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Metal Chelation Behavior of Hop Acids in Buffered Model Systems

As part of the importance of metal ions for the brewing process and continuing interest in the hop acids' interactions with metal ions, the complexation behavior of hop acids towards different metal ions was investigated. Hop α-acids were shown to be capable of forming complexes with Cu^{2+} and Fe^{2+} ions and showed no complexation of Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} as tested using UV-VIS spectroscopy in solutions at an ionic strength of 0.1 mol dm-3 and containing 10 % (v/v) ethanol. In separate trials, Fe^{2+} was found to be capable of binding 3 mol α-acid at pH 4.3 and pH 5.5, and 2 mol α-acid at pH 6.2 and pH 8.2 while α-acids, in turn, were only able to bind one mol Fe^{2+}. Cu^{2+}, Fe^{2+} and Fe^{3+} ions could be removed from solutions by complexation with hop α-acids, iso-α-acids, and β-acids and subsequent filtration using 0.45 µm cellulose membrane filters, while Ca^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} ions were not affected and remained in the solutions. The hop acids' capability to remove Cu^{2+} and Fe^{3+} was higher at higher pH values and was in the order α-acids > β-acids > iso-α-acids with the exception of Fe^{3+} and pH 4.3 where the iso-α-acids' effect was more pronounced than the β-acids'. For Fe^{2+}, the order was α-acids > iso-α-acids > β-acids. A mixture of all hop acids had the highest effect. The hop acids' complexation characteristic can be considered beneficial for wort or beer quality as 'unwanted' metal ions are affected while vital metal ions are not, which e.g. favors beer flavor stability.

Descriptors: Hop bitter acids, complexation, metal ions, UV-VIS, ICP-OES

1 Introduction

Humulones or hop α-acids ((6*S*)-3,5,6-Trihydroxy-2-(3-methylbutanoyl)-4,6-bis(3-methylbut-2-en-1-yl)cyclohexa-2,4-dien-1-one) and lupulones or hop β-acids (3,5-dihydroxy-2-(3-methylbutanoyl)-4,6,6-tris(3-methylbut-2-enyl)cyclohexa-2,4-dien-1-one) are organic acids which have their only origin in the resin of the hop plant (*Humulus lupulus*). Both possess cyclic β,β'-triketone moiety which is present in many biologically active components of medicinal plants. First evidence of these compounds was found in 1885 [1]. Isohumulones or iso-α-acids (3,4-Dihydroxy-5-(3-methylbut-2-enyl)-2-(3-methyl-1-oxobutyl)-4-(4-methyl-1-oxopent-3-enyl)-1-cyclopent-2-enone) are derivatives of humulones and are formed by an acyloin ring contraction when exposed to heat or to strong alkaline conditions [2]. The iso-α-acids' absolute configuration remained speculative over years but recently, Urban and co-workers [3] discovered that earlier proposed structures from *Alderweireldt* et al. [4] were incorrect and reported the correct absolute configuration of (+)-cis-*iso*-α-acids to be (4*S*, 5*R*).

Hop bitter acids are commonly used in the brewing industry because iso-α-acids contribute to beer bitterness as do derivatives of lupulones [5, 6]. Many in vivo and in vitro studies also attested the hop bitter acids health beneficial and antibacterial effects which makes their usage interesting for the pharmaceutical industry and for the food industry [7, 8]. For beer production, the hop bitter acids' effect is even broader than only providing bitterness as they contribute to the beer's foam stability [9], antimicrobial activity [10] and oxidative stability [11–15]. They e.g. act as mobile-carrier ionophores thus inhibiting the growth of beers-spoilage bacteria or help to protect beer against oxidative deterioration reactions [16]. Recently, investigators examined the hop acids' antioxidant behavior more detailed and found one possible explanation by which hop α-acids exert their activities is by complexing ferrous iron thus diminishing the activation of oxygen and its catalytic function in the Fenton reaction system [11, 14, 15]. In this context, modifications of the hopping technology were shown to directly impact the iron content in wort and beer [13]. Also the trans-isohumulones' ionophoric and therefore antibacterial properties were demonstrated to depend on their ability to complex metal ions [10, 16].

A large number of metal complexes have been studied with regards to their interesting and important characteristics. The nature, coordination and composition of metal ions and their ligands determine their chemical and physical properties and defined metal-ligand complexes can thus exhibit applications in clinical, analytical, and industrial processes. Studies on transition metal compounds of Schiff base ligands are reported to be of great significance due to their spectral properties and wide applications [17, 18] or are used as model molecules for biological oxygen carrier systems [19]. Furthermore, metal complexes can possess more active antibacterial properties [20, 21] or show greater antioxidant activity [15, 22, 23] than the single free ligands.

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Most metal ions are only present in traces in wort and beer but still have a considerable role for the brewing process and for the finished product as they influence the pH of mash, wort and beer, and are important yeast nutrients, affect foam stability, or are involved in flavor deterioration reactions [24]. There is a certain balance of metal ions being released in brewing by-products and brought in from the raw materials. Malt was revealed to be the biggest source of metal ions during beer production but ultimately, only little amounts 'survive' into the finished beer [25]. Studies from *Svendsen* and *Lund* [26] demonstrated that metal ions were mostly found as negatively or positively charged complexes at sizes of 4–12 kDa and they suggested that phenols or phytic acid may be the predominant free ligands.

The complexation of certain metal ions can be considered advantageous for the beer quality as e.g. flavor deterioration reactions are diminished [13, 15]. Copper ions were claimed to selectively complex volatile sulphur compounds thus affecting beer flavor [27]. Magnesium and calcium affect the isomerisation kinetics and configuration ratio of hop α-acids to cis- and trans-iso-α-acid [2] while chromium (III), copper (II), lead (II) and mercury (II) salts of α-acids showed no formation of iso-α-acids under heat exposure [28]. Aluminium was additionally found to have a positive effect on iso-α-acid formation during wort boiling conditions by *Jaskula* et al. [29]. Though, complexation reactions can also be unfavorable such as e.g. the complexation and precipitation of some metal ions may lower the concentration of important yeast nutrients [30, 31]. Non-complexed iron or copper ions may have a negative effect on beer quality because of their influence on oxygen activation by electron transfer and the catalyzed generation of reactive hydroxyl radicals by the Fenton-reaction system [32, 33]. Complexes of, in particular, ferrous iron ions with polyphenols and/or proteins (polyphenol-protein complexes) were proven to be partly responsible for the formation of chill-haze during storage of beer after the consumption of the endogenous antioxidative potential [34, 35].

Investigating the complexation behavior of organic molecules towards metal ions is difficult and several methods exist with partly ambiguous outcomes. The stability, type, and structure of complexes between ligands and metal ions can vary and is mostly dependent on the amount and coordination of the ligands' valence electrons and the metal ion. Flavonoids were demonstrated to be good iron chelators and their chelation sites were studied in detail by first-principle electronic structure calculations based on density functional theory (DFT). Three quercetine molecules were found to bind to one iron ion and the 3-hydroxyl-4-carbonyl group was the optimal chelation site for all flavonoids measured. *El Hajji* et al. [36] used UV-visible spectroscopy measurements and albeit found 1:1 complexes of quercetine and iron or copper ions in acidic and neutral solutions. Gallic acid, an organic acid which is inter alia found in hops as part of the tannin fraction, was investigated with regards to its complexing behavior towards ferric iron and binary 1:2 (Fe^{3+}:gallic acid) complexes were stated by *Fazary* et al. [37]. *Lu* et al. [38] however found a 1:1 complex when mixing ferrous iron with gallic acid. *Blanco* et al. [39] were the first to investigate the kinetics and thermodynamics of 2-acetyl-1,3-cyclohexanedione-Fe^{3+} complexes in 1:1 ratios and reported that both Fe^{3+} and its conjugated base, $Fe(H_2O)_5OH^{2+}$, can react in a reversible pathway with the enol tautomers of those hop-related ligands. In a similar study, *Hynes*, Blanco, and *Mooney* [40] also examined 2-acetyl-1,3-cyclohexanedione-Cu^{2+} complexes and found that copper can react with both the enol and the keto tautomer of the ligand.

As part of the importance of metal ions for the brewing process and continuing interest in the hop acids' interactions with metal ions, in this work, firstly, the complexation behavior of hop α-acids towards different metal ions was screened by measuring the hop α-acids' spectra changes after incubation with metal ions using UV-VIS spectroscopy. Additionally, hop α-acids, β-acids, and iso-α-acids were mixed with metal ions and prior to measuring residual metal ion concentrations in the solutions by inductively coupled plasma-optical emission spectroscopy (ICP-OES), potential complexes were removed by filtration. Furthermore, the stochiometry of hop α-acid-Fe^{2+} complexes at different pH values was examined using UV-VIS spectroscopy again.

2 Materials and Methods

2.1 Chemicals

Acetic acid (glacial) and sodium acetate trihydrate were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Ethanol, magnesium(II)sulfate heptahydrate, and potassium chlorite were obtained from ChemSolute (Renningen, Germany). Calcium chlorite, copper(II)sulfate pentahydrate, iron(II)sulfate heptahydrate, iron(III)chloride hexahydrate, manganese(II)sulfate monohydrate, and zinc(II)sulfate heptahydrate were purchased from Merck KGaA, Darmstadt, Germany. All chemicals were of highest purity available. All solutions were made with double-distilled water and prepared freshly every day. Purified hop α-acids (86.4% purity), hop β-acids (69.5 % purity), and hop iso-α-acids (30.4 % purity) were supplied courtesy from Hopsteiner (Mainburg, Germany).

2.2 Determination of metal concentrations in test samples

Metal concentration was measured using an iCAP 6200 inductively coupled plasma-optical emission spectroscopy (ICP-OES) system fitted with a CID 86 detector and argon as the carrier gas. The following parameters were used for the measurements: RF power: 1150 W; argon gas flow rates: auxiliary 0.5 L/min, nebulizer 0.5 L/min; sample flow rate: 4.0 mL/min. The analytical wavelengths

Table 1 Emission lines used for the quantification of Ca, Cu, Fe, Mg, Mn, and Zn by ICP-OES

Element	Emission lines used [nm]
Calcium	318.1; 317.9
Copper	324.7
Iron	239.5; 259.9
Magnesium	279.0; 279.8
Manganese	260.5
Zinc	202.5; 213.8

used for the determination are stated in table 1. A six-point calibration curve ranging from 0–1 mg/L for the individual metal ions was used to quantify the test sample's concentration ($r^2 > 0.99$).

2.3 Spectrophotometric measurements

Spectra of the complexes of metal ions or hop α-acids individually, or mixtures of substances were examined with the spectrophotometer (Lambda 25, Perkin Elmer, Waltham, USA). The absorption spectra of single substances or mixtures were recorded at 220–800 nm. Additionally, complexes of Fe^{2+} with α-acid were measured at the wavelength of 550 nm.

2.4 Complexation behavior of hop α-acids towards various metal ions

The tendency of hop α-acids to complex various metal ions was tested by mixing 100 µM of hop α-acids with 100 µM of either Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} metal salts. After incubation for 1 hour at room temperature in buffered model solutions (0.1 M sodium acetate buffer, pH 5.2, 10 % (v/v) ethanol), the mixtures absorption spectra were recorded at 220–800 nm. The hop acids were pre-dissolved in ethanol prior to addition. Changes in the absorption spectra of mixtures as opposed to sole hop α-acids imply a change in their chemical configuration and therefore suggest a complexation.

In separate trials, 60 µM α-acids were mixed with 0, 30, 60, and 100 µM Fe^{2+} and the spectra were again recorded using UV-VIS spectroscopy after 1 hour incubation at room temperature. Additionally, sample mixtures were filtered through a 0.45 µm cellulose syringe filter and the spectra were recorded again to test if the filtration step removes the complexes.

2.5 Complexation tendencies of hop α-acids, hop β-acids, hop iso-α-acids or mixtures of hop acids with various metal ions

50 µM of Ca^{2+}, Cu^{+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} was mixed individually with 50 µM of hop α-acids, hop β-acids, hop iso-α-acids, or a mixture of 50 µM of each hop acid in buffered model solutions (0.1 M sodium acetate buffer, 10 % (v/v) ethanol) at pH 4.3 or pH 5.7. For ferrous and ferric iron, the trials were additionally carried out at pH 5.2. Samples with only ethanol added served as a blank. After 1 hour incubation at room temperature, reaction mixtures were filtered using 0.45 µm cellulose syringe filters to remove hop acid-metal ion complexes and the solutions' remaining metal ion concentrations were quantified by ICP-OES.

2.6 Stochiometry of hop α-acid-Fe^{2+} complexes at different pH values

The stochiometry of the hop α-acid-Fe^{2+} complex was examined by applying the mole-ratio method [41, 42], and 25 µM of Fe^{2+} was mixed with 0–175 µM of hop α-acids prior to incubating for 1 hour at room temperature in

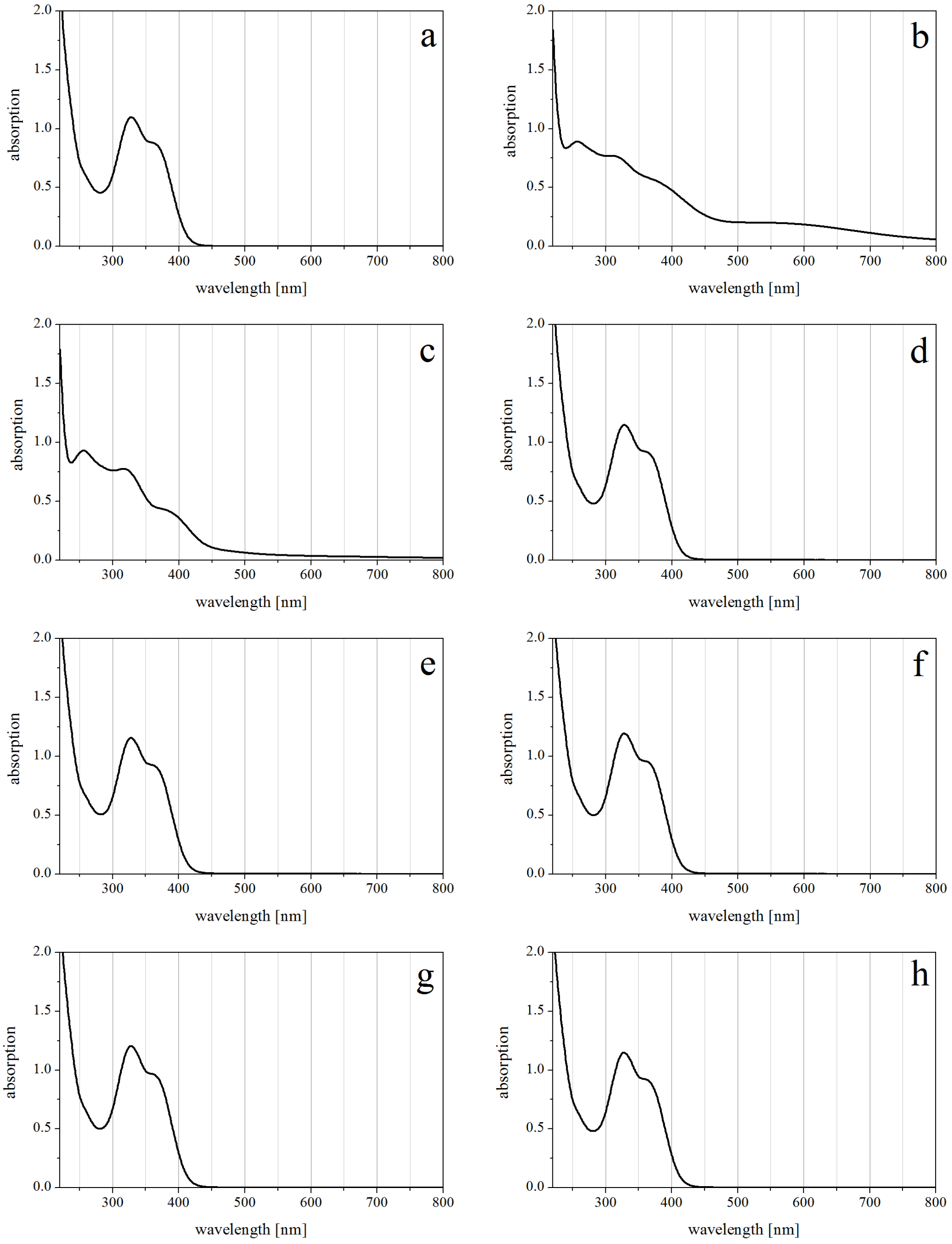

Fig. 1 UV-VIS absorption spectra of 100 µM sole hop α-acids (a) and equimolar mixtures of hop α-acids with metal ions (100 µM each); b: α-acid-Fe^{2+}; c: α-acid-Cu^{2+}; d: α-acid-K^{+}; e: α-acid-Mn^{2+}; f: α-acid-Zn^{2+}; g: α-acid-Mg^{2+}; h: α-acid-Ca^{2+}. Substances were mixed and incubated for 1 hour at room temperature at pH 5.5 before the spectra were recorded

Table 2 Metal ion concentrations in supernatants after mixing with hop acids, incubation for 1 hour at room temperature, and filtration

		concentration in µM				
metal ion added	pH	no hops added	α-acids added	β-acids added	iso-α-acids added	hop acid mixture added[a]
Fe^{2+}	4.3	55.7	48.3	51.8	49.4	32.4
	5.2	48.6	33.5	39.0	32.7	19.4
	5.7	58.4	24.8	37.2	21.6	17.9
Fe^{3+}	4.3	58.0	27.0	50.0	46.7	4.9
	5.2	42.0	7.3	19.7	20.8	3.2
	5.7	17.7	4.9	10.0	17.7	1.4
Cu^{2+}	4.3	42.3	10.8	39.0	40.3	5.2
	5.7	34.3	5.4	28.6	40.7	0.6
Ca^{2+}	4.3	69.0	70.1	73.9	71.3	73.0
	5.7	72.7	67.9	70.6	73.9	69.0
Mg^{2+}	4.3	41.8	43.9	41.2	41.7	45.0
	5.7	42.7	41.1	42.4	46.6	40.4
Mn^{2+}	4.3	42.1	41.8	41.3	41.3	41.1
	5.7	41.4	40.9	40.7	41.2	39.5
Zn^{2+}	4.3	45.1	48.1	48.9	49.0	48.0
	5.7	49.8	48.6	49.0	48.6	45.9

[a] 50 µM of α-acid, β-acid, iso-α-acid were mixed resulting in a total addition of 150 µM.

buffered model solutions (0.1 M sodium acetate buffer, 10 % (v/v) ethanol). The solutions' pH was adjusted to pH 4.3, pH 5.5, pH 6.2, or pH 8.2 using 0.1 M HCl or 0.1 M NaOH. The absorbance of the hop α-acid-iron-complex was recorded at 550 nm.

3 Results and Discussion

The hop α-acids' ability to react with different metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}) was screened by observing potential changes in the absorption spectra after mixing and incubation with metal ions. Figure 1 depicts the absorption spectra of the hop α-acid-metal salt mixtures at 220–800 nm after 1 hour incubation at room temperature.

Clearly, only Fe^{2+} and Cu^{2+} (Fig. 1, b and c) mixed individually with α-acids resulted in a decline of the α-acids' spectra in the range of ca. 310–390 nm while an additional maximum peaking at 255 nm and broad additional absorption spectra ranging from 390–800 nm could be observed. Mixing α-acids with Cu^{2+} yielded a lower absorption in the range of 390-800 nm as compared to Fe^{2+}. These shifts are caused by a conjugative effect when complexes are formed due to the formation of a new ring involving the metal ion. Most likely, the α-acids' cyclic β,β'-triketone moiety acts as the binding site forming a monodentate complex [39]. Spectral changes indicate the formation of complexes with both Fe^{2+} and Cu^{2+} while all other metal ions tested showed no spectra changes and therefore no complex formation at the experiment conditions tested. The spectrophotometric shifts when mixing iron ions with hop α-acids are in accordance with results from ref. [15].

It was further tested if filtration with a 0.45 µm cellulose syringe filter quantitatively removes α-acid-Fe^{2+}-complexes, and leaves behind uncomplexed α-acids. Mixing 60 µM α-acids with 0, 30, 60, and 100 µM Fe^{2+} yielded the spectra changes depicted in figure 2a. At these conditions, the reactions' isobestic point was found to lie at a wavelength of ca. 314 nm. This wavelength is also used as reference point in the analytics of hop acids by HPLC [43] as the concentration-dependent absorbance of these compounds remains constant independent of e.g. impurities of the samples with metal ions.

Increasing the Fe^{2+} concentration also resulted in an increase of the α-acid-Fe^{2+}-complex until a maximum appeared to be reached at a Fe^{2+} concentration of 60 µM and consequently, further Fe^{2+} additions did not yield an additional absorption increase at 550 nm.

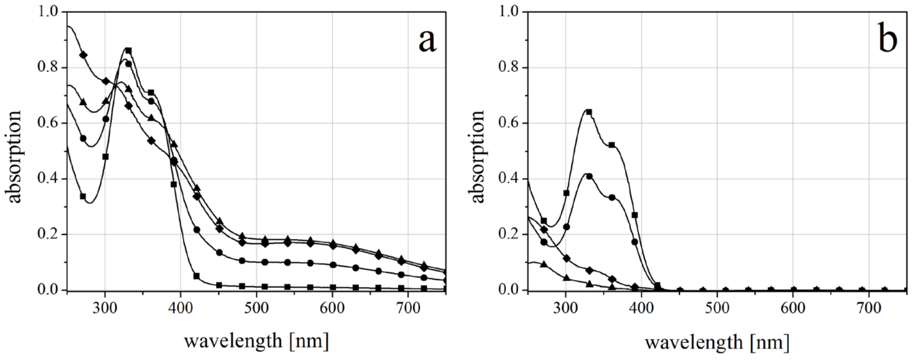

Fig. 2 UV-VIS spectra of α-acid-iron-complexes at different iron concentrations and effect of filtration. a: unfiltered sample mixtures; b: filtered sample mixtures. 60 µM α-acids were mixed with 0 µM Fe^{2+} (–■–), 30 µM Fe^{2+} (–●–), 60 µM Fe^{2+} (–◆–), and 100 µM Fe^{2+} (–▲–), incubated for 1 hour at room temperature, and the absorption spectra were recorded

After filtering the samples using a 0.45 µm cellulose membrane filter, no more absorption bands at 500–750 nm were detectable which is evidence that the α-acid-Fe^{2+}-complexes were removed (Fig. 2b). Residual, uncomplexed α-acids were not filtered out from the solutions and the characteristic wavelength could therefore be identified at 250–440 nm. Fe^{2+} additions at 60 or 100 µM produced further absorption bands at 250–430 nm which increased with increasing Fe^{2+} addition. These absorbencies can be traced back to the Fe^{3+} hydroxo species' charge-transfer bands [44] as formed by autoxidation of Fe^{2+} to Fe^{3+} and subsequent reaction with oxygen and water.

In a next step, α-acids, β-acids, and iso-α-acids were incubated with various metal ions in order to examine differences in complexation behaviors of hop acids and additionally screen for tendencies to complex and remove different metal ions by filtration. Trials were carried out in buffered model solutions at pH 4.3 and pH 5.7 to simulate the beer's or wort's pH conditions, respectively. After incubation of the mixtures to allow potential complex formation, the complexes were removed by filtration using 0.45 µM cellulose filters and the metal ion concentrations were quantified in the supernatants. For ferrous (Fe^{2+}) and ferric iron (Fe^{3+}), the trials were additionally carried out at pH 5.2. The results are depicted in table 2.

It is noteworthy, that the blank samples deviated from the targeted concentration of 50 µM which may be traced back to varying purities of the chemicals used. Additionally, it was observed that the Fe^{3+} concentration in the blank at pH 5.2 and pH 5.7 was lower than that of Fe^{2+}. Fe^{3+} can hydrolyze at pH > 2 forming colloidal gels which results in precipitation of hydrous oxides [44]. At these experimental conditions, it can't therefore be excluded that Fe^{3+} precipitated during the incubation time to some extent. The condition-dependent tendencies of Fe^{3+} to hydrolyze and to precipitate make it difficult to study this ion. The effects seen were still unavoidable because one of the study's goals was to investigate the metal ion's behaviors at pH values relevant for beer production. From the observations, it seems evident that the ability of hop acids to chelate iron ions appeared to be pH dependent in the order pH 4.3 < pH 5.2 < 5.7, and was stronger towards Fe^{3+} than towards Fe^{2+} ions.

Hop α-acids showed the strongest tendency to complex metal ions, followed by hop β-acids and iso-α-acids, with the exception of Fe^{2+} where the order was α-acids > iso-α-acids > β-acids. Also, for Fe^{3+} at pH 4.3, iso-α-acids showed a slightly better effect and stronger complexation than β-acids. A mixture of all hop bitter acids yielded additional effects and the highest rate of iron precipitation; though, it must be considered that in total 150 µM of hop acids were present in the solutions. According to *Spetsig, L.* [45], hop β-acids have a very limited solubility (1.5 mg/L at 25 °C and pH 4.3) and are even poorer soluble than α-acids. It remains open if the β-acids' effect is improved at wort boiling conditions because the hop β-acids' solubility is increased at higher temperatures. Though, the limited solubility of β-acids is clearly the reason why they are precipitated during wort boiling and are mostly not detected in non-dry-hopped beers [24].

In addition to Fe^{2+}/Fe^{3+} ions, Cu^{2+} ions were also removed from the solutions by the hop acids' action which is in accordance to the findings from the spectrophotometric trials (Fig. 1). Hop α-acids were again strongest in activity while β-acids and iso-α-acids showed no or only little effectiveness at both pH values measured.

All the other metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}) screened in this experiment were hardly or not affected, and α-, β- and iso-α-acids only removed iron and copper ions from the solutions. These results are in conformity with findings from ref. [13] where *Kunz* and co-workers also observed that iron was affected by hop α-acids while zinc ions remained unaffected. *Schurr* et al. [46], on the contrary, found manganese-binding activity of iso-α-acids and *Simpson* and *Hughes* [47] attested binding of trans-iso-α-acids to K^+ ions when divalent or trivalent cations were present. It is also known that earth alkaline metals, e.g. Mg^{2+}, affect the isomerisation of α-acids to iso-α-acids [24] which suggests binding of hop α-acids to these ions.

From the data gathered thus far, it can be concluded that the complexation characteristics of hop bitter acids can be considered beneficial for the wort and beer quality because metal ions which are claimed to promote beer flavor deterioration reactions are complexed while vital metal ions, such as e.g. Zn^{2+}, a key yeast nutrient [24], remain in the solutions.

Sole hop α-acids don't show absorbance at 550 nm and the wavelength of 550 nm can therefore be used as a measure to determine the α-acid-Fe-complex stochiometry. This characteristic was used in supplementary trials in which the stochiometry of the α-acid-Fe^{2+}-complex was studied.

Hop α-acids present only one acidic proton and can therefore only form monodentate complexes with metal ions. The possibility that more than one Fe^{2+} ion bind to one molecule α-acid was still tested at pH 4.3. Increasing the Fe^{2+} concentration from 25 to 250 µM Fe^{2+} and mixing with 25 µM α-acids however yielded no increase of the complexes' absorption at 550 nm (data not shown) and therefore confirmed literature claims from ref. [39]. At Fe^{2+} excess,

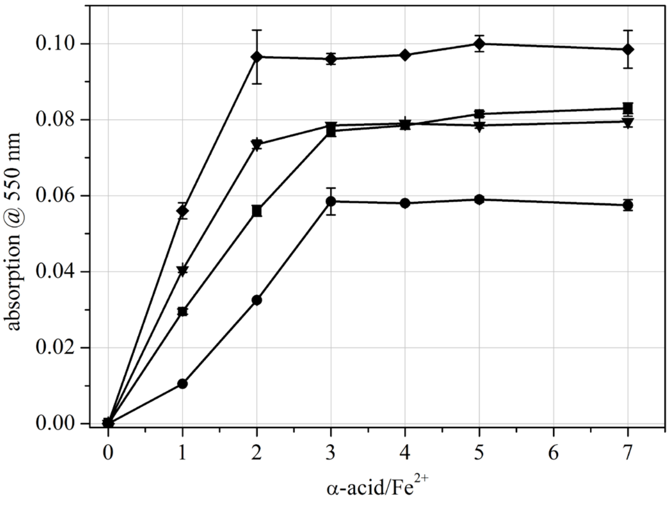

Fig. 3 **Hop α-acid-iron ratio as a function of pH. Fe^{2+} (25 µM) was mixed with 0–175 µM of α-acids and incubated for 1 hour in 0.1 M sodium acetate buffer at pH 4.3 (–■–), pH 5.5 (–●–), pH 6.2 (–▼–), and at pH 8.2 (–◆–). Mean values ± 1 standard deviation of a triplicate determination are presented**

even haze formation, and after longer incubation, the emergence of dark-colored clusters of amorphous particles which rose in size and ultimately precipitated was observed. Precipitations when mixing α-acid with iron were also observed by Jaskula et al. [29]. Sole Fe^{2+} ions in solution did not form such precipitates. It is therefore likely that α-acids together with Fe^{2+} can form amorphous clusters in a complex interplay, though, only at high iron overshoot.

Fe^{2+}, in contrast to α-acids, may be capable of forming polynuclear coordination complexes with organic molecules. This possibility was therefore tested with α-acids at pH 4.3, pH 5.5, pH 6.2, and pH 8.2. The experiment's results are depicted in figure 3.

The maximum absorption of the α-acid-iron-complex in the pH range of 4.3 and 5.5 was at a ratio of 3:1 (α-acid:Fe) and no further absorbance increase was seen when increasing the ratio to 4:1 or higher, suggesting a maximum occupation ratio of 3 to 1. The maximum occupation changed when increasing the pH when increasing the pH, and at pH 6.2 and pH 8.2, at maximum and at pH 6.2 and pH 8.2, maximum two mol α-acid could bind to one mol iron thus implying a maximum saturation of two mole α-acid per mol iron at this pH range.

Binding of ligands to metal ions and their reactions are very complex and depend on many factors. The hydration of iron ions in aqueous solutions is strongly dependent on the solution's pH, and the iron ions' occupancy with OH^- ions increases with increasing pH. Consequently, in the pH ranges measured in this study (pH 4.3–8.2), Fe^{2+} has more binding sites at lower pH values, and, in turn, less binding sites are available at higher pH values. Lower pH values should therefore allow more ligands to bind to iron than higher pH values.

This effect is opposed by the ligands' characteristic to act as a base or Lewis base because they can donor electrons. In dependency of the ligands' pK_a value, ligands are protonated in a milieu below their pK_a value and their binding sites can thus be occupied by protons. As related to hop α-acids, pH values > 5.0 (pK_a of α-acids [48]) are accordingly beneficial for complexation reactions because more binding sites of the ligands or α-acids are available. The pH dependent complex formation is therefore generally influenced by two factors which are promoting the ligand-metal ion reactions or are counteracting the complex formation: the pH dependent occupancy of metal ions by hydratation and the concurrence principle between protons and metal ions at the ligand's binding sites.

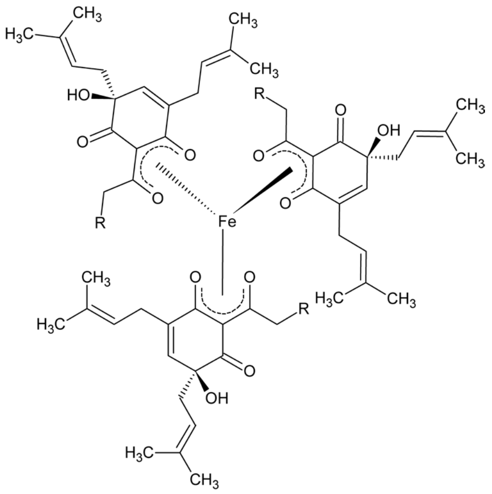

Fig. 4 Proposed schematic structure of α-acid-Fe^{3+} complexes at a ratio 3:1

Hop α-acids are anticipated to be only present in their monoenol form at 0–25 °C, they possess only one acidic proton, and therefore can only donate one electron for pairing to form a covalent bond with the metal centre of the complex [39]. Complex formation and covalent binding of three mol α-acid and 1 mol iron therefore requires the presence of three electrons to be shared from iron. Consequently, only Fe^{3+} can be present in the complex and not Fe^{2+}, at least at these pH conditions of pH 4.3 and pH 5.5. This is in accordance with claims from *Wietstock* and *Shellhammer* [15] who anticipated that Fe^{2+}, once it forms a complex with hop α-acids, is readily oxidized to Fe^{3+}, and that this is predominant oxidation state of iron in the complex.

Based on the findings from this study and literature data, a proposed schematic structure of hop α-acids and iron at a ratio of 3:1 is depicted in figure 4. The delocalized electron from the α-acid's cyclic β,β'-triketone skeleton forms a covalent bond with the metal ion thus resulting in an uncharged complex.

4 Conclusions

Outcomes from this study elucidate the complex reactions between hop acids and metal ions at beer- or wort-like pH conditions. Fe^{2+}, Fe^{3+}, and Cu^{2+} were complexed by hop α-, β-, and iso-α-acids while Ca^{2+}, K^+, Mg^{2+}, Mn^{2+}, or Zn^{2+} were unaffected. The stoichiometry of hop-α-acid-Fe-complexes was found to be pH-dependent and was 3:1 (α-acid:Fe) at pH 4.3 and pH 5.5, and 2:1 (α-acid:Fe) at pH 6.2 and pH 8.2. The hop acids' complexation characteristic can be judged advantageous as 'unwanted' metal ions are affected while 'vital' metal ions are not. This study thus provides new information and relevant insights into complexation characteristics of hop acids. Deductions from this study can therefore benefit the hops and brewing industry in terms of e.g. hop utilization or industrial applications.

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