II)		127.6	125.5	123.5	125.4	116.3	114.8	127.1	117.0	63.5	13.4

The larger the decrease in metal ion concentration after filtration, the more intensely shaded the cell.

Copper behaves very similarly to iron with tannic acid, in terms of diminishment after filtration (see Table 5: big reduction at pH 5.60 and significantly less at pH 4.30). However, when comparing the absorbances, copper behaves differently than both iron ions. Whereas a clear signal change is seen at pH 5.60 (Fig. 4e), one does not take place at pH 4.30 (Fig. 4f).

Concerning the question on whether the formed complexes are large enough to be filtered out: table 5 depicts all the sole metal ion ICP-OES results, featuring the mean residual metal ion concentrations (in µmol/) found in the filtrate, after running the metal-chelator mixtures through a 0.2 µm filter. Table 6 presents the results of the metal ion mix.

The first column shows the quantified metal levels without any addition of chelator. Ideally, these values should all deviate around 250 µmol/l, which seems to generally be the case (considering also the varying purities of the used chemicals). However, for both Fe-species, values appeared noticeably lower at pH 5.60. This can be explained due to formation iron-hydroxides [13, 25, 26]. With Visual MINTEQ, it was calculated that – in absence of chelators and under buffer 1 (pH 5.60) conditions – 19.9 % of filterable $[Fe(OH)_2]^+$ is formed, but only for Fe^{III} (0.0 % for Fe^{II}). It thus seems that Fe^{II} is capable of oxidizing to Fe^{III} under these conditions – albeit slowly [27, 28].

This precipitation of iron-hydroxides does not happen as considerably in acidic media or when a chelator is present [13, 29, 30].

This may be the reason why Fe^{II} is the most commonly found iron-species in beer [31, 32], as it is low in pH and contains endogenous chelators. Although wort is less acidic than beer, iron-hydroxide formation is unlikely, because of many chelators naturally being present.

EDTA makes strong complexes with Fe, Ca, Zn, Mg, Mn, Cu [33, 34], which is also seen from the calculations in table 4. However, the EDTA-complexes are small enough to penetrate the filter, which is the reason why we see only small drops in metal levels after filtration.

There are no noteworthy metal decreases with citric and tartaric acid. Good iron-catching compounds are quercetin (especially Fe^{III}) [35], chlorogenic acid (ditto) [36, 37], ferulic acid [38], gallic acid (solely Fe^{III}) [39, 40], and tannic acid [41, 42]. The latter performs extraordinarily well for both iron species and copper [41–43]. Gallic acid also chelates copper, but not as effectively [44]. Judging from these results, tannic acid seems the most promising compound in terms of large complex-making capability. This is consistent with tannic acid possessing multiple phenolic hydroxyl groups (Fig. 2), which allows for copious metal-chelator cross-links. Phytic acid is the only chelator binding with zinc in a way that it becomes filterable [45, 46]. As zinc is important for yeast health [47], this would be unfavourable in brewing practice. Edney and team made worts from different low-phytate barleys and saw significantly higher levels of zinc and magnesium present [48]. Because of this mineral absorption tendency, phytic acid is sometimes referred to as an anti-nutrient.

In table 5, we also behold a clear connection between a more alkaline pH and the forming of better/larger complexes (with chlorogenic acid as the sole exception, because of its low pK_a, and only for Fe^{II}). pH has a big impact on chelation behaviour, as it determines the ionic species of the chelating agent (a chelator must be ionized to be active) as well as the speciation and hydrolysis products of the metal ions. Complexes are less stable in acidic systems, due to protonation of ligand functional groups; and the protons will compete with the metal ions for binding. Thus, in terms of pH, wort is generally a better environment for complex formation than beer [13, 17, 49].

Unfortunately, manganese did not form filterable complexes with any of the chelators. In contrast to the other transition metals, Mn^{II} is known for its rather weak ability to form organic complexes [50, 51]. However, in the presence of other metal ions (mix), a reduction in Mn by phytic acid did take place (Table 6).

Phytic acid is also the only chelator that shows highly erratic behaviour compared to what is seen with the single metal ions (Table 5). Whereas most chelators retain (e.g. tannic acid) or even lose (e.g. gallic acid) some of their catching potential in the metal ion mix, phytic acid suddenly exceeds in capturing Fe, Ca, Zn, Mn, and Cu (especially at pH 5.60). On suspicion that Zn^{II} played an important role in this, a follow-up chelation test was conducted with zinc being left out of the ion mix (data not shown). The results, however, were not significantly different from what was seen with the mix including zinc; except for the Ca^{II} being noticeably higher. That observation, together with the fact that calcium is known to form stable bridge structures with phosphates [52–54], makes calcium the likely cause of the decreased dialysability seen with the metal ion mix and phytic acid.

4 Conclusions

Nine compounds (EDTA, citric acid, tartaric acid, quercetin, chlorogenic acid, ferulic acid, gallic acid, phytic acid, and tannic acid) were screened for their complexing capacities with seven metal ions (Fe^{II}, Fe^{III}, Cu^{II}, Mn^{II}, Ca^{II}, Zn^{II}, Mg^{II}; both separately and mixed) in both a ‘wort’ and ‘beer’ buffer solution (pH 5.60, 0.0 vol% ethanol and pH 4.30, 5.0 vol% ethanol, respectively). The aim was to find compounds that can aid brewers in producing a more flavour stable beer, by lowering the stale-inducing transition metals.

Amongst the tested chelators, there is a definite pH effect where they seem to make larger complexes (as appraised by filterability of the complexes) with metal ions at pH 5.60 (the approximate acidity of wort) than at 4.30 (the approximate acidity of beer).

Most chelators do not cause a decrease in the beneficial metal ions (Ca, Zn, Mg), with phytic acid being the exception by chelating i.a. zinc. Tannic acid is by far the best performing chelator in terms of making large complexes with both Fe^{II}, Fe^{III} and Cu^{II}. And although in this study it primarily shows this behaviour at the wort pH of 5.60, filtration trials have shown that tannic acid is able to form iron-complexes in finished beer as well [42].

Iron reducing effects were also witnessed with chelators like quercetin, chlorogenic acid, and gallic acid. The last one, however, did not bind Fe^{II}. Apart from tannic acid, gallic acid was also the only compound capable of chelating out copper (albeit to a lesser extent). This is in line with tannic acid being composed out of eight to nine gallic acid molecules, bound to a central glucose [55].

As mentioned, almost any concentration decrease was witnessed at pH 5.60. Noticeable exceptions were chlorogenic acid, which succeeds in binding Fe^{II} at the more acidic pH of 4.30, and phytic acid with zinc. Manganese is, unfortunately, never significantly removed by any of the tested chelators. The best effect is seen with gallic acid. This stability of manganese is also seen in the brewing process, where it is found in the final beer at higher concentrations, relative to the other heavy metals (such as iron and copper), which are more readily removed during wort boiling and fermentation [56].

In the metal ion mix trial, gallic acid does not chelate copper anymore, chlorogenic acid chelates Fe^{II} slightly better, and tannic acid takes away some zinc; all at pH 5.60. Apart from that, phytic acid is the only chelator that is acting remarkably differently compared to its chelating behaviour seen with the single metal ions, suddenly chelating out every metal ion except for Mg^{II}. Calcium is thought to lie at the basis of this effect. These results are in accordance with how phytic acid behaves in more complex food systems [57, 58].

The high tendency of tannic acid to form large, precipitating complexes with both iron and copper ions could be used to remove these pro-oxidative metals from wort (and beer). It is highly likely for the resulting beer to achieve an improved oxidative stability, which would benefit brewers and consumers alike. Hence, follow-up studies will be conducted to check whether the complexing tendencies of the most successful chelators can be replicated in wort during mashing, and how we can potentially optimize their chelation towards catalytic transition metals.

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5 References

1. Preedy, V.R.: Beer in Health and Disease Prevention, Elsevier, 2008.
2. Aquilani, B.; Laureti, T.; Poponi, S. and Secondi, L.: Beer choice and consumption determinants when craft beers are tasted: An exploratory study of consumer preferences, Food Quality and Preference, **41** (2015), pp. 214-224.
3. Andersen, M.L.; Outtrup, H. and Skibsted, L.H.: Potential antioxidants in beer assessed by ESR spin trapping, Journal of Agricultural and Food Chemistry, **48** (2000), no. 8, pp. 3106-3111.
4. 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.
5. Kuchel, L.; Brody, A.L. and Wicker, L.: Oxygen and its reactions in beer, Packaging Technology and Science, **19** (2006), no. 1, pp. 25-32.
6. Fridovich, I.: Oxygen: How Do We Stand It?, Medical Principles and Practice, **22** (2013), no. 2, pp. 131-137.
7. Kaneda, H.; Kobayashi, N.; Takashio, M.; Tamaki, T. and Shinotsuka, K.: Beer Staling Mechanism, Master Brewers Association of the Americas Technical Quarterly, **36** (1999), no. 1, pp. 41-47.
8. Miller, D.M.; Buettner, G.R. and Aust, S.D.: Transition metals as catalysts of 'autoxidation' reactions, Free Radical Biology and Medicine, **8** (1990), no. 1, pp. 95-108.
9. 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.
10. Porter, J.R. and Bamforth, C.W.: NOTE: 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.
11. Wietstock, P.C. and Shellhammer, T.H.: Chelating Properties and Hydroxyl-Scavenging Activities of Hop α- and Iso-α-Acids, Journal of the American Society of Brewing Chemists, **69** (2011), no. 3, pp. 133-138.
12. Wietstock, P.C.; Baldus, M.; Öhlschläger, M. and Methner, F.: Hop Constituents Suppress the Formation of 3-Methylbutanal and 2-Furfural in Wort-Like Model Solutions, Journal of the American Society of Brewing Chemists, **75** (2017), no. 1, pp. 41-51.
13. Wietstock, P.C.; Kunz, T.; Pereira, F. and Methner, F.-J.: Metal Chelation Behavior of Hop Acids in Buffered Model Systems, BrewingScience, **69** (2016), no. 9/10, pp. 56-63.
14. Kunz, T.; Frenzel, J.; Wietstock, P.C. and Methner, F.-J.: Possibilities to improve the antioxidative capacity of beer by optimized hopping regimes, Journal of the Institute of Brewing, **120** (2014), no. 4, pp. 415-425.
15. Wietstock, P.C.; Kunz, T. and Methner, F.-J.: Influence of Hopping Technology on Oxidative Stability and Staling-Related Carbonyls in Pale Lager Beer, BrewingScience, **69** (2016), no. 11/12, pp. 73-84.
16. Plavšić, M.; Ćosović, B. and Lee, C.: Copper complexing properties of melanoidins and marine humic material, Science of The Total Environment, **366** (2006), no. 1, pp. 310-319.
17. Jacobsen, T. and Lie, S.: Metal binding in wort – an evaluation of practical stability constants, EBC Proceedings of the European Brewery Convention Congress, Nürnberg, Germany, 1979, pp. 117-129.
18. Aron, P.M.: The Effect of Hopping Technology on Lager Beer Flavor and Flavor Stability and the Impact of Polyphenols on Lager Beer Flavor and Physical Stability, 2011.
19. Wannenmacher, J.; Gastl, M. and Becker, T.: Phenolic Substances in Beer: Structural Diversity, Reactive Potential and Relevance for Brewing Process and Beer Quality, Comprehensive Reviews in Food Science and Food Safety, **17** (2018), no. 4, pp. 953-988.
20. Buettner, G.R.: The Pecking Order of Free Radicals and Antioxidants: Lipid Peroxidation, α-Tocopherol, and Ascorbate, Archives of Biochemistry and Biophysics, **300** (1993), no. 2, pp. 535-543.
21. Buettner, G.R. and Jurkiewicz, B.A.: Catalytic Metals, Ascorbate and Free Radicals: Combinations to Avoid, Radiation Research, **145** (1996), no. 5, pp. 532-541.
22. Baron, C.P.; Refsgaard, H.H.F.; Skibsted, L.H. and Andersen, M.L.: Oxidation of bovine serum albumin initiated by the Fenton reaction--effect of EDTA, tert-butylhydroperoxide and tetrahydrofuran., Free radical research, **40** (2006), no. 4, pp. 409-17.
23. Gutteridge, J.M.C.: Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate., Biochemical Journal, **224** (1984), no. 3, pp. 761-767.
24. Dwyer, F.P. and Mellor, D.P.: Chelating Agents and Metal Chelates, Elsevier, 1964.
25. Garrido-Ramírez, E.G.; Theng, B.K.G. and Mora, M.L.: Clays and oxide minerals as catalysts and nanocatalysts in Fenton-like reactions - A review, Applied Clay Science, **47** (2010), no. 3-4, pp. 182-192.
26. Khoe, G.H.; Brown, P.L.; Sylva, R.N. and Robins, R.G.: The hydrolysis of metal ions. Part 9. Iron(III) in perchlorate, nitrate, and chloride media (1 mol dm^{-3}), Journal of the Chemical Society, Dalton Transactions, (1986), no. 9, pp. 1901-1906.
27. Cornell, R.M. and Schwertmann, U.: The Iron Oxides, Wiley, Weinheim, FRG, 2003.
28. Welch, K.D.; Davis, T.Z. and Aust, S.D.: Iron Autoxidation and Free Radical Generation: Effects of Buffers, Ligands, and Chelators, Archives of Biochemistry and Biophysics, **397** (2002), no. 2, pp. 360-369.
29. Richter, G.W. and Solez, K.: Transition Metal Toxicity, Academic Press, 1990.
30. Li, Y.; Bachas, L.G. and Bhattacharyya, D.: Selected Chloro-Organic Detoxifications by Polychelate (Poly(acrylic acid)) and Citrate-Based Fenton Reaction at Neutral pH Environment, Industrial & Engineering Chemistry Research, **46** (2007), no. 24, pp. 7984-7992.
31. Khalafi, L.; Doolittle, P. and Wright, J.: Speciation and Determination of Low Concentration of Iron in Beer Samples by Cloud Point Extraction, Journal of Chemical Education, **95** (2018), no. 3, pp. 463-467.
32. Filik, H. and Giray, D.: Cloud point extraction for speciation of iron in beer samples by spectrophotometry, Food Chemistry, **130** (2012), no. 1, pp. 209-213.
33. Norvell, W.A. and Lindsay, W.L.: Reactions of EDTA Complexes of Fe, Zn, Mn, and Cu with Soils1, Soil Science Society of America Journal,

33 (1969), no. 1, p. 86.

34. Hart, J.R.: Ethylenediaminetetraacetic Acid and Related Chelating Agents, Ullmann's Encyclopedia of Industrial Chemistry, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2011.
35. Hajji, H. El; Nkhili, E.; Tomao, V. and Dangles, O.: Interactions of quercetin with iron and copper ions: Complexation and autoxidation, Free Radical Research, **40** (2006), no. 3, pp. 303-320.
36. Hynes, M.J. and O'Coinceanainn, M.: The kinetics and mechanisms of reactions of iron(III) with caffeic acid, chlorogenic acid, sinapic acid, ferulic acid and naringin, Journal of Inorganic Biochemistry, **98** (2004), no. 8, pp. 1457-1464.
37. Andjelkovic, M.; Van Camp, J.; De Meulenaer, B.; Depaemelaere, G.; Socaciu, C.; Verloo, M. and Verhe, R.: Iron-chelation properties of phenolic acids bearing catechol and galloyl groups, Food Chemistry, **98** (2006), no. 1, pp. 23-31.
38. Das, U.; Manna, K.; Khan, A.; Sinha, M.; Biswas, S.; Sengupta, A.; Chakraborty, A. and Dey, S.: Ferulic acid (FA) abrogates γ-radiation induced oxidative stress and DNA damage by up-regulating nuclear translocation of Nrf2 and activation of NHEJ pathway, Free Radical Research, **51** (2017), no. 1, pp. 47-63.
39. Powell, H. and Taylor, M.: Interactions of iron(II) and iron(III) with gallic acid and its homologues: a potentiometric and spectrophotometric study, Australian Journal of Chemistry, **35** (1982), no. 4, p. 739.
40. Fazary, A.E.; Taha, M. and Ju, Y.-H.: Iron Complexation Studies of Gallic Acid, Journal of Chemical & Engineering Data, **54** (2009), no. 1, pp. 35-42.
41. Hem, J.D.: Complexes of Ferrous Iron With Tannic Acid, Water Supply Paper, **1459** (1960), no. D, pp. 75-94.
42. Formanek, J.A. and Bonte, P.: Use of Tannic Acid in the Brewing Industry for Colloidal and Organoleptic Stability, Technical Quarterly, **54** (2017), no. 1, pp. 11-16.
43. Andrade, R.G.; Dalvi, L.T.; Silva, J.M.C.; Lopes, G.K.B.; Alonso, A. and Hermes-Lima, M.: The antioxidant effect of tannic acid on the in vitro copper-mediated formation of free radicals, Archives of Biochemistry and Biophysics, **437** (2005), no. 1, pp. 1-9.
44. Nenadis, N.; Lazaridou, O. and Tsimidou, M.Z.: Use of Reference Compounds in Antioxidant Activity Assessment, Journal of Agricultural and Food Chemistry, **55** (2007), no. 14, pp. 5452-5460.
45. Crea, F.; De Stefano, C.; Milea, D. and Sammartano, S.: Speciation of phytate ion in aqueous solution. Thermodynamic parameters for zinc(II) sequestration at different ionic strengths and temperatures, Journal of Solution Chemistry, **38** (2009), no. 1, pp. 115-134.
46. Crea, F.; De Stefano, C.; Milea, D. and Sammartano, S.: Formation and stability of phytate complexes in solution, Coordination Chemistry Reviews, **252** (2008), no. 10-11, pp. 1108-1120.
47. Stehlik-Tomas, V.; Zetic, V. and Stanzer, D.: Zinc, copper and manganese enrichment in yeast *Saccharomyces cerevisae*, Food Technology and Biotechnology, **42** (2004), no. 2, pp. 115-120.
48. Edney, M.J.: Effect of Low-Phytate Barley on Malt Quality, Including Mineral Loss, During Fermentation, Journal of the American Society of Brewing Chemists, **65** (2007), no. 2, pp. 81-85.
49. Catapano, M.; Tvrdý, V.; Karlíčková, J.; Migkos, T.; Valentová, K.; Křen, V. and Mladěnka, P.: The Stoichiometry of Isoquercitrin Complex with Iron or Copper Is Highly Dependent on Experimental Conditions, Nutrients, **9** (2017), no. 11, p. 1193.
50. 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.
51. Pohl, P.: Determination and fractionation of metals in beer: A review, Food Additives & Contaminants: Part A, **25** (2008), no. 6, pp. 693-703.
52. Odani, A.; Takamido, R. and Yamauchi, O.: Phytate, an environmental phosphate from grain source. Metal complex formation and degradation by phytase, Journal of Inorganic Biochemistry, **67** (1997), no. 1-4, p. 378.
53. Andrews, M.; Briones, L.; Jaramillo, A.; Pizarro, F. and Arredondo, M.: Effect of calcium, tannic acid, phytic acid and pectin over iron uptake in an in vitro Caco-2 cell model, Biological Trace Element Research, **158** (2014), no. 1, pp. 122-127.
54. Trela, B.C.: Iron stabilization with phytic acid in model wine and wine, American Journal of Enology and Viticulture, **61** (2010), no. 2, pp. 253-259.
55. Siebert, K.J. and Lynn, P.Y.: Effect of Protein-Polyphenol Ratio on the Size of Haze Particles, Journal of the American Society of Brewing Chemists, **58** (2000), no. 3, pp. 117-123.
56. 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.
57. Cheryan, M. and Rackis, J.J.: Phytic acid interactions in food systems, C R C Critical Reviews in Food Science and Nutrition, **13** (1980), no. 4, pp. 297-335.
58. Wanasundara, P.K.J.P.D. and Shahidi, F.: Process-Induced Compositional Changes of Flaxseed, 1998, pp. 307-325.

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