Free Radical-Mediated Formation of Aroma-Active Aldehydes During Beer Production and Storage and Anti-Staling Effects of the Hop Dosage

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“Ich kenne die ganze Welt.”

(Jakob, 4 Jahre)

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Abstract

Several chemical and molecular changes result in beer off-flavors which become recognizable after a certain storage time thereby reducing the consumer's acceptance. Elucidating factors and reactions which adversely affect or promote beer staling is therefore of major interest for the brewing industry. The objective of this work was to elucidate the role of reactive oxygen species for the formation of aroma-active aldehydes as well as to characterize and optimize the anti-staling effects of the hop dosage ultimately aiming to improve the resistance of beer against staling.

A pathway for the hydroxyl and hydroxyethyl radical-mediated formation of aroma-active aldehydes by oxidative degradation of their parent amino acids was first investigated in model systems, and its relevance was subsequently examined during beer storage using response surface methodology. The Maillard reaction and consecutive Strecker degradation as initialized from non-oxidative xylose and glucose decomposition as well as the factor Fe^{2+} concentration were found irrelevant for Strecker aldehyde formation during beer storage; yet, a linear relationship between Strecker aldehydes formed and total packaged oxygen as well as amino acid concentration could be identified. Oxygen-involvement in the *de novo* formation of phenylacetaldehyde and benzaldehyde from phenylalanine during beer storage was demonstrated using labeling experiments. Additional lab-scale experiments suggested a relevance of amino acid oxidative degradation during wort production. Albeit varying the concentration of Fe^{2+}, a catalyst in radical generation, was found irrelevant for Strecker aldehyde formation within the scope of the beer storage experiments, its withdrawal from reactions by treatment of beers with excess EDTA was perceived to significantly diminish Strecker aldehyde levels. As related to the findings, diminishing oxygen uptake, lowering amino acid concentrations, and rendering iron harmless during beer production and storage can be anticipated to be key strategies to diminish staling.

In an attempt to find approaches to counteract staling, antioxidative properties of hop acids and the hop dosage were scrutinized. Hop α-acids were found to complex prooxidative metal ions thereby counteracting free radical formation and promoting autoxidation of Fe^{2+} to Fe^{3+} thus stabilizing iron in its higher, less harmful, valence state. Accordingly, next to suppressing free radical formation and withdrawing iron from potential reaction partners, one of the main functionality of hop α-acids was found to lie in their capability to counteract recycling of Fe^{3+} to Fe^{2+} consequently improving oxidative beer stability. UV-VIS measurements suggested a covalent binding between Fe^{3+} and hop α-acids and the formation of a complex at a ratio of 3:1

(α-acid:Fe^{3+}). While α-, β-, and *iso*-α-acids were shown to complex Cu^{2+}, Fe^{2+} und Fe^{3+} ions, no complexation of Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} ions was noticed. The hop acid's selective complexation behavior can be regarded as very advantageous for beer quality as prooxidative transition metal ions are rendered harmless without adversely affecting the wort's or beer's ionic composition.

Testing different hop constituents in wort-like model syst0ems during incubation at 100 °C demonstrated a high efficacy of hop α- and β-acids in suppressing the formation of 2-furfural and 3-methylbutanal, while *iso*-α-acids were shown to be less effective. Hop α-acids were investigated in greater detail, and α-acid-induced suppression of 3-methylbutanal formation could be partly traced back to the hop α-acids' capability to functionally complex Fe^{2+}-ions and scavenge organic, carbon-centered radicals thereby suppressing free radical-mediated oxidative degradation of leucine. Constantly allocating fresh α- and β-acids can therefore be seen as a key strategy to counteract staling aldehyde formation during wort production.

The knowledge could be successfully adapted to semi-technical beer production and an increased oxidative stability and resistance to staling of beers was achieved by optimization of the hopping technology as verified analytically and sensorial. The malt bill and wort matrix were noticed being crucial for the state of iron and efficacy of the hop dosage. The hopping technology should therefore be adapted to the wort matrix and initial malt bill when aiming to achieve the best optimal utilization. Modestly lower bitter substance yields were found when dosing α-acids later which may be balanced by higher hop dosages.

In sum, knowledge about yet unexplored beer staling mechanisms and the anti-staling efficacy of hops, particularly hop acids, could be expanded. Outcomes from this work take the brewing and beverage industry a step further towards producing beers with a higher resistance against staling, from which eventually not only the brewing industry but also customers will benefit.

Zusammenfassung

Eine Vielzahl an chemischen und molekularen Vorgängen führt zum Auftreten von Fehlaromen während der Lagerung von Bier und zu einer verringerten Akzeptanz bei Konsumenten. Wissen um Faktoren, die die Geschmacksstabilität von Bier beeinflussen, ist damit von großem Interesse für viele Brauereien. Ziel der vorliegenden Arbeit war es, die Rolle von reaktiven Sauerstoffspezies in Bildungsreaktionen von Aroma-aktiven Aldehyden aufzuklären, sowie die antioxidative Wirkungsweise von Hopfen, insbesondere Hopfensäuren zu erörtern und optimierte Hopfengaben zu erarbeiten.

Ein vorgeschlagener Mechanismus zur Bildung Aroma-aktiver Streckeraldehyde durch Hydroxyl- und Hydroxyethylradikal-induzierten, oxidativen Abbau von Aminosäuren wurde zunächst in Modellsystem bestätigt, und dessen Relevanz für die Bieralterung anschließend mittels Oberflächenwirkungsversuchsplänen untersucht. Während der nicht-oxidative Abbau von Glucose und Xylose im Rahmen der Maillardreaktion sowie eine Veränderung der Fe^{2+}-Konzentration nachweislich keinen Einfluss auf die Bildung von Streckeraldehyden hatte, konnte ein linearer Einfluss der Faktoren Sauerstoffgehalt und Aminosäurekonzentration auf die Bildung von Streckeraldehyden während der Bierlagerung belegt werden. Die sauerstoffabhängige Nachbildung von Phenylacetaldehyd und Benzaldehyde aus Phenylalanin während der Lagerung konnte mittels Isotopenmarkierungsexperimenten nachgewiesen werden. Zusätzliche Experimente im Labormaßstab deuteten darauf hin, dass der Sauerstoff-induzierte Abbau von Aminosäuren ebenso bei der Würzeproduktion eine bedeutende Rolle spielt. Dessen ungeachtet, dass der Faktor Fe^{2+}-Konzentration bei den durchgeführten Experimenten nicht signifikant war, führte die Zugabe von EDTA zu einer deutlichen Abnahme an Streckeraldehyden nach der Lagerung. Die Vermeidung von Sauerstoffeintrag, Maßnahmen die den Aminosäuregehalt in Würze und Bier erniedrigen, sowie Substanzen, die der prooxidativen Wirkung von Übergangsmetallionen entgegenarbeiten, sind daher in Bezug auf die Minimierung von Alterungserscheinungen als besonders zielführend anzusehen.

Zur Erarbeitung von Strategien, die der Bieralterung entgegenstehen, wurde die antioxidative Wirkungsweise von Hopfensäuren in weiteren Versuchen eingehend untersucht. Das antiradikalische Verhalten von α-Säuren konnte auf deren funktionelle Komplexierung von prooxidativen Übergangsmetallionen zurückgeführt werden. Hierbei wurde insbesondere für α-Säuren die Autoxidation von Fe^{2+} zu Fe^{3+} und Stabilisierung von Eisen im höheren Valenzniveau nachgewiesen. Neben der Radikalunterdrückung und dem Entzug von Eisen von anderen Reaktionspartnern, kann die antioxidative Wirkungsweise von α-Säuren

dementsprechend darauf zurückgeführt werden, dass sie der Reduktion von Fe^{3+} zu Fe^{2+} entgegenwirken. Mittels UV-VIS-Messungen konnte anschließend die Struktur eines α-Säure-Fe^{3+}-Komplexes im Verhältnis 3:1 (α-Säure:Fe^{3+}) vorgeschlagen werden. Während Cu^{2+}-, Fe^{2+} und Fe^{3+}-Ionen von α-, β-, und *iso*-α-Säuren komplexiert wurden, konnte keine Komplexierung der Metallionen Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+} und Zn^{2+} nachgewiesen werden. Das selektive Komplexierungsverhalten von Hopfensäuren kann daher in Bezug auf die Bierqualität als sehr förderlich angesehen werden, da prooxidative Metallionen komplexiert werden ohne dass dabei die ionische Komposition der Würze oder des Bieres nachteilig beeinflusst wird.

Weiterführende Versuche in Bierwürze-ähnlichen Modelllösungen zeigten auf, dass α- and β-Säuren signifikant der Bildung von 3-Methylbutanal und 2-Furfural entgegenwirken, während *iso*-α-Säuren keinen Effekt zeigten. Die Wirkung der α-Säure wurde dabei eingehender untersucht und es konnte belegt werden, dass insbesondere der Radikal-induzierte, oxidative Abbau von Leucin zu 3-Methylbutanal durch Fe^{2+}-Komplexierung und das Abfangen organischer Radikale unterdrückt wurde. Die stetige Gewährleistung adäquater α- und β-Säurekonzentrationen während der Würzebereitung kann daher als zielführend betrachtet werden, um der Bildung von Alterungsaldehyden entgegenzuwirken.

Die Resultate wurden schließlich erfolgreich auf die semi-technische Bierproduktion angewandt. Optimierte Hopfengaben konnten erarbeitet und deren positive Wirkung auf die Geschmacksstabilität und Verzögerung von Alterungserscheinungen analytisch und sensorisch bestätigt werden. Die Modifikationen der Hopfengabe führten jedoch zu geringeren Bitterstoffgehalten im Bier, die durch leicht höhere Hopfengaben ausgeglichen werden müssten. Die Hopfengaben sollten dabei methodisch der jeweiligen Würzematrix angepasst werden, um eine größtmögliche Effektivität in Bezug eine Stabilitätssteigerung zu erzielen.

Zusammenfassend wurden in dieser Doktorarbeit das Wissen um oxidative Bieralterungsmechanismen und die antioxidative Wirkungsweise von Hopfensäuren erweitert. Darauf aufbauend konnten praktikable Vorschläge erarbeitet werden, um Biere mit einer gesteigerten Resistenz gegen Alterungserscheinungen zu produzieren, von denen nicht nur die Brau- und Getränkeindustrie, sondern letztendlich auch die Endverbraucher profitieren.

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Abbreviations Used

2-DR	*2-desoxyribose*
AG	*aminoguanidine*
CCRD	*central composite rotatable design*
cp.	*compare*
DAB	*diaminobenzene*
DLG	*Deutsche Landwirtschaftsgenossenschaft*
DPPH	*2,2-diphenyl-1-picrylhydrazyl*
EAP	*endogenous antioxidative potential*
EDTA	*ethylenediaminetetraacetic acid*
e.g.	*exempli gratia*
etc.	*et cetera*
ESR	*electron spin resonance*
et al.	*et alii*
GC	*gas chromatography*
ICP-OES	*inductively coupled plasma optical emission spectrometry*
MS	*mass spectrometry*
PBN	*N-t-butyl-α-phenylnitrone*
POBN	*α-(4-pyridyl-1-oxide)-N-tert-butylnitrone*
RSM	*response surface methodology*
ref.	*reference*
ROS	*reactive oxygen species*
SAFE	*solvent assisted flavor evaporation*
TBI	*thiobarbituric acid index*
TU Berlin	*Technische Universität Berlin*

1 Introduction

Beer is one of the oldest 'crafted' beverages known to mankind. Its multifaceted flavor is derived from numerous substances all of which are present in beer in a complex composition and interplay. Though, as related to the beer's flavor, the chemical composition is very fragile and is continuously changing. The term beer flavor instability became therefore a commonly used terminology. During the process step of maturation, one of the final steps during beer production, flavor changes are considered advantageous because the so-called green-beer character is lost and negative off-notes are vanished. Though, at the latest from the point when the beer is filled into bottles, cans, or kegs, upcoming flavor changes are regarded as being adverse for its quality, and beer is therefore best consumed fresh.

These molecular changes are a consequence of numerous intrinsic and extrinsic critical factors. Beer is not in a chemical or molecular equilibrium after bottling and, in similarity to a closed thermodynamic system, whose static state is not yet reached, it underlies constant chemical transformations and reactions striving for the state of the lowest entropy. This can be also considered as the intrinsic beer imbalance. Additionally, a beer bottle, can, or keg, is also connected to the environment, and several critical extrinsic factors adversely affect the beer system. Amongst these factors, above all, storage temperature and oxygen ingress through the packaging are probably most critical for flavor deterioration processes. Though, also other factors such as light exposure, mechanical factors (e.g. agitation during transport), or reactions with the packaging itself come into play.

The final condition of a beer when consumed is therefore unambiguously a consequence of virtually all raw materials used for beer production, every single production step beginning from malt production and ending with packaging, each single factor during transport and distribution, and, finally, a matter of how the consumers store the beer at home. Accordingly, to approach the problem of beer flavor instability, there is unsurprisingly an endless number of starting points. Only a fundamental understanding about the significance, interrelationship and effect size of the different factors, reactions and reaction partners, as well as the chemical and molecular changes, allow directed and efficient actions for improving and guaranteeing beer quality, eventually meeting the consumer's demands for an immaculate, fresh-tasting beer.

Approaches certainly must be taken step-wise as the possibilities and factor combinations are endless and decades of brewing-related research still haven't come close to solving the problem of beer flavor instability, yet the knowledge has been expanded and several critical factors and

reactions were identified to play a role in the intricate task to investigate and improve beer flavor stability.

In this dissertation work, for the first time in brewing-related research, a pathway for the formation of aroma-active aldehydes as induced from amino acid oxidative degradation by reactive oxygen species (ROS) was proposed and its impact and relevance during beer deterioration processes was examined. Furthermore, antioxidative and antiradical hop-derived substances were elucidated and hopping technologies were developed and applied, ultimately aiming at an improved quality of beer and delayed or diminished appearance of staling-related compounds during beer storage.

In the following subchapters, a brief recapitulation of published literature with regards to staling-related compounds as well as factors and reactions which were reported to promote and/or impede beer flavor stability will be presented. Because comprehensive and contemporary reviews about beer deteriorating processes and off-flavor compounds are accessible from Baert et al.[19], De Schutter et al.[60], or Vanderhagen et al.[260], the emphasis and coverage of this literature review is confined to critical factors, staling-related compounds, molecular changes, and antioxidative substances, which are closely affiliated with the content and scope of this dissertation work.

1.1 Staling-related compounds in beer and their origin

There are many possible sources for beer off-flavors which are not necessarily all in responsibility of the breweries and beers can for instance be exposed to unusual high temperatures during transport (e.g. during oversea transport[200]) or when stored under falsely conditions in the market (e.g. temporary storage on the yard in the sun). Also, beer lines in pubs can be filthy and the beer thus takes on microbial-derived off-flavors before poured into the glass.[209] In addition to the importance of the perceived stale flavor of beer arising during storage, beer quality is also adversely affected during storage because the beer's bitterness impression changes, its characteristic body is lost, an increase in color can be observed, and chill haze or permanent haze are formed.[39, 49, 56, 120, 142, 148, 150, 151, 159, 177, 229, 230]

Taken together, the fundamental decline of beer quality during ageing can be summed up to essentially, for one thing, the development of a stale flavor, and, at the same time, to the loss of beer-typical characteristics such as its mouthfeel and its physical appearance. Yet, because consumers can usually not define the origin of such flaws and may blame it to the producer, brewers owe their consumers to do the best they can to minimize defects and to delay staling

whenever possible. As the focus of this work lies clearly on the emergence of stale-flavor compounds during beer production and storage, only compounds with regards to stale flavor and their origin shall be presented in this chapter.

Thorough research of the last decades made it possible to identify numerous flavor-active compounds as contributing to beer staling character. Naoki Hashimoto was in fact the first to find that off-flavors must be derived from compounds with carbonyl moiety as addition of 2,4-dinitrophenyl hydrazine diminished the stale flavor impression of beer.[106] Amongst those compounds, aldehydes, esters, furan and furanols, cyclic acetals, heterocyclic carbonyls, pyrazines, sulfur-containing compounds, and lactones could later be identified as potentially contributing to the stale flavor of beer.[19, 60, 260] Their origin is not always fully understood; though, given their chemical structure, there is a consensus that mostly oxidative processes must account for their formation. In fresh beer, aldehyde levels are rather low, though during ageing their concentrations increase and their aroma impressions therefore become perceptible. Albeit many compounds still don't exceed their individual thresholds in aged beer, their contribution to stale flavor impressions was discussed on basis of combinatory or synergistic effects.[94, 140] The formation and liberation of aldehydes during storage is therefore considered one of the main causes for beer flavor deterioration.[19, 49, 140, 169-171, 218, 219, 234, 261]

The molecular character of derived substances, the response to the presence or absence of oxygen during beer production or storage as well as the mutual dependency on the availability of reaction partners, allowed the identification of several pathways. In ref. 19, 60 and 260, an overview of known substances, their flavor thresholds, their odor or flavor impressions, and their pathways of origin can be found. Table 1 provides a brief outline of important carbonyl compounds and their possible molecular origin. Because the aforementioned reviews are contemporary, this chapter is only giving a brief overview of known pathways, point out new findings, and is then again only devoted to selected compounds, pathways, and newer findings which are related to the scope of this dissertation work.

Table 1: Important malt-derived carbonyl compounds in wort and beer.[19, 60, 260]

Strecker (or Strecker-like) degradation of amino acids	Fatty acid oxidation	Maillard reaction	Carotenoid degradation
2-Methylpropanal	*trans*-2-nonenal	2-Furfural	β-Damascenone
2-Methylbutanal	Hexanal	5-Hydroxymethyl furfural	
3-Methylbutanal	2,4-Decadienal	Acetylfuran	
Methional		Furfurylethylether	
Phenylacetaldehyde		γ-Nonalacton	
Benzaldehyde			

With regards to the occurrences producing an increase of staling aldehyde concentrations during beer storage, there is a consensus now that compounds can be released from a bound state and can be formed *de novo* during storage. It is unclear, though, as to which mechanisms contribute most. As related to a possible *de novo* formation, Hashimoto and colleagues suggested potential pathways. Because the yeast can reduce aldehydes to their corresponding higher alcohols, it seems reasonable that, vice versa, higher alcohols can be oxidized again to aldehydes during storage of beer. In 1972, Hashimoto and co-workers postulated that this reaction is catalyzed by melanoidins[107], and reported five years later that derived aldehydes can undergo aldol condensation to give unsaturated aldehydes with stale-flavor, such as e.g. the reaction of heptanal and acetaldehyde would give *trans*-2-nonenal.[108] These pathways are questionable considering the low concentrations of aldehydes in beer and the yield of this reaction found in model solutions (~0.2 %).[27] Furthermore, according to theoretical considerations from Barker et al.[27], because of the abundance of ethanol in beer, acetaldehyde as derived from ethanol oxidation is the only aldehyde which is likely to be produced by oxidation, and oxidants would be completely consumed by the oxidation of ethanol.

Hashimoto et al.[109] further postulated that oxidative cleavage of *iso*-α-acids gives rise of volatile carbonyl compounds with various chain lengths. In fact, they found that beers brewed without hops hardly developed any stale flavor. Yet, this observation was disproved in numerous other studies and hopped beers were clearly shown to be more resistant against staling.[14, 91, 127, 149, 173, 179] Also, anticipations from De Clippeeler that oxidation products of *iso*-α-acids can be the source for Strecker-like degradation of amino acids was first shown to be valid in model

systems but then refuted again by herself to be of importance during actual beer storage.[54] Nevertheless, as recently revisited by Rakete and co-workers, also carboxylic acids as derived from hydrolytic cleavage of *iso*-α-acids under oxidative conditions may contribute to off-flavors in beer.[210, 286]

The Strecker degradation of amino acids as induced by α-dicarbonyl compounds from the Maillard reaction is certainly a possible origin for the emergence of Strecker aldehydes during wort production and beer storage.[36, 61, 173, 224, 243] The reaction starts with the nucleophilic addition of an unprotonated amino group to a carbonyl group and, after forming a hemiaminal and an imine as intermediates, results in an α-ketoamine and a Strecker aldehyde.

While it is unambiguous that the formation α-dicarbonyl compounds and consecutive Strecker degradation of amino acids occurs during mashing and wort boiling because of the high temperatures, adding amino acids to beer was also reported to result in higher concentrations of aldehydes after prolonged storage. The formation of Strecker aldehydes during storage was greatly enhanced by oxygen exposure of the beers and catalyzed by the presence of iron and copper ions.[31] Because of the amounts of Strecker aldehydes detected during beer storage, Thum et al.[249] claimed that Strecker degradation is only from importance for beer staling at high amino acid concentrations. An explanation for the strong response of Strecker aldehydes to oxygen exposure and to the presence of transition metal ions is only inadequately discussed and is underexplored in published literature.

Studies from Bravo et al.[36, 37] imply that the Maillard reaction is occurring during storage of beer at relatively mild conditions such as storing beers at 28 °C. Yet, the relevance of these findings is uncertain. In their investigations from 2002, Bravo and colleagues tested trapping agents to inhibit consecutive reactions of Maillard reaction intermediates and found that formation of 5-hydroymethylfurfural was indeed blocked and staling of beers was retarded.[37] However, the researchers could not rule out that the used trapping agent, aminoguanidine (AG), formed stable adducts with α,β-unsaturated carbonyls itself thus not being effective by blocking dicarbonyls but by eradicating flavor-active compounds instead. In a similar study, they therefore tested 1,2-diaminobenzene (1,2-DAB) as a trapping agent to evaluate the role of α-dicarbonyls in Strecker aldehyde formation during beer storage because as opposed to AG, 1,2-DAB is not capable of forming stable adducts with sensory-active aldehydes. Though, only three odorants were significantly affected by the treatment, furaneol, *trans*-2-nonenal, and phenylacetaldehyde. Given the relatively harsh storage conditions (60 °C, 3 days), and since Strecker aldehydes other than phenylacetaldehyde were unaffected from the treatments as well

as that effects may additionally be partly seen due to complex formation with HSO_3^-, the role of the Maillard reaction in Strecker aldehyde emergence during storage is uncertain. Still, later experiments from Rakete, Klaus, and Glomb confirmed partly that the Maillard reaction occurs during storage of beers at 50 °C, and an oxidative pathway for the formation of 2-furfural during storage of beer as derived from oligosaccharide oxidation, subsequent hydrolytic β-dicarbonyl cleavage, cyclization and dehydration of the resultant 3-deoxypentoson could be proposed.[211]

In addition to the classical α-dicarbonyl-initiated Strecker degradation, Strecker aldehydes are now also believed to being originated from so-called Strecker-like degradation reactions amongst which α-unsaturated carbonyl compounds serve as the oxidizing substrate. The reaction of the isoprenyl side chain of *iso*-α-acid oxidation products with amino acids represents one example of such a Strecker-like reaction yielding Strecker aldehydes.[54] However, this particular pathway was found irrelevant during actual beer staling. Schieberle and Komarek[222] suspected that ROS or riboflavin can initiate *ortho*-quinone formation via oxidation of the catechin *ortho*-dihydroxy-ring system which, in turn, has α-dicarbonyl-like moiety and can therefore result in Strecker-like degradation of amino acids. In fact, work from George P. Rizzi supports these findings as he found *ortho*-quinones as derived from oxidation of certain polyphenols with catechol (1,2-dihydroxylbenzene) moiety being capable of initiating degradation of amino acids thereby forming their corresponding volatile aldehydes.[215] Additionally, Cremer et al.[48] also found that Amadori compounds (intermediates of the Maillard reaction) can instigate Strecker degradation of amino acids thereby forming aroma-active aldehydes. As recently discovered, also lipids or lipid derivatives were found being a potential source for Strecker-like degradation.[293] Proof of the relevance of these pathways for beer ageing is outstanding thus far.

Given the complexity of the beer matrix and the multitude of suggested pathways, there is naturally controversy about the routes which ultimately result in the emergence of staling aldehydes during beer storage. There is arising evidence that the aforementioned pathways may have only limited relevance, and several studies suggest now a release of aldehydes from a bound state. In fact, Suda et al.[243] discovered that ca. 85 % of aldehydes detected in beer were already formed during wort boiling while only 15 % were formed *de novo* during storage. Three potential mechanisms supporting the theory of bound-state aldehydes were proposed: the adduct formation between aldehydes and bisulfite, Schiff base (imine) formation between aldehydes and amino acids or proteins, and as recently detected, the reaction of aldehydes with the amino acid cysteine thereby forming stable 2-substituted 1,3-thiazolidine-4-carboxylic

acids. While the theory of imine formation was found irrelevant by Baert et al.[20], it is evident that both bisulfite and thiazolidine adducts are reasonable alternatives to proposed pathways for a *de novo* formation of aldehydes. However, also existence of precursor substances may serve as an explanation for the emergence of aldehydes during storage of beer. Transition metal ion-mediated oxidative degradation of Amadori compounds as formed from an amino acid and a sugar source represents one possible pathway.[115]

Reasons enough, the brewing process is crucial for the liability of a beer to develop a stale flavor. During mashing, the oxidation or of poly-unsaturated fatty acids produces numerous negative aroma-active compounds. As many enzymes are still active during mashing and lautering, both autoxidation or enzyme-catalyzed oxidation are discussed to account for the fatty acid oxidative degradation.[25, 65, 66, 227] *trans*-2-Nonenal is probably the most known compound derived from these pathways. It is suspected being responsible for the often mentioned 'cardboard'-flavor of beer.[125, 218] Its emergence during storage was anticipated to be derived from precursor substances,[67, 160, 266] also because labeled oxygen added to beer before storage was not incorporated into the arising carbonyl.[192] Its negative flavor was found to increase over time[49] but was also found to decrease again when aged further.[169, 295, 296]

At elevated temperatures, e.g. during mashing, lautering, and wort boiling, certainly the Maillard reaction probably accounts for many of the possible precursor substances and reducing heat load during beer production is considered one of the most important steps to improve beer quality.[59, 172, 195] Yet, also the performance of the fermentation and the activity of the yeast evidently define the quality of a beer,[165] not only because many aroma-active compounds are formed in the course of fermentation[39] but also because of the reduction of unwanted compounds[203, 217] as well as the formation of SO_2, a very potent antioxidant in beer.[11, 134, 136, 152, 154, 252]

1.2 Fate of oxygen and oxygen-derived radicals in beer ageing

Oxygen is a *Janus*-faced factor during beer production and storage. It is clearly essential, e.g. for germination of the barley embryo during malting[88, 284], or for yeast growth and particularly cell membrane framework[39, 110]; yet, oxygen ingress is mostly considered to be adverse for beer quality when occurring during beer production or storage.

Several authors found a clear connection between elevated oxygen levels during production and packaging and an increase in the amount of aroma-active aldehydes formed during storage.[31, 62, 173, 188, 219, 222] Oxygen which is diffusing through crown cork liner polymers is readily

consumed by the beer matrix[114] and consequently yields oxidation of the beers.[65, 173, 248] Particularly, Strecker aldehydes were therefore reported being typical "oxidation indicators".[188] In addition to potential *de novo* formation from degradation of *iso*-α-acids[109] or oxidation of higher alcohols[107], oxidation of reversible complexes or precursors such as e.g. bisulfite-aldehyde adducts[20, 27, 136] or Amadori compounds[115], respectively, and following release of aldehydes may serve as an explanation for the oxidation-dependent emergence of these volatile aldehydes during storage. Exposing beers to oxygen and supplementing beers with the Fenton reagents H_2O_2 and Fe^{2+} produced very similar ageing scores and profiles, though,[219] thus pointing to the involvement of oxygen-derived radicals in ageing reactions. Obvious proof of a direct oxidation of beer constituents yielding Strecker aldehydes as induced by reactive oxygen species is clearly lacking in published literature.

Per chemical definition, oxidative processes comprise all reactions at which an electron transfer between two compounds and thus a change of their oxidation number occurs, and consequently, oxygen is not necessarily involved in all oxidation reactions. However, because of its harmful character, oxygen is considered the main initiator and culprit of oxidative reactions during beer production and storage, also because little contaminations with oxygen, e.g. diffusion through the crown cork, yet appear to have a high impact on product quality.

Contemporary scientific knowledge describes the route of oxygen in biological systems as follows: Atmospheric oxygen is relatively stable and non-reactive because it is predominantly present in a triplet state, i.e. in this particular state, the electron configuration of the oxygen molecule features two unpaired electrons with parallel spin occupying two different π*-orbitals or 2p-shells. Unlike most other organic molecules which are present in singlet-state (electrons in anti-parallel spin), this unusual electron configuration prevents, according to the Pauli Exclusion Principle, a direct reaction with most organic molecules. Triplet-oxygen can however be activated and readily react with molecules in a doublet state, such as radicals, thereby forming new radicals. However, for reactions with most organic molecules to take place, the electron configuration of triplet-oxygen must be changed into singlet state. The energy for this to happen can come from photochemical activation by light irritation or from reduction by strong reducing substances. Oxygen is then transferred into an active, reactive, and mostly radical-type species, the so-called reactive oxygen species (ROS).

In fact, during the last three decades of brewing-related research, ROS were identified as one of the primary factors contributing to beer deterioration processes.[26, 47, 76, 132, 134, 151, 154, 251] Because of its negative redox potentials (-0.33 V), a strong reducing agent is needed to activate

oxygen.[102] In wort or beer, most of all, the Fenton and Haber-Weiss-reaction are thought to be responsible for oxygen activation and consecutive reactions. Transition metal ions such as iron and copper act as catalysts in these reactions and are also claimed to play a central role in the activation of oxygen.[124, 131, 134, 296] Though, on a second thought, the process of oxygen activation appeared to be more multifaceted and a complex electron chain in which polyphenols, *iso*-α-acids, reductones, further Maillard reactions intermediates, or in mash or wort, also enzymic systems can participate.[24, 30, 124, 134, 196, 24150] The activation energies for this intricate interplay of different constituents varies, and the temperature plays a distinct role in providing the activation energy needed which is probably one of the reasons why different storage temperatures result in different ageing profiles.[86, 133, 219] Once oxygen is activated, a spontaneous complex reaction cascade with oxygen-derived radicals and non-radical oxidizing intermediates follows. From triplet oxygen, first, the superoxide anion ($^{\bullet}O_2^-$) is formed, which can be protonated giving rise of the perhydroxyl radical ($^{\bullet}OOH$). $^{\bullet}O_2^-$ can however also be further reduced to the peroxide anion ($^{\bullet}O_2^{2-}$) which is readily protonated again giving H_2O_2. Within the Fenton or Haber-Weiss-reaction, ultimately, the highly-reactive hydroxyl radical ($^{\bullet}OH$) is generated. The redox potentials of the different intermediates in this reaction chain increase with proceeding reduction of the different oxygen species starting from -0.33 V ($^3O_2$) and ending at +2.33 V ($^{\bullet}OH$). The higher the redox potential, the higher is the tendency of a substance to oxidize molecules with lower redox potentials. The oxidizing agent is concomitantly reduced. Because of its high redox potential, $^{\bullet}OH$ is one of the most reactive radicals known and is capable oxidizing of virtually all organic substances spontaneously.[71, 99, 102] In Figure 1, a schematic overview of oxygen activation with arising intermediates and the consecutive reaction of the hydroxyl radical with ethanol in beer forming the hydroxyethyl radical is depicted.

While Bamforth and Parsons[26] were the first to mention free radicals in the context of beer staling, in 1988, Kaneda and co-workers[132] were able to analytically identify $^{\bullet}OH$ in beer by using electron spin resonance (ESR) spectroscopy. The recognition of the existence of $^{\bullet}OH$ in beer gave then the initial spark and the origin and the impact of ROS were elucidated with great effort. Evidence of the existence of hydrogen peroxide (H_2O_2) in beer was accomplished ca. 10 years later from Uchida and Ono.[254] Because ethanol is, after water, in beer the most abundant organic constituent, hydroxyl radicals are believed to readily react with it thereby forming the hydroxyethyl radicals whose existence was eventually detected in beer by Andersen and Skibsted.[12] Passionate research in the last decades allowed further characterizing the role of ROS, antioxidants, and prooxidants on beer staling using mostly ESR spectroscopy with

different spin trap agents[9-11, 16-18, 47, 75, 76, 79-82, 113, 119, 132, 133, 135, 137, 139, 149, 150, 152, 154-156, 166, 175, 191, 251-254, 274] or chemulinescence measurements.[133, 296, 297] Clearly, ROS can be anticipated to react with a multitude of beer constituents resulting in adverse changes of the sensory profile of beers.

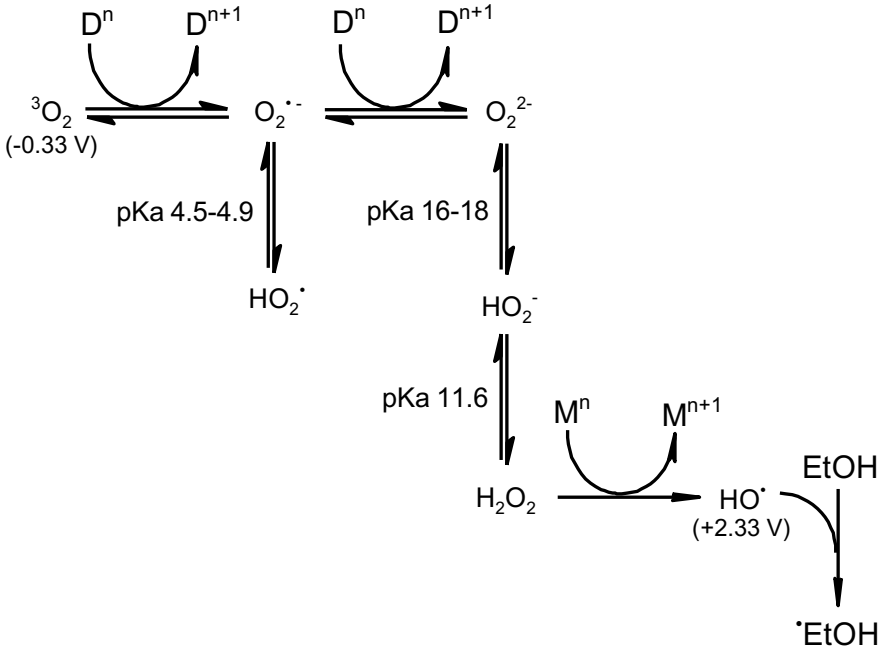

Figure 1: Oxygen activation and consecutive reactions in beer. D = electron donor, e.g. polyphenols, transition metal ion, etc.; M = transition metal ion.[50, 102, 124, 132]

1.3 Antioxidants in beer and the protective properties of hops

Fundamental knowledge about pathways and mechanisms of ROS also allowed to direct research towards identifying and characterizing the role of several beer antioxidants and prooxidants. In attempts to counteract beer flavor alterations, substances potentially retarding or blocking beer deterioration reactions were discovered and potential culprits were detected and ascertained. Naturally, higher transition metal ion concentrations and higher oxygen concentrations yield higher levels of free radicals thus deteriorating beer quality.[131, 133, 134] Though, on closer examination, the situation appears to be more complex. Depending on interactions of substances with the reaction kinetics, the oxidative chain reaction can be blocked, retarded, or promoted at different points (Figure 2).

For the efficacy of an antioxidant, the environmental conditions (pH, polarity, availability of reaction partners, etc.) as well as the concentration of the antioxidants are of major importance.[100] There is additionally indication that synergistic effects and regeneration of antioxidants may be of bigger importance than the effectiveness of the individual substances.[180]

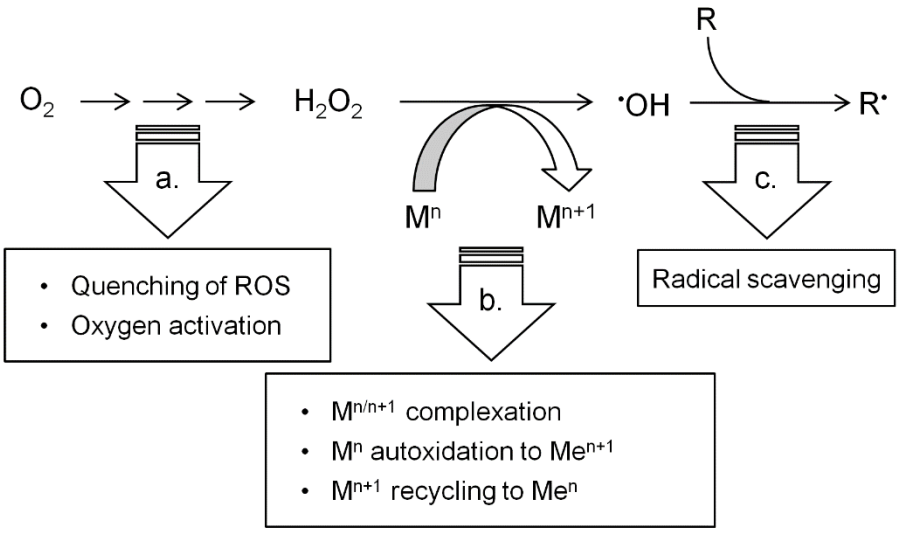

Figure 2: Schematic overview of ROS formation and potential antioxidative or prooxidative mechanisms. The pathways illustrate the mechanism of ROS quenching/oxygen activation (a), possible interactions with metal (M) ions (b), and radical scavenging (c). R = organic substrate.

ROS are usually not always formed immediately once ageing of beer starts but after a definite time period. The so-called 'lag-time' or the 'endogenous antioxidant potential (EAP)' of beer are analytical tools providing information about the time point at which free radicals are generated from the beer matrix. At this, sulfur dioxide (SO_2) was reported to be one of the most important beer constituents[11, 134, 136, 152, 154, 252] and its potential role as an antioxidant was verified in numerous studies. At the pH conditions of beer, SO_2 is predominantly present in its non-volatile conjugated base, HSO_3^-.[136, 271] Its antioxidative character is described foremost by its ability to quench H_2O_2, or other ROS (Figure 2, pathway a) thus rendering them harmless. Secondly but surely not less importantly, HSO_3^- has the capability to reversibly bind aroma-active carbonyl compounds thereby suppressing their adverse aroma impressions.

Metal ion complexation (Figure 2, pathway b) is very intricate and can have both antioxidative or prooxidative effects. The nature and concentration of the ligand, the stoichiometry of the formed complex, and the geometry of the complex itself have a great effect on radical formation.[101, 181] Complexation of metal ions can turn them into a state which is inert to participate in redox reactions or, conversely, make them available to them. Ligands often possess the characteristic to render metal ions in a certain valence state which can have prooxidative or antioxidative consequences depending on whether the reduced or the oxidized form of the transition metal ion will be promoted.[124, 272] Oxygen, nitrogen, and sulfur atoms of molecules are most commonly the metal ligands. Complexing agents with oxygen atoms serving as the ligand tend to stabilize iron in its oxidized form, Fe^{3+}, thus decreasing the

reduction potential of the iron. Opposite, nitrogen- or sulfur-ligands are likely to stabilize Fe^{2+}, thus increasing the reduction potential of iron. Yet, due to the process of complexation even both anti- and prooxidative effects can be caused from the same ligand, depending on the concentration or ligand-to-metal ion ratio.[41, 237] Additionally, the accessibility of the metal ion in the complex plays a major role, and complexation agents may be capable of 'shielding' the transition metal ion from partaking in reactions with potential reaction partners (e.g. H_2O_2) thereby preventing redox reactions of the transition metal ion.[28, 35, 289] A further consequence of ligand-metal ion interactions comprises the ability of a complexation agent to remove metal ions bound to other molecules thus preventing them from undergoing site-localized reactions with the molecules.[97] Conversely, metal ions may also ease the reaction of molecular oxygen with biomolecules by acting as a bridge between them, as both oxygen and biomolecules can e.g. bind to iron through their *d* orbitals thus removing oxygen's spin restriction (see also chapter 1.2).[101, 181]

The effectiveness of ascorbic acid added as a potential preservative was shown to be closely connected to the beer matrix and the beer's SO_2 concentration.[174] Because of its reductone moiety, ascorbic acid has the potential of reducing metal ions to their lower valence state thus making them capable of acting as catalysts in the Fenton reaction again[113, 155, 191] which may further explain its partly prooxidative character. This or a similar behavior was also suspected to be the case for other intermediates of the Maillard reaction and for certain polyphenols, as there was reported both antioxidative[1, 15, 157, 161, 178, 179, 268, 269] and prooxidative[9, 10, 45, 46, 50, 147, 191, 220] behavior. Iron is believed to be primarily existent in its lower valence state, Fe^{2+}, in wort or beer, which has important consequences for its behavior and reactivity in beer staling mechanisms as it is always present in its harmful state, at least as long as reducing substances are present.[113, 131, 134, 153, 155, 166] The substances' strong reducing potential may furthermore also trigger activation of ground-state oxygen to its reactive counterparts (ROS), which adds further prooxidative characteristics and consequences.[2, 34, 42, 44, 124, 220, 292]

Scavenging of $^{\bullet}OH$ (Figure 2, pathway c) is also discussed as one important feature of antioxidants. The unpaired electron is captured by the antioxidant and must be stabilized in an inert state.[100] This characteristic is attributed to many polyphenols as they are capable of forming stable phenoxy radicals which are resonance-stabilized.[7, 8] Anthocyanidins may furthermore be capable of donating an electron (accompanied by a hydrogen nucleus) to a free radical from their hydroxyl groups. The electron, in turn, stabilizes the free radical thereby rendering it less harmful. The polyphenol is becoming an aroxyl radical which is more stable due to resonance-stabilization than the free radical prior to its reduction. Halliwell et al.[100]

doubted that any antioxidant would be capable of scavenging •OH directly (Figure 2, pathway c) at noteworthy levels, though. They anticipated that this demanded unrealistically high concentrations of antioxidants as the reactivity of •OH is too high and reaction rates with target molecules were too fast. According to Halliwell et al.[100], suppression of •OH is more likely to be derived from preventing the catalytic action of transition metal ions. Furthermore, the researchers suspected that transition metal ion complexation does not necessarily yield suppression of •OH generation but rather defines the location of their appearance. Because of the physical proximity of •OH formation and the antioxidants, it is likely that •OH reacts then with the antioxidants instead of being released into solution.

Synergistic effects between antioxidants for wort or beer are indeed also conceivable. However, the knowledge of these interactions is very limited in beer systems. Yet, Rogers and Clarke[216] indeed suspected a possible regeneration of thiols in beer by SO_2. Recent work from Lund et al.[166] supports this hypothesis. Proteins bearing thiol groups are believed to be potent antioxidants. Cystein, thioredoxin, and glutathione were, amongst complex proteins, identified as such thiols in beer.[216] For cystein, again also prooxidative effects were reported as its strong reducing potential may again recycle transition metal ions to their lower valence state or trigger oxygen activation.[124] Particularly in interplay with copper ions, cystein was found to produce significant amounts of H_2O_2 and consecutively trigger dimethylsulfide oxidation to dimethylsulfoxide.[23]

Despite the aforementioned intricate situation and potential prooxidant behavior of some compounds, the antioxidant pool of beer comprises, in addition to SO_2, reductones, certain Maillard reaction products, polyphenols, and thiol-containing proteins. Amongst the discussion and discovery of antioxidants, hops were also found being a rich source of antioxidants and the high potential of hops to preserve beer was used already many years ago when beers were exported overseas.

The role of hops in beer brewing is pivotal. In addition to their preservative effects, they are commonly added to the boiling wort because of their vital contribution to the bitter taste and their distinct aroma. Chemically, dried hops are constituted of numerous different organic compounds from which the hop resins, hop essential oils, and hop phenolic material are probably most essential for beer quality.[39] Basic hop chemistry[38, 57, 58, 242], the composition of hop products[29, 112, 117, 193], the hop compounds' genesis during brewing and storage[39, 57, 104, 105, 121, 122, 126-128, 223, 242, 263], and contribution of different hop constituents to beer bitterness[83, 84, 118,

[143, 144, 167, 168, 199], aroma[85, 92, 123, 130, 170, 171, 201, 202, 207, 213, 239, 240, 258], beer foam[6, 22, 90, 116, 146, 184, 232, 233], or antimicrobiological activities[6, 225, 228, 236] are well documented in the literature.

Even though one may find hops and their constituents very well investigated in last decades, ongoing research still reveals new outcomes or rectifies earlier findings. In 2013, Urban and colleagues[256] discovered that earlier proposed structures of *iso*-α-acids from Alderweireldt et al.[3] were incorrect and reported the correct absolute configuration of (+)-*cis*-*iso*-α-acids to be (4*S*, 5*R*). The chemodiversity of key bitter compounds in hops was recently expanded again by unknown compounds[64], and particularly the hard resin of hops attracted much interest as it contributes aroma and bitterness to beer.[4, 5, 63, 246, 247] Arising popularity in cold hopped beers furthermore triggered to delve deeply in the contribution and interplay of hop essential oils, and technological factors affecting hoppy aroma and aroma perception.[73, 74, 123, 206, 207, 213, 240, 245, 258, 259, 277-279, 287] Furthermore, other than beer-related research, investigations about the physiological relevance of hop constituents constantly unearth yet unknown beneficial properties, particularly of xanthohumol, a unique prenylflavonoid present in the hops plant.[141, 163, 257, 262, 270, 290, 291]

In addition to investigating the hops' bittering, aroma, anti-microbiological, health beneficial, and foam-enhancing properties, researchers discovered many antioxidative and antiradical compounds in hops. Albeit it seems unambiguous that unhopped beers are clearly subordinate in terms of resistance against staling and storage-induced appearance of off-flavor compounds in comparison to hopped beers[14, 91, 127, 149, 173, 179], there are contradictory results in terms of the impact of the hop product and to whether hop-derived polyphenols or the compounds contained in the soft resins of hops make up the primary origin of the protective properties of hops.[14, 157, 161, 178, 179, 274] Most studies in fact claim that the hop phenolic fraction represents the key origin of the hops' antioxidant properties.[129, 145, 157, 158, 161, 179, 185] Though, these studies often utilized artificial radical species such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical, the 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet}$) radical, or used assays for determining physiological relevance. The meaningfulness of these assays for beer staling mechanisms may be questionable. Artificial radicals can have different reaction rates or prefer different reaction partners[78, 183, 288] than naturally occurring radicals, and falsely conclusions may thus be drawn. Secondly, environmental conditions, such as pH, temperature and attacking sites of the marker molecules, are often different in assays than those in real conditions, which may affect the effectiveness of an antioxidant.[100] These assays may therefore not always reveal the 'true picture', and should always be used in combination with other assays or applied in experiments with practical relevance. Yet, Liu et al.[162] were able to show that DPPH$^{\bullet}$

scavenging correlated well with staling taste test score of beer after storage, which is proof for the applicability of this assay for determining factors affecting beer ageing. However, DPPH• shows no reaction with SO_2[133], which is a very strong antioxidant in beer, and its application may therefore be limited again. To correctly assess antioxidants, one should therefore keep in mind how the assay used works, and that it may not be able to display all efficacies of an antioxidant, thus not coming up with falsely conclusions.

Numerous studies also reported findings opposite from the widespread opinion that hop polyphenols make up the origin of the hops' anti-staling distinctiveness, and found also potent antioxidants in the soft resin fraction of hops.[91, 149, 164, 179, 197, 244, 250, 274] Studying the hop acids' antioxidant behavior is in fact difficult as they show only very limited solubility in aqueous solutions[235] which is a prerequisite for some assays, e.g. for determining superoxide dismutase activity according to Oyanagui and Sato.[198] Not earlier than 1993, Oyaizu et al.[197] were probably the first to identify that methanol extracts from hops possessed antioxidant activity. Tagashira et al.[244] indicated two years later that hop α-acids and β-acids possess remarkable DPPH• scavenging potential and inhibit lipid peroxidation. Because DPPH• scavenging in naturally occurring hop acids was detected but not in artificially prepared analogues lacking the 5-hydroxyl group, this group was claimed to be the active site for its scavenging activity. Since all hop acids analogues with *β,β'*-triketone moiety featured lipid peroxidation inhibitory activity, this group was suspected to be responsible for this characteristic. Liu et al.[164] used a β-carotene-linoleic acid model and oxidative decay of 1,10-phenanthroline for measuring the antioxidative activity and •OH scavenging activity of hop oils and hop acids and found the highest antioxidant activity and •OH scavenging activity for hop oils and α-acids, followed by β-acid and *iso*-α-acids and derivatives. Hot water extracts of different hop cultivars containing readily water-soluble polyphenols and flavonoids showed lower •OH scavenging activity than ethanol or CO_2 extracts. Consequently, Liu et al.[164] concluded that higher hop bitter acid contents in hop varieties result in stronger antioxidant activities thereof. These data clearly contradict claims from Lermisieu et al.[157] who stated the preserving efficacy of hops can be solely traced back to hop polyphenols presence.

Ting and co-workers[250] confirmed earlier results using the DPPH• assay again, and additionally applied ESR spectroscopy with N-tert-Butyl-α-phenylnitrone (PBN) as a spin trap to assess the hop constituents' effect on wort boiling conditions and in beer. During wort boiling and in beer, hop α-acids were the most potent radical suppressant followed by hop pellets and hop CO_2 extract. Polyphenols as contained in the pellets had no additive effect. Colupulone, an analogue of hop β-acids, showed moderate suppression of radicals while the *iso*-α-acids' effectiveness

was negligible. These results were later confirmed by Wietstock et al.[274] who were additionally able to show that dosing hops to wort yielded consequently also lower staling aldehyde levels. Ting et al.[250] explained the hop constituents' effectiveness, particularly of α-acids, by their capability to form stable phenoxy radicals which can act directly as antioxidants, by their complexation functionality towards transition metal ion, and from their ability to block or inhibit non-oxidative α-dicarbonyl reactions. Though, it should be noted that these assumptions had rather theoretical character and no direct evidence of the designated activities were delivered. A further elucidation of the hop acids' mode of action was still outstanding.

A Serbian researcher team determined the antioxidant activity of hop cones and commercially available hop products by different antioxidant assays.[91] Unsurprisingly, hop-derived total phenolics were significantly correlated with $DPPH^{\bullet}$ quenching and yet, α-acids were found to significantly scavenge H_2O_2 as measured by a direct current polarographic method. H_2O_2 scavenging and $DPPH^{\bullet}$ scavenging activity were consequently completely lacking significant correlation. Even though hop phenolics were also found in further experiments to be superior in a ferric reducing antioxidant power (FRAP) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging, Gorjanović et al.[91] still suspected that hop α-acids make up the fraction in hops with the stronger preserving effectiveness. Recently, de Almeida and co-workers[52] investigated the reactivity of hop β-acids towards the predominant radical in beer, the 1-hydroxyethyl radical. They identified the three prenyl-side chains as the reaction sites thereby arousing resonance-stabilized radicals. These radicals, in turn, were supposed to further react with molecular oxygen, $^{\bullet}OH$, or 1-hydroxyethyl radicals thus giving rise of β-acid oxidation products. A similar mechanism was described for *iso*-α-acid oxidative decomposition by 1-hydroxyethyl radicals.[53] Although de Almeida's objective was not to elucidate antioxidative behavior, deductions from these data still point to the antioxidative properties of hop β-acids.

Assays for determining antioxidant activities of test compounds can deliver useful information about activities of individual compounds and their distinctiveness. But when working in a complex wort or beer matrix with numerous constituents, it eventually counts if staling is suppressed and beer flavor is enhanced by their addition. The 'true' effectiveness of an antioxidant can be only ascertained by actual brewing trials, followed by ageing of the beers, and sensorial and analytical characterization of off-flavors. Despite the consensus that the hop dosage is considered to have protective effects, there are only comparatively few studies available attesting the counteracting effect of the hop dosage on the emergence of ageing-related compounds during ageing.[14, 173, 179, 274]

In her dissertation work, Aron[14] performed semi-technical brewing trials and found that unhopped control beers clearly scored highest in ageing score and in levels of aldehydes as compared to beers produced with hop components added. All kettle hopping regimes reduced the concentration of potentially prooxidative iron and copper ions while pelletized hops showed the greatest effect. Spent hops and pelletized hops were highest in FRAP, though ESR data was contradictory and beers high in hop polyphenols were lowest in flavor stability. In terms of total staling aldehydes measured, beers produced with pelletized hops were again lowest in concentration, followed by beers dosed with CO_2 extract and spent hop material. The protective effect of hops was therefore concluded to be best when using whole hop products. Czech researchers also performed different hopping technologies during semi-technical scale beer production and used sole CO_2 extract, hop pellets, or combinations of both.[179] $DPPH^{\bullet}$ reducing activity was significantly correlated with 2-furfural and *trans*-2-nonenal after forced ageing, and dosing hop pellets either at the onset of boil or in distributed dosages was superior in terms of sensorial ageing scores and staling aldehyde levels measured. In fact, dividing the total hop mass dosed appeared to be superior in terms of flavor stability as opposed to a single dose. Again, in accordance with Aron's work,[14] hop products containing both hop acids and hop polyphenols were concluded as being preferably used. Wietstock et al. [274] as well as Kunz and co-workers[149] yet came upon the presumption that despite hop polyphenols possess antioxidative potential and undeniably contribute to beer flavor stability, hop α- and β-acids still make up the hop fraction with the highest antioxidative potential. Their investigations therefore utilized solely hop CO_2 extract when performing brewing trials. A clear correlation between hop mass dosed and suppression of staling aldehydes[274] as well as the positive effects of dividing the hop dosage or the application of modified hop products on beer flavor stability was unveiled using ESR spectroscopy.[149] These findings taken together suggest that there is a great potential of the hop dosage, and furthermore suggest a further optimization of the hop dosage in terms of improving resistance of beers against oxidation. Fundamental knowledge about pathways and the hops' mode of action is a premise for approaching optimization steps.

2 Research justification and objectives of this study

Flavor instability of beer remains one of the most challenging quality problems in the brewing industry. Substantial understanding of fundamental reactions and molecular interactions is a base premise to approach the problem of this issue. Despite ongoing years and endeavors of research as related to the molecular processes occurring during beer storage, knowledge about the processes is still underrepresented in published literature, particularly as related to the consequences of reactive oxygen species, their relationship with the formation of off-flavor compounds during beer storage, and the anti-staling effect of hops, particularly hop acids. Breweries, who work according to the regulations of the German purity law, are not allowed to add preservatives to their beers, and beer production is limited to the four raw materials water, malt, hops, and yeast. Beer quality and particularly, oxidative beer flavor stability is therefore determined by the choice of the raw materials as well as by technological factors. Hops were discovered to possess potent antioxidative compounds and the hop dosage has clearly a decisive impact on the endogenous antioxidative potential of beer. The efficacy of the hop dosage as related to the blockage or inhibition of oxidative reactions in wort and beer, and the associated suppression of staling-related compounds are yet underexplored in brewing-related literature. Optimization of the hop dosage directed to improving the oxidative flavor stability of beer is still outstanding.

The first part of this dissertation work therefore aimed to close a knowledge gap and link the detrimental role of reactive oxygen species directly to the formation and appearance of staling-compounds during storage of beer. Trials were first carried out in buffered model solutions and subsequently, findings were transferred to actual beer production and storage. As a consequential step, and linked but not necessarily limited to the findings and proposed pathways from the first part of the thesis, **the second part** of this thesis focused on the suppression of oxidative processes yielding off-flavor compounds in beer by revealing, classifying, and exploiting the hops' antioxidative potential. At a glance, the present dissertation work comprised the following steps:

- Identification of an ROS-induced pathway yielding staling compounds during wort production and beer storage
- Detection and classification of antioxidative substances in hops and investigation of their pertinent modes of effectiveness
- Verification and application of the findings to the brewing process

In Figure 3, a schematic overview of the initial starting problem, the problem-solving approach, and the aim of the study are shown and put into context.

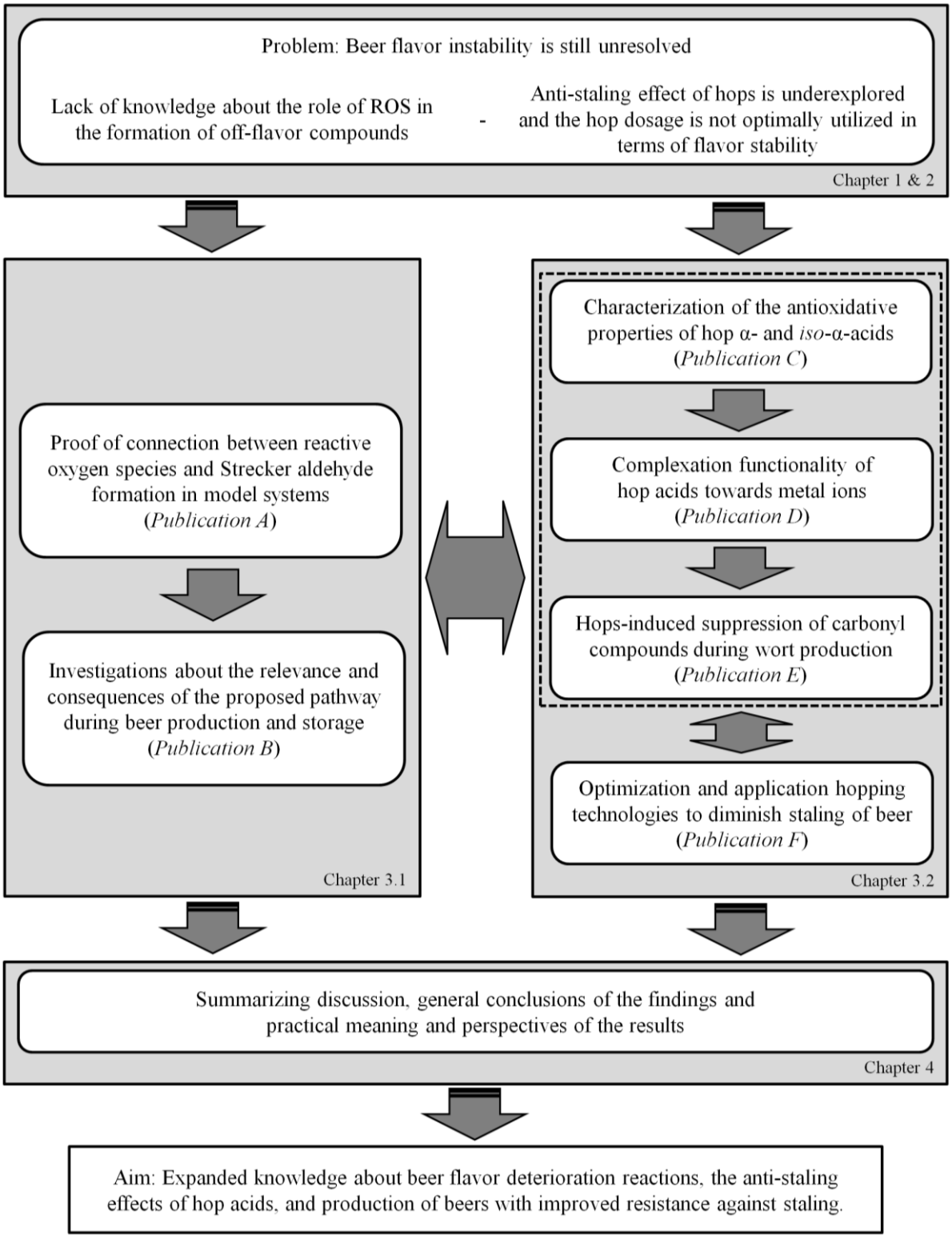

Figure 3: Overview about the structure of the thesis, the initial problem, the problem-solving strategy, and the aim of the dissertation work.

3 Publications

The results of the single examinations of this thesis were published in six international peer-reviewed journals. All publications were originated from solitary experiments, the publications' findings and outcomes are closely related, though. In publication A and B, the work on elucidating an oxidative pathway for the formation of Strecker aldehydes by oxidative degradation of amino acids during beer storage and wort production is presented. Publications C, D, E, and F address the antioxidative properties of hops, their mode of diminishing aldehyde formation, and finally, their optimized application in the brewing process.

The experimental work for the publications A, B, D, E, and F was accomplished at the Technische Universität Berlin under supervision of Prof. Dr.-Ing. Frank-Jürgen Methner during the period of the years 2011-2016. Publication C originated from experimental work conducted at the Oregon State University, Corvallis, OR, USA, under supervision of Prof. Dr. Thomas H. Shellhammer during the year 2010. Publication D was published in shared first-authorship with Thomas Kunz.

The own-share of all publications in sum is ca. 75-80 %. Ideas for the publications A, B, D, E, and F were created in cooperation with Prof. Dr.-Ing. Frank-Jürgen Methner. The idea for publication C was developed together with Thomas H. Shellhammer during the period of residence at the Oregon State University. Publication D was published in shared first-authorship with Thomas Kunz. The own share of both first-authors is therefore ca. 50 %. The experiments were planned, executed, and interpreted together. Except for publication D, the first author was solely responsible for the planning, experimental design, execution, and evaluation of the experiments. The practical examinations were done solely or with assistance from student associates. The lab of the chair of brewing science at the Technische Universität Berlin provided analytical support.

The publications shall now be presented in the following chapters 3.1 and 3.2. They are presented in coherent order and not arranged according to the dates of publication. In chapter 4, the publications' most significant results and findings are recapitulated, their interrelationship is explicitly pointed out and discussed, critical points are addressed, and the works' significance for the brewing industry is reflected.

3.1 Evidence of an oxygen radical-mediated formation of aroma-active aldehydes from amino acid precursors during beer production and storage

Publication A

Comprehensive literature research delivered a coherent pathway for the oxidative degradation of amino acids by hydroxyl radicals yielding Strecker aldehydes formerly postulated by Stadtman and co-workers[238]; yet, their work was carried out at human-physiological conditions and with the focus on biological consequences.

In an attempt to find a reasonable explanation for the detrimental role of oxygen and ROS during beer production and storage, in a consequential step to Stadtman's work, it was therefore tested if this pathway was also applicable to beer-like environments and to identify the reaction products as derived from amino acid oxidative degradation by ROS-attack. The complex beer matrix was reduced to buffered model systems. The complete description, the results, and the discussion of this study can be found in the publication

"Formation of Aldehydes by Direct Oxidative Degradation of Amino Acids via Hydroxyl and Ethoxy Radical Attack in Buffered Model Solutions"[283] *(Publication A).*

Taken together the findings, it could be evidently demonstrated that oxidative degradation of amino acids by the Fenton reagents H_2O_2 and Fe^{2+} yields their pertinent Strecker aldehydes in a beer-like environment. Furthermore, a clear relationship between hydroxyl and hydroxyethyl radical levels and Strecker aldehydes was found. A reaction route relevant for beer deterioration reactions could be postulated.

P. C. Wietstock, and F.-J. Methner

Formation of Aldehydes by Direct Oxidative Degradation of Amino Acids via Hydroxyl and Ethoxy Radical Attack in Buffered Model Solutions

The formation of aldehydes in bottled beer is promoted by the presence of oxygen and transition metal ions. In this paper, a so far unrevealed pathway to explain this phenomenon is presented. Leucine, isoleucine, and phenylalanine were oxidized by H_2O_2-Fe^{2+} in 'beer-like' buffered model solutions (pH 4.5; 5 % (v/v) ethanol) at room temperature thereby yielding 3-methylbutanal, 2-methylbutanal, phenylacetaldehyde, and benzaldehyde, respectively, as measured and identified by solvent extraction and HRGC-MS. Further trials revealed that the aldehydes formed were significantly correlated with radical concentration as determined by electron spin resonance (ESR) spectroscopy indicating that hydroxyl radicals (•OH) and ethoxy radicals (EtO•) are involved in the pathway. A reaction route for 'beer-like' model systems is featured and confirmed by a storage trial in which a steady increase of aldehydes over a time span of 18 days could be demonstrated.

Descriptors: aldehydes, amino acids, electron spin resonance spectroscopy, hydroxyl radicals

1 Introduction

Beer instability has many faces and can be divided into a number of categories such as physical-, flavor-, microbiological-, foam-, and light (in)stability. Non-oxidative and microbiological deterioration processes are most widely under control and have been conquered by technical improvements and cautious industrial hygiene. Next to a myriad of flavor-active compounds deriving from the yeast metabolism contributing to the perception of bottled beer, unwanted off-flavors arise during storage which is a result of complex reactions occurring in the final product. Estery and floral aromas that are initially recognized as pleasant will decrease. The bitterness quality and sulfury notes decline while other off-flavor notes like bready-, sweet-, caramel-, and sherry-like notes are recognized [17, 20, 45, 67, 68, 71]. Research from the last years and decades gave evidence that carbonyl compounds are primarily responsible for the appearance of such off-flavors as they exceed their individual flavor thresholds during storage or are thought to be involved in a synergistic interplay [35]. Although an uncountable number of compounds are involved in the perception of stale flavor, researchers agreed that specific aldehydes provoke such in bottled beer and are consequently used as markers for analytically identifying and monitoring stale flavor during storage. The individual aldehydes greatly vary in their contribution to the aged odor and flavor of beer. In comparison to other carbonyls, (E)-2-nonenal has a very low determined flavor threshold of 0.03–0.05 ppb [57, 66] and is accompanied with the so-called "cardboard" or "bready" flavors [6, 7, 34, 38, 46]. The odor thresholds of 2-furfural and 5-hydroxymethylfurfural were determined with odor thresholds of 15.16 and 35.78 ppm, respectively, and are perceived as caramel-like and bready [27, 46, 57]. Additionally, *De Clippeleer* et al. [19] demonstrated that 2-furfural has a remarkable effect on beer astringency and mouthfeel even when present in a sub-threshold flavor concentration of 400 ppb. Further compounds comprise the group of the so-called Strecker aldehydes which involves 2-methylpropanal (86 ppb*; grainy, fruity), 2-methylbutanal (45 ppb*; almond-like, malty), 3-methylbutanal (56 ppb*; malty, chocolate-, cherry-, almond-like), methional (4.2 ppb; like cooked potatoes, worty), phenylacetaldehyde (105 ppb; flowery, hyacinth-, roses-like), and benzaldehyde (515 ppb; almond-, cherry-, stone-like) [46, 57]. Their respective odor thresholds (marked with an asterisk), flavor thresholds and descriptors are mentioned in parentheses.

In beer and during beer production, all these compounds are formed in interplay of many constituents from the raw materials (malt, hops, water, yeast) and through complex reactions in which factors like temperature, oxygen, light irradiation, enzymatic, and pro-oxidative and antioxidative interactions are involved. Enzymatic oxidation by oxygen oxidoreductases, and photo- and autoxidation of unsaturated fatty acids, such as linoleic and linolenic acid, contribute to the formation of fatty acid derivatives such as (E)-2-nonenal [21, 34, 61] and hexanal [24]. The non-enzymatic browning or Maillard-reactions occur primarily during wort boiling in beer production and give rise to many different products. One of the most-established and surveyed reaction in this context is the reaction of an amino acid with an α-dicarbonyl compound yielding Strecker aldehydes which contain one carbon atom less than

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the respective amino acid ('Strecker degradation'). Although all amino acids can theoretically react in such way, only the Strecker aldehydes from valine (2-methylpropanal), leucine (3-methylbutanal), isoleucine (2-methylbutanal), methionine (methional) and phenylalanine (phenylacetaldehyde and benzaldehyde) are relevant in beer because of these amino acids concentrations in beer and their Strecker degradation products' flavor thresholds [46, 57]. According to *Coghe, Derdelinckx* and *Delvaux* [15], the Maillard reaction mainly commences at elevated temperatures (above 50 °C) and in a slightly acidic pH range (pH 4–7). There is evidence that Maillard reactions also occur at lower temperatures, e.g. during beer storage, thereby consequently contributing to the appearance of off-flavors [10, 11]. However, outcomes from this study are partly ambiguous because evidence was based on blocking α-dicarbonyls with aminoguanidine [10]. In addition to rendering α-dicarbonyls 'harmless', aminoguanidine is also capable of reacting with monocarbonyls directly [1], accordingly reducing stale aroma in beer.

The rate of beer staling and the appearance of off-flavors are often linked to the presence of oxygen and transition metal ions in bottled beer. In particular Strecker aldehydes were found in higher concentrations when oxygen was present at elevated levels [54]. *Clapperton* [14] observed that the ribes flavor in beer was closely connected to the amount of air in the headspace. *Blockmans, Devreux*, and *Masschelein* [9] added the amino acids valine and leucine to beer and noticed increased concentrations of the corresponding aldehydes, 2-methylpropanal and 3-methylbutanal, when oxygen was present. This effect was strengthened by addition of copper and iron ions. Studies from *Miedaner, Narziss* and *Eichhorn* [51] verified these findings and observed also higher concentration of Strecker aldehydes when oxygen was abundant in bottled beer while other carbonyls, e.g. 2-furfural, were unaffected. It is not fully understood which reactions are accountable for these observations. The Strecker degradation of amino acids cannot be held solely accountable for this phenomenon. *Hashimoto* and *Eshima* [30, 31] proposed the melanoidin-catalyzed oxidation of relevant higher alcohols and the aldol condensation of unsaturated aldehydes as further potential pathways. *Barker* et al. [7] doubted their relevance for beer storage and anticipated that aldehydes are mainly formed during wort boiling, subsequently binding to bisulfite during fermentation and forming reversible complexes which are then released during storage. The existence of sulfite-adducts was also studied and confirmed by *Kaneda* et al. [39]. *Baert* et al. [4, 5] studied these possibilities in greater detail and furthermore suspected that imine adducts can be also be a source of carbonyls. *Suda* et al. [62] added C^{13} labeled amino acids to filtered wort before boiling for 90 minutes and claimed that 85 % of the Strecker aldehydes present after 2 weeks at 37 °C aging were derived from wort boiling and 15 % were derived from Strecker degradation in the bottled beer itself. A potential reaction pathway involving oxygen was proposed by De Clippeleer [18] in which the oxidative degradation products of isohumulones, the hydroxy-allo-isohumulones, act in a Strecker-like reaction thereby yielding the corresponding aldehydes. However, this reaction pathway which has been postulated from studies in model systems may be negligible in beer because of the low oxygen concentrations in beer. Further studies in model systems revealed that transition metal ion-catalyzed oxidation of Amadori-compounds also contributes to the formation of Strecker aldehydes [32, 56]. It remains open, though, if these reactions are also applicable for beer systems.

The role of oxygen and transition metal ions is closely connected to the discovery of reactive oxygen species (ROS) [37, 64, 65]. Studies using electron spin resonance (ESR) spectroscopy helped to verify their existence and further elucidate their influencing factors during beer production and in the final product [2, 3, 25, 41–44, 47–50, 55, 63, 72]. ROS, and in particular the highly reactive hydroxyl radical ($^{\bullet}OH$), are formed in the Fe- and Cu-catalyzed Fenton- and Haber-Weiss-reaction systems. Hydroxyl radicals, in turn, react nonspecifically with organic beer constituents but in principal with ethanol thereby generating ethoxy radicals ($EtO^{\bullet}$) because ethanol is, after water, the second most occurring organic compound in beer [3].

Stadtman and *Berlett* [60] were the first to describe a pathway involving transition metal-catalyzed oxidation of amino acids, and found ammonium ions, α-keto acids, CO_2, oximes and aldehydes or carboxylic acids with one carbon atom less as the corresponding amino acids as the major reaction products. The oxidation products of leucine were studied in greater detail and 3-methyl-2-oxobutyric acid, 3-methylbutanoic acid ethyl ester and 3-methylbutanal were identified as its derived oxidation products [59]. At acidic pH ranges, this pathway leading to the formation of derived aldehydes, ammonium ions and CO_2 appears more likely to happen [16, 59]. All these trials, however, were carried out within the scope of cell ageing at a physiological pH and without the presence of ethanol.

There is evidence that 'beer-radicals' are closely connected to beer deterioration reactions; however, the direct impact of those radicals for beer flavor deterioration reactions such as the formation of staling-related aldehydes has not been elucidated thus far. Both the existence of oxygen-derived 'beer-radicals' and the published claims that more Strecker aldehydes are formed when elevated levels of oxygen are present in bottled beer support the conclusion of the existence of a direct oxidation of amino acids by radical attack as proposed by Stadtman and Berlett [60]. The objective of the study presented was to investigate if the mechanism as described by Stadtman and Berlett [60] is also applicable for a 'beer-like' model system with a particular focus on the concomitant formation of staling-related aldehydes.

2 Materials and Methods

Chemicals. Acetic acid (glacial), α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN), iron(II)sulfate heptahydrate, iron(III)chloride hexahydrate, leucine, and phenylalanine were obtained from Sigma Aldrich Inc., Steinheim, Germany. Hydrogen peroxide and isoleucine were purchased from AppliChem GmbH, Darmstadt, Germany. Ethylenediaminetetraacetic acid (EDTA), sodium acetate trihydrate, sodium carbonate, and sodium sulfate were obtained from Merck KGaA, Darmstadt, Germany. Diethylether and anhydrous ethanol were purchased from VWR international GmbH, Darmstadt, Germany. All chemicals were of analytical grade or higher. All solutions were made with double-distilled water and prepared freshly every day.

2.1 Determination of volatile carbonyls in buffered model solutions

Aldehyde concentrations were determined following a modified literature procedure described by *Engel, Bahr* and *Schieberle* [23]. This procedure uses the solvent assisted flavor evaporation (SAFE) technique and high resolution gas chromatography (HRGC) together with mass spectrometry (MS) analysis. In the present study, the distillation step using the SAFE apparatus was skipped because no non-volatile interfering material was expected in the buffered model solutions. An aliquot (100 mL) of sample was spiked with 1 µg of pentanal as an internal standard (IS). The sample was subsequently extracted twice with 150 mL diethyl ether, washed twice with a 0.5 M Na_2CO_3 solution and water, respectively, dried over Na_2SO_4 for 1 h, and concentrated to 5 mL using a Vigreux column at a temperature of 46–48 °C. A sample (1 µL) of the concentrated distillate was applied via a cold injection system (Gerstel, Mülheim, Germany) in 10:1 split mode to a gas chromatograph (6890, Agilent Technologies, Waldbronn, Germany) fitted with a capillary column (VF-5 MS, 60 m x 0.25 mm, 0.25 µm film, Varian, Darmstadt, Germany). The following temperature program was used for the GC oven: after 12 min at 35 °C, the oven temperature was raised to 150 °C at a rate of 12 °C/min and then to 250 °C at 30 °C/min where it was held for 5 min. The flow rate of the helium carrier gas was 0.6 mL/min. The MS analysis was performed by an MSD 5973 mass spectrometer (Agilent Technologies, Waldbronn, Germany). Mass spectra in the electron impact mode (MS/EI) were generated at 70 eV using selected ion monitoring (SIM). The retention times and monitored ions are depicted in table 1. For the quantification of the aldehydes, separate calibration curves for 3-methylbutanal, 2-methylbutanal, benzaldehyde, and phenylacetaldehyde were determined at final concentrations ranging from 0 to 1000 ppb. The compounds were solved individually in the buffer/ethanol mixture and, after following the extraction procedure as described above, measured by using HRGC-MS. All calibrations produced a linear response with an r^2 value > 0.97 over the whole concentration range analyzed.

Table 1 Retention times (t_R) and parameters for selected ion monitoring of the measured aldehydes

		Selected ion monitoring, m/z	
Name	t_R [min]	ion 1	ion 2
3-Methylbutanal	10.6	58	86
2-Methylbutanal	11.1	58	86
Pentanal (IS)	13.1	58	86
Benzaldehyde	23.3	106	77
Phenylacetaldehyde	25.1	91	120

2.2 Determination of •OH and EtO• concentration in sample solutions

The concentration of •OH and EtO• in the samples were determined by using ESR spectroscopy. The principle of the measurement relies on spin-trapping with α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN) and measuring ESR signal intensities using an ESR spectrometer (e-scan, Bruker, BioSpin, Rheinstetten, Germany). The ESR spectrometer was used with the following settings: centre field: 3.475 G; attenuation: 0 dB; sweep width: 100 G; receiver gain: 2.0×10^3; resolution: 512; modulation amplitude: 1.49 G; modulation frequency: 86 kHz; conversion time: 10 ms; time constant: 40 ms; scans: 50. Typical reactions were started by adding 1 mL of a 50 mM H_2O_2 solution to a 9 mL aliquot of the reaction mixtures to be assayed. The ESR measurement was started immediately after the H_2O_2 solution was added. The ESR signal intensity was evaluated automatically by using the software WinEPR, version 4.3 (Bruker Biospin GmbH, Rheinstetten, Germany). The samples' radical concentration is determined as ESR signal intensity. All measurements were carried out at ambient temperature (20–22 °C).

2.3 Influence of ethylenediaminetetraacetic acid (EDTA)-Fe^{2+} ratio on radical formation in buffered model solutions

An ethylenediaminetetraacetic acid (EDTA)-Fe^{2+} complex was used in this study as Fenton reagent for the generation of radicals. The stoichiometry in this complex has a great influence on the effectiveness of radical generation [8, 28]. To study this effect in further detail, a trial was conducted in which solutions of EDTA and $FeSO_4 \times 7\ H_2O$ were mixed and pre-incubated for 5 minutes at different ratios. The tested ratios were 0:100 µM, 100:100 µM, 200:100 µM, and 100:200 µM (EDTA:Fe^{2+}). The trials were conducted in buffer/ethanol mixtures (acetate buffer, pH 4.5, 0.2 mM; 5 % (v/v) ethanol) containing 7.5 mM POBN, and reactions were started by addition of 5 mM H_2O_2. The ESR settings and measurement procedure were the same as described above.

2.4 Studies of aldehyde formation from amino acids (AA) in buffered model solutions

Aldehyde formation in model solutions from AA by direct oxidation via a Fenton reagent was elucidated by preparing reaction mixtures containing 5 % (v/v) of anhydrous ethanol, 100 µM $FeSO_4 \times 7\ H_2O$, and 5 mM of the assayed AA. Typical reactions were started by adding 10 mL of a 300 mM H_2O_2 solution to 90 mL aliquots of the reaction mixtures. The reaction mixtures were then incubated for 1 hour at 20 °C and aldehydes were isolated and determined using the procedure described above.

To elucidate the dependency of aldehyde formation from •OH/EtO• concentration, two experiments were carried out.

In a first set of experiments, the dependency of aldehyde formation from radical concentration was elucidated with the same buffered model solutions as described above. However, in this experiment, instead of adding $FeSO_4 \times 7\ H_2O$ at a fixed concentration, a solution containing 0–200 µM of an EDTA-Fe^{2+} complex (EDTA:Fe^{2+}, 1:1) was added with the goal to vary the radical concentration. Mixing Fe^{2+} with EDTA in a ratio of 1:1 and using this complex should diminish Fe^{2+} autoxidation to Fe^{3+} while hydroxyl radicals are still formed from this complex. As a reference, one sample was prepared by pre-incubated Fe^{3+} with EDTA instead of Fe^{2+} because Fe^{3+} is not capable of acting catalytically in the Fenton reaction. The final H_2O_2 concentration in the model systems used in these trials was 5 mM. Reactions were carried out for 5 days

at 20 °C in the dark and the samples' aldehyde concentrations were determined as described above using diethylether extraction followed by HRGC-MS.

In a separate trial, the test solutions' $^{\bullet}OH/EtO^{\bullet}$ concentrations were determined using ESR measurement as described above. Prior to the measurement, sample solutions were prepared by adding 5 % (v/v) of ethanol, 0200 µM of EDTA-Fe^{2+} (EDTA:Fe^{2+}, 1:1), and 7.5 mM POBN to an acetate buffer (pH 4.5, 20 mM). The reactions were started again by adding 5 mM H_2O_2.

To study the effect of increasing $EtO^{\bullet}$ concentrations on the formation of aldehydes, a second set of experiments was carried out. The previously described assay was modified and the ethanol concentration was adjusted to 0–10 % (v/v) in steps of 2.5 % (v/v) while all the other parameters were kept constant (100 µM EDTA-Fe^{2+} (EDTA:Fe^{2+}, 1:1), 5 mM of the assayed AA). The test samples' aldehyde concentration was determined after 5 days storage at 20 °C in the dark. In a separate trial, the reaction mixtures' $^{\bullet}OH$-/$EtO^{\bullet}$-POBN concentration was determined again by using the same ESR settings and experiment design as described above; however, with the modification that the EDTA-Fe^{2+} concentration was kept constant at 100 µM, and the samples' ethanol concentrations were adjusted from 0–10 % (v/v) in steps of 2.5 % (v/v). All trials were done in triplicate.

2.5 Monitoring the rate of the formation of aldehydes by oxidative degradation of AAs in buffered model solutions

The rate of the formation of aldehydes in buffered model solutions was examined by dissolving 5 mM AAs (leucine, isoleucine, phenylalanine), 100 µM EDTA-$FeSO_4 \times 7\,H_2O$ (EDTA:Fe^{2+}, 1:1), and 5 mM H_2O_2 in 100 mL of a buffer/ethanol mixture (acetate buffer, pH 4.5, 0.2 mM; 5 % (v/v) ethanol) and storing these mixtures for 18 days at room temperature. In total, 6 swing top bottles (V = 180 mL) were prepared. The level of aldehydes was determined from the single bottles after 0, 1, 2, 3, 12, and 18 days with the method described above. For the bottle representing day 0, no H_2O_2 was added and the bottle was measured directly after preparing the samples assuming that no reactions occurred, yet.

3 Results and Discussion

Beer is a very complex matrix and it is thus practically impossible to examine and to give proof of a single reaction pathway. In the present study, the complex beer matrix was simplified and reduced to the presence of AAs and Fe^{2+}. Furthermore, a 0.2 mM acetate buffer at a pH of 4.5 with 5 % (v/v) ethanol was used as the base. Typical reactions were then started by the addition of 5 mM H_2O_2 to the reaction mixtures containing 100 µM Fe^{2+} and 5 mM of AAs. In a first trial, leucine was added to the reaction mixture and incubated for 1 hour at 20 °C. The volatiles from this solution were isolated by solvent extraction, and the isolate was evaluated using HRGC-MS in SIM mode. From this isolate, 3-methylbutanal (t_R = 10.6; m/z = 58/86) was identified as the prevalent peak (Fig. 1, II) and an amount of 0.56 ± 0.11 µM was determined (n = 2). A sample where no leucine was added served as a reference (Fig. 1, I). Further trials were carried out where Fe^{2+} or H_2O_2 were omitted from the test solution in individual trials and double-distilled water was added instead. The omission of Fe^{2+} or H_2O_2 led to a drastic decrease of the amounts of 3-methylbutanal being detected by 83.8 % and 96.7 %, respectively, as compared to the trial where both substances were included. This finding implies that both play a major role in the reaction leading to the formation of 3-methylbutanal. The observations further signify that H_2O_2 may be more reactive in the formation of 3-methylbutanal indicating a direct oxidative degradation of leucine by H_2O_2. A further trial where solely leucine and neither Fe^{2+} nor H_2O_2 was added still revealed a diminutive peak at a retention time of 10.6 minutes indicating that the product which was used for the study was already contaminated with minimal amounts of 3-methylbutanal. The concentration of this peak was determined as 0.015–0.023 µM indicating a contamination of the product by 0.005 %.

Phosphate buffer (0.2 mM; pH 4.5) with 5 % (v/v) ethanol was also tested in the assay and yielded 18.2 % lower amounts of 3-methylbutanal as compared to the use of an acetate buffer (data not shown). The reason may lie in the phosphate buffer's ability

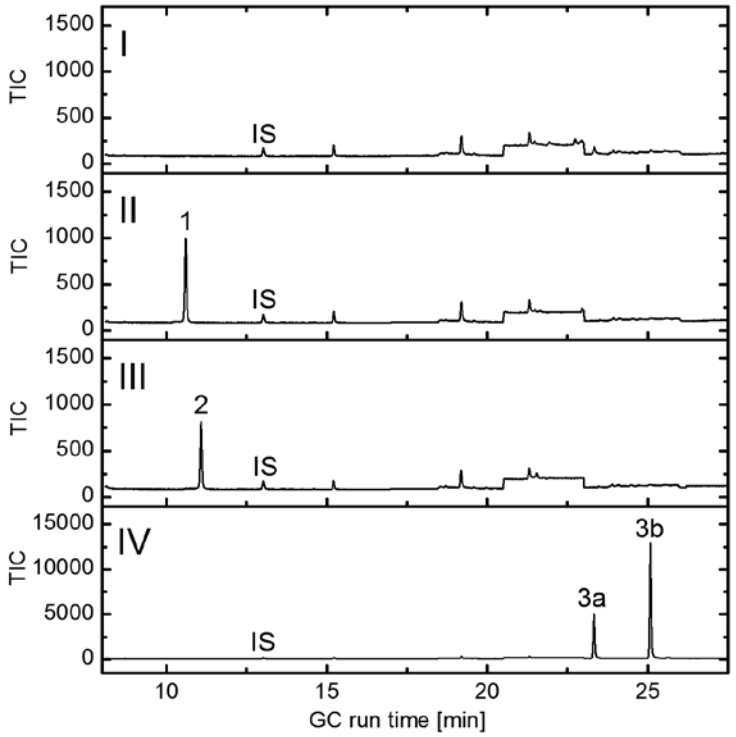

Fig. 1 HRGC-MS chromatograms of the reaction products of Fe^{2+}-H_2O_2 without addition of amino acids (I), and with addition of leucine (II), isoleucine (III), and phenylalanine (IV), respectively. The samples were incubated for 1 hour at 20 °C in a buffer/ethanol mixture (acetate buffer pH 4.5 0.2 mM; 5 % (v/v) ethanol) and isolated by extraction with diethylether. The MS was operated in EI mode (70 eV) using selected ion monitoring.

1: 3-methylbutanal, t_R = 10.6 min, m/z = 58/86;

2: 2-methylbutanal, t_R = 11.1 min, m/z = 58/86;

3a: benzaldehyde, t_R = 23.3 min, m/z = 106/77;

3b: phenylacetaldehyde, t_R = 25.9 min, m/z = 91/120;

IS: pentanal, t_R = 13.1 min, m/z = 58/86

to enhance Fe^{2+} autoxidation to Fe^{3+} [70] thereby decreasing the Fe^{2+} concentration available for the Fenton reaction.

As a complement to the previous trial where leucine was tested, isoleucine and phenylalanine were also examined in individual experiments and incubated for 1 hour at 20 °C. From the isolates, 2-methylbutanal at a concentration of 0.81 µM was obtained when isoleucine was added (Fig. 1, III), and, interestingly phenylacetaldehyde (1.66 µM) and benzaldehyde (6.78 µM) were found when phenylalanine was added to the test solutions (Fig. 1, IV). The appearance of benzaldehyde as derived by oxidative degradation from phenylacetaldehyde was reported recently by *Chu* and *Yaylayan* [13]. The pathway introduced by them also involves an oxygen-induced free-radical mechanism and therefore confirms the observations from this study.

Taken together, these results imply that hydroxyl radicals ($^{\bullet}OH$) which are generated in the Fenton reaction with Fe^{2+} as a catalyst are involved in these reactions. By adding H_2O_2 to the model solutions, the Fenton reaction cascade is initiated and $^{\bullet}OH$ radicals are formed. Ethanol is a major quencher of these radicals and because it is the most abundant compound in the model solutions used, it will be the primary reactant of $^{\bullet}OH$, yielding ethoxy radicals ($EtO^{\bullet}$) [3, 43].

To study the role of $^{\bullet}OH$ and $EtO^{\bullet}$ in these reactions, two additional trials were carried out using the same experiment design as described in the materials and methods section. In a first experiment, varying concentrations of $^{\bullet}OH/EtO^{\bullet}$ radicals were produced by adding different quantities of the Fenton reagent EDTA-Fe^{2+} to the reaction mixtures. This complex was used because pre-incubating Fe^{2+} with EDTA diminishes its autoxidation to Fe^{3+}. Furthermore, iron can provoke site localized reactions with the compound of interest when bound to it [12, 58]. By binding Fe^{2+} to EDTA prior to adding it, these site localized reactions are prevented while Fe^{2+} is still capable of acting as a catalyst in the Fenton reaction thus allowing the 'production' of radicals. For reviews on Fenton chemistry and the efficiency of chelators please consult the following references [29, 70, 74].

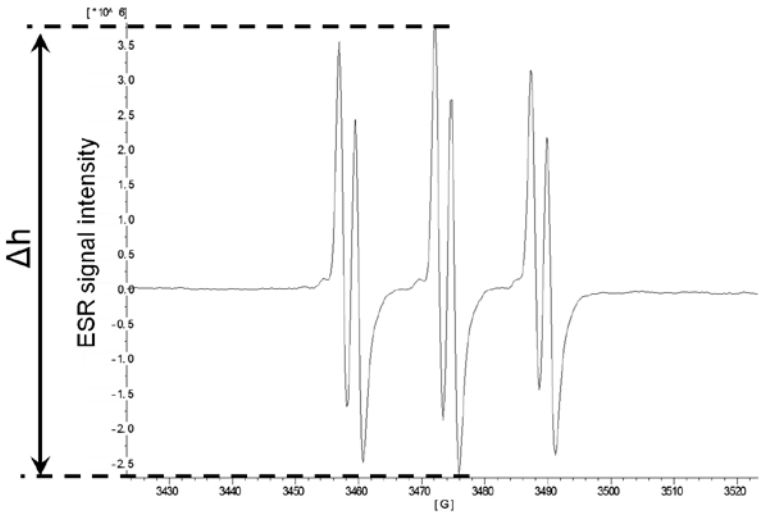

Fig. 2 ESR spectrum of •OH/EtO•-POBN spin trap adducts at room temperature following addition of POBN (7.5 mM), EDTA-$FeSO_4$ × 7 H_2O (EDTA:Fe^{2+}; 100:100 µM), and H_2O_2 (5 mM) to a buffer/ethanol mixture (acetate buffer, pH 4.5, 0.2 mM; 5 % (v/v) ethanol)

The stoichiometry of the EDTA-Fe^{2+} complex has a great influence on the efficiency of radical formation [8, 28]. Therefore, preliminary trials were carried out to study the effect of the EDTA-Fe^{2+} stoichiometry on radical formation efficiency in greater detail. Figure 2 shows an ESR signal as received when measuring $^{\bullet}OH/EtO^{\bullet}$-POBN adducts [3, 43]. The height of the signal (Δ h) is proportional to the concentration of formed spin adducts in the system and was consequently used to evaluate the sample's radical concentration.

Table 2 Influence of EDTA-Fe^{2+} ratio on ESR signal intensity [a]

ESR signal intensity			
no EDTA	EDTA:Fe^{2+} ratio		
	100:100 µM (1:1)	100:200 µM (1:2)	200:100 µM (2:1)
736990 ± 12098	674164 ± 10145	311066 ± 8527	701026 ± 6888

[a] Data represents the means of a triplicate experiment ± 1 standard deviation.

The data as shown in table 2 demonstrate that equimolar EDTA-Fe^{2+} ratios of 100:100 µM resulted only in a slightly diminished formation of radicals (8.5 %) as compared to adding 100 µM Fe^{2+} only. Supermolar EDTA-Fe^{2+} ratios (EDTA:Fe^{2+}; 200:100 µM) yielded a distinct reduction of the ESR signal intensity by 57.8 % while submolar ratios (EDTA:Fe^{2+}; 100:200 µM) resulted again in only a slight decrease of 4.9 % as compared to adding Fe^{2+} only. Others found similar results [28].

The ratio of 1:1 (EDTA:Fe^{2+}) was finally chosen to study the dependency of aldehyde formation from $^{\bullet}OH/EtO^{\bullet}$ attack because all the ferrous iron is then complexed while an adequate quantity of radicals is produced. EDTA-Fe^{2+} was added in concentrations of 0–200 µM to the reaction mixtures containing AAs and 5 % (v/v) ethanol and the reactions were started again by adding 5 mM H_2O_2. After incubation for 5 days at 20 °C in the dark, the sample solutions were extracted and the isolates were measured using HRGC-MS. In a separate trial, the radical generation from these samples was determined using ESR spectroscopy as described in the materials and methods section. The results are depicted in figure 3. The concentrations of phenylacetaldehyde and benzaldehyde were divided by a factor 10 for a better visibility in the graph.

As expected, the four aldehydes 3-methylbutanal, 2-methylbutanal, phenylacetaledehyde, and benzaldehyde were detected in the isolates. Their concentrations followed a linear response ($r^2 > 0.97$) and were increased with higher EDTA-Fe^{2+} concentrations, except for benzaldehyde which showed no linear behavior ($r^2 = 0.57$) and no remarkable increase. The same behavior was observed for the ESR signal intensity and therefore the free radical concentration ($r^2 > 0.99$). Replacement of Fe^{2+} by Fe^{3+} revealed only little amounts of 0.02 µM of the aldehydes being detected. Most likely, the detected aldehyde concentrations can be traced back to impurities of the AA products used because Fe^{3+} is thought to be incapable of acting catalytically in the Fenton reaction. This was strengthened by the observations from an ESR experiment

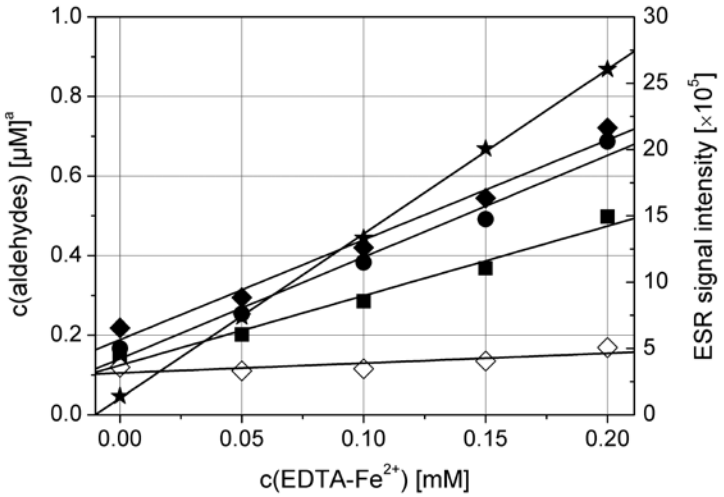

Fig. 3 **Dependency of ESR signal intensity (★), and the formation of 3-methylbutanal (■), 2-methylbutanal (●), benzaldehyde (◇), and phenylacetaldehyde (◆), from the EDTA-Fe^{2+} concentration (0–200 µM) in buffered model solutions (acetate buffer, pH 4.5, 0.2 mM; 5 % (v/v) ethanol). The model solutions contained 5 mM of leucine, isoleucine, and phenylalanine, respectively, and reactions were started by the addition of 5 mM H_2O_2. The reaction mixtures were incubated for 5 days at 20 °C in the dark. The ESR signal intensity was measured by adding 0–200 µM of EDTA-Fe^{2+} (EDTA:Fe^{2+}, 1:1) and 7.5 mM POBN to an acetate buffer (pH 4.5, 20 mM) containing 5 % (v/v) of ethanol. The reactions were started by adding 5 mM H_2O_2 and the ESR measurement was started immediately. The ESR measurements were carried out at ambient temperature (20–22 °C). The lines represent linear fittings of the data.**

[a] The concentrations of benzaldehyde and phenylacetaldehyde were divided by a factor 10 to fit them into the graph

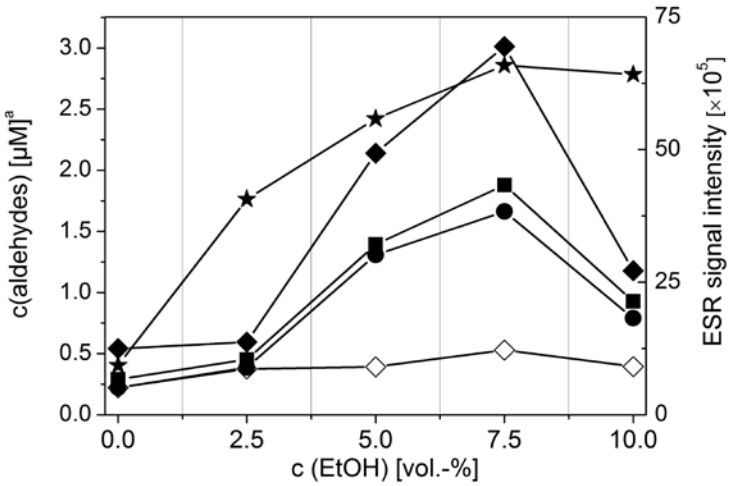

Fig. 4 **Dependency of ESR signal intensity (★), and formation of 3-methylbutanal (■), 2-methylbutanal (●), benzaldehyde (◇), and phenylacetaldehyde (◆), respectively, from ethanol concentration (0–10 % (v/v)) in buffered model solutions (acetate buffer, pH 4.5, 0.2 mM). The model solutions contained 100 µM EDTA-Fe^{2+}, 5 mM of leucine, isoleucine, and phenylalanine, respectively, and reactions were started by the addition of 5 mM H_2O_2. The reaction mixtures were incubated for 5 days at 20 °C in the dark. The ESR signal intensity was measured by adding 0–10 % (v/v) of ethanol, 100 µM of EDTA-Fe^{2+} (EDTA:Fe^{2+}, 1:1), and 7.5 mM POBN to an acetate buffer (pH 4.5, 20 mM). The reactions were started by adding 5 mM H_2O_2 and the ESR measurement was started immediately. The ESR measurements were carried out at ambient temperature (20–22 °C). The lines represent linear fittings of the data.**

[a] The concentrations of benzaldehyde and phenylacetaldehyde were divided by a factor 10 to fit them into the graph

where no ESR signal could be detected when using Fe^{3+} instead of Fe^{2+} (data not shown).

Correlating the concentrations of 3-methylbutanal, 2-methylbutanal, and phenylacetaldehyde with the ESR signal intensity and therefore with the free radical concentration yielded Pearson correlation coefficients > 0.98 indicating a clear relationship between radical concentration and aldehyde formation. Interestingly, leucine and isoleucine seem to have a similar and 10-fold lower trapping capacity for $^{\bullet}OH/EtO^{\bullet}$ attack than that of phenylalanine. Benzaldehyde was formed in higher concentrations than 3-methylbutanal and 2-methylbutanal but in lower concentration as compared to phenylacetaldehyde. The explanation may lie in its formation indirectly from phenylacetaldehyde. The correlation of benzaldehyde concentration with ESR signal intensity still yielded a Pearson correlation coefficient = 0.83, suggesting that radicals are involved in the abstraction of the carboxyl group from phenylacetaldehyde thereby forming benzaldehyde. The demonstrated results are consistent with published claims that amino acids, such as phenylalanine are better `sinks' for radical attack [26, 69] but also imply that leucine and isoleucine are susceptible too for $^{\bullet}OH/EtO^{\bullet}$ attack thereby forming the corresponding aldehydes.

The influence of the ethanol concentration and therefore the reactivity of AAs towards $EtO^{\bullet}$ attack was tested in a second set of experiments. The same experiment design was used again with the modification that in this trial, the ethanol concentration was increased stepwise from 0 to 10 % (v/v) and the EDTA-Fe^{2+} concentrations and ratio was kept constant at 100 µM. The concentration of radicals was determined again in a separate experiment by using ESR spectroscopy.

The aldehyde concentrations in the isolates after 5 days of storage at 20 °C show a clear trend and increase with higher ethanol concentration up to a concentration of 7.5 % (v/v) (Fig. 4). At 10 % (v/v) ethanol concentration, the concentration dropped by 49.4 %, 47.5 %, 39.1 %, and 74.5 % for 3-methylbutanal, 2-methylbutanal, phenylacetaldehyde, and benzaldehyde, respectively. When taking this data point not into account, the aldehydes' ethanol dependent behavior can be described by a linear behavior for the individual aldehydes (3-methylbutanal, $r^2 > 0.94$; 2-methylbutanal, $r^2 > 0.93$; phenylacetaldehyde, $r^2 > 0.93$; benzaldehyde, $r^2 > 0.91$). The ESR signal intensity showed the same trend as the aldehydes and also followed a linear response ($r^2 > 0.91$) up to an ethanol concentration of 7.5 % (v/v). An explanation for the increase in ESR signal intensity lies in the stability of the POBN spin trap adducts. The spin trap adducts of $EtO^{\bullet}$ with POBN have a greater half-life period (ca. 16 minutes) than those of $^{\bullet}OH$ with POBN (< 1 minute) [43, 47]. Up to an ethanol concentration of 7.5 % (v/v), in addition to the POBN-$HO^{\bullet}$ adducts, more POBN-$EtO^{\bullet}$ adducts are formed which 'survive' the way to the ESR's measuring chamber and the measurement span consequently producing an increased ESR signal intensity. As the ethanol concentration of 7.5 % (v/v) is exceeded, it is assumed that ethanol provokes a dilution effect

thereby causing a diminished radical formation (unpublished work; details are available from Thomas Kunz on request). A further increase of ethanol concentration to 10 % (v/v), led to a leveling off of the ESR signal intensity This dilution effect may also clarify the decrease in the formation of aldehydes at this data point.

Excluding the data point at 10 % (v/v) ethanol revealed significant correlations between the ethanol concentration and the aldehyde concentrations (Pearson correlation coefficients > 0.95) and the ESR signal intensity and the aldehyde concentrations (Pearson correlation coefficients > 0.86) indicating that the AAs used in this study are also susceptible by $EtO^{\bullet}$ attack.

To exclude the possibility that the ethanol additions caused an alkaline pH shift which may affect the occurring reactions, the test solutions' pH was also measured. A slight pH increase by 0.08 units from 0 to 10 % (v/v) could be observed. It is unlikely that this minimal pH shift is accountable for the observations.

Taking together, these data give evidence of a reaction route for the formation of the aldehydes 3-methylbutanal, 2-methylbutanal, phenylacetaldehyde, and benzaldehyde in a beer-like model system via direct oxidation of leucine, isoleucine, and phenylalanine, respectively, by attack from $^{\bullet}OH/EtO^{\bullet}$ without α-dicarbonyls present. A proposal of the reaction route was adapted from Stadtman [47, 59] and is depicted in figure 5 with leucine as an example: Catalyzed by Fe^{2+}, H_2O_2 decomposes to ^{-}OH and $^{\bullet}OH$ (Fenton reaction), from which $^{\bullet}OH$ further reacts with organic radicals ($R^{\bullet}$) such as ethanol. The primary attack on leucine (I) by $^{\bullet}OH/R^{\bullet}$ involves abstraction of a hydrogen atom from the α-carbon to form a carbon-centered radical (II) (reaction a). O_2 addition yields a peroxy radical derivative (III) (reaction b), which, upon reaction with a superoxide anion radical (or its conjugate acid, $HOO^{\bullet}$) leads to the production of O_2 and an alkylperoxide derivative (IV) (reaction c). An imino derivative (V) can then be formed by spontaneous decomposition of the alkylperoxide (reaction d) thereby dissociating H_2O_2. The imino derivative, in turn, can undergo hydrolysis to form an ammonium ion, carbon dioxide, and the aldehyde 3-methylbutanal (V) (reaction e).

The rate at which a chemical reaction proceeds is typically influenced by the amount of each reactant present, the ambient reaction conditions such as pH, and the temperature of the reaction. Based on the results and depicted reaction route, it may be assumed that the rate of attack of $^{\bullet}OH/EtO^{\bullet}$ on leucine, isoleucine, and phenylalanine is constant with time, as long as AA, Fe^{2+}, H_2O_2 and ethanol are present, and that the evolving aldehydes are a measure of the $^{\bullet}OH/EtO^{\bullet}$ attack. The rate at which the Fenton reaction occurs is strongly pH-dependent [40, 52, 73] but generally is completed within the order of seconds or minutes [22, 36, 73]. Therefore, the H_2O_2 present should be consumed rapidly. Yet, with proposal of the reaction route (Fig. 5) it can be anticipated that the H_2O_2 formed in reaction d becomes available for the Fenton reaction again and, as long as reactants are present, the reaction starts over again.

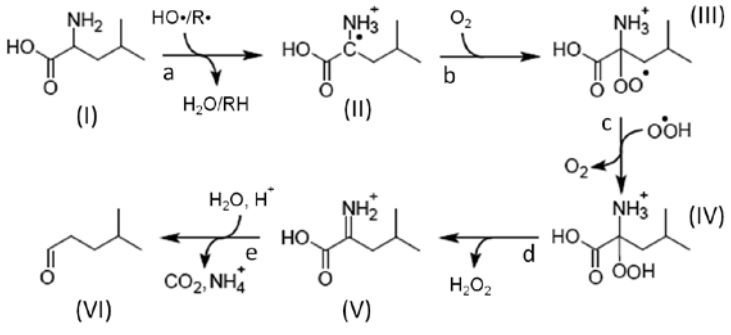

Fig. 5 Formation of 3-methylbutanal via oxidative degradation of leucine by $^{\bullet}OH/EtO^{\bullet}$ attack (modified reaction route adapted from Stadtmann [59]).

To test this hypothesis, the time-dependent reaction course over a span of 18 days was investigated again working with similar reaction conditions as in the previous trials and measuring aldehyde concentrations after 0, 1, 2, 3, 12, and 18 days.

Over the time-span measured, a steady increase of aldehydes could be observed (Fig. 6) which strengthens the previously proposed theory. Certainly, higher concentrations of H_2O_2 and Fe^{2+} or higher temperatures will affect the rate at which these reactions occur. Stadtman and Berlett [60] observed in their investigations that the metal-catalyzed oxidation of leucine is almost completely dependent of the presence of bicarbonate ions such as $NaHCO_3$. This may be an explanation for the overall low yields of the reaction products (aldehydes) found in the present study. As bicarbonate ions are present in beer, further trials should be carried out under addition of bicarbonate ions to the reaction mixture and examining the effect on the reaction rate.

In the series of experiments presented in this study, all reactions were started by the addition of H_2O_2 and with higher concentrations

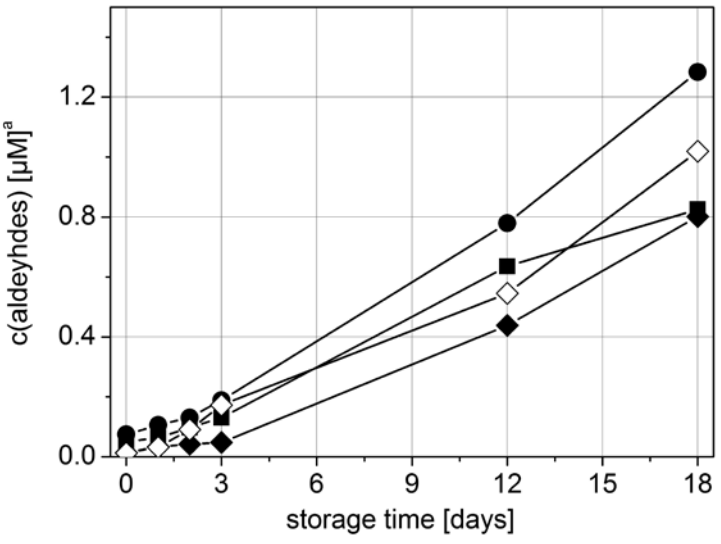

Fig. 6 Time-course of the formation of 3-methylbutanal (■), 2-methylbutanal (●), benzaldehyde (◇), and phenylacetaldehyde (◆), respectively, in buffered model systems (acetate buffer, pH 4.5, 0.2 mM, 5 % (v/v) ethanol). The solutions contained 5 mM leucine, isoleucine, and phenylalanine, respectively, 100 µM EDTA-$FeSO_4$ × 7 H_2O (EDTA:Fe^{2+}, 1:1), and were started by the addition of 5 mM H_2O_2. For the bottle representing day 0, no H_2O_2 was added and the bottle was measured directly after preparing.

[a] The concentrations of benzaldehyde and phenylacetaldehyde were divided by a factor 10 to fit them into the graph.

of AAs and Fe^{2+} than supposed to be in beer. In beer, however, the appearance and formation of H_2O_2 is believed to be a product of various oxidation systems in which oxygen, transition metal ions, ethanol, polyphenols, hop bitter acids, and melanoidins play a role [33, 53]. Furthermore, oxidative reactions are the results of a very complex interplay in which antioxidants and pro-oxidants such as sulfite, ascorbic acid, reductones, etc. are involved. Accordingly, outcomes from this study certainly not reflect 'real' conditions which disputes to some extent the adaptability and relevance of this reaction route for beer. Yet, the knowledge of the existence of radicals in beer and the observation that more aldehydes are formed during beer aging in the presence of oxygen and promoted by elevated iron levels support the relevance of this pathway.

4 Conclusions

The series of experiments presented, demonstrated that an additional mechanism leading from α-amino acids to aldehydes in 'beer-like' model solutions exists in addition to the already published mechanisms such as the Strecker degradation of amino acids. Deductions from this study relate the role of 'beer-radicals' with aldehyde formation and consequently provide a possible explanation for increased Strecker aldehyde concentrations in aged beer when elevated oxygen levels were present. This information can therefore contribute to some extent to the clarification of the processes which promote the formation of aroma-active aldehydes in bottled beer and other beverages. The results obtained from this study demand continued research aiming to further clarify the role of this pathway and gain more insight into the relevance of this radical-dependent mechanism as related to aldehyde formation in beer and other foods. The final goal of this research, certainly, is to facilitate the preparation of beers with an enhanced shelf-life and consistency to allow a longer lasting pleasant flavor.

5 Literature

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Publication B

In a next step, the relevance of the pathway as proposed in publication A[283] was scrutinized during beer production and storage. Detectable intermediates in the Strecker or Strecker-like reaction are similar whether they are derived from 'ordinary' Strecker degradation[61, 224], by Strecker-like reactions[215, 222], or by the proposed reaction route from the previous work.[283] Furthermore, radical intermediates of the proposed reaction cascade are too short-lived and can thus not be identified with existing techniques. Verification of the relevance of the pathway as proposed in ref. 283 was thus not achievable by identification of associated reaction products or intermediates in an actual beer environment. In an attempt to produce evidence to confirm or disprove the existence of a ROS-mediated oxidative degradation of amino acids in a beer system, it was thus decided to vary beer storage parameters potentially being relevant or irrelevant for the pathway and use statistical methods to narrow down the probability of this pathway to be responsible for the emergence of staling-compounds during beer ageing. Response surface methodology along with labeling experiments was decided to be most suitable for approaching this task. Consecutive storage trials were planned and conducted in a step-to-step approach. The complete description of the experiments and the outcomes were published in the *Journal of Agriculture and Food Chemistry*:

"Relevance of Oxygen for the Formation of Strecker Aldehydes During Beer Production and Storage"[281] *(Publication B).*

The current knowledge as related to beer ageing has been confirmed and was expanded in the present study by additional information resulting from the application of as yet unexplored experiments, but, more importantly, new detailed views were presented by the elaboration of special methodologies that were introduced. Clear prove of the proposed pathway was not achieved as the beer matrix is by far too complex and too many reaction routes are imaginable. Yet, deductions from the study point unmistakably to the well-known truth that eliminating oxygen entry and lowering amino acid concentrations whenever possible in the course of beer production should be the least goal when aiming to improve beer flavor stability. Yet, the conclusive information gained in this first part of the dissertation work allows drawing conclusions about how measures should be taken to block or abate the oxidative chain reactions ultimately preserving the beer's fresh taste. In relation to that but not necessarily limited to it, unveiling and exploiting the great potential and efficacy of hops was goal of subsequent work and second part of this dissertation work.

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Relevance of Oxygen for the Formation of Strecker Aldehydes during Beer Production and Storage

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Supporting Information

ABSTRACT: Off-flavor in beer is often associated with the appearance of staling aldehydes. In this study, the factors amino acid concentration, carbohydrate concentration, Fe^{2+} concentration, and oxygen concentration were investigated in terms of their effect on the formation of carbonyl compounds during storage using response surface methodology. From all factors tested, only amino acid concentration and oxygen concentration promoted Strecker aldehyde formation during storage, while all other carbonyls measured were unaffected. A mixture of glucose/xylose, representing carbohydrate sources, as well as Fe^{2+} concentration were insignificant factors, though carbohydrate additions exhibited a significant role in the formation of 2-furfural. De novo formation of phenylacetaldehyde from phenylalanine during beer storage was observed using labeling experiments and a linear relationship between Strecker aldehydes formed and total packaged oxygen was identified. Capping beers with oxygen barrier crown corks and addition of 10 mg/L EDTA to beers effectively diminished Strecker aldehyde formation. Oxygen was additionally shown to significantly promote Strecker aldehyde formation during sweet wort production. A pathway for the reactive oxygen species-induced degradation of amino acids yielding Strecker aldehydes was proposed and was further scrutinized in buffered model solutions. The insignificant role of Fe^{2+} in the response surface experiments is discussed.

KEYWORDS: *beer, wort production, oxygen, reactive oxygen species, Strecker aldehydes*

INTRODUCTION

The stale character of food is a quality issue for many producers and distributors. It is caused by certain carbonyl compounds emerging during storage and, once they exceed their flavor thresholds, producing distinct off-flavors. Despite decades of research, flavor instability is still a major problem for many breweries. Reactions leading to the formation or liberation of off-flavor compounds during beer storage were studied intensively in the last decades and many pathways, and sources of off-flavors have been suggested. For reviews, see refs 1 and 2.

Among those pathways, there is an ongoing debate as to whether there is a release of certain compounds from a bound-state or if there is a de novo formation of those compounds in the bottled beers. While the theory about a release from a bound state seems to manifest itself,[3−5] there is also still evidence that reactions as related to a de novo formation of aldehydes occurs in bottled beer.[6−10] Suda et al.[11] reported that 85% of Strecker aldehydes present in beer after aging were derived from wort production, while 15% were formed de novo during the aging process. In terms of a formation during storage, Bravo et al.[12] claimed that the Maillard reaction occurs to some extent during storage of Pilsner lager type beers thereby causing the deterioration of the beer's freshness. This is supported by findings from Rakete et al.[13] who reported the formation of Maillard reaction products from oligosaccharides during beer storage. A potential role of free radicals in beer staling was initially investigated by Bamforth and Parsons,[14] and their harmful character was further verified by others.[15−19] Molecular oxygen is activated in a complex interplay thereby forming various oxygen radicals (e.g., $O_2^{\bullet-}$, $OH^{\bullet}$, and $HO_2^{\bullet}$) and nonradical oxidizing agents (e.g., H_2O_2), all of which are termed reactive oxygen species (ROS). Transition metal ions such as iron and copper play a central role in these reaction cascades as they act as catalysts in the so-called Fenton reaction ultimately yielding hydroxyl radicals which can subsequently react with ethanol forming hydroxyethyl radicals.[17] While oxygen exposure and oxidative conditions were reported to promote beer staling,[7,20−23] the reasons for these findings remain speculative. Hashimoto et al.[9] suggested that the oxidation products of isohumulones (the bitter components in beer) can yield volatile aldehydes, but this pathway was found irrelevant by De Clippeleer.[24] In a different study, Hashimoto et al.[8] intended that the oxidation of higher alcohols in a complex interplay with melanoidins yields the corresponding aldehydes, though this pathway was doubted by Devreux, Blockmans, and van de Meersche[25] because they observed the requirement of light and found inhibitory effects of polyphenols. *ortho*-Quinones as derived from oxidation of certain polyphenols with the catechol (1,2-dihydroxybenzene) moiety was found to be capable of initiating Strecker-like reactions with amino acids thereby forming volatile aldehydes.[26] Schieberle and Komarek[7] reported that ROS and riboflavin may play a central role in this mechanism during beer storage. Wietstock and Methner[10] proposed a pathway for the formation of Strecker aldehydes by hydroxyl or hydroxyethyl radical attack of their parent amino acids thereby proposing a direct connection between ROS and the formation of off-flavor

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compounds. In fact, Saison et al.[23] discovered that the formation of Strecker aldehydes during beer aging was significantly promoted by adding H_2O_2 and Fe^{2+} or exposing samples to oxygen, and that aging characteristics were alike. Narziß et al.[22] and Blockmans et al.[21] also discovered a promoting effect of oxygen during beer aging on Strecker aldehydes. Studies from Schieberle and Komarek[7] even imply that the influence of oxygen exposure to beer is greater than the impact of temperature.

Toward the objective to deploy and differentiate pathways contributing to the formation of carbonyl compounds during beer production and storage, in this work, response surface methodology (RSM) was applied and was complemented with trials in buffered model systems. In consecutive trials, the factors amino acid concentration, carbohydrate concentration, Fe^{2+} concentration, and oxygen were tested in central composite rotatable designs (CCRD) on their impact to promote the formation of carbonyl compounds during beer storage. As yet unexplored de novo Strecker aldehyde formation in the bottles during aging was additionally assessed by labeling experiments. The relevance of oxygen for the formation of carbonyl compounds during wort production was also tested. The objective of this study was therefore to gain insight into the detrimental role of beer constituents and particularly oxygen-derived reactions on the formation of Strecker aldehydes during beer storage and wort production. Outcomes from this study are not necessarily limited to beer production and beer storage per se, and results or adoptions from this research thus help the brewing and food industry to understand and optimize their production and packaging, certainly with the ultimate goal to improve and preserve food quality.

MATERIALS AND METHODS

Chemicals. Acetic acid (glacial), benzaldehyde, ethyl nicotinate, 2-furfural, γ-nonalacton, heptanal, iron(II)sulfate hepta hydrate, methional, 2-methylbutanal, 3-methylbutanal, octanal, pentanal, phenylacetaldehyde, sodium acetate trihydrate, and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) were purchased from Sigma-Aldrich Inc., Steinheim, Germany. Glucose, sodium carbonate, sodium sulfate, and xylose were obtained from Merck KGaA, Darmstadt, Germany. Diethyl ether and anhydrous ethanol were purchased from VWR international GmbH, Darmstadt, Germany. All chemicals were of analytical grade or higher. All aqueous solutions were made with double-distilled water and prepared freshly every day. Solutions of iron(II)sulfate hepta hydrate were additionally prepared with degassed water.

Wort and Beer Analysis. Extract (2.9.2.3), color (2.12.2), pH (2.13), free amino nitrogen (2.6.4.1.1), total polyphenols (2.16.1), and bitter units (2.17.1) were analyzed according to MEBAK.[27] The numbers in parentheses indicate the method used. Total packaged oxygen in beers was also measured according to MEBAK,[27] method 2.28.1.1.2, using a DIGOX 6.1 apparatus (Dr. Thiedig, Berlin, Germany). SO_2 in beer was analyzed using continuous flow analysis (CFA, Skalar Analytical B.V., Breda, Netherlands) as described in ref 28.

Quantitation of Amino Acids. Amino acids were quantitated by applying the EZ:Faast GC-FID kit for free amino acid analysis from Phenomenex (Torrance, CA, USA) according to the instructions provided by the manufacturer. The principle of the method relies on solid-phase extraction and subsequent derivatization of amino acids with propyl chloroformate. Quantitation of derivatized amino acids was done using a HP 5890 series II (Agilent, Santa Clara, CA, USA) equipped with a ZEBRON ZB-AAA column (10 m × 0.25 mm, Phenomenex, Torrence, CA, USA). The following temperature program was used: 110 °C for 1 min, ramped at 22 °C/min to 320 °C, held for 1 min. The temperature of the injection port was 250 °C, and the detector temperature was 320 °C. Nitrogen was used as the carrier gas at a total flow of 25 mL/min. Sample volumes of 2 μL were injected using an HP 7673 autosampler (Agilent, Santa Clara, CA, USA) in 1:15 (v/v) split mode.

Determination of Iron Concentrations in Beers. Iron concentration in beer was quantitated 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 used for the determination of iron were 239.5 and 259.9 nm. A six-point calibration curve was used to quantitate the concentration of the samples. The calibration was done matrix-matched in beer to deplete influences of the samples' organic matrices. The calibration curve showed coefficients of determination of >0.99.

Quantitation of Aldehydes by Solvent-Assisted Flavor Evaporation (SAFE)-GC/MS. Solvent assisted flavor evaporation (SAFE) according to Engel, Bahr, and Schieberle[29] and high resolution gas chromatography (HRGC) together with mass spectrometry (MS) analysis were used to measure staling aldehyde concentration in beers and worts. For the detailed procedure and equipment used, see ref 30. For retention times and target ions used for quantitation, see Table S1.

Determination of Radical Levels in Worts and Beers Using ESR Spectroscopy. The determination of the beer's endogenous antioxidative potential (EAP value) and radical formation was analyzed using electron spin resonance (ESR) spectroscopy according to MEBAK, method 2.15.3.[27] ESR spectra were obtained using an X-band spectrometer (e-scan, Bruker BioSpin, Rheinstetten, Germany) with the following settings: center field, 3475 G; attenuation, 0 dB; equivalent to 8.492 mW; sweep width, 14 G; receiver gain, 2.0×10^3; resolution, 512; modulation amplitude, 1.49 G; modulation frequency, 86 kHz; conversion time, 10 ms; time constant, 40 ms; scans, 25.

Effect of Glucose/Xylose, Amino Acid, and Fe^{2+} Levels on Carbonyl Formation. The effect of the independent factors carbohydrate concentration, Fe^{2+} concentration, and amino acid concentration on the formation of carbonyl compounds was evaluated using a CCRD. The design matrix was a 2^3 factorial design with 6 central points to calculate the pure error and 6 axial points at the extreme levels. The extreme levels were chosen with $\alpha = \pm 1.414$, thus allowing rotability of the system. Table 1 shows the levels used. A commercially available filtered lager-type beer (11.5°P, 4.9% v/v alcohol, pH 4.41, 32 bitter units, and 48 μg/L iron) available in 500 mL bottles was used as the base beer for the trials. The bottle volumes were exactly adjusted to 490 mL using a bottle-check filling quantity template (DIN A 82, Deutscher Brauer-Bund e.V., Berlin, Germany) prior to spiking. The stock solutions were prepared in concentrations that allowed a 1 mL addition to the beers to achieve the desired levels as given in Table 1. The bottles were carefully opened and, upon completion of the additions, were carefully hit at the bottle's body to provoke beer foam to rise up in order to drive out oxygen from the bottle headspace. Once the foam was at the level of the bottle opening, the bottles were quickly capped again. The bottles were then stored for 18 months at 20 °C in the dark. Upon completion of storage, carbonyl compounds were analyzed using SAFE-GC/MS.

Table 1. Levels Used for a Central Composite Rotatable Experimental Design to Study the Effect of the Independent Factors Glucose/Xylose Concentration, Amino Acid (AA) Concentration, and Fe^{2+} Concentration

factors	$-\alpha$	−1	0	+1	$+\alpha$
c(glucose/xylose) [mg/L]	131.82	200	300	400	468.18
c(AA) [mg/L]	6.36	20	40	60	73.64
c(Fe^{2+}) [μg/L]	0	20	50	80	100

Monitoring Carbonyl Formation as a Function of Amino Acids, Fe^{2+}, and Oxygen Exposure. To investigate the effect of

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Table 2. Overview of the Set of Samples for Investigating the Influence of Oxygen Exposure, Leu Additions, and Fe^{2+} Additions on the Formation of Carbonyl Compounds during Beer Storage[a]

	monitoring during 30 weeks of storage				measurement upon completion of 30 weeks storage		
	no additions (reference)	Leu	Leu/O_2	$Leu/O_2/Fe^{2+}$	AA mixture	AA mixture/O_2	AA mixture/O_2/Fe^{2+}
Leu addition	-	25 mg/L	25 mg/L	25 mg/L	20 mg/L	20 mg/L	20 mg/L
Ile addition	-	-	-	-	20 mg/L	20 mg/L	20 mg/L
Phe addition	-	-	-	-	20 mg/L	20 mg/L	20 mg/L
Fe^{2+} addition	-	-	-	500 μg/L	-	-	500 μg/L
Exposed to oxygen	-	-	√	√	-	√	√

[a]AA = amino acid.

amino acid additions, Fe^{2+} additions, or oxygen exposure during beer storage, a commercially available Pilsner beer (11.7°P, 4.9% v/v alcohol, pH 4.44, 28 bitter units, 53 μg/L iron) was treated as follows: first the bottles were carefully opened, and 1 mL of amino acids (Leu, Ile, and Phe) and/or Fe^{2+} stock solution was added such that the amounts given in Table 2 were achieved in the beers. Overfoaming as described previously was applied to diminish oxygen. One set of bottles was prepared as a reference by adding 2 mL of degassed double-distilled water and bringing the bottles to an overfoam to diminish oxygen. To assess the influence of oxygen exposure, overfoaming was omitted, and bottles were closed directly after spiking. Oxygen concentration was measured directly after capping from 6 randomly collected bottles of each treatment and using a DIGOX 6.1 apparatus. Oxygen levels were 53.1 ± 3.3 μg/L when bottles were foamed over and 2990.8 ± 177.3 μg/L, when bottles were capped with air left in the bottle headspace. The formation of carbonyl compounds was monitored periodically during 30 weeks of storage at 28 °C in the dark from the reference and from bottles where only Leu or Leu and Fe^{2+} were added. The bottles with Leu, Ile, and Phe added were only measured upon completion of 30 weeks of storage. Furthermore, radical formation and the endogenous antioxidative potential as well as the SO_2 content were measured from the fresh samples by ESR spectroscopy or CFA, respectively. The recovery rate of amino acids and iron additions were additionally checked and were 98.7 ± 8.3% and 96.6 ± 0.8%, respectively.

Labeling Experiments and Impact of Total Packaged Oxygen and Fe^{2+}. A CCRD was used again to investigate the influence of the independent factors total packaged oxygen and Fe^{2+} on the formation of carbonyl compounds in beer. The design matrix was a 2^2 factorial design with 5 central points to calculate the pure error and 4 axial points at the extreme levels (α = 1.414). The complete design consisted of 13 experimental points. The levels used are shown in Table 3. The same commercially available lager-type beer

Table 3. Levels Used for the Central Composite Rotatable Experimental Design to Study the Effect of the Independent Factors Fe^{2+} Concentration and Oxygen Concentration in the Atmosphere during Bottling on the Formation of Carbonyl Compounds during Beer Storage

factors	$-\alpha$	−1	0	1	$+\alpha$
Fe^{2+} addition [μg/L]	no addition	73.0	250.0	427.0	500.0
oxygen conc. [%]	<0.01	3.07	10.50	17.92	20.99

as in the previous trial was used. Because carbonyl compounds are reported to be released from a bound state such as, e.g., from bisulfate complexes,[2,4,31] and because SO_2 is capable of suppressing the formation of ROS,[18,32,33] the beer was stored prior to spiking at 28 °C in the dark until the beer's initial SO_2 content of 2.3 mg/L was <0.1 mg/L. The SO_2 content was checked periodically until, after 20 weeks at 28 °C, the target value of <0.1 mg/L was reached. Then, the sample bottles were finally prepared in a glovebox under nitrogen atmosphere ($c(O_2)$ < 0.01%). First, the bottle volume was exactly adjusted to 490 mL using a bottle-check filling quantity template (DIN A 82, Deutscher Brauer-Bund e.V., Berlin, Germany). To examine a potential de novo formation of carbonyl compounds during storage as, e.g., initiated by ROS, D^5-labeled phenylalanine at a concentration of 20 mg/L was added to all beers. The D^5-labeled phenylalanine and Fe^{2+} stock solutions were prepared such that an amount of 1 mL to the beer achieved the target concentration.

The desired total packaged oxygen in the bottles (Table 3) was achieved by adjusting the atmosphere in the glovebox to different oxygen saturations. The chamber was therefore shortly opened at one vent, and oxygen from the ambient air was allowed to diffuse into the glovebox. The vent was then closed again, and the atmosphere was allowed to equilibrate for 15 min. Three fans installed in the chamber ensured a quick equilibration and a consistent distribution of the ambient air in the chamber's atmosphere. The desired atmosphere was then maintained and checked frequently during spiking. Upon completion, all bottles were capped with crown corks and were stored at 28 °C in the dark for 12 weeks. Carbonyl compounds were quantitated periodically during storage and upon completion of the storage trial using SAFE-GC/MS. For monitoring during storage, bottles with 10.5% oxygen saturation and 250 μg/L Fe^{2+} as well as bottles filled at <0.01% oxygen in the atmosphere and without Fe^{2+} added were prepared separately. Fe^{2+} additions and the addition of phenylalanine-D^5 showed again very good recovery of >95%.

A preliminary trial was conducted in which the procedure for adjusting the total packaged oxygen was validated to be used in the trials. Different oxygen atmospheres were adjusted as described above, and the bottles were uncapped and kept open in the chamber for 5 min until closed and capped again. Subsequently, the total packaged oxygen was immediately measured from 6 bottles. Clearly, a straight linear response (r^2 > 0.99) could be observed, and the total packaged oxygen concentration increased with rising oxygen concentration in the atmosphere, thus demonstrating that the procedure was applicable to be used in the trials. The total packaged oxygen in the beers plotted over the oxygen concentration in the atmosphere is shown in Figure S1.

Influence of EDTA and Oxygen Barrier Liner Crown Corks during Beer Storage. The influence of excess EDTA and minimized oxygen exposure were assessed in a separate trial. The same procedure as described before was used, and beer samples were prepared in a glovebox. To scrutinize the effect of minimized oxygen entry through the crown cork, oxygen barrier crown corks made up from high-density polyethylene were used, and beers were bottled under limited oxygen atmosphere (<0.01% O_2).

To further investigate the role of iron during storage, 10 mg/L EDTA, a good iron chelator, were added to beers, and bottles were capped at 10.5% of oxygen in the atmosphere. This concentration was reported to be sufficient in blocking or diminishing beer staling in ref 21. After 12 weeks of storage at 28 °C, carbonyl compounds were analyzed. Three individual bottles were prepared and analyzed separately.

Model Studies. A buffered model system (0.1 mM acetate buffer, pH 4.3, 5.0% v/v ethanol) was used to identify the role of Strecker aldehyde formation from the amino acids Leu, Ile, and Phe with only Fe^{2+} and oxygen present. All solutions were prepared to give the desired levels of 25 mg/L of each amino acid and 100 μg/L of Fe^{2+} in the samples. Samples were then purged with nitrogen for at minimum 30 min prior to filling in 0.33 L glass bottles resulting in final oxygen

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Table 4. *F*- and *p*-Values of Carbonyl Compounds (Responses) As Formed during Storage of Beers with the Independent Factors Carbohydrate Concentration (Mixture of Glucose and Xylose), Fe^{2+} Concentration, and Amino Acid Concentration (Mixture of Leu, Ile, and Phe)[a]

	3-methylbutanal		2-methylbutanal		phenylacetaldehyde		2-furfural	
factor	*F*-value	*p*-value[b]	*F*-value	*p*-value[b]	*F*-value	*p*-value[b]	*F*-value	*p*-value[b]
carbohydrate concentration[b]							4.59	0.046
amino acid concentration[c]	30.21	<0.0001	121.1	<0.0001	8.29	0.010		

[a]Only responses are shown where significant models at $p < 0.05$ were found. All model types were linear. [b]A mixture of glucose and xylose was added. [c]A mixture of Leu, Ile, and Phe was added.

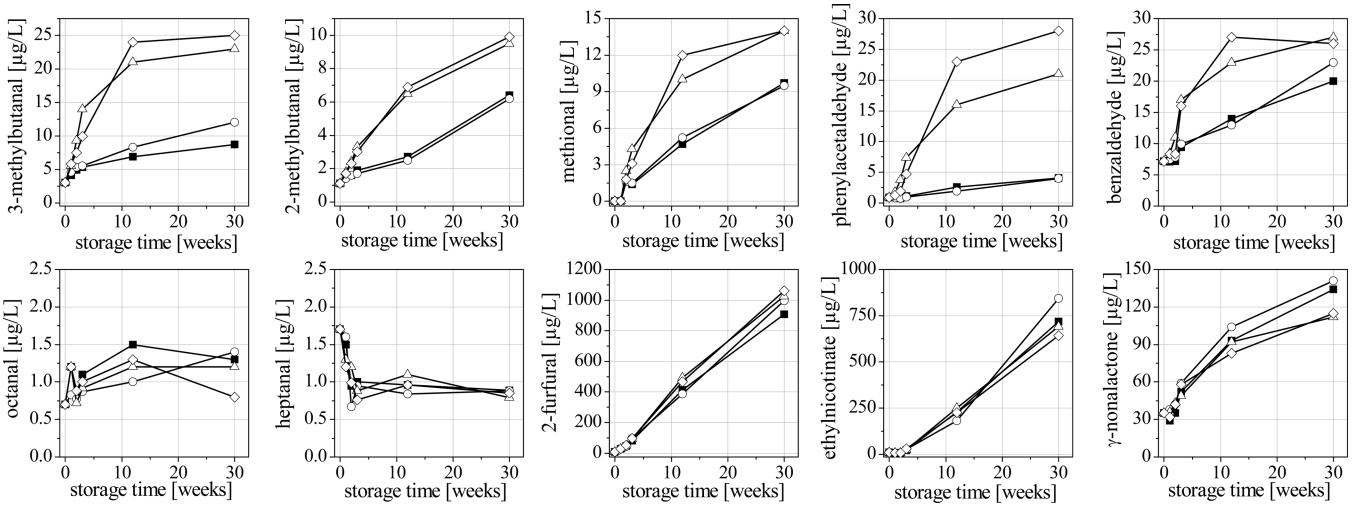

Figure 1. Effect of adding Leu, Fe^{2+}, and air exposure on the formation of carbonyl compounds during the storage of beer at 28 °C for 30 weeks. (■) no additions, no air exposure; (○) addition of 25 mg/L Leu; (◊) addition of 25 mg/L Leu and exposing samples to air; (△) addition of 25 mg/L Leu and 500 µg/L Fe^{2+}, and exposing samples to air.

concentrations of 490 ± 53 µg/L as measured from 6 separate bottles directly after sample preparation. The bottles were then stored for 6 months at 28 °C in the dark, and carbonyl compounds were subsequently analyzed by SAFE-GC/MS.

Impact of Oxygen during Mashing on Carbonyl Formation. The influence of oxygen during mashing and mash separation was investigated by conducting mashing experiments either under nitrogen atmosphere or under air atmosphere in a glovebox (Toepffer Lab Systems, Göppingen, Germany). For both set of trials, the glovebox was used to ensure that the same conditions were kept during both set of trials. Pilsner malt was milled 1 day prior to mashing and was vacuum-packed directly upon completion of milling to minimize oxygen. For mashing under anaerobic conditions, the glovebox was flushed overnight with nitrogen gas achieving an oxygen saturation in the chamber's atmosphere of below 0.01% as measured by a VisiFerm oxygen probe (Hamilton Germany GmbH, Höchst im Odenwald, Germany). During mashing, a constant slight positive pressure of nitrogen gas was maintained. All mashing experiments were then conducted under continuous stirring using a laboratory masher (Bender & Hohbein, Bruchsal, Germany). Worts were produced isothermally by mixing well 80 g of fine grist with 320 mL of double-distilled water at a temperature of 65 °C and holding that temperature for 90 min. For anaerobic mashing, all water used for mashing-in and rinsing was deaerated by boiling for 30 min prior to starting the experiments. After 90 min, the mashes were heated to 78 °C and then directly filtered using paper filters. The mashing beakers were additionally rinsed with 100 mL of double-distilled water (T = 78 °C) which was also added on top of the filter. The filtrates were collected in 250 mL Schott bottles. During filtration, the funnels were covered with watch glasses to diminish potential evaporation of volatiles. The filtration lasted for ca. 45 min. When filtration was completed, the bottles were closed tightly, and bottles were immediately cooled in an ice bath and then further cooled to 2 °C and kept at this temperature until analysis. From the sweet worts, carbonyl compounds were analyzed by SAFE-GC/MS. Additionally, original extract, pH, color, free amino nitrogen content, and total polyphenol content were determined. Three individual experiments were conducted and analyzed.

Impact of Oxygen during Heating of Wort on Carbonyl Formation. The influence of oxygen during the heating phase of sweet wort to start of boil was examined in experiments where the same base wort was flushed with nitrogen or air and subsequently heated to start of boil. Sweet worts were produced as described before using a laboratory masher. In total, 12 batches of sweet wort were produced, combined, and cooled to 2 °C after filtration. The sweet wort was then mixed well, aliquots of 250 mL were filled in 250 mL Schott bottles, and the worts were subsequently flushed with nitrogen at a flow of 100 mL/min for 2 h to displace the dissolved oxygen. A reference was flushed with air and at the same flow rate to guarantee that all worts were handled similarly. The temperature was kept at 2 °C during flushing to diminish evaporation of volatiles. The potential effect of the flushing procedure was checked by sampling aliquots of 100 mL from the bottles and analyzing the wort's amount of carbonyl compounds. Upon completion, the bottles were closed tightly and were placed in a 20 °C water bath which was immediately heated to 100 °C under continuous shaking at 100 rpm. The heating lasted 60 min. After incubating the bottles at 100 °C for another 5 min, the bottles were carefully placed in an ice bath for 30 min and then kept at 2 °C until further analyzed. Samples from the base wort before flushing, after the flushing procedure, and after heating were collected, and carbonyl compounds were quantitated using SAFE-GC/MS. Additionally, original extract, pH, and color were analyzed from the worts after heating. All trials were carried out in triplicate.

Statistical Evaluation. Response surface experimental design and statistical analysis was performed using Design-Expert software (Ver. 7.0.0, Stat-Ease, Inc., MN, USA). The statistical significance of the different factors and their interaction were determined using analysis of variance (ANOVA). A backward elimination was applied to the data

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in which all blocks and forced terms were fit to the data first. According to the principle of hierarchy, nonsignificant terms were kept in the model if they were contained in other interaction terms that were found to be significant. This procedure is considered to be a robust choice since all model terms will be given a chance to be included in the model.[34] For difference testing, *t* test analysis was performed using XLSTAT software (Ver. 2014.5.03., Addinsoft, Andernach, Germany).

RESULTS AND DISCUSSION

Effect of Glucose/Xylose, Amino Acids, and Fe^{2+} on Carbonyl Formation. Reactive intermediates of the Maillard reaction such as α-dicarbonyls can act as oxidizing agents thereby provoking the decarboxylation of amino acids which usually yields, after hydrolysis of the resulting imine, an α-keto amine and a so-called Strecker aldehyde.[35] In a first set of experiments, the involvement of the Maillard reaction in carbonyl formation as induced from glucose or xylose degradation during beer storage was tested because it represents one hypothesis for the appearance of carbonyl compounds during beer aging[12,13] which may, in turn, also promote the formation of Strecker aldehydes.

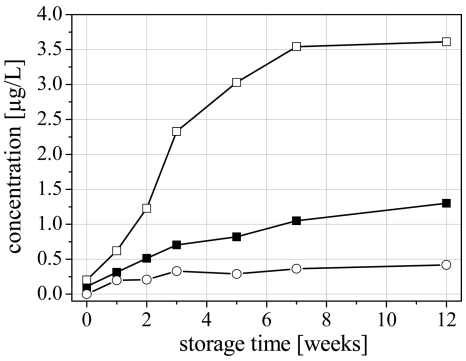

Figure 2. Time-course formation of phenylacetaldehyde-D^5 (■/□) and benzaldehyde-D^5 (○) during storage dependent on initial oxygen concentration during bottling with oxygen present in the bottle headspace (open symbols) and with oxygen minimized (solid symbols). Benzaldehyde-D^5 was not detected in beers where oxygen exposure was diminished, and no data is therefore shown. Bottles were stored for 12 weeks at 28 °C.

Interestingly, only the mixture of the amino acids Leu, Ile, and Phe added to the beers produced significant responses for the Strecker aldehydes 3-methylbutanal, 2-methylbutanal, and phenylacetaldehyde (Table 4). None the Strecker aldehydes were influenced by the carbohydrate additions or by Fe^{2+}, and these factors were thus removed from the models by backward elimination. 2-Furfural was the only carbonyl compound which was influenced by the carbohydrate additions; however, it showed the lowest model *F*-value of 4.99 and concomitantly the highest *p*-value of 0.039. Xylose was reported in ref 36 to induce 2-furfural formation which sufficiently explains this outcome, also because xylose is an abundant carbohydrate in beer,[37] though also hexoses were reported to be a possible source of 2-furfural formation.[13]

For the compounds methional, benzaldehyde, heptanal, octanal, γ-nonalacton, and ethyl nicotinate, no significant models were found which implies that none of the factors used in this design was affecting their formation during beer storage.

The individual samples varied only little in terms of their concentration of carbonyl compounds after storage (Table S2). For all responses where significant models were found, the model data were satisfactory, though the coefficients of variation (R^2) were low for some of the compounds and ranged from 0.20 (2-furfural) to 0.87 (2-methylbutanal) (Table S3). This may be explained by the little variation within the individual samples in terms of their concentrations of carbonyl compounds after storage and the boundaries of the CCRD. Still, the adequate precision, which is a measure of the range of the predicted response relative to its associated error, was always >4, thus implying adequate signals. Furthermore, raw R^2 was always close to adjusted R^2, and this was in turn in reasonable agreement with the predicted R^2, indicating that the models were applicable.

These data taken together suggest that the Maillard reaction is not responsible for the formation of Strecker aldehydes, at least not as induced from the carbohydrate mixture and levels of glucose/xylose tested in this trial, and for the storage temperature of 20 °C and storage period of 18 months. The effectiveness of the availability of amino acids on the formation of their concomitant Strecker aldehydes is an indication that a de novo formation of Strecker aldehydes occurs during storage and that this reaction is affected by the availability of its precursors.

Relevance of Oxygen and Amino Acid Availability for Strecker Aldehyde Formation. The effects of oxygen exposure were tested next in a series of experiments and combined with additions of Fe^{2+} and Leu additions. Air in the bottle headspace as well as adding Leu clearly yielded an increased concentration of the Strecker aldehydes 3-methylbutanal (+187%), 2-methylbutanal (+55%), methional (+44%), phenylacetaldehyde (+64%), and benzaldehyde (+583%), as compared to the reference (Figure 1). Leu addition resulted in a higher formation of 3-methylbutanal (+38%) even at minimized oxygen concentrations and had no effect on the other carbonyl compounds measured.

In accordance with the trials where Leu was added, the addition of 20 mg/L of Leu, Ile, and Phe simultaneously added to the beers before storage was started caused an increase of 3-methylbutanal (+13%), 2-methylbutanal (+19%), and phenyl-

Table 5. *F*- and *p*-Values of Carbonyl Compounds (Responses) As Formed during Storage of Beers with the Independent Factors Oxygen Concentration and Fe^{2+} Concentration[a]

	3-methylbutanal		2-methylbutanal		methional		phenylacetaldehyde		benzaldehyde		D^5-labeled phenylacetaldehyde	
factor	*F*-value	*p*-value[b]	*F*-value	*p*-value[b]	*F*-value	*p*-value[b]	*F*-value	*p*-value[b]	*F*-value	*p*-value[b]	*F*-value	*p*-value[b]
oxygen concentration	80.75	<0.0001	62.36	<0.0001	50.71	<0.0001	78.98	<0.0001	144.24	<0.0001	97.94	<0.0001
Fe^{2+} concentration	3.58	0.0879										

[a]Only responses are shown where significant models at $p < 0.05$ were found. All model types were linear. [b]Probability of the *F*-value. It is the probability of getting an *F*-value of this size if the term did not have an effect on the response.

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Table 6. Effect of Oxygen Barrier (OB) Liner and 10 mg/L EDTA on the Formation of Carbonyls during 12 Week Storage at 28 °C[a]

compound	carbonyls in μg/L			
	reference (fresh)	reference (aged)	OB liner	EDTA addition
3-methylbutanal	4.9 ± 0.7	7.5 ± 0.1	6.4 ± 0.2	5.5 ± 0.5
2-methylbutanal	2.4 ± 0.4	4.7 ± 0.4	4.0 ± 0.2	2.9 ± 0.1
methional	2.8 ± 1.0	4.9 ± 1.0	3.1 ± 0.3	4.2 ± 0.2
phenylacetaldehyde	8.3 ± 1.5	14.2 ± 0.1	12.4 ± 0.9	10.0 ± 0.7
benzaldehyde	1.2 ± 0.3	2.0 ± 0.1	1.8 ± 0.2	1.4 ± 0.2
2-furfural	169.8 ± 11.9	474.5 ± 35.9	490.8 ± 35.9	468.9 ± 20.2
γ-nonalacton	4.9 ± 1.6	160.2 ± 10.6	169.8 ± 32.2	163.5 ± 16.5
ethylnicotinate	46.8 ± 5.3	76.8 ± 5.9	77.7 ± 18.2	72.1 ± 7.2
heptanal	<1.0	<1.0	<1.0	<1.0
octanal	1.1 ± 0.1	1.1 ± 0.3	1.2 ± 0.6	1.3 ± 0.0
phenylacetaldehyde-D^5	<1.0	1.6 ± 0.3	<1.0	<1.0
benzaldehyde-D^5	<1.0	<1.0	<1.0	<1.0

[a]Mean values ±1 standard deviation of a triplicate experiment are shown.

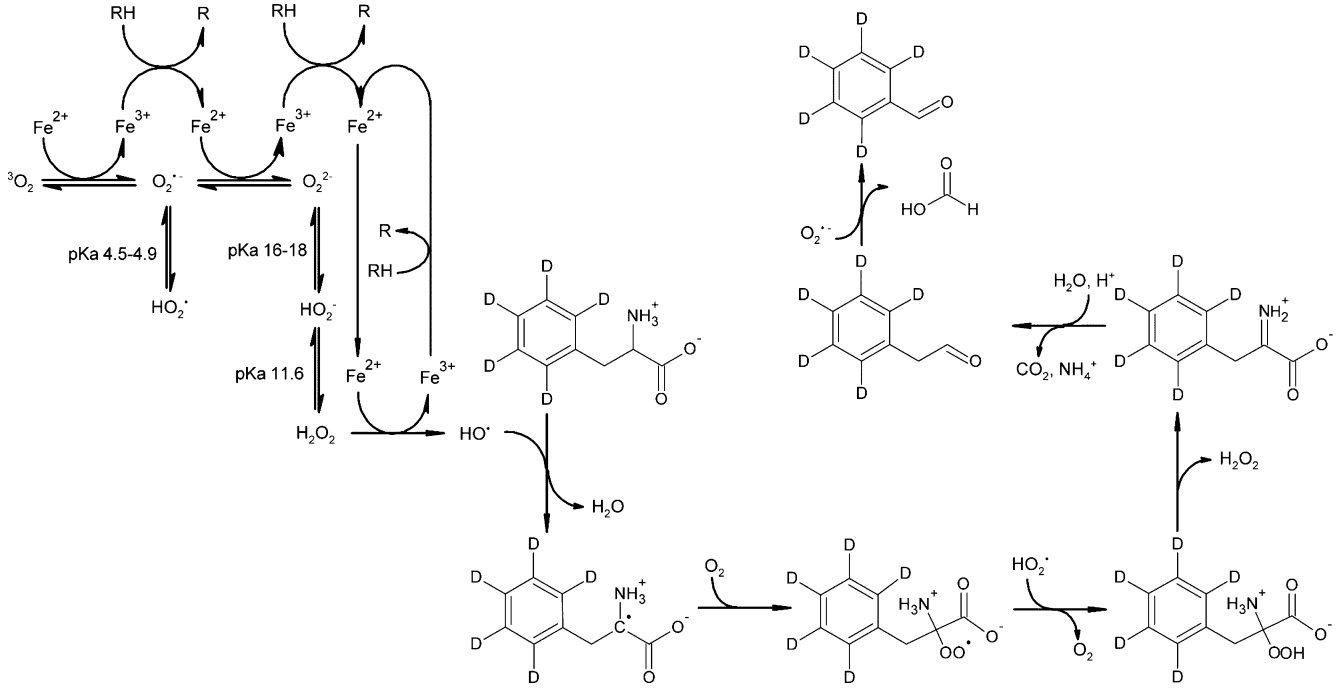

Figure 3. Proposed pathway for the oxygen-induced formation of phenylacetaldehyde-D^5 and benzaldehyde-D^5 from phenylalanine-D^5 in beer during storage. RH = reducing substances in beer. Pathways were adapted and modified from refs 10, 33, and39.

acetaldehyde (+21%) after 30 weeks of storage when the beers were bottled without air in the headspace. In beers with air in the bottle headspace, in accordance with the previous trial, a strong increase of 3-methylbutanal (+141%), 2-methylbutanal (+88%), phenylacetaldehyde (+136%), benzaldehyde (+559%), and methional (+34%) was observed.

Adding 500 μg/L of Fe^{2+} had no effect, and similar levels of Strecker aldehydes were found at the end of storage. 2-Furfural, heptanal, octanal, γ-nonalacton, and ethyl nicotinate were unaffected from the different treatments.

The initial SO_2 concentration of the beers was 0.8−1.0 mg/L as measured directly after the spiking. When air was left in the headspace, the initial SO_2 was consumed rapidly and was below 0.1 mg/L already after 2 weeks of storage. When oxygen was minimized during spiking, the SO_2 concentration also decreased during storage and was <0.1 mg/L after 6 weeks of storage. In accordance with literature data,[38] the beer's SO_2 concentrations showed a good correlation with the beer's EAP-value of 40 and 42 min for the reference sample and for the sample where only Leu was added, respectively (Figure S2). Exposing samples to air after spiking yielded a direct increase of radical formation once the ESR measurement was started. When Fe^{2+} was added, a higher radical concentration as indicated by the T_{400}-value (radical concentration after 400 min of measurement) was observed, while all other samples measured showed similar T_{400}-values. It is noteworthy to mention that ESR measurements are carried out under oxygen atmosphere, and oxygen therefore never becomes a limiting factor for the reaction cascade eventually yielding hydroxyl and hydroxyethyl radicals in beer systems. In bottled beer, though, oxygen is usually consumed very rapidly during storage and then only diffuses through the crown cork liner polymers into the beers. Oxygen can therefore be anticipated to be a limiting factor for radical formation in beers, and Fe^{2+}, because it serves

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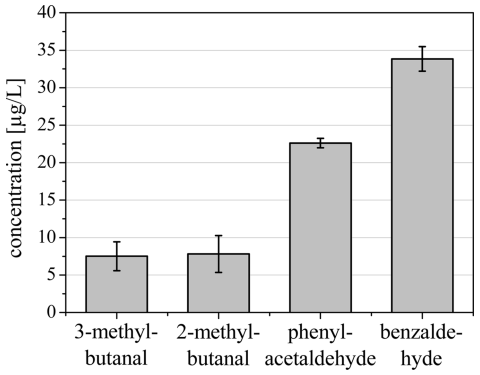

Figure 4. Strecker aldehyde formation in buffered model solutions (pH 4.3, 5% v/v ethanol) containing 25 mg/L Leu, Ile, Phe, and 100 μg/L Fe^{2+}. Bottles were stored for 6 months at 28 °C in the dark. Mean values are presented. Error bars represent ±1 standard deviation, n = 3.

Table 7. Influence of Oxygen during Mashing on Wort Analytical Parameters and Carbonyl Compounds in Sweet Wort[a]

		mashing	
		under O_2 atmosphere	under N_2 atmosphere
Wort Analysis			
extract	[% wt./wt.]	14.0 ± 0.05	13.9 ± 0.23
pH	[-]	5.51 ± 0.01	5.50 ± 0.01
color	[°EBC]	4.6 ± 0.0	4.1 ± 0.1 a
free amino nitrogen	[mg/L]	209.7 ± 5.6	218.9 ± 5.95
total polyphenols	[mg/L]	148.5 ± 2.5	188.0 ± 6.6 a
Carbonyl Compounds			
3-methylbutanal	[μg/L]	75.1 ± 5.2	26.8 ± 2.4 a
2- methylbutanal	[μg/L]	29.7 ± 1.8	20.3 ± 1.7 b
methional	[μg/L]	39.7 ± 2.3	13.2 ± 1.1 a
phenylacetaldehyde	[μg/L]	47.0 ± 2.8	14.4 ± 0.9 a
benzaldehyde	[μg/L]	7.1 ± 0.4	3.9 ± 0.3 a
2-furfural	[μg/L]	81.5 ± 3.4	78.2 ± 7.8
γ-nonalacton	[μg/L]	1.9 ± 0.2	1.9 ± 0.2
ethylnicotinate	[μg/L]	<1.0	<1.0
heptanal	[μg/L]	<1.0	<1.0
octanal	[μg/L]	<1.0	<1.0

[a]Mean values ±1 standard deviation of a triplicate experiment are shown. Different letters indicate the significant difference of mean percentages between samples by Student's t test at the 99.9% confidence level (a) and 99.0% confidence level (b).

as a catalyst, only eases the activation of oxygen and formation of ROS. As opposed to ESR measurements, it does not necessarily yield an increased formation of ROS, at least as long as oxygen is the reaction's limiting factor.

Oxygen-Promoted de Novo Formation of Carbonyl Compounds during Beer Storage. D^5-labeled phenylacetaldehyde and D^5-labeled benzaldehyde were detected in the beers and showed a clear dependency of the initial oxygen concentration before storage (Figure 2). Their appearance can be explained by degradation of phenylalanine-D^5 to phenylacetaldehyde-D^5 which can be further decarboxylated to give benzaldehyde-D^5.[39] In accordance to previous data (cp. Figure 1), all Strecker aldehydes showed a clear response to total packaged oxygen concentration and followed a sigmoidal curve during storage when oxygen was present, and a rather linear increase when bottles were prepared at minimized oxygen exposure (Figure S3).

From the factors total packaged oxygen and Fe^{2+} tested, only initial oxygen concentration was significant for the responses 3-methylbutanal, 2-methylbutanal, methional, phenylacetaldehyde, benzaldehyde, and phenylacetaldehyde-D^5 at model F-values ranging from 42.17 to 144.24 (Table 5). An increasing oxygen concentration resulted in an increase of the amount of Strecker aldehydes being formed during storage. Fe^{2+} concentration was insignificant for all compounds and was eliminated by backward elimination at $\alpha > 0.10$ from the models with the exception of 3-methylbutanal, where it was kept in the model because it showed a p-value of 0.0879. For raw data from the CCRD experiment, see Table S4. ANOVA analysis of the experimental data from the RSM experiment indicated significant linear models for the responses 3-methylbutanal, 2-methylbutanal, methional, phenylacetaldehyde, and phenylacetaldehyde-D^5 (Table S5). For the responses benzaldehyde-D^5, 2-furfural, heptanal, octanal, γ-nonalacton, and ethylnicotinate, no significant model terms could be found indicating that oxygen and Fe^{2+} concentration do not affect the formation of these carbonyl compounds during beer storage.

The emergence and increase of phenylacetaldehyde-D^5 from phenylalanine-D^5 is a clear evidence for the de novo formation of those compounds in bottled beer during storage, and its response to initial oxygen concentration is further proof for the involvement of oxygen in the reactions eventually yielding phenylacetaldehyde. For all other Strecker aldehydes, a similar behavior can be anticipated. The reason that benzaldehyde-D^5 was not significant may be explained by the fact that it was detected only at very low concentrations of <1.0 μg/L.

It is noteworthy to mention that in all trials, Fe^{2+} additions had no effect on the amounts of carbonyl compounds found at the end of storage, even though Fe^{2+} acts as a catalyst in the Fenton reaction system. However, because it can be anticipated that oxygen is always the rate limiting factor in bottled beer, only little amounts of Fe^{2+} are supposed to be needed to promote ROS formation and concomitant radical attack on amino acids ultimately yielding aldehydes. Furthermore, as proposed by Kunz et al.,[40] Fe^{2+}, once it is oxidized to Fe^{3+}, is rapidly reduced again to its lower valence state, thus being available again for its catalytic action in the formation of ROS. Because reducing substances are usually present in high concentrations in fresh beer and thus in high excess as compared to Fe^{3+}, iron is practically only present in its lower valence state, Fe^{2+}, at least as long as reducing substances are present.[41] This hypothesis is also supported by findings from Lund et al.[42] who showed that oxygen activation was independent from the amount and valence state of iron added to beers.

However, adding EDTA showed a strong effect on the amount of Strecker aldehydes detected after storage. Formation of phenylacetaldehyde-D^5 was prevented completely when EDTA was added. As opposed to the RSM experiments where addition of Fe^{2+} was found insignificant for the formation of Strecker aldehydes, EDTA is capable of complexing transition metals,[43] thus withdrawing iron and other transition metal ions entirely from potential reaction partners. Hence, it hinders Fe^{2+} to act as a catalyst in the Fenton reaction system and prevents Fe^{3+} recycling to Fe^{2+}. Consequently, ROS formation is completely suppressed,[41] and consecutive reactions are diminished which may serve as a reasonable explanation for

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Table 8. Influence of Oxygen during Heating to the Onset of Boil of Sweet Wort on Wort Analytical Parameters and Carbonyl Compounds in Sweet Wort[a]

			after purging		after heating	
		sweet wort	with air	with N_2	under O_2 atmosphere	under N_2 atmosphere
Wort Analysis						
extract	[% wt./wt.]	12.69 ± 0.01	n.m.	n.m.	n.m.	n.m.
pH	[-]	5.57 ± 0.02	n.m.	n.m.	5.57 ± 0.01	5.57 ± 0.01
color	[°EBC]	7.4 ± 0.1	n.m.	n.m.	8.1 ± 0.2	7.4 ± 0.0 a
free amino nitrogen	[mg/L]	249.8 ± 3.1	n.m.	n.m.	248.3 ± 4.0	248.7 ± 2.0
total polyphenols	[mg/L]	132.2 ± 1.1	n.m.	n.m.	121.7 ± 0.6	135.6 ± 1.1 a
Carbonyl Compounds						
3-methylbutanal	[μg/L]	34.2 ± 0.1	29.4 ± 2.0	26.7 ± 2.0	51.4 ± 2.7	39.5 ± 2.2 a
2- methylbutanal	[μg/L]	16.6 ± 0.7	13.1 ± 1.0	11.3 ± 1.0	21.4 ± 2.2	16.7 ± 1.6 b
methional	[μg/L]	32.3 ± 1.7	29.0 ± 2.3	29.5 ± 2.3	29.1 ± 0.7	26.0 ± 0.7 a
phenylacetaldehyde	[μg/L]	32.1 ± 2.5	30.7 ± 2.3	31.7 ± 2.3	39.9 ± 0.5	34.7 ± 0.7 a
benzaldehyde	[μg/L]	7.0 ± 0.6	7.4 ± 0.4	7.7 ± 0.4	7.8 ± 0.3	6.4 ± 0.5 b
2-furfural	[μg/L]	43.2 ± 3.7	46.2 ± 2.4	48.1 ± 1.7	48.9 ± 0.7	52.4 ± 3.4
γ-nonalacton	[μg/L]	3.0 ± 2.9	2.9 ± 0.3	2.8 ± 0.3	2.2 ± 0.1	2.4 ± 0.2
ethylnicotinate	[μg/L]	<1.0	<1.0	<1.0	<1.0	<1.0
heptanal	[μg/L]	<1.0	<1.0	<1.0	<1.0	<1.0
octanal	[μg/L]	1.7 ± 0.2	1.7 ± 0.1	2.0 ± 0.1	1.4 ± 0.2	1.4 ± 0.2

[a]Mean values ±1 standard deviation of a triplicate experiment are shown. n.m. = not measured. Different letters indicate the significant difference of the mean percentages between samples after heating by Student's *t* test at the 99.9% confidence level (a) and 99.0% confidence level (b).

the observations. These findings are in good agreement with literature data.[21]

Using an oxygen barrier crown cork clearly diminished Strecker aldehyde levels (Table 6) probably because diffusion through the crown cork liner material is prevented. Using these crown corks can therefore be considered to be a good measure to diminish beer staling.

Deductions from these data therefore point clearly to the relevance of an ROS-induced pathway as proposed by Wietstock and Methner[10] as being responsible for the de novo formation of Strecker aldehydes in bottled beer. In consideration of all findings, a pathway for the formation of phenylacetaldehyde-D^5 and benzaldehyde-D^5 during beer storage including the activation of oxygen, following ROS formation, Fe^{3+} recycling, and phenylalanine-D^5 oxidative degradation was proposed and is shown in Figure 3.

Though, it is noteworthy that from 20 mg/L of phenylalanine-D^5 spiked into the beer, at maximum 6.3 μg/L (0.03%) phenylacetaldehyde-D^5 was formed during storage, while from 28.5 mg/L phenylalanine already present in the beer, a proportionally higher amount of 24.8 μg/L (0.09%) of phenylacetaldehyde was detected. There may therefore also other pathways being involved in Strecker aldehyde such as, e.g., the liberation from 2-substituted 1,3-thiazolidine-4-carboxylic acid complexes as proposed by Baert et al.[3,5] It is questionable, though, if these complexes are vulnerable to oxidation. 2-Furfural, for instance, which was clearly not dependent on initial oxygen concentration in this study, is also bound in these complexes according to Baert et al.,[5] and should therefore also respond to oxygen if these complexes were prone to oxidation; though, this was not the case. Yet, also oxidation of higher alcohols[6] or direct oxidative degradation of Amadori compounds already present in beer[44] are possible contributors to the apparent response of Strecker aldehydes to oxygen.

Model Studies. To further scrutinize the relevance of an oxidative degradation of amino acids[10] in terms of contributing to a de novo formation of Strecker aldehydes during beer storage, a trial in buffered model solutions was carried out with only the reactions partners amino acids, oxygen, and Fe^{2+} present. As shown in Figure 4, 3-methylbutanal, 2-methylbutanal, phenylacetaldehyde, and benzaldehyde were found in the distillates after storage at concentrations ranging from 7.5 (3-methylbutanal) to 33.8 μg/L (benzaldehyde), thus implying that Strecker aldehyde formation can occur at these experimental conditions such as only when the reaction partners amino acids, Fe^{2+}, and oxygen present. Interestingly, benzaldehyde was detected in a higher proportional percentage in relation to phenylacetaldehyde, as it was detected in beer. This may be attributed to less reaction partners of ROS other than phenylacetaldehyde, in particular of the superoxide anion ($O_2^{-\bullet}$).[39]

Relevance of Oxygen for the Formation of Carbonyls during Wort Production. Because the detrimental role of oxygen may not be limited to beer storage, the relevance of oxygen for the formation of Strecker aldehydes during wort production such as mashing and heating to start of boil was also tested. During wort boiling, oxygen solubility is very low because of the high temperatures, and this process step was therefore not assessed. The impact of wort aeration was also not investigated because it was demonstrated to be irrelevant for beer aging by Depraetere and co-workers.[45]

Oxygen presence had a considerable effect on the carbonyl pattern in sweet wort and in particular the Strecker aldehydes 3-methylbutanal, 2-methylbutanal, methional, phenylacetaldehyde, and benzaldehyde were higher when oxygen was present (Table 7). Oxygen absence or presence had also a significant effect on pH, color, and amount of total polyphenols. The effect on malt polyphenols was also found by Stephenson et al.[46] and may be explained oxidative polymerization, insolubilization and removal of these substances during the filtration step. The sweet wort's extract and content of free amino nitrogen were not affected influenced by the absence or presence of oxygen.

The same tendencies were seen when sweet worts were heated to the onset of boil with and without oxygen presence, and Strecker aldehydes were higher when oxygen was present

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in the wort samples (Table 8). However, the effect was less pronounced as it was during mashing which may be explained the shorter exposure time as well as higher temperatures and concomitant lower oxygen solubility. Total polyphenol levels and color were again significantly affected, while pH and free amino nitrogen content were not. Purging with oxygen or nitrogen did not result in significant differences between samples, yet it caused a loss of volatiles, in particular of 2-methylbutanal and 3-methylbutanal. The effects of oxygen on the formation of carbonyls as seen in mashes and in sweet worts during the heating phase may be again partly explained by the ROS-induced oxidative degradation of amino acids, though because of the complexity of the mashes in their composition also other pathways such as enzymic processes[46] cannot be excluded. Narziß, Reicheneder, and Bauer[47] found also that beers which were produced under oxygen avoidance were superior as related to their analytical and sensory quality which confirms the outcomes from this study.

All data found in this study are in good agreement with published literature where also a clear effect of oxygen on Strecker aldehyde formation and beer staling was found.[7,22,23] Yet, this effect is not limited to beer production or beer storage, and also wine was reported to undergo flavor changes dependent on oxygen exposure.[48−50] Interestingly, de novo formation of aldehydes was observed when SO_2 was depleted in the wines[49] which goes along with the generation and emergence of ROS. Similar pathways as occurring in beer can be anticipated.

In this study, for the first time, evidence supporting a mechanism for the ROS-induced oxidative degradation of amino acids yielding a de novo formation of Strecker aldehydes during beer storage is presented. Outcomes from this study certainly do not imply that this mechanism is solely accountable for the emergence of Strecker aldehydes during beer production and storage, and ultimately, a mixture and interplay of different pathways and proposed reactions can be anticipated. Yet, the importance of hydroxyl and hydroxyethyl radicals and the clear relationship between Strecker aldehyde formation and total packaged oxygen point to the existence and relevance of this pathway during beer aging. This study therefore helps to further clarify food staling mechanisms, particularly, as related to the oxidative degradation of amino acids and concomitant formation of Strecker aldehydes during food processing and storage.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b03502.

Retention times and m/z ratios used for quantitating carbonyl compounds by GC/MS; carbonyl concentrations of beers from a central composite rotatable design experiment with the independent factors carbohydrate, amino acid, and Fe^{2+} concentration; model data for the responses from a central composite rotatable design experiment with the independent factors carbohydrate concentration and amino acid concentration; carbonyl concentrations of beers from a central composite rotatable design experiment with independent factors oxygen concentration and Fe^{2+} concentration; total packaged oxygen as a function of oxygen concentration in the atmosphere during bottling; ESR data of fresh beer samples without additions; with addition of Leu only; addition of Leu and oxygen exposure; addition of Leu, Fe^{2+}, and oxygen exposure; time-course formation of carbonyl compounds during storage depending on initial oxygen and Fe^{2+} concentration during bottling (PDF)

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Notes

The authors declare no competing financial interest.

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Publication B

Supplementary Material

Table S1: Retention times and *m/z* ratios used for quantitating carbonyl compounds by GC/MS.

Substance	t_R [min][a]	*m/z*
3-methylbutanal	10.15	58
2-methylbutanal	10.63	58
pentanal (IS)	12.96	58
2-furfural	19.05	96
heptanal	21.26	70
methional	21.50	104
benzaldehyd	23.03	106
octanal	23.78	84
phenylacetaldehyd	24.79	91
ethyl nicotinate	27.61	106
γ-nonalacton	29.55	85

[a]: t_R = retention time.

Table S2: Carbonyl concentrations (responses) of beers from a central composite rotatable design experiment with the independent factors carbohydrate, amino acid, and Fe^{2+} concentration. Beers were stored for 18 months of storage at 20 °C. 3-MB = 3-methylbutanal, 2-MB = 2-methylbutanal, MET = methional, PAL = phenylacetaldehyde, BZALD = benzaldehyde, FUR = 2-furfural, NON = γ-nonalacton, NIC = ethyl nicotinate, HEP = heptanal, OCT = octanal.

Independent factors			Responses									
Carbohydrate addition[a] [mg/L]	AA addition[b] [mg/L]	Fe^{2+} addition [µg/L]	3-MB [µg/L]	2-MB [µg/L]	MET [µg/L]	PAL [µg/L]	BZALD [µg/L]	FUR [µg/L]	NON [µg/L]	NIC [µg/L]	HEP [µg/L]	OCT [µg/L]
300	40	50	7.8	5.2	7.0	11.0	3.4	742.2	16.8	429.7	1.3	4.9
300	40	50	6.7	4.8	6.0	10.0	2.9	540.5	14.5	373.8	1.6	2.5
300	40	50	8.0	5.4	6.6	10.0	2.6	608.0	15.9	377.0	1.5	2.6
300	40	50	7.6	5.4	5.4	9.4	2.3	514.2	13.9	329.1	< 1.0	2.1
300	40	50	7.2	4.9	4.8	7.2	1.8	427.0	13.1	369.5	< 1.0	1.2
300	40	50	7.0	5.0	4.7	9.1	2.5	496.0	15.7	370.6	< 1.0	1.7
200	20	20	6.3	3.9	6.3	6.5	2.5	468.2	14.0	328.5	1.4	2.3
200	60	20	7.9	5.9	5.8	10.0	2.8	548.8	30.9	383.4	1.5	3.3
200	20	80	6.8	4.1	5.7	7.2	2.5	570.4	14.6	373.2	1.7	3.2
200	60	80	9.3	6.8	5.2	8.6	2.4	506.1	16.5	325.8	5.7	2.5
400	20	20	7.6	4.4	5.1	7.4	1.8	541.0	17.6	387.8	0.9	1.3
400	60	20	8.8	6.6	5.4	8.3	2.2	615.0	12.8	325.6	1.2	2.0
400	20	80	6.6	4.0	5.9	9.1	2.9	650.4	12.9	335.1	1.3	2.5
400	60	80	9.5	6.8	5.9	12.0	2.6	598.7	21.2	317.9	< 1.0	2.0
300	40	100	9.7	6.5	4.8	8.4	2.3	492.0	12.0	288.8	1.2	1.9
300	40	0	9.1	5.8	5.0	8.7	1.9	502.6	15.8	259.0	1.1	1.7
300	6.36	50	6.2	3.2	5.0	6.7	2.3	531.9	10.0	294.5	1.4	1.9
300	73.64	50	11.0	8.5	4.9	9.3	1.8	508.3	12.9	272.0	1.3	2.2
131.82	40	50	7.8	5.4	4.3	7.3	1.9	399.0	7.7	269.1	1.3	6.1
468.18	40	50	7.7	5.4	5.5	9.0	2.2	548.2	15.6	250.3	1.2	5.8

a: A mixture of glucose and xylose was added. The concentrations represent the concentration added for each carbohydrate individually.

b: A mixture of leucine, isoleucine, and phenylalanine was added. The concentrations given represent the concentration added from each amino acid individually.

Table S3: Model data for the responses from a central composite rotatable design experiment with the independent factors carbohydrate concentration (mixture of glucose and xylose) and amino acid concentration (mixture of Leu, Ile, Phe). Only responses are shown where significant models at $p < 0.05$ were found. All model types were linear.

	Responses			
Values	3-methylbutanal	2-methylbutanal	phenylacetaldehyde	2-furfural
R^2	0.63	0.87	0.32	0.20
Adjusted R^2	0.61	0.86	0.28	0.16
Predicted R^2	0.54	0.84	0.16	0.04
Adequate precision	15.82	31.67	8.29	6.17
Model F-value	30.21	121.1	8.29	4.59
Lack of fit F-value	3.17	4.03	0.89	0.20

Table S4: Carbonyl concentrations (responses) of beers from a central composite rotatable design experiment with the independent factors oxygen concentration and Fe^{2+} concentration. Beers were stored for 12 weeks at 28 °C. 3-MB = 3-methylbutanal, 2-MB = 2-methylbutanal, MET = methional, PAL = phenylacetaldehyde, BZALD = benzaldehyde, PAL-D^5 = phenylacetaldehyde-D^5, BZALD-D^5 = benzaldehyde-D^5, FUR = 2-furfural, NON = γ-nonalacton, NIC = ethyl nicotinate.

Independent factors		Responses									
$c(O_2)$ [%]	Fe^{2+} addition [µg/L]	3-MB [µg/L]	2-MB [µg/L]	MET [µg/L]	PAL [µg/L]	BZALD [µg/L]	PAL-D5 [µg/L]	BZALD-D5 [µg/L]	FUR [µg/L]	NON [µg/L]	NIC [µg/L]
< 0.01	250.0	7.2	4.4	2.7	10.0	1.8	1.6	< 1.0	474.0	73.0	159.7
3.07	427.0	9.3	5.6	4.0	11.4	2.9	1.9	< 1.0	487.1	90.2	213.1
3.07	73.2	9.4	5.5	3.3	10.9	2.6	1.9	< 1.0	444.4	73.3	149.9
10.5	0.0	12.1	7.0	4.5	15.6	5.4	3.3	< 1.0	476.3	75.3	171.8
10.5	250.0	11.1	6.0	3.9	14.3	4.9	2.8	< 1.0	451.3	71.3	162.5
10.5	250.0	13.7	7.5	5.0	16.0	6.3	3.4	< 1.0	520.3	61.0	117.5
10.5	250.0	13.1	7.2	6.4	18.1	6.4	3.7	< 1.0	561.4	94.0	209.6
10.5	250.0	13.0	6.3	4.0	18.3	6.1	3.6	< 1.0	543.1	66.4	249.8
10.5	250.0	13.1	7.5	5.1	14.1	5.4	2.9	< 1.0	481.9	73.2	163.7
10.5	500.0	13.7	7.3	4.0	14.6	6.1	3.0	< 1.0	494.2	57.1	110.0
17.92	427.0	16.9	8.5	7.1	20.5	9.9	5.0	< 1.0	544.0	86.4	195.2
17.92	73.2	16.8	8.7	6.3	21.1	11.0	5.1	< 1.0	568.8	79.4	184.7
20.99	250.0	17.6	9.2	8.2	25.9	12.9	6.3	< 1.0	469.3	62.8	190.1
Fresh beer	-	4.9	2.4	< 1.0	1.1	1.8	< 1.0	< 1.0	169.8	4.9	48.2

Table S5: Model data for the responses from a central composite rotatable design experiment with the independent factors oxygen and Fe^{2+} concentration. Only responses are shown where significant models at $p < 0.05$ were found. All model types were linear. 3-MB = 3-methylbutanal, 2-MB = 2-methylbutanal, MET = methional, PAL = phenylacetaldehyde, BZALD = benzaldehyde, PAL-D^5 = phenylacetaldehyde-D^5.

	Responses					
Values	3-MB	2-MB	MET	PAL	BZALD	PAL-D^5
R^2	0.89	0.85	0.82	0.88	0.93	0.90
Adjusted R^2	0.87	0.84	0.81	0.87	0.92	0.89
Predicted R^2	0.80	0.82	0.76	0.83	0.90	0.87
Adequate precision	18.71	20.13	18.16	22.66	30.62	25.23
Model F-value	42.16	62.36	50.71	78.98	144.24	97.94
Lack of fit F-value	0.35	0.80	0.93	0.81	0.19	0.39

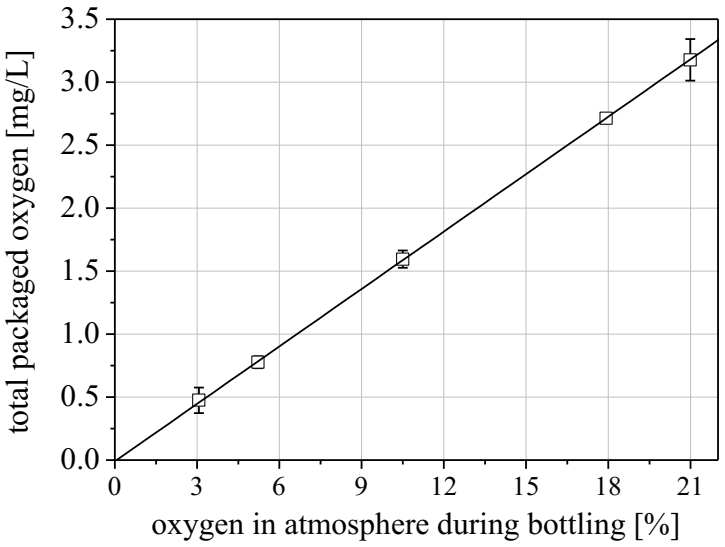

Figure S1: Total packaged oxygen as a function of oxygen concentration in the atmosphere during bottling. Mean values are presented. Error bars represent ± 1 standard deviation, n = 6.

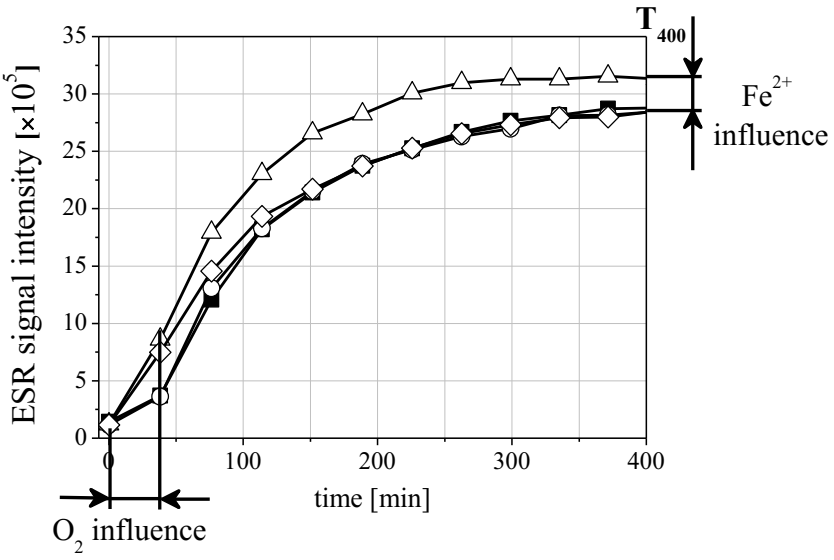

Figure S2: ESR data of fresh beer samples with the following treatments: no additions, no air exposure (■), addition of 25 mg/L leucine, no air exposure (○); addition of 25 mg/L leucine and exposing samples to air (◇); addition of 25 mg/L leucine and 500 µg/L Fe^{2+}, and exposing samples to air (△).

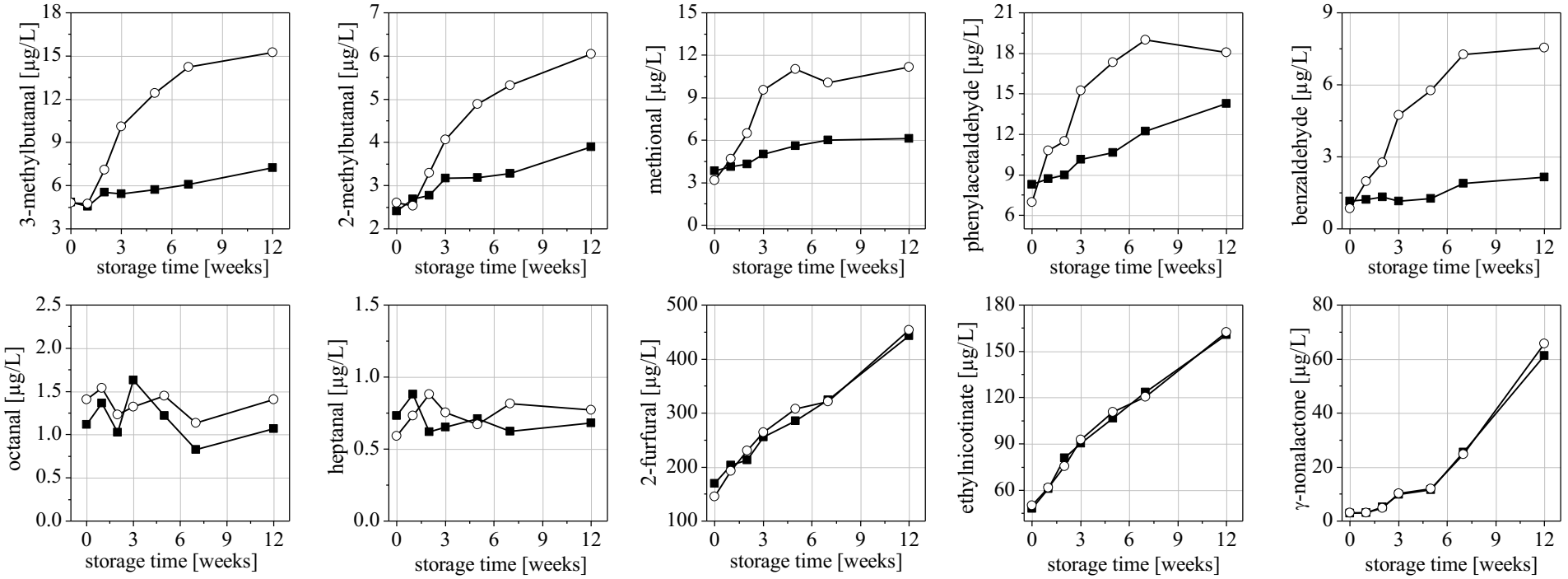

Figure S3: Time-course formation of carbonyl compounds during storage in dependency of initial oxygen and Fe^{2+} concentration during bottling. (○): With 10.5 % oxygen present in bottle headspace and 250 µg/L Fe^{2+} iron added; (■): with oxygen minimized and no Fe^{2+} added. Bottles were stored for 12 weeks at 28 °C.

3.2 Analytical-chemical characterization of the anti-staling effects of the hop dosage and application to the brewing process

Publication D and F of the thesis were conducted within the scope and work load of the AiF project “Variationen des Hopfenmanagements zur gezielten Ausfällung oxidationsfördernder Metallionen im Verlauf des Brauprozesses“ (AiF 17439 N) which was supported via AiF within the program for promoting the “Industrielle Gemeinschaftsforschung (IGF)” of the German Ministry of Economics and Energy (BMWi).

Publication C

Antioxidants are substances that can block or abate oxidative reactions, though the way of exerting their mode of action is often different which has important consequences to how and when antioxidants should be applied to products or how they can be effectively preserved. In the last two decades, the protective effects of hops were thoroughly investigated chemically and analytically (see chapter 1.3). The potential of hop acids is underexplored. In order to scrutinize the hop acid’s antiradical characteristics, in the publication

“Chelating Properties and Hydroxyl-Scavenging Activities of Hop-α and Iso-α-Acids”[276] *(Publication C).*

the hop acids’ mode of suppressing oxidative reactions was examined. Findings from this study expanded the knowledge about the antiradical activity of particularly hop-α and *iso*-α-acids. Their effectiveness in diminishing oxidative degradation of 2-desoxyribose (a marker compound for hydroxyl radical attack) could be traced back to their complexation functionality towards Fe^{2+} ions thereby promoting its autoxidation to Fe^{3+} while hydroxyl radical scavenging capability was not observed.

Chelating Properties and Hydroxyl-scavenging Activities of Hop α- and Iso-α-acids

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ABSTRACT

Metal chelation and scavenging of highly reactive hydroxyl radicals are commonly proposed to explain the antioxidant behavior of hop acids. In the current study, an assay measuring the oxidative degradation of 2-deoxyribose (2-DR) by hydroxyl radicals was used to elucidate these mechanisms and examine the origin of the ability of hop acids to prevent oxidative damage. Hop α- and iso-α-acids were able to prevent 2-DR oxidative degradation; however, α-acids appeared to be 60% more effective than iso-α-acids. The ability of hop acids to inhibit 2-DR oxidative degradation could be inversely correlated with Fe^{2+} concentration, which suggests that they prevent 2-DR oxidation by chelating iron. Moreover, it was demonstrated that these hop acids were not capable of scavenging hydroxyl radicals. Further trials showed that hop α- and iso-α-acids enhanced Fe^{2+} autoxidation to Fe^{3+} when complexing it and, thus, lowered the catalytic function of the iron in the Fenton reaction system.

Keywords: Chelation, Flavor stability, Hops, Iron, Radical scavenging

RESUMEN

Quelación de metales y la compactación de los radicales hidroxilo altamente reactivos son comúnmente propuestos para explicar el comportamiento antioxidante de los ácidos del lúpulo. En el presente estudio, un ensayo de la medición de la degradación oxidativa de 2-desoxirribosa (2-DR) por los radicales hidroxilo fue usado para elucidar los mecanismos y examinar el origen de la capacidad de los ácidos del lúpulo para prevenir el daño oxidativo. Los α- y iso-α-ácidos del lúpulo fueron capaces de evitar que 2-DR degradación oxidativa, sin embargo, α-ácidos que parecía ser un 60% más efectivo que el iso-α-ácidos. La capacidad de los ácidos del lúpulo para inhibir 2-DR degradación oxidativa podría ser inversamente correlacionada con la concentración de Fe^{2+}, lo que sugiere que impiden la oxidación 2-DR por quelantes de hierro. Por otra parte, se ha demostrado que estos ácidos del lúpulo no eran capaces de eliminar los radicales hidroxilos. Estudios posteriores demostraron que los α- y iso-α-ácidos de lúpulo mayor auto-oxidación de Fe^{2+} a Fe^{3+} cuando complejos y, por tanto, disminución de la función catalizadora del hierro en el sistema de reacción de Fenton.

Palabras claves: Eliminación de radicales libres, Estabilidad del sabor, Hierro, Lúpulo, Quelación

The hydroxyl radical is regarded as a serious, harmful species in aqueous solutions. There is evidence that free radicals, primarily highly reactive hydroxyl radicals, play a major role in beer deterioration (3,24,42). The presence of transition metals, such as iron and copper, facilitates these reactions by acting catalytically. Iron may easily oscillate between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states; Fe^{2+} ions are relatively more reactive and thereby contribute to the formation of active oxygen species via the Fenton and Haber-Weiss reactions (21,23,46). Oxygen participates in these reactions, and as it successively passes through the superoxide, peroxide, and hydroxyl forms, its reactivity and harmful characteristics increase (5). In packaged beer excessive amounts of oxygen and high iron levels may cause a rapid change in aroma and taste. Although it is not possible to completely inhibit beer staling reactions, minimizing the formation of reactive oxygen species (ROS) in beer and wort by reducing oxygen pickup during the process and lowering the metal ion concentration can result in a remarkable improvement in beer flavor stability (4).

Flavor deterioration in food is ultimately an interplay between antioxidants and pro-oxidants that influence oxidative reactions in a positive or negative manner, respectively. Pro-oxidants can promote staling reactions by reducing metal ions in their ferrous iron state, making them available as catalysts for radical generation. In contrast, antioxidant activity can involve the capture of free radicals or ROS. Additionally, it is believed that chelation of iron inhibits its catalyzing action in the Fenton reaction, and therefore, strong metal chelators are thought to be good antioxidants in systems where iron is present. However, the role of metal chelators as antioxidants is complex; for instance, Chvátalová et al (10) found that polyphenols with catechol and galloyl moiety could form chelates with ferrous iron, thereby increasing the rate of Fe^{2+} autoxidation. However, polyphenols bearing a catechol group also reduced ferric iron to ferrous iron, thereby promoting its catalytic action in the Fenton reaction.

The Fenton reaction system and the efficiency of •OH scavengers depend greatly on reaction conditions, such as pH (12,34,39), and there are potentially different kinds of oxidizing iron species, such as ferryl ions, being formed (27,36,37). There is evidence that the nature and concentration of the ligand (chelator) and the stoichiometry of the chelator-iron complex have a great effect on the efficiency of radical formation. The explanation appears to lie in the accessibility of hydrogen peroxide to the iron ion, which in turn is related to the number of ligation sites occupied by the chelator (6, 8,45). Another mechanism involves chelators that are able to remove iron bound to other molecules and thereby prevent it from undergoing site-localized reactions with these molecules. However, the scavenger must have a higher binding affinity for the metal than the ligand (15). Therefore, an ideal chelator for preventing Fenton chemistry must chelate and stabilize iron in a redox state that is inert to either oxidation or reduction by commonly encountered reducing agents.

Recently, investigators examined the antioxidant ingredients in hops to elucidate the underlying mechanisms by which they exert their activities. Hops (*Humulus lupulus*) are exclusively used to provide aroma and bitterness in beer. The brewer's major interest lies in hop bitter acids, the α-acids, which isomerize during wort boiling and form bitter tasting iso-α-acids (11). Even though β-acids are sparingly soluble in wort and beer, there is evidence that they may enhance beer flavor stability because they show antioxidative properties (41,44).

Liu et al (29) investigated the antioxidative activity of hop acids by measuring the absorbance of a 1,10-phenanthroline–Fe^{2+} system (1,10-phenanthroline is an indicator for oxidation events) to determine the hydroxyl-scavenging activity of hop acids and to evaluate their antioxidative activity based on coupled oxidation of a β-carotene–linoleic acid model system. Ting et al (41) used elec-

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tron spin resonance (ESR) spectroscopy coupled with a 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay to investigate the antioxidative activity of individual hop acids. As a result of these studies, the antioxidative properties of hop acids were correlated with their iron-chelating properties (7,41), hydroxyl radical-scavenging activities (29), and radical-stabilizing β,β′-triketone moieties (40). Further, the cyclic β,β′-triketone structure of hop acids appears to play a major role in their ability to form complexes with metal ions; however, they present only one acidic proton and, subsequently, cannot form polynuclear coordination compounds (7). There is evidence that certain hop polyphenols also have strong antioxidative properties (13,28,32), although to what extent polyphenols contribute to beer flavor stability is still a topic of considerable debate (1).

Predicting and examining the antioxidative properties of either the anti- or pro-oxidative behavior of individual compounds is a topic of great interest within the food and brewing industries, and there is a large variety of methods from which to choose. ESR spectroscopy has become an important tool for measuring the concentrations and natures of free radicals in food systems (2,3,41,44), although it is not always capable of elucidating the underlying mode of action of a potential antioxidant. Assays using free stable radicals, such as DPPH, are widely used (25,35); however, results from this type of assay partially contradict other results (41, 44). One reason may be that these radicals favor specific reaction partners.

Lopes et al (30) studied the effect of tannic acid using a 2-deoxyribose (2-DR) assay and a ferrozine assay and showed its ability to remove Fe^{3+} from EDTA, thereby forming a complex that inhibits hydroxyl radical formation. Hermes-Lima et al (18) also used these assays to demonstrate the ability of pyridoxal isonicotinoyl hydrazone to enhance the rate of ferrous ion oxidation to ferric ion, suggesting that it decreases the concentration of Fe^{2+} available for the Fenton reaction.

In the current study, the mode of action of hop α- and iso-α-acids was studied. The purpose was to examine whether their antioxidative behavior is derived from their ability to chelate iron ions and thereby inhibit hydroxyl radical formation, or if they are able to quench the hydroxyl radicals produced by Fenton's reagent.

EXPERIMENTAL

Reagents and Solutions

2-DR, $FeCl_3$, thiourea, thiobarbituric acid (TBA), hydrogen peroxide, ferrozine, acetic acid (glacial), acetonitrile, methanol, and ethanol were purchased from VWR International. Sodium acetate trihydrate, sodium phosphate monobasic, sodium phosphate dibasic, and $FeCl_2$ were purchased from Sigma-Aldrich. All reagents were of analytical grade or higher. All solutions were made with deionized water (Milli-Q [MQ], Millipore Corporation). Purified α-acids (86.4% purity) and iso-α-acids (30.17% purity) were supplied by Hopsteiner. Hop acids were stored in a freezer and a solution of acetonitrile/water (50:50, vol/vol) and were prepared fresh every day.

2-DR Oxidative Degradation Assays

2-DR oxidative degradation was used as a measurement for determining a test compound's antioxidative potential and mechanism (16). The principle of the assay relies on the quantification of the 2-DR oxidative degradation product, malonaldehyde, by its condensation with TBA and measuring the absorbance of the reaction product at 532 nm. To elucidate and differentiate the antioxidative mechanism of the test samples, two sets of experiments were carried out.

Typical reactions for determining the ability of a test compound to prevent •OH radical formation by chelating iron were started by mixing 0–200 μ*M* test sample with Fe^{2+} (5 and 10 μ*M*) and incubating the mixture for 10 min to allow the formation of Fe^{2+}-test sample complexes. Solutions contained 5 m*M* 2-DR, 100 μ*M* H_2O_2, and 20 m*M* acetate buffer (pH = 5.5). Reactions were carried out at room temperature and stopped by the addition of 4% (vol/vol) phosphoric acid followed by 1% TBA (wt/vol in 50 m*M* NaOH). After boiling for 15 min, absorbance was measured at 532 nm with a spectrophotometer (UV-1700, Shimadzu Corp.).

To elucidate the •OH radical-scavenging activities of the test samples, formation of the Fe^{2+}-sample complex was prevented by mixing EDTA (75 μ*M*) with Fe^{3+} (50 μ*M*) and preincubating the mixture for 10 min at room temperature to allow the formation of Fe^{3+}-EDTA complexes. Solutions contained 5 m*M* 2-DR and 20 m*M* acetate buffer (pH 5.5). After adding test samples (0 and 100 μ*M*), formation of •OH radicals from the Fenton reagent Fe^{3+}-

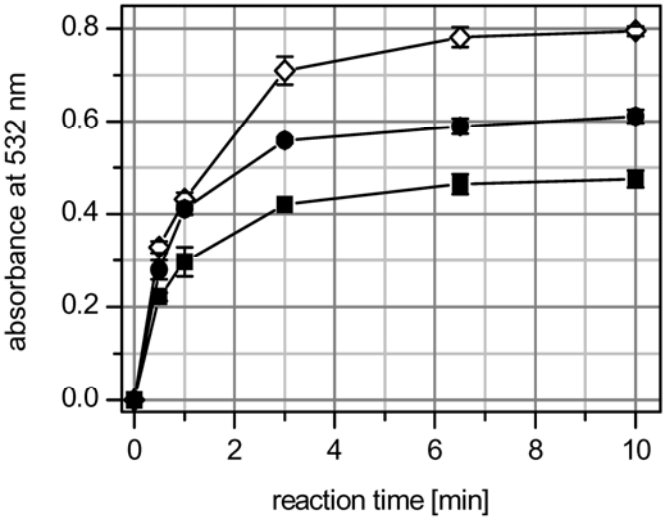

Fig. 1. Time course of 2-deoxyribose (2-DR) oxidative degradation in the absence (◇) or presence of 100 μ*M* α-acids (■) and iso-α-acids (●). Solutions contained 5 m*M* 2-DR, 20 m*M* acetate buffer (pH 5.5), and 100 μ*M* H_2O_2. Reactions were started by the addition of 10 μ*M* Fe^{2+}. Error bars represent standard ± 1 deviation; *N* = 4.

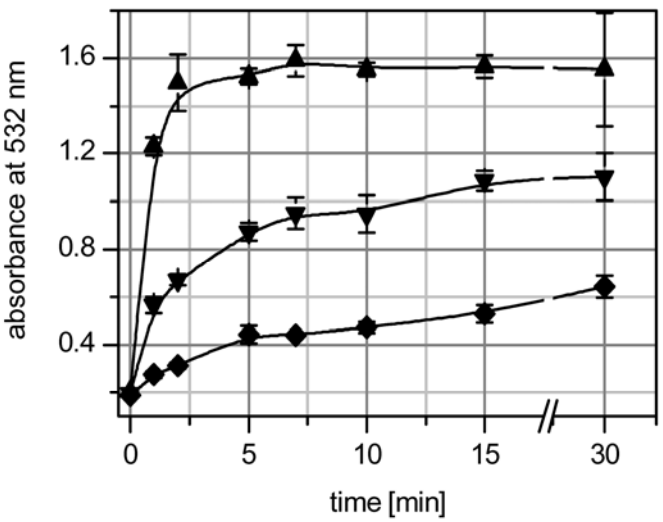

Fig. 2. Time course of 2-deoxyribose (2-DR) oxidative degradation at pH 6.5 (▲), 5.5 (▼), and 4.3 (◆). Solutions contained 5 m*M* 2-DR, 20 m*M* acetate buffer (pH 5.5), and 100 μ*M* H_2O_2. Reactions were started by the addition of 10 μ*M* Fe^{2+}. Error bars represent standard ± 1 deviation; *N* = 4.

EDTA was started by adding 100 μ*M* ascorbate to the reaction mixture and incubating it under oxygen atmosphere for 60 min at 60°C. Reactions were stopped and measured as described earlier.

Absorption Spectra

Spectra of the complexes of test samples with Fe^{2+} and Fe^{3+} were examined with the spectrophotometer (UV-1700, Shimadzu). The complexes were incubated as described in the ferrozine assay and measured with Fe^{2+} or Fe^{3+} and, in the absence of iron, against a blank containing water.

Fe^{2+} Concentration Measurement

Fe^{2+} concentration was measured according to the method of Carter (9). The principle of the assay relies on the formation of a stable complex between ferrous iron and ferrozine, with its absorbance maxima at 562 nm. First, different concentrations of the test sample (0.01–2 m*M*, 1,000-μL final volume) were incubated for 10 min at room temperature with 200 μL of $FeCl_2$ solution (5 μ*M* Fe^{2+} in MQ water) in 3,800 μL of acetate-buffered media (buffer concentration = 100 m*M*, 5% ethanol [vol/vol], pH 5.5). The reaction was initiated by the addition of 4 mL of ferrozine (2 m*M* in MQ water). After 10 min, absorbance at 562 nm was read with the spectrophotometer (UV-1700, Shimadzu). All given concentrations are final concentrations.

RESULTS AND DISCUSSION

A 2-DR assay was used to study the ability of hop α- and iso-α-acids to inhibit hydroxyl radical formation via the Fenton reaction. The pentose sugar 2-DR is likely to react with hydroxyl radicals and form a mixture of products. When heated with TBA at low pH, some or all of these products react, forming a pink chromogen that can be measured by its absorbance at 532 nm. Thus, generation of these adducts can be used as a simple assay for hydroxyl radical control and, therefore, for determining the antioxidative behavior of test compounds.

In preliminary trials, methanol, ethanol, and acetonitrile were tested for use as solvents for the hop acids in the 2-DR assay. Ethanol and methanol inhibited 2-DR oxidative degradation to a high degree, even at low concentrations (0.01–0.2%, vol/vol), and, thus, were not used in the assay (data not shown). Ethanol is a major quencher of hydroxyl radicals and may form the radical 1-hydroxyl ethyl (3), which appeared to be unreactive with the indicator molecule 2-DR. A similar mechanism can be assumed for methanol. Acetonitrile affected the assay only marginally and, therefore, was used to dissolve the hop acids.

The use of acetate and phosphate buffers was tested as well. The phosphate buffer affected both assays and yielded no reproducible results. One reason may be its ability to enhance Fe^{2+} autoxidation in aqueous solutions (43). The acetate buffer showed no effects and, thus, was used in the assays (data not shown).

A time-course experiment to determine the oxidative degradation of 2-DR induced by 20 μ*M* Fe^{2+} and 100 μ*M* H_2O_2 with and without addition of 100 μ*M* hop acids indicated that both hop acids were able to reduce 2-DR damage; however, α-acids appeared to be more effective than iso-α-acids (Fig. 1). When individually examined, α- and iso-α-acids that were added to the incubation media inhibited 2-DR oxidative degradation to an extent that remained unchanged for 60 min (data not shown).

The Fenton reaction rate appeared to be slower and, thus, partially contradicted the results of Jiang et al (22), who found that hydroxyl radical production at pH 7.4 was very fast and usually completed by 1 sec after mixing Fe^{2+} with H_2O_2. In the current study, the time course for 2-DR oxidative degradation was on the order of minutes as opposed to seconds. As pH decreased from 6.5 to 4.3, the reaction rate slowed considerably (Fig. 2). This outcome has been confirmed by other studies (12,34), which showed that the Fenton reaction rate is depends strongly on the pH of the system.

To elucidate and differentiate the antioxidative mechanisms of the hop acids, two sets of experiments were carried out. In the first set of trials, hop acids were preincubated with ferrous iron to allow the formation of hop acid-iron complexes to determine whether hydroxyl radicals were generated from these complexes, thereby causing 2-DR damage. In the second trial, hydroxyl radicals were produced from the Fenton's reagent EDTA-iron complex, and the ability of the hop acids to scavenge these radicals, thereby preventing 2-DR oxidative degradation, was investigated.

Inhibition of 2-DR Damage by Fe^{2+}-Hop Acid Complexes

The first step in these experiments was to allow the formation of Fe^{2+}-hop acid complexes. Increasing concentrations of α- and iso-α-acids (0–200 μ*M*) were mixed with ferrous iron and incubated to allow the formation of Fe^{2+}-hop acid complexes. In terms of inhibiting 2-DR oxidative degradation, both hop acids were more effective antioxidants with 5 μ*M* Fe^{2+} than with 10 μ*M* Fe^{2+} (Fig. 3). The 50% effective concentration (EC_{50}) values (Table I) suggest that iso-α-acids were approx. 60% less efficient in preventing 2-DR oxidative degradation than were α-acids. The effect of hop acids on suppression of 2-DR oxidative degradation was compared with the iron chelator EDTA. EDTA is a typical iron chelator, and its role in Fenton reactions has been well documented. It can either stimulate or inhibit oxidation depending on the iron/EDTA ratio, causing inhibition of oxidation at ratios higher than 1:1 and stimu-

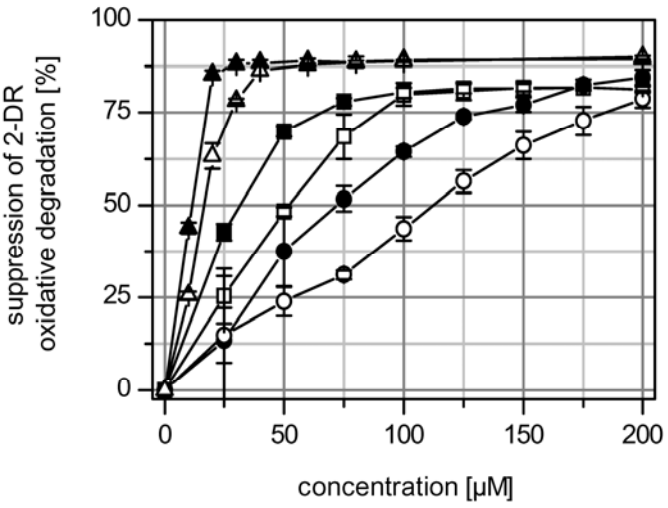

Fig. 3. Effect of EDTA (▲/△), α-acid (■/□), and iso-α-acid (●/○) concentrations on suppression of 2-deoxyribose (2-DR) oxidative degradation. Samples were preincubated for 10 min with 5 μ*M* (solid symbols) and 10 μ*M* (open symbols) Fe^{2+} before the reaction was started by addition of 100 μ*M* H_2O_2. Error bars represent standard ± 1 deviation; $N = 4$.

TABLE I
EC_{50} (50% Effective Concentration) Values of Hop α-Acids, Iso-α-acids, and EDTA at 5 and 10 μ*M* Fe^{2+}[a]

	EC_{50} (μ*M*)		
c(Fe^{2+})	α-Acids	Iso-α-acids	EDTA
5 μ*M*	29.0	69.9	5.6
10 μ*M*	47.2	113.0	19.6

[a] α-Acids, iso-α-acids, and EDTA (0–200 μ*M*) were mixed with ferrous iron and incubated to allow the formation of Fe^{2+}-hop acid complexes. Reactions were started by adding 100 μ*M* H_2O_2 to the incubation mixture and were carried out in an acetate buffer system at pH 5.5.

lation of oxidation at lower ratios (6,14). The protective effect of EDTA was more efficient in inhibiting 2-DR oxidative degradation than the hop acids, but its efficiency diminished with increasing iron levels (Fig. 3; Table I): doubling of the iron concentration resulted in a 250% increase in the EDTA EC_{50} value but only a 60% increase in both hop acids EC_{50} values. None of the substances tested were capable of completely inhibiting 2-DR oxidative degradation. Both effects (lower efficiencies at higher iron concentrations and the inability of the substances to completely inhibit oxidative degradation) may be explained by Fe^{2+} side reactions with the indicator molecule or the formation of other oxidants that can react with 2-DR (15,39).

The concentration dependency of Fe^{2+} on oxidative degradation of 2-DR that was preincubated with 40 and 100 μ*M* hop acids to allow the formation of Fe^{2+}-hop acid complexes is shown in Figure 4. Again, complete inhibition of 2-DR oxidative degradation was not achieved even at very low Fe^{2+} concentrations. The dosage of 100 μ*M* α-acids showed the strongest antioxidant behavior and the efficiency of α-acids did not decline until a concentration of 10 μ*M* Fe^{2+} was reached, suggesting an interaction at a ratio of 10:1 (α-acids/Fe^{2+}). After reaching 10 μ*M* Fe^{2+}, a linear decline in suppression of 2-DR oxidative degradation was observed. Increasing the Fe^{2+} concentration and preincubating with 40 μ*M* α-acids and 100 μ*M* iso-α-acids resulted in a nearly identical linear response, and the suppression efficiency of hop acids declined until 0% inhibition was reached. This suggests again that α-acids were approx. 60% more effective than iso-α-acids. The data also showed a strong inverse correlation ($P < 0.0001$) between the effectiveness of hop acids in preventing 2-DR damage and iron concentration, indicating a clear interaction between the hop acids and ferrous iron.

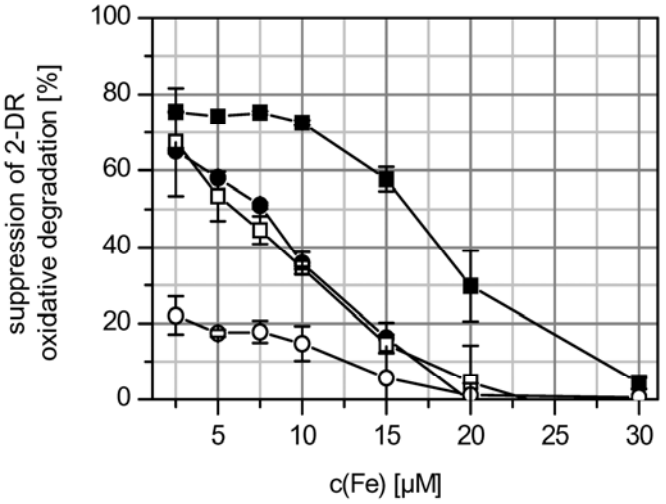

Fig. 4. Impact of Fe^{2+} concentration on suppression of 2-deoxyribose (2-DR) oxidative degradation when incubated with 40 μ*M* (□) and 100 μ*M* (■) α-acids and 40 μ*M* (○) and 100 μ*M* (●) iso-α-acids. Samples were preincubated for 10 min with Fe^{2+} before the reaction was started by addition of 100 μ*M* H_2O_2. Error bars represent standard ± 1 deviation; $N = 4$.

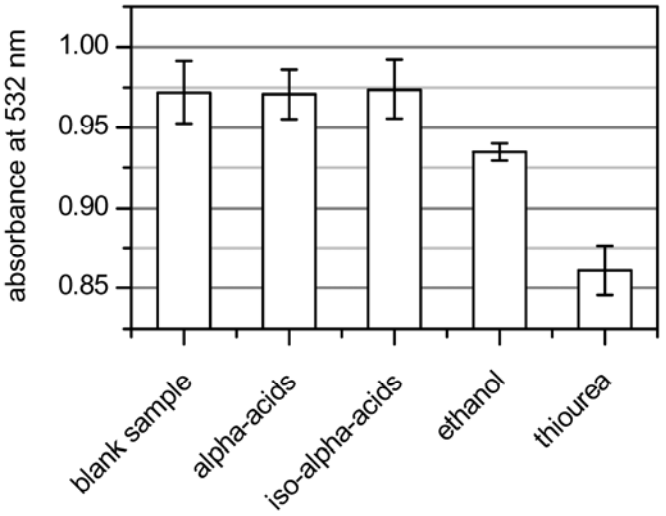

Fig. 5. Hydroxyl radical-scavenging activities of α-acids, iso-α-acids, ethanol, thiourea, and a blank. Solutions contained Fe^{3+}/EDTA (50:75 μ*M*), 100 μ*M* test sample, and 20 m*M* acetate buffer (pH 5.5). Reactions were started by addition of ascorbate (100 μ*M*) and were incubated for 1 hr at 60°C under oxygen atmosphere. Error bars represent standard ± 1 deviation; $N = 6$.

Hydroxyl Radical-scavenging Activity of α- and Iso-α-acids

As a complement to the previous experiments, the formation of hop acid-iron complexes was prevented by preincubating ferric iron with EDTA to allow the formation of EDTA-Fe^{3+} complexes. After addition of 100 μ*M* α- and iso-α-acids, the reaction was started by addition of ascorbate, which reduced the iron to its lower valence state (Fe^{2+}) and made it available for catalytic action in the Fenton reaction. Hydroxyl radicals were generated by the presence of atmospheric O_2 via the Fenton reaction during incubation at 60°C.

The trials clearly showed that the addition of hop acids to the incubation media had no effect on the oxidative damage of 2-DR. Thus, hop acids appeared to be incapable of scavenging hydroxyl radicals (Fig. 5). In contrast, the published literature indicates that α-acids are able to scavenge other radicals (e.g., DPPH•), whereas iso-α-acids are incapable of scavenging other radicals (41). Moreover, ESR experiments have shown that iso-α-acids may act as prooxidants (44).

Taken together, these results strongly suggest that the main mechanism by which both α- and iso-α-acids suppress 2-DR oxidative damage is not by trapping •OH but by chelating Fe^{2+}. There are two possible mechanisms. It is possible that they enhance the rate of Fe^{2+} autoxidation to Fe^{3+}, thereby decreasing the Fe^{2+} concentration available for the Fenton reaction, or they may form a complex with Fe^{2+} that is not accessible for H_2O_2 and, therefore, does not par-

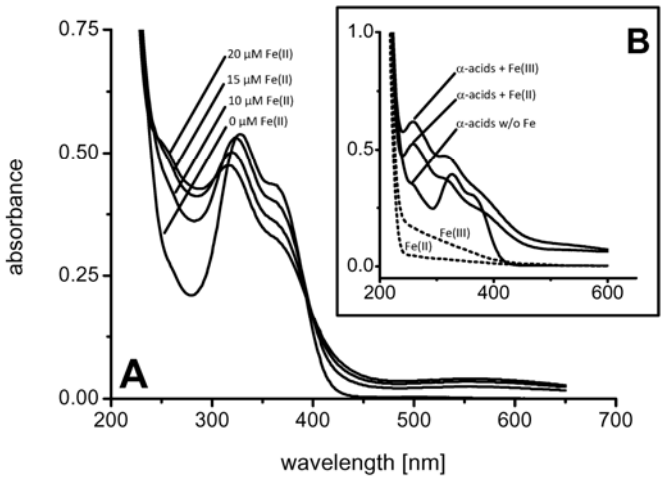

Fig. 6. A, Spectra of complexes formed between 50 μ*M* α-acids and 0, 10, 15, and 20 μ*M* Fe^{2+}. Sample solutions were preincubated for 10 min at room temperature prior to measurements and were performed in 20 m*M* acetate buffer (pH 5.5). **B,** Spectra of 30 μ*M* α-acids preincubated for 10 min at room temperature in acetate buffer (pH 5.5) with 30 μ*M* Fe^{2+} and Fe^{3+}.

ticipate in the Fenton reaction. Further trials were performed to distinguish between these two mechanisms.

Enhancement of Fe^{2+} Autoxidation by Hop α- and Iso-α-acids

Mixing 50 μ*M* hop acids individually with 0–20 μ*M* Fe^{2+} in acetate buffer (pH 5.5) and incubating the mixture for 10 min resulted in a shift and decline in the spectra of the α-acids in the range of 320–330 and 360–365 nm, and the formation of two new peaks at 260 and 560 nm was observed (Fig. 6). The spectra of the iso-α-acids showed only minor changes; their absorbance bands peaking at 255 nm declined only slightly, and a marginal increase in the range of 325–500 nm was observed (data not shown). Incubation of α-acids with 50 μ*M* Fe^{2+} resulted in nearly the same spectra as for α-acids that were incubated with 50 μ*M* Fe^{3+} (Fig. 6B). The difference in the absorbance of the spectra may be explained by the higher absorbance of Fe^{3+} compared with Fe^{2+}; both were measured as a blank in the test buffer. It is clear from Figure 6B that α-acids reacted with both oxidation states of iron. The lack of major differences between the two complexes may be explained by oxidation of Fe^{2+} to Fe^{3+} or the reduction of Fe^{3+} to Fe^{2+} induced by the α-acids.

To examine these possibilities, ferrozine was used to measure the levels of Fe^{2+} in the test solutions after preincubation with different concentrations of hop acids. Ferrozine is the disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine. This compound reacts with divalent iron to form a stable magenta complex that peaks at 562 nm and can be used for the direct determination of ferrous iron (38). Ferrozine does not form this colored complex with ferric iron. EDTA is known to enhance Fe^{2+} autoxidation to Fe^{3+} (43) and, therefore, was used as a reference.

Figure 7 shows the ability of hop acids to suppress the reaction between ferrozine and Fe^{2+}. Both hop α- and iso-α-acids decreased the level of Fe^{2+} available for the formation of the complex with increasing concentrations. Hop α-acids appeared to lower the Fe^{2+} concentration more efficiently compared with iso-α-acids. However, the activity of α-acids declined with increasing concentration, and a 30:1 (α-acids/Fe^{2+}) ratio was needed to completely inhibit the formation of ferrozine-Fe^{2+} complexes. This outcome contradicts the results of Blanco et al (7), who found that the enol tautomers of 2-acetyl-1,3-cyclohexanedione derivatives, such as α- and iso-α-acids, formed 1:1 complexes with Fe^{3+} in aqueous solutions. Compared with the hop acids, EDTA decreased the Fe^{2+} concentration most efficiently.

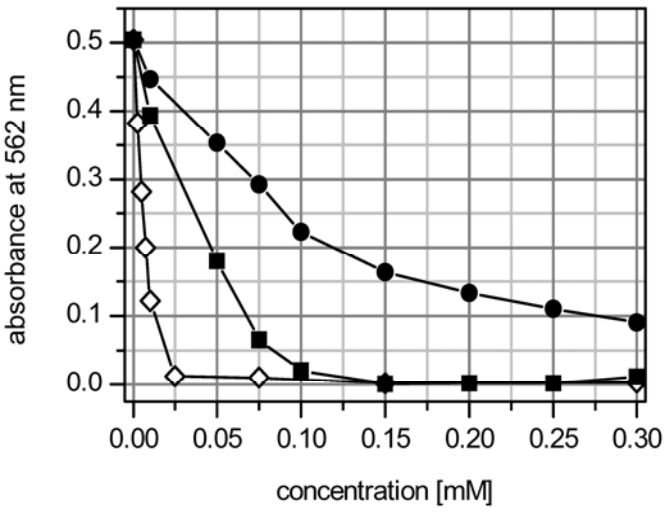

Fig. 7. Impact of α-acid (■), iso-α-acid (●), and EDTA (◇) concentrations on Fe^{2+} concentration. Samples were incubated for 10 min with 5 μ*M* Fe^{2+} before absorbance at 562 nm was measured. $N = 4$.

A correlation analysis of this set of data with the data from the 2-DR assay (Fig. 3, samples incubated with 5 μ*M* Fe^{2+}) yielded high correlation coefficients ($P < 0.0001$), which again confirmed that the antioxidant behavior of hop acids was derived from their ability to interact with Fe^{2+}. Furthermore, the data suggest that the hop acids increased the rate of Fe^{2+} autoxidation to Fe^{3+} when complexing it, because ferrozine reacts only specifically with Fe^{2+} and not with Fe^{3+}. This assumption is supported by other studies that found that oxygen ligands such as α- and iso-α-acids (7) prefer Fe^{3+} and, thus, decrease the iron's reduction potential. These chelators promote the oxidation of Fe^{2+} to Fe^{3+}, but with a potential concomitant reduction of molecular oxygen to partially reduced highly ROS (33). By enhancing Fe^{2+} autoxidation, hop acids may provoke the activation of oxygen when generating ROS, which may lead, in turn, to oxidative damage in beer. However, with the conditions used in the experiments performed in this study, we found that both α- and iso-α-acids exhibited only antioxidative behavior. This finding contradicts earlier studies using ESR spectroscopy in which we observed that iso-α-acids acted as pro-oxidants when added to wort (44). Others studies found that *trans*-iso-α-acids did not have any effect on oxidative stability when added to wort (41). Conversely, the published literature indicates that adding hops markedly diminishes the formation of staling aldehydes in bottled beer during storage (31,44). Apart from this, it should be considered that although iso-α-acids acted as antioxidants in our experiment their oxidative degradation may not only decrease beer bitterness (26) but also may yield carbonyl compounds of various chain lengths that could impact beer flavor (17). Furthermore, photo-oxidative degradation of iso-α-acids can form precursors of 3-methylbut-2-ene-1-thiol, which has a "skunky" odor that is readily detected at very low concentrations (19,20).

CONCLUSIONS

This study showed that the ability of hop acids to suppress the formation of hydroxyl radicals via the Fenton reaction was derived from their ability to interact with Fe^{2+}, thereby enhancing its autoxidation to Fe^{3+} and, thus, reducing its catalytic function. Both hop α- and iso-α-acids were incapable of scavenging highly reactive hydroxyl radicals; however, the published literature indicates that α-acids are able to scavenge other radicals (e.g., DPPH•), whereas iso-α-acids have been found to be incapable of scavenging other radicals. Although beer staling underlies more complex mechanisms, the ability of hop acids to interact with iron seems to play a major role in producing beers with greater flavor stability.

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Publication D

While the aforementioned work[276] provided evidence for the mode by which hop acids suppress radical reactions, just as they are capable of complexing iron ions, the complexation characteristics of hop acids towards other metal ions is inadequately represented in published literature. Towards the aim to deploy how hop constituents, and in particular hop α-, β-, and *iso*-α-acids, interact with different metal ions and potentially affect beer quality, the hop acids' complexation functionality towards different metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}) was investigated in buffered model solutions. Additionally, the hop acid-Fe-complex chemistry was further examined. The detailed work on this subject can be taken from the *BrewingScience* article

"Metal Chelation Behavior of Hop Acids in Buffered Model Systems"[282] (Publication D).

This work confirmed the earlier findings from publication C[276] and additionally expanded it by the knowledge that only the transition metal ions iron and copper are complexed by hop acids spectra while Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} ions remain unaffected at conditions relevant for brewing such as e.g. the pH of the solution. The pH was in fact found to remarkably influence the reaction between hop acids and iron ions. The results and the effects are discussed in detail in the published work.[282] A structure for the hop α-acid-Fe-complex could be proposed.

With regards to the focus and goal of this dissertation work, the hops' complexation activity can be considered advantageous for wort and beer quality. Undesired metal ions are removed or stabilized in a less harmful state, while other metal ions remain unaffected. This information allows a target-oriented application and modification of hop additions during beer production without having to fear negative consequences.

Wietstock, P. C., Kunz, T., Perreira, F. and Methner, F.-J.

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.

Authors

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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

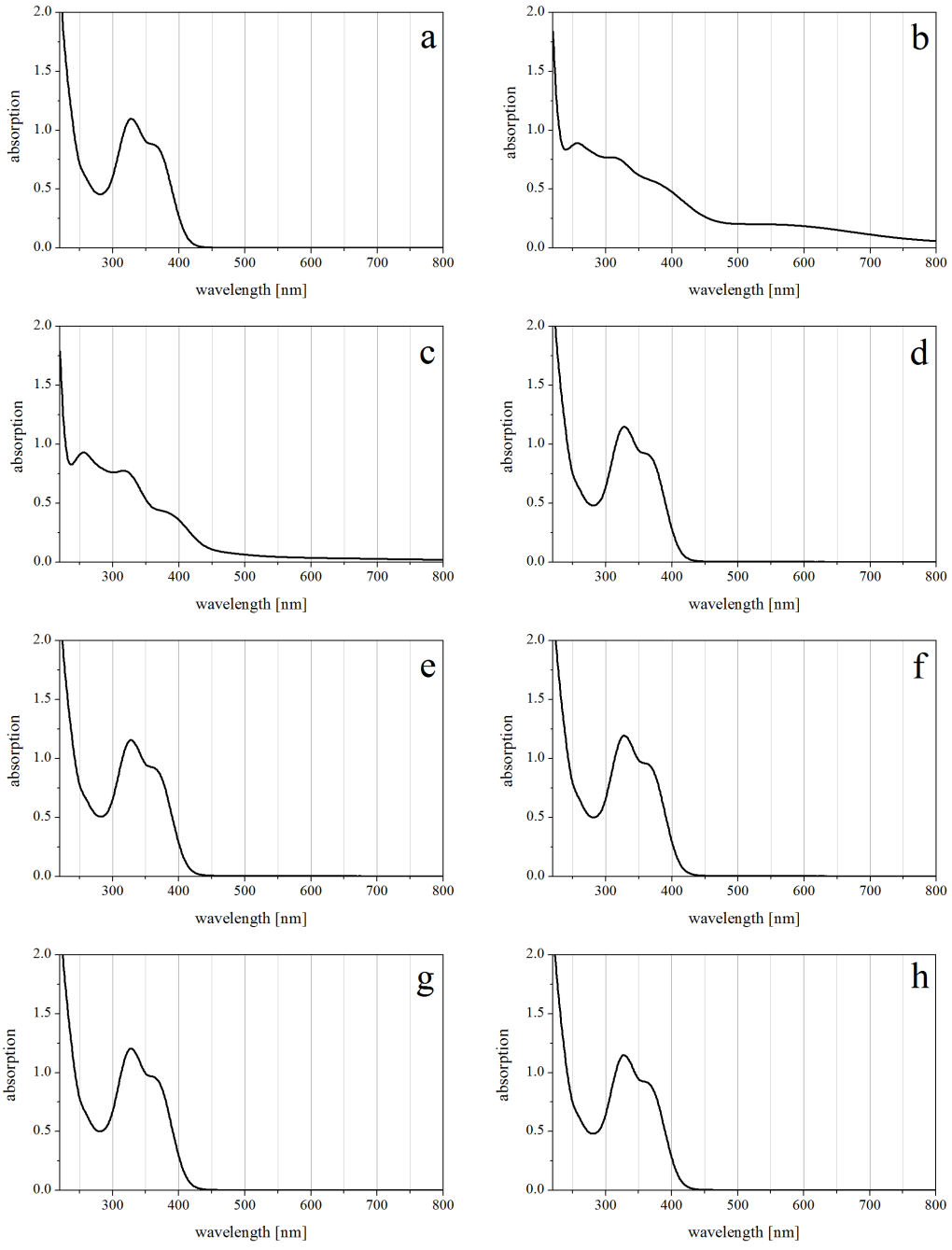

Fig. 1 **UV-VIS absorption spectra of 100 µM sole hop α-acids (a) and equimolar mixtures of hop α-acids with metal ions (100 µM each); b: α-acid-Fe^{2+}; c: α-acid-Cu^{2+}; d: α-acid-K^{+}; e: α-acid-Mn^{2+}; f: α-acid-Zn^{2+}; g: α-acid-Mg^{2+}; h: α-acid-Ca^{2+}. Substances were mixed and incubated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

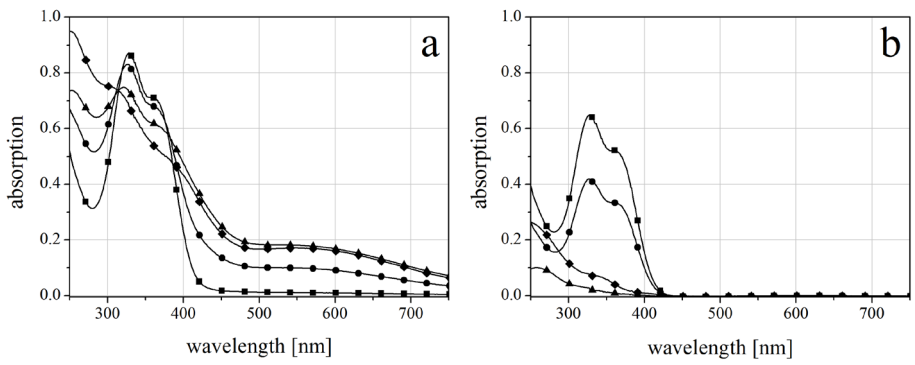

Fig. 2 UV-VIS spectra of α-acid-iron-complexes at different iron concentrations and effect of filtration. a: unfiltered sample mixtures; b: filtered sample mixtures. 60 µM α-acids were mixed with 0 µM Fe^{2+} (–■–), 30 µM Fe^{2+} (–●–), 60 µM Fe^{2+} (–◆–), and 100 µM Fe^{2+} (–▲–), incubated for 1 hour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

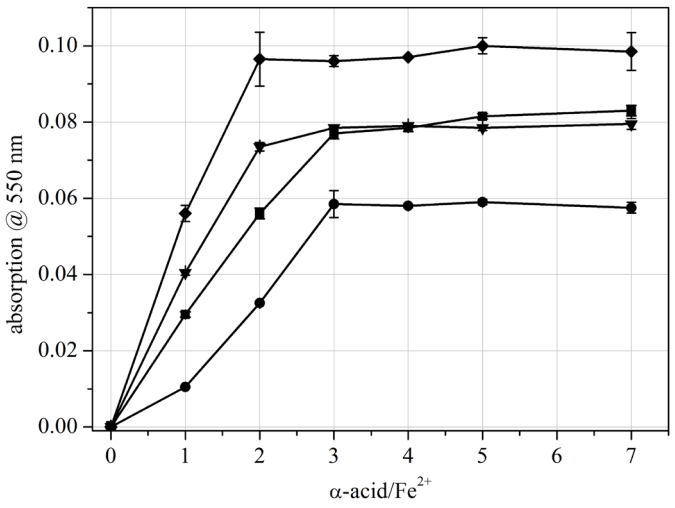

Fig. 3 Hop α-acid-iron ratio as a function of pH. Fe^{2+} (25 µM) was mixed with 0–175 µM of α-acids and incubated for 1 hour in 0.1 M sodium acetate buffer at pH 4.3 (–■–), pH 5.5 (–●–), pH 6.2 (–▼–), and at pH 8.2 (–◆–). Mean values ± 1 standard deviation of a triplicate determination are presented

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

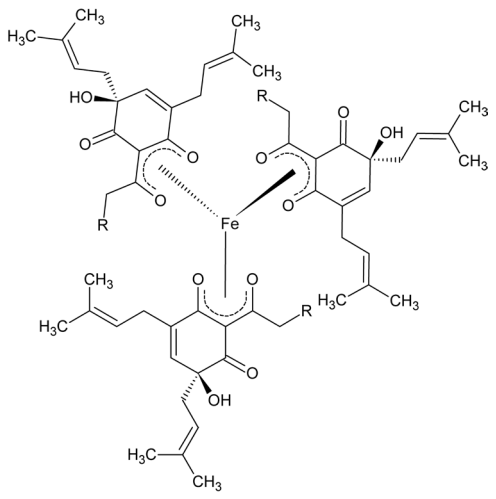

Fig. 4 Proposed schematic structure of α-acid-Fe^{3+} complexes at a ratio 3:1

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.

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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Publication E

It is evident, also from the previous work[281], that staling-related compounds are derived to some extent from *de novo* reactions during beer storage, but a major part may also be originated from an intrinsic aldehyde pool present in wort and beer which is stocked up in the course of beer production. Consequently, every production step eventually impacts the final beer quality. This clearly suggests addressing the problem of beer flavor instability early on during the brewing process such as already during mashing and wort boiling.

In order to gain knowledge about the hops' ability and mode to diminish staling compounds during wort production, experiments in wort-like model systems were conducted. For the complete work on this topic, see publication

"Hop Constituents Suppress the Formation of 3-Methylbutanal and 2-Furfural in Wort-Like Model Solutions"[273] (Publication E).

While all hop constituents tested (α-acids, *iso*-α-acids, β-acids, tetrahydro-*iso*-α-acids, or spent hop residues from the CO_2 extraction process) diminished 2-furfural formation, though to a varying degree, only α-acids and β-acids significantly diminished 3-methylbutanal formation from its parent amino acid leucine. This behavior may also be valid for other staling aldehydes.

Findings from this study expanded the current knowledge by processes during wort boiling and furthermore substantiated the strong effectiveness of the hop dosage in diminishing aldehyde formation. The directed allocation of α-acids and β-acids during wort production can therefore be regarded as a key strategy to diminish staling.

Hop Constituents Suppress the Formation of 3-Methylbutanal and 2-Furfural in Wort-Like Model Solutions

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ABSTRACT

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The ability of hop constituents to suppress the formation of 3-methylbutanal and 2-furfural in wort-like model systems containing maltose, leucine, and Fe^{2+} was investigated. From all constituents tested (α-acids, β-acids, iso-α-acids, tetrahydro-iso-α-acids, and spent hops), solely α-acids and β-acids were capable of significantly suppressing the formation of 3-methylbutanal as derived from degradation of leucine. 2-Furfural was also found in the model solutions after incubation, and its formation was diminished by all hop constituents but to a varying degree. A central composite rotatable design was employed, and from all factors tested (maltose, leucine, Fe^{2+}, and α-acids), only maltose and leucine were found to be significant model terms ($P < 0.05$) for the formation of 3-methylbutanal, whereas α-acids also showed effectiveness but at a lower confidence level ($\alpha = 0.1063$). Maltose and α-acid concentrations were significant model terms for the formation of 2-furfural. Although Fe^{2+} concentration was an insignificant factor for aldehyde formation, complexing it with ethylenediaminetetraacetic acid still inhibited aldehyde formation. When no maltose was present, 3-methylbutanal was also shown to be derived from leucine oxidative degradation by hydroxyl radical attack. Hop α-acids were demonstrated to significantly abate this particular pathway by Fe^{2+} complexation and hydroxyl radical scavenging.

Keywords: Hops, Hop α-acids, Iron, Maillard reaction, Carbonyls, Response surface methodology

Carbonyl compounds can be formed via different pathways during food processing. Their associated aromas are often described as undesirable in the finished product or can even be dangerous to health (72); however, on the contrary, the aroma and taste of many food products could not be imagined without their presence (13). In beer, carbonyl compounds, as derived from the Maillard reaction or other pathways, also play an important role in its distinct aroma and flavor. For detailed and contemporary reviews on pathways yielding carbonyl compounds during the processing and storage of beer, see Vanderhaegen et al. (75), de Schutter et al. (25), and Baert et al. (4).

Among all those pathways, the Maillard reaction has been studied intensively in the past, but it is still far from being completely understood because of its complexity. The Maillard cascade commences with a nucleophilic addition of the amino group of an amino compound to the reducing end of the open-chain conformation of a reducing sugar. In a consecutive step, the Strecker degradation or Strecker-like reaction between amino acids and α-dicarbonyls or α-unsaturated carbonyl compounds, respectively, yields the formation of Strecker aldehydes, which can produce unwanted aromas in beer (28,66,71). As related to the appearance and formation of carbonyl compounds during malt and beer production, it is clear that the nonenzymatic browning reaction or Maillard reaction occurs to the highest extent during kilning of green malt, and then later during mashing and wort boiling, because of the elevated temperatures of >50°C during these process steps (17). However, there is also evidence that the Maillard reaction occurs to some extent in the finished beer during storage (11,62). Once carbonyl compounds are formed, they are reported to exist in a bound state and/or are liberated during storage (5,6), but also de novo formation, for example, by oxidative degradation of iso-α-acids (36), melanoidin-mediated oxidation of higher alcohols (35), or free radical–initiated oxidative degradation of amino acids in bottled beer (80,81) has been suggested.

Metal salts are commonly found in foods and beverages. Even though they are mostly low in concentration, they still have a great impact because they alter the chemical reactivity or induce or catalyze certain reaction cascades. Considerable efforts were made to identify the role of particularly iron and copper ions in the so-called Fenton and Haber-Weiss reaction, and researchers agreed on their catalytic role in forming certain radicals, which are highly reactive, thus yielding decomposition or oxidation (42,43, 80,81). The metal ions' fundamental behavior in affecting certain pathways and/or the composition of the end products was also reported in connection with the Maillard reaction (48,60,63,64), which can be of use because certain Maillard reaction products are prevented (32), but which can also be unwanted because, for example, certain unfavorable reactions are accelerated (31). Undoubtedly, the reactivity of the different metal ions greatly varies (59), and monitoring and controlling their presence or absence in food systems is important for preserving quality.

With regard to beer production, carbonyl compounds as derived from, for example, the Maillard reaction products can be wanted or unwanted. They impart aroma, color, and full-bodied flavor to the final beer, in particular in dark beers (17–19,22), but also produce off-flavors, especially during storage (4,21,25,54,66,67,71, 75,76). To have a better control of the formation of aldehydes during beer production, next to practical measures such as minimizing heat load during wort production, preventing oxygen uptake during beer processing and bottling, diminishing iron and copper ion entry through raw materials, or maintaining "good" yeast management, researchers also attempted to add specific trapping agents such as aminoguanidine to beer to bind α-dicarbonyls, thereby enhancing flavor stability (11).

A great natural source for increasing beer shelf life was also found to lie in hops (46,55,57,78). Although the protective and conserving effect of hops was already known and used for centuries during beer production and transport, researchers were recently able to further elucidate the antistaling effect of hops and traced it back to the hop α- and β-acids' unique molecular structure, which makes them effective antioxidants. In a more detailed view, hop acids were reported to suppress the formation of hydroxyl radicals ($^{\bullet}OH$) by complexing iron and copper ions (catalysts in the Fenton and Haber-Weiss reaction) (79) or were claimed to act as phenoxy radicals, which can directly operate as antioxidants (74). Other than hop acids, polyphenols were also indicated as potential antioxidants (3,9,51,52), but results were partly contradictory because some polyphenols were also reported to be prooxidative or not reactive (1,41,68). Most of these studies

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were carried out in model systems to identify the mode of action by which hops exert their antioxidative potential, but hop additions were also found to remarkably diminish formation of staling aldehydes during beer storage compared with an unhopped beer (3,55,57,78). Yet, still only a little is known about the mode of action by which hops and hop constituents directly abate the formation of carbonyl compounds during wort production and beer processing.

Wort is a complex matrix consisting of many compounds derived from water, malt, and hops, and it is therefore practically impossible to examine single effects or modes of action of any substance of interest. When working in complex matrices, one approach is therefore to build empirical models by changing independent or dependent factors of interest and subsequently identifying the significant factors and anticipating pathways.

Response surface methodology (RSM) is a powerful tool for which the relationship between one or multiple responses and several variables can be explored. It was developed by Box and co-workers (10) and has been widely adopted in numerous studies since then (14,26,45,61,65). The first step in RSM is to plan a series of experiments with set factor levels and to measure the values of responses under defined conditions. These planned experiments are known as the response surface design, and they can vary based on the number of factors and the desire for geometry of the design space. Toward the objective to deploy empirical models to experimental data, linear or square polynomial functions are utilized.

Central composite rotatable designs (CCRDs) are special cases of RSM designs. For a CCRD, second-order and lower polynomials are used to describe the relationship between factors and responses. For each response, a model equation for the factors using regression analysis and analysis of variance (ANOVA) can be fit. This equation can then be used to help predict the response values for specific values of input factors and, furthermore, estimate optimized concentrations or conditions for the factors within the range of experimental design to minimize or maximize responses. For a more comprehensive overview about RSM designs and CCRD see reference 58.

In an attempt to elucidate the hops' capability and mode of action to diminish the appearance of carbonyl compounds during the process of wort production, in this work, the complex wort matrix was reduced to a phosphate buffer system at wort pH 5.5 with the presence of maltose, leucine, and ferrous iron (Fe^{2+}), all of which are present in wort. Typical experiments were then carried out at wort boiling temperatures of 100°C for 90 min and in hermetically closed bottles to prevent evaporation of potential reaction products. 2-Furfural, as derived from maltose degradation, and 3-methylbutanal, as derived from the Strecker degradation of leucine, were expected to be found in the model solutions. As a first step, the effect of hops, as related to their capability to diminish the formation of those aldehydes in the used model system, was verified, and subsequently, the effectiveness of different hop constituents was tested and compared. Based on these findings, RSM and a CCRD were used to identify the relationships and interactions between the independent factors maltose concentration, leucine concentration, hop α-acid concentration, and Fe^{2+} concentration on the dependent responses 2-furfural and 3-methylbutanal as measured by solvent-assisted flavor evaporation (SAFE) gas chromatography/mass spectrometry (GC/MS). Additional trials were subsequently conducted to further elucidate the observations. This work features new insights into how hop constituents abate the formation of carbonyl compounds during wort boiling conditions and which hop constituents are most effective. Deductions from this study therefore help to understand the mechanisms by which hops exert their protective effect and, furthermore, give indications of how to maximize the hops' effectiveness.

EXPERIMENTAL

Chemicals and Materials

Benzaldehyde, ethylenediaminetetraacetic acid (EDTA), 2-furfural, iron(II) sulfate heptahydrate, leucine, maltose, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde, and phosphate buffer salts were purchased from Sigma-Aldrich (Steinheim, Germany). Anhydrous acetonitrile, anhydrous ethanol, and diethyl ether were obtained from VWR International (Darmstadt, Germany). Disodium hydrogen phosphate dihydrate and sodium dihydrogen phosphate monohydrate were purchased from Merck (Darmstadt, Germany). H_2O_2 was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). All chemicals were of analytical grade or higher. All aqueous solutions were made with bidistilled water and prepared freshly every day. Solutions of iron(II) sulfate heptahydrate were additionally prepared with degassed water.

Hop pellets (U.S. Bravo pellets type 90, 15.2% wt/wt α-acids, crop 2013), spent hops (residues from the CO_2 extract production process, hop acids < 0.5% wt/wt), and purified hop extracts (α-acids, 89.5% wt/wt; β-acids, 60.8% wt/wt; iso-α-acids, 30% wt/wt; and tetrahydro-iso-α-acids, 10% wt/wt) were provided by Hopsteiner (Mainburg, Germany).

Thiobarbituric Acid Index

The thiobarbituric acid index (TBI) of heated mixtures was determined according to the Mitteleuropäische Brautechnische Analysenkommision (MEBAK) method (56).

Total Nitrogen and Free Amino Nitrogen Contents of Spent Hops Material

The total nitrogen content of spent hops material was determined according to the MEBAK method (56). The free amino nitrogen content of the used spent hops material was measured by preparing a hot water extract of the spent hops. Approximately 10 g of spent hops material was therefore mixed with 250 mL of phosphate buffer (0.1M, pH 5.5) and subsequently heated for 30 min at 95°C under continuous shaking in a water bath. After filtering using paper filters and cooling in a water bath to 20°C, the free amino nitrogen content of aliquots of the filtrate was measured by a modified MEBAK (56) method using a continuous flow analyzer (Skalar Analytical B.V., Breda, the Netherlands). The sample treatment and procedure were used because they reflect the same practice and best comparability as when heating the hops material during the experiments.

Quantitation of Amino Acids in Spent Hops

Amino acids in spent hops were measured by using the EZ:Faast amino acid kit (Phenomenex, Torrance, CA, U.S.A.). All reagents and materials were provided in the test kit. Briefly, the hot water extracts from the spent hops material were prepared as described earlier for the free amino nitrogen analysis. Then, the test kit procedure was followed according to the manufacturer's instructions, and 100 μL of the sample was mixed with 100 μL of internal standard (200 μM norvaline in 0.01M HCl) and then passed carefully through a solid-phase extraction (SPE) tip. The SPE material was then washed and eluted using the provided EZ:Faast reagents in two steps. After following derivatization with a reagent containing propyl chloroformate, the derivatives were further extracted by addition of a chloroform/isooctane mixture, and 0.1M HCl was added. The derivatized amino acids in the organic layer were then quantitated by GC with a flame ionization detector on an HP 5890 series II GC (Agilent, Santa Clara, CA, U.S.A.) equipped with a ZEBRON ZB-AAA column (10 m × 0.25 mm, Phenomenex). The following temperature program was used: 110°C for 1 min, ramp at 22°C/min to 320°C, and hold for 1 min. The temperature of the injection port was 250°C, and the detector temperature was 320°C.

Nitrogen was used as the carrier gas at a total flow of 25 mL/min. Sample volumes of 2 µL were injected using an HP 7673 autosampler (Agilent) in split mode (1:15, v/v).

Quantitation of Aldehydes by SAFE-GC/MS

SAFE according to the method of Engel et al. (29) and high-resolution GC (HRGC) with MS analysis were used to measure staling aldehyde concentration in test samples. An aliquot (100 mL) of test sample was passed through a folded paper filter and spiked with 1 µg of pentanal as an internal standard. The sample was extracted twice with 150 mL of diethyl ether. To remove the nonvolatile material, the unified extracts were distilled under high vacuum by means of a SAFE apparatus. The distillate was washed twice with a 0.5M Na_2CO_3 solution and water, dried over Na_2SO_4, and concentrated to 5 mL using a Vigreux column. A sample (1 µL) of this concentrated distillate was applied via a cold injection system (Gerstel, Mülheim, Germany) in 10:1 split mode to a GC (6890, Agilent Technologies, Waldbronn, Germany) fitted with a capillary column (VF-5 MS, 60 m × 0.25 mm, 0.25 µm film, Varian, Darmstadt, Germany). The following temperature program was used for HRGC: after 12 min at 35°C, the oven temperature was raised to 150°C at a rate of 12°C/min and then to 250°C at 30°C/min, and it was held at 250°C for 5 min. The flow rate of the helium carrier gas was 0.6 mL/min. The MS analysis was performed by an MSD 5973 MS (Agilent Technologies). Mass spectra in the electron impact mode were generated at 70 eV using selected ion monitoring. The retention times (t_R) and *m/z* ratios used for quantitation of aldehydes are presented in Table I.

Quantitation of Iron

Iron 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: radio-frequency power, 1,150 W; argon gas flow rates, auxiliary 0.5 L/min, nebulizer 0.5 L/min; and sample flow rate, 4.0 mL/min. The analytical wavelengths used for the determination of iron were 239.5 and 259.9 nm. A six-point calibration curve was used to quantify the test samples' concentrations. The calibration curves showed good linearity ($R^2 > 0.99$).

Statistical Evaluation

Response surface experimental design and statistical analysis were performed with Design-Expert software (version 7.0.0, Stat-Ease, Minneapolis, MN, U.S.A.). The statistical significance of the different factors and their interaction was determined with ANOVA. For difference testing, *t* test analysis was performed with XLSTAT software (version 2014.5.03, Addinsoft, Andernach, Germany).

Effect of Hop Pellet Additions on TBI and Aldehyde Formation

In a first approach, the effect of hops on the formation of carbonyl compounds in model systems was tested. Wort-like model solutions were prepared by adding 12 g/L of maltose solely or 12 g/L of maltose and 461 mg/L of leucine to a phosphate buffer (0.1M, pH 5.5). Subsequently, aliquots of 100 mL of these mixtures were incubated for 90 min at 100°C with and without the addition of 83.7 mg of hop pellets, achieving a final concentration of 127.2 mg/L of hop α-acids in the mixtures. The bottles were closed with polytetrafluoroethylene-coated silicone seals to avoid evaporation of volatiles during incubation. Hop pellets were chosen in this experiment because they represent the complex mixture of all main hop constituents contained in hops such as hop acids and the hop phenolic fraction. Directly after heating, all samples were cooled in an ice bath, and the mixtures' 3-methylbutanal and 2-furfural concentrations were quantitated by SAFE-GC/MS. Additionally, the samples' TBIs were assessed. The experiment was carried out in triplicate.

Effect of Hop Acids and Spent Hops Material on Aldehyde Formation

The effects of adding 127 mg/L of hop acids (α-acids, iso-α-acids, β-acids, and tetrahydro-iso-α-acids) or 2.54 g/L of spent hops (residues from CO_2 extraction) during heat holding were tested in the same base model solutions as used before but solely with 12 g/L of maltose and 46.1 mg/L of leucine mixed together. All hop acids were predissolved in ethanol. Stock solutions were prepared in concentrations that allowed a 1 mL addition to the beers to achieve the desired concentration. The amount of 2.54 g/L of spent hops was chosen because it represents the amount of hop phenolic material in pelletized hops when also 127 mg/L of α-acids were to be dosed and pelletized hops with 5% wt/wt α-acids were assumed to be used. Samples for which no hops were added were used as controls. In the control samples and in the samples for which spent hops were added, 1 mL of ethanol was also added. All sample mixtures were incubated for 90 min in a 100°C water bath in hermetically closed Duran bottles (Wertheim am Main, Germany). Directly after heating, all samples were cooled in an ice bath, and the mixtures' 3-methylbutanal and 2-furfural concentrations were quantitated by SAFE-GC/MS. All experiments were carried out in triplicate.

Effect of Maltose, Leucine, Fe^{2+}, and Hop α-Acids on Aldehyde Formation

RSM was employed to investigate the relationships and interactions between the independent factors maltose, leucine, Fe^{2+}, and hop α-acids on the dependent responses 2-furfural and 3-methylbutanal. The experimental design used was a CCRD. The design matrix was a factorial three-level, four-factor design with six central points to calculate the pure error and eight axial points at the extreme levels ($\alpha = 1.414$), thus allowing rotatability of the system. The concentrations of each component were coded at five levels ($-\alpha$, −1, 0, 1, $+\alpha$), and the levels used were selected to cover a range of concentrations that were to be found during production of a pale lager beer (Table II) (2). The complete RSM experimental design consisted of 30 experimental points and is provided in Table III. Base solutions were prepared at levels of 0–200 g/L of maltose in 0.1M phosphate buffer at pH 5.5. Then, hop α-acids (predissolved in ethanol) and leucine and iron(II) sulfate

TABLE I
Retention Times (t_R) and *m/z* Ratios for Quantitation of Aldehydes by Solvent-Assisted Flavor Evaporation GC/MS

Substance	t_R (min)	*m/z*
3-Methylbutanal	10.15	58
2-Methylbutanal	10.63	58
Pentanal (internal standard)	12.96	58
2-Furfural	19.05	96
Benzaldehyde	23.03	106
Phenylacetaldehyde	24.79	91

TABLE II
Concentrations and Levels of the Different Variables Used in the Central Composite Rotatable Design

Variables	$-\alpha$	−1	0	1	$+\alpha$
Leucine (mg/L)	0	12.5	25.0	37.5	50.0
Hop α-acids (mg/L)	0	37.5	75.0	112.5	150.0
Maltose (g/L)	0	50.0	100.0	150.0	200.0
Fe^{2+} (µg/L)	0	250.0	500.0	750.0	1,000.0

heptahydrate (both predissolved in bidistilled water) were added such that adding 1 mL to the maltose mixture achieved the target concentration. All final sample mixtures were then incubated in hermetically closed Duran bottles for 90 min at 100°C in a water bath. Directly after heating, all samples were cooled in an ice bath, and the mixtures' 3-methylbutanal and 2-furfural concentrations were quantitated by SAFE-GC/MS.

In additional trials, the influence of Fe^{2+} on the formation of carbonyl compounds was reassessed by incubating mixtures of 12 g/L of maltose and 25 mg/L of leucine (center point) with 500 µg/L of Fe^{2+} or 10 mg/L of EDTA, individually. The sample preparation and treatment were the same as described before, and samples were heated in hermetically closed bottles for 90 min at 100°C. A sample without Fe^{2+} or EDTA added served as a reference. The concentrations of 3-methylbutanal and 2-furfural were quantitated by SAFE-GC/MS after heat exposure and cooling down.

Formation of 3-Methylbutanal by Leucine Oxidative Degradation

In the absence of sugar-derived α-dicarbonyls, 3-methylbutanal may also be formed by oxidative degradation of leucine through hydroxyl radical attack (80, 81) or by thermally induced oxidative decarboxylation of amino acid–iron complexes (59). To test the possibility and relevance of these pathways for 3-methylbutanal formation in model systems, 100 mg/L (0.762 mM) of leucine was heated to 100°C in the presence of 558.5 µg/L (10 µM) or 5,585.0 µg/L (100 µM) of Fe^{2+}, and this temperature was kept for 90 min. Additionally, reference samples were prepared without Fe^{2+} or with 29.214 mg/L (100 µM) of EDTA added. The addition of EDTA was intended to complex any residual metal ions in the solution. The trials were conducted in hermetically closed bottles. The buffer system used was the same as described earlier. After cooling down, the concentration of 3-methylbutanal from model mixtures was quantitated by SAFE-GC/MS. All concentrations given are final concentrations in the sample mixtures. All experiments were carried out in triplicate.

Suppression of Leucine Oxidative Degradation by Hop α-Acids

Two modes of action by which hop α-acids suppress leucine oxidative degradation that yields 3-methylbutanal are conceivable: complexation of iron ions, thus preventing them from partaking in oxidation reactions, and scavenging of hydroxyl radicals (78). To further elucidate the hop α-acids' efficacy and discriminate between both pathways, two sets of trials were conducted. Because hop α-acids isomerize to iso-α-acids when exposed to heat, as opposed to the previous trials, samples were incubated at 20°C, and the oxidative chain reaction ultimately yielding 3-methylbutanal was started by addition of H_2O_2.

In a first set of experiments, suppression of 3-methylbutanal formation by Fe^{2+} complexation was tested by allowing complex formation between hop α-acids and Fe^{2+}. Fe^{2+} (100 µM) was therefore mixed with 300 µM hop α-acids in buffered model systems (0.02M phosphate buffer, pH 5.5, 10% v/v acetonitrile) already containing 0.762 mM leucine, and it was subsequently incubated for 1 h at 20°C to allow complex formation. Reactions were then started by addition of 5 mM H_2O_2 to the model mixtures and incubating for 24 h at 20°C; they were stopped by rapidly cooling the mixtures to 0°C in an ice bath. Aldehydes were determined by SAFE-GC/MS on the same day.

To examine the possibility that hop α-acids block or inhibit leucine oxidative degradation and consecutive 3-methylbutanal formation by hydroxyl scavenging, complex formation between hop α-acids and Fe^{2+} was prevented by complexing Fe^{2+} with EDTA prior to adding hop α-acids. Hydroxyl radicals can still be generated from this EDTA-Fe^{2+} complex, although further complexation of Fe^{2+} is prevented (34). Fe^{2+} (100 µM) was therefore preincubated with 104 µM EDTA for 15 min at 20°C to allow the formation of EDTA-Fe^{2+} complexes. After preincubation, test mixtures containing 0.762 mM leucine and 300 µM hop α-acids were incubated again for 1 h at 20°C, and 5 mM H_2O_2 was added to start the reactions. Sample mixtures were subsequently allowed to react for 24 h at 20°C and were stopped and measured as described in the previous trial. All concentrations given are final

TABLE III
Response Surface Design with Central Composite Rotatable Design (CCRD) Points, Sequence Numbers, and Concentrations[a]

CCRD point	Run sequence	Leucine (mg/L)	α-Acids (mg/L)	Maltose (g/L)	Fe^{2+} (µg/L)
Central point	3,6,14,19, 22, 24	(0) 25.0	(0) 75.0	(0) 100.0	(0) 500.0
Factorial point	1	(–1) 12.5	(–1) 37.5	(+1) 150.0	(–1) 250.0
Factorial point	2	(+1) 37.5	(–1) 37.5	(–1) 500.0	(–1) 250.0
Factorial point	4	(–1) 12.5	(–1) 37.5	(–1) 500.0	(+1) 750.0
Factorial point	5	(–1) 12.5	(+1) 112.5	(–1) 500.0	(–1) 250.0
Factorial point	7	(+1) 37.5	(+1) 112.5	(+1) 150.0	(–1) 250.0
Factorial point	8	(+1) 37.5	(–1) 37.5	(+1) 150.0	(+1) 750.0
Factorial point	9	(+1) 37.5	(+1) 112.5	(–1) 50.0	(+1) 750.0
Factorial point	10	(–1) 12.5	(+1) 112.5	(+1) 150.0	(+1) 750.0
Factorial point	11	(–1) 12.5	(+1) 112.5	(–1) 50.0	(+1) 750.0
Factorial point	12	(–1) 12.5	(–1) 37.5	(–1) 50.0	(–1) 250.0
Factorial point	13	(+1) 37.5	(–1) 37.5	(+1) 150.0	(–1) 250.0
Factorial point	15	(+1) 37.5	(+1) 112.5	(+1) 150.0	(+1) 750.0
Factorial point	16	(+1) 37.5	(–1) 37.5	(–1) 50.0	(+1) 750.0
Factorial point	17	(–1) 12.5	(–1) 37.5	(+1) 150.0	(+1) 750.0
Factorial point	18	(+1) 37.5	(+1) 112.5	(–1) 50.0	(–1) 250.0
Factorial point	20	(–1) 12.5	(+1) 112.5	(+1) 150.0	(–1) 250.0
Axial point	21	(0) 25.0	(0) 75.0	(–α) 0.0	(0) 500.0
Axial point	23	(0) 25.0	(0) 75.0	(+α) 200.0	(0) 500.0
Axial point	25	(0) 25.0	(–α) 0.0	(0) 100.0	(0) 500.0
Axial point	26	(+α) 50.0	(0) 75.0	(0) 100.0	(0) 500.0
Axial point	27	(0) 25.0	(+α) 150.0	(0) 100.0	(0) 500.0
Axial point	28	(0) 25.0	(0) 75.0	(0) 100.0	(+α) 1,000.0
Axial point	29	(–α) 0.0	(0) 75.0	(0) 100.0	(0) 500.0
Axial point	30	(0) 25.0	(0) 75.0	(0) 100.0	(–α) 0.0

[a] The numbers in parentheses represent the coded levels (–1, 0, +1, +α, and –α). The α values represent the extreme values at α = 1.414, thus allowing rotatability of the system.

concentrations in the sample mixtures. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

To study the effect of hops on aldehyde formation, the complex wort matrix was reduced to the presence of one sugar, one amino acid, and phosphate salts, all of which are present in wort (12). Even though pentoses are more effective in the Maillard reaction than hexoses (49), maltose was chosen as the sugar because it represents the carbohydrate most abundant during wort boiling (12). Leucine was chosen as the amino compound. The Strecker aldehyde from leucine, 3-methylbutanal, is well known for its significant odor (7) and is claimed to be a key aroma compound of many foods and raw materials (8,27,33,54,66,67), which made it suitable for its usage in this study. At later stages of this study, in addition to hop constituents, Fe^{2+} was incorporated in the experiments because this metal ion is known to promote the Maillard reaction (39), to trigger oxidation reactions (43), and was also reported to interact with hop acids (79).

Effect of Addition of Hop Pellets or Different Hop Constituents on Aldehyde Formation

The effect of adding hop pellets type 90 to model buffer solutions containing maltose only or maltose and leucine was tested first to identify their capability to abate the formation of respective carbonyl compounds as derived from the Maillard reaction. The TBI is used as an indicator for thermal load of wort or beer (24). Thiobarbituric acid reacts with carbonyl compounds to form a colorant that can be measured photometrically. A high TBI therefore indicates that more carbonyl compounds are present.

As observable in Table IV, incubating samples with solely maltose or maltose and leucine resulted in similar TBIs. Adding hop pellets to these mixtures appeared to diminish the TBI number by 14.5–16.6%, indicating that lower amounts of carbonyl compounds were formed. Because the TBI is a rather unspecific method, the carbonyl compounds were additionally determined using SAFE-GC/MS.

From solutions in which only maltose was heated, 2-furfural was identified as the prevalent peak at an amount of 125.0 ± 22.7 µg/L (Table IV). 2-Furfural is mainly derived from pentose degradation (20,40) but was recently also found to be formed in an oxidative pathway of the Maillard reaction from hexoses (62), which can explain the observation made during this experiment. When maltose and leucine were heated together, in addition to 2-furfural, also 3-methylbutanal, the Strecker aldehyde from its parent amino acid leucine, was found in the sample mixtures. Even though maltose was present in approximately 1,000 times higher concentrations than leucine in the initial solutions, the model mixtures still contained about three times higher concentrations of 3-methylbutanal than of 2-furfural after incubation. The comparatively lower levels of 2-furfural compared with 3-methylbutanal may be explained by the incapability of maltose to react in the Maillard reaction when present in its acetal form. The reducing open ring form (aldehyde) is only very low at these reaction conditions (50). D-Glucose was, for example, found to be present in the aldehyde form at only 0.004% in aqueous solutions at 30°C (82).

Adding hop pellets type 90 appeared to abate these reactions and yielded a reduction of 51.6 ± 4.0 µg/L (41.3%) of 2-furfural when incubated together with maltose only, and 98 ± 39.1 µg/L (24.6%) and 41.2 ± 4.3 µg/L (37.3%) of 3-methylbutanal and 2-furfural, respectively, when incubated with solutions of maltose and leucine (Table IV). This confirms findings from references 3, 55, 57, and 78, which were carried out during actual production of beers, and thus further imparts that the used model buffer system was applicable for investigating the effectiveness and principle by which hops attenuate the reactions that yield carbonyls during wort and beer production.

Next, the effects of single hop constituents were tested individually. Hop α-acids, β-acids, iso-α-acids, tetrahydro-iso-α-acids, or 2.54 g/L of spent hops (residues from CO_2 extraction) were added to mixtures already containing 120 g/L of maltose and 46.1 mg/L of leucine and were incubated. Spent hops were included because they represent the polar fraction of the hop plant, including, for example, a multiplicity of hop polyphenols.

From all hop constituents tested, solely α-acids and β-acids were capable of significantly suppressing the formation of 3-methylbutanal (Fig. 1). 2-Furfural was also found in the model solutions after incubation, and its formation was significantly diminished

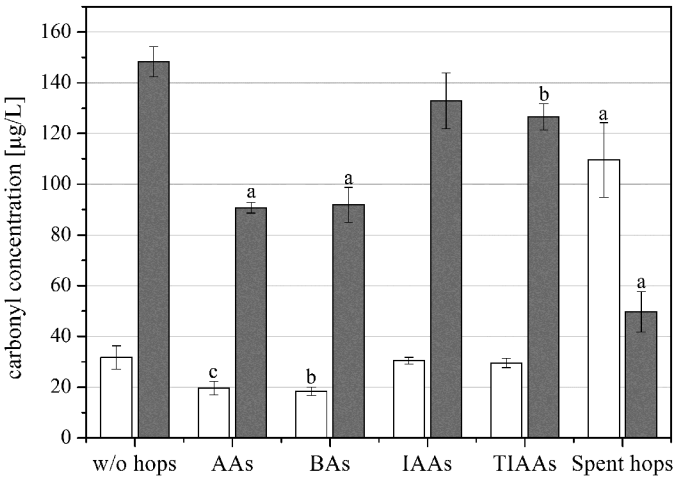

Fig. 1. 3-Methylbutanal (white bars) and 2-furfural (gray bars) in model mixtures containing 120 g/L of maltose and 46.1 mg/L of leucine with and without (w/o) the addition of 127 mg/L of hop α-acids (AA), β-acids (BA), iso-α-acids (IAA), tetrahydro-iso-α-acids (TIAA), or 2.54 g/L of spent hops. Mean values are presented. Error bars represent ±1 standard deviation of a triplicate experiment. Different letters above bars indicate significant difference of mean percentages between samples and the reference (without hops) by a Student's *t* test at the 99.9% confidence level (a), 99% confidence level (b), and 95% confidence level (c).

TABLE IV
Thiobarbituric Acid Index (TBI) Numbers as Well as 3-Methylbutanal and 2-Furfural Concentrations in Model Solutions Containing Maltose Solely, Maltose and Hop Pellets, Maltose and Leucine, or Maltose, Leucine, and Hop Pellets[a]

Parameter	Maltose only	Maltose + hop pellets	Maltose + leucine	Maltose + leucine + hop pellets
TBI	10.4 ± 0.2	8.7 ± 0.3	10.5 ± 0.1	9.0 ± 0.1
3-Methylbutanal (µg/L)	<1	<1	397.7 ± 53.0	299.7 ± 39.1[b]
2-Furfural (µg/L)	125.0 ± 22.7	73.4 ± 4.0[b]	110.5 ± 7.4	69.3 ± 4.3[b]

[a] Sample mixtures were incubated for 90 min at 100°C. Mean values ± standard deviations are presented. $N = 3$.
[b] Significant difference of mean percentages between maltose only and maltose + hop pellets, as well as between maltose + leucine and maltose + leucine + hop pellets, by a Student's *t* test at the 95% confidence level.

by all hop constituents but to a varying degree. Spent hops were, for example, able to abate 2-furfural formation by 66.5 ± 16.1%, whereas iso-α-acids and tetrahydro-iso-α-acids only diminished its formation by 10.5 ± 8.3 and 14.7 ± 4.1%, respectively. α-Acids and β-acids ranged in between iso-α-acids, tetrahydro-iso-α-acids, and spent hops as, respectively, 38.9 ± 2.3 and 38.0 ± 7.4% less 2-furfural was detected in the mixtures after heat exposure. Remarkably, addition of spent hops resulted in high amounts of 3-methylbutanal and an increase of 245.7 ± 13.4% compared with the model mixture to which no hops were added (reference), and also other aldehydes were present (2-methylbutanal, 16.8 ± 1.2 μg/L; phenylacetaldehyde, 3.1 ± 0.6 μg/L; benzaldehyde, 3.2 ± 0.2 μg/L; and octanal, 2.1 ± 0.3 μg/L). The spent hops starting material was therefore tested for the presence of those aldehydes. It was accordingly extracted with diethyl ether: the extracts were purified by SAFE, and aldehydes were quantitated by GC/MS. Minimal amounts of aldehydes, as also found in the model solutions after incubation, were detected in the spent hops prior to incubation but not in such amounts that it could potentially result in the concentrations found in the model mixtures (data not shown).

It was therefore suspected that potential precursors such as, for example, leucine, isoleucine, or phenylalanine entered the solutions by adding the spent hops to the model mixtures, thus yielding the corresponding aldehydes during heating. The total nitrogen content of the spent hops material and free amino nitrogen content of a hot water extract made from the spent hops material were therefore determined and showed a total nitrogen content of 4.70 ± 0.02 g/100 g in dry matter (d.m.) spent hops, and a free amino nitrogen content of 475.4 ± 2.8 mg/100 g of spent hops (d.m.). Leucine, isoleucine, and phenylalanine were detected in the sample mixtures at concentrations of 11.4, 7.6, and 11.7 mg/L, respectively, among other amino acids such as alanine (96.6 mg/L), glycine (9.3 mg/L), valine (16.8 mg/L), threonine (20.8 mg/L), proline (45.6 mg/L), and glutamine (42.4 mg/L). This confirms the initial assumption that precursor substances were present when spent hops material was added to the model mixtures, and it may therefore explain the higher amounts of the aforementioned aldehydes detected after incubation. The observation that amino compounds were brought in by adding the spent hops material to the model mixtures may also serve as an explanation of why the furfural levels were remarkably lowered compared with adding hop α- and β-acids. 2-Furfural can be derived from oxidation of maltose to maltosone, formation of 1,2-enediol, and cleavage yielding 3-deoxypentosone, which then cyclizes and is dehydrated to give 2-furural (62). Because amino compounds, as brought in by the addition of the spent hops, are potential reaction partners of the intermediates from 2-furfural formation via nucleophilic attack, the formation of 2-furfural is assumed to be diminished.

Factors Affecting Aldehyde Formation in Model Systems Studied by RSM

Because hop α-acids were clearly effective in significantly diminishing the formation of both 2-furfural and 3-methylbutanal, and because of their importance for beer production and their high abundance during wort boiling, hop α-acids were chosen as the hop constituent for the response surface experimental design. Maltose, leucine, Fe^{2+}, and hop α-acids were tested in ranges that are commonly found during wort production for pale beers.

The statistical significance of the different factors and their interaction was determined with ANOVA. For detailed calculations of the model data, please consult reference 58. As the first step, a backward elimination was applied to the data. During this procedure, the full model was reduced step-wise by the model terms with the highest partial probability (*P* value). The regression procedure stopped when a term with a criterion of $\alpha = 0.1$ was reached. According to this principle of hierarchy, nonsignificant terms were kept in the model if they were contained in other interaction terms that were found to be significant. This procedure is considered to be robust because all model terms will be given a chance to be included in the model (16,58). During this step, the factor "Fe^{2+} concentration" was removed from both models, 3-methylbutanal and 2-furfural, because it was found to be insignificant at $\alpha = 0.1$ for 3-methylbutanal and 2-furfural formation under these experimental conditions. When processing the 2-furfural response, leucine concentration was additionally eliminated from the model at $\alpha = 0.1$. For all remaining independent variables and both dependent responses, 3-methylbutanal and 2-furfural, linear models explained the experimental data best with significant ($P < 0.05$) model *F* values of 56.94 and 93.94 for 3-methylbutanal and 2-furfural, respectively. Furthermore, all models had insignificant lack of fit at the 95% confidence level relative to the pure error, which signified that the variation in the model points did not significantly differ from the variation of the replicated points.

The coefficient of determination (R^2) was 0.8768 for 3-methylbutanal, which implied that the sample variation of 87.68% for 3-methylbutanal formation was attributed to the significant independent variables maltose concentration, leucine concentration, and hop α-acids, and only 12.32% of the total variation was not explained by the model. The 2-furfural model exhibited an R^2 of 0.8826, thus suggesting that 88.26% of the 2-furfural formation could be ascribed to the significant independent factors maltose concentration and hop α-acid concentration, and only 11.74% of the data's total variation was not explained by the model (Table V). Adjusted R^2 corrects R^2 for the sample size and number of terms in the model and should be close to the R^2 that was given for both models (58). The predicted R^2 (amount of variation in predicted model) of 0.7884 for 3-methylbutanal formation and 0.8208 for 2-furfural formation was in reasonable agreement with the adjusted R^2 of 0.8614 and 0.8732, respectively.

Adequate precision compares the range of the predicted values at the design points to the average prediction error. Ratios greater than 4 indicate adequate model discrimination (58). The adequate precision yielded values of 22.511 and 25.341 for 3-methylbutanal and 2-furfural, respectively, indicating adequate signals.

All these data taken together demonstrate a satisfactory adjustment of both linear models to the experimental data and therefore point to the applicability of the models to be used to study the factors in the model system.

Next, the *F* values of the factors in the models were determined. The factor *F* values indicate how large the factor variability is relative to the variability of the group means. If the variances are close to the same, the ratio will be close to 1, and it is less likely that any of the factors have a significant effect on aldehyde concentration (58). The factors maltose and leucine concentration were significant model terms ($P < 0.05$) for 3-methylbutanal concentration, with the highest *F* values of 15.99 and 152.02, respectively (Table VI). Hop α-acid concentration exhibited an *F* value of 2.82 and was calculated to be insignificant at the 95% confidence interval. However, the hop α-acids' *P* value of 0.1063

TABLE V
ANOVA Model Data for Response Surface Reduced Linear Models

Values	3-Methylbutanal	2-Furfural
R^2	0.8768	0.8826
Adjusted R^2	0.8614	0.8732
Predicted R^2	0.7884	0.8208
Adequate precision	22.511	25.341
Model *F* value	56.94	93.94
Lack of fit *F* value	5.27	2.02

still implied effectiveness to some degree, although at a lower confidence level. Because of that and because hop constituents were reported to abate the formation of 3-methylbutanal during actual beer production (55,57,78), it was decided to keep this variable in the model. For 2-furfural concentration, maltose and hop α-acid concentration were both significant model terms with *F* values of 160.54 and 27.34, respectively.

Under the experimental conditions used, and in this particular model system, the formation of 3-methylbutanal (equation 1) and 2-furfural (equation 2) was found to be best predicted by the following models:

$$\text{3-met} = -5.3520 + 1.3471 \cdot A - 0.0611 \cdot B + 1.0923 \cdot C \quad (1)$$

$$\text{2-fur} = 351.86 - 54.61 \cdot B + 132.33 \cdot C \quad (2)$$

where 3-met is 3-methylbutanal concentration (μg/L), 2-fur is 2-furfural concentration (μg/L), *A* is leucine concentration (mg/L), *B* is hop α-acid concentration (mg/L), and *C* is maltose concentration (g/L).

For graphical visualization, the three-dimensional response surface curves were plotted against the most significant model terms, which were maltose and leucine concentration in the case of 3-methylbutanal, and maltose and hop α-acid concentration in the case of 2-furfural. As shown in Figure 2, leucine concentration had clearly the highest effect on 3-methylbutanal concentration in the mixtures after incubation, as also implied by its model *F* value (Table VI). A high maltose concentration and a high leucine concentration yielded the highest 3-methylbutanal concentration. Increasing maltose concentrations had obviously the highest effect on 2-furfural formation, whereas higher dosages of hop α-acids resulted in a clear decrease of 2-furfural (Fig. 3).

Altogether, these results indicate that the Maillard reaction is the main pathway for the formation of 3-methylbutanal and 2-furfural under these experimental conditions. Hop α-acids seem to abate these reactions by as yet unexplored modes of action. All trials implicated an insignificance of Fe^{2+} on the formation of those aldehydes under these experimental conditions; however, it was reported to affect hydroxymethylfurfural formation (30).

In wort production, sugars, amino acids, and hop constituents are supposed to be always present in an excess compared with the levels of iron (2). The relative levels of iron are infinitely low, and it may thus be possible that the outcome of the RSM underestimated the impact of the transition metal. To reassess the iron's influence, excess EDTA, a good iron chelator, was used, and its function in the model system at 12 g/L of maltose and 25 mg/L of leucine (reference) was tested. Additionally, an experiment with 500 μg/L of Fe^{2+} added to the model mixtures was conducted.

No significant difference at the 95% confidence level was found between the sample for which only 12 g/L of maltose and 25 mg/L of leucine were added (reference; 3-methylbutanal, 22.3 ± 0.5 μg/L; 2-furfural, 189.1 ± 14.5 μg/L) and the sample for which 500 μg/L of Fe^{2+} was added (3-methylbutanal, 22.1 ± 1.8 μg/L; 2-furfural, 216.0 ± 10.6 μg/L). The model solution for which iron was complexed with EDTA showed significantly ($P < 0.05$) lower amounts and a reduction of the formation of 3-methylbutanal and 2-furfural by 7.9 ± 0.5 μg/L (35.5%) and 90.1 ± 1.0 μg/L (48.7%), respectively, compared with the reference sample. This observation implies that the complexation of Fe^{2+} by EDTA in fact attenuated aldehyde formation. Because of the observation that complexing Fe^{2+} by EDTA yielded in fact a diminished aldehyde formation, it was suspected that there were already minimal

TABLE VI
ANOVA Data for the Dependent Significant Factors of the Response Surface Reduced Linear Models[a]

	3-Methylbutanal		2-Furfural	
Factor	***F* value**	***P* value**	***F* value**	***P* value**
Maltose	15.99	<0.0001	160.54	<0.0001
Leucine	152.02	<0.0001	…	…
Hop α-acids	2.82	0.1063[b]	27.34	<0.0001

[a] *P* value is probability of the *F* value. It is the probability of getting an *F* value of this size if the term did not have an effect on the response. Insignificant model terms were eliminated by backward selection at α = 0.1.

[b] The factor hop α-acid concentration was kept in the model even though it slightly exceeded the confidence level of α = 0.1. Explanations are given in the text.

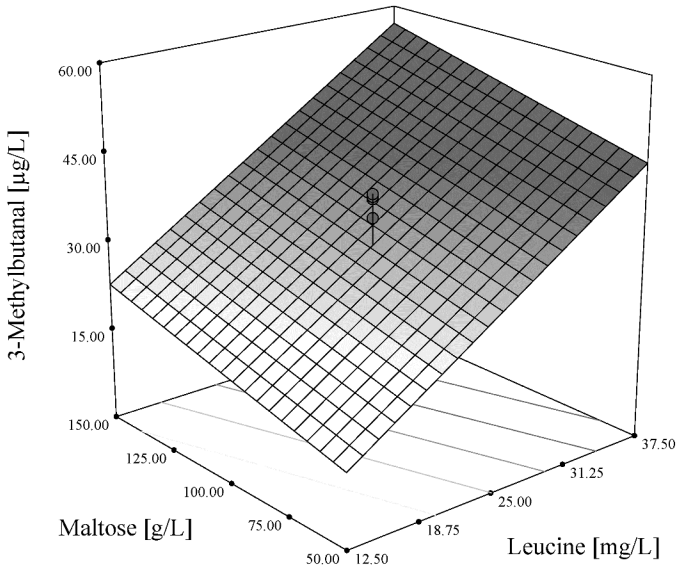

Fig. 2. Three-dimensional response surface diagram for the response 3-methylbutanal and the effect of the independent factors maltose concentration and leucine concentration. The factor Fe^{2+} concentration was removed from the model by backward elimination at α = 0.1, and hop α-acid concentration was kept at its center level (75 mg/L).

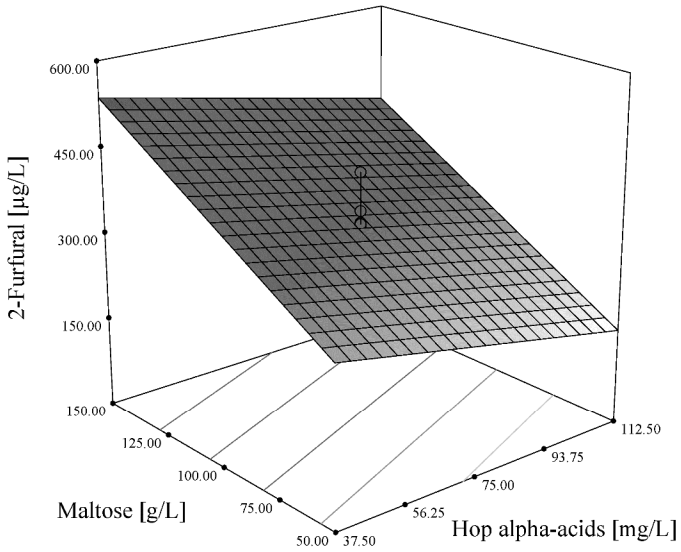

Fig. 3. Three-dimensional response surface diagram for the response furfural and the effect of the independent factors hop α-acid concentration and maltose concentration. The factors Fe^{2+} concentration and leucine concentration were removed from the model by backward elimination at α = 0.1.

amounts of iron present in the model system, leading to the formation of both 3-methylbutanal and 2-furfural. Testing all possible sources for iron contamination with ICP-OES indeed revealed an influx of 30 µg/L of iron through the phosphate buffer salts used for the trials, which may additionally explain the insignificance of Fe^{2+} in the RSM experiments.

Taking these data together, it is likely that Fe^{2+} indeed affects the reaction, although only small amounts of Fe^{2+} are most probably sufficient to promote all possible reactions so that the presence of additional Fe^{2+} did not result in significant changes. This is also in agreement with literature data in which transition metals such as iron were reported to affect certain oxidative pathways of the Maillard reaction and their products (30,39,48). Iron can easily transition between its two valence states (Fe^{2+} and Fe^{3+}) and, for example, can be reduced from Fe^{3+} to Fe^{2+} by certain intermediates of the Maillard reaction with reductone moieties, such as enediols (47). Compared with the levels of iron, such reducing substances can be anticipated as being present in high excess compared with iron, and moreover, Fe^{3+} reduction is anticipated to proceed rapidly (47). It is therefore likely that iron is predominately present as Fe^{2+}, which then again promotes the Maillard reaction as reported (30,37,48). Compared with the RSM experiments in which addition of Fe^{2+} was found to be insignificant for the formation of 3-methylbutanal and 2-furfural, the effectiveness of EDTA in suppressing aldehyde formation can be explained by its ability to complex transition metal ions, thus withdrawing them entirely from potential reaction partners. Hence, it prevents Fe^{2+} from interacting with the Maillard reaction and concomitantly prevents Fe^{3+} from recycling to Fe^{2+}. In consequence, consecutive reactions are diminished, which may serve as a reasonable explanation for the observations. The role and behavior of iron ions in the model systems used is in reasonable agreement with literature data (38,41,42,53,80).

Oxidative Degradation of Leucine by Fenton Reagents and Effect of Hop α-Acids

Amino acids are usually stable and remain intact for long periods even at high temperatures when no catalytic action of a decarboxylation enzyme or an oxidizing agent is involved (70). Only a little is known about a direct degradation of amino acids in a Strecker-like pathway without α-dicarbonyls or reactive carbonyl compounds being present, though. Wietstock and Methner (81) reported that Strecker aldehydes can be derived from amino acid oxidative degradation by hydroxyl and hydroxyethyl radical attack. This pathway was recently verified to be of relevance for beer aging and during wort production (80). For the pathway and more detailed information, see references 81 and 80. Additionally, Nashalian and Yaylayan (59) reported that aldehydes can be formed by transition metal–assisted thermal decarboxylation. Instead of catalyzing the formation of hydroxyl radicals that then yield the decomposition of amino acids as proposed by Wietstock and coworkers (80,81), Nashalian and Yaylayan (59) suggested that the metal ion forms a complex with the amino acid itself, which subsequently undergoes thermal decomposition through an intramolecular redox reaction, thereby forming a metal-ion-centered radical cation and a carboxyl amine radical. Both products are then converted into further intermediates, ultimately yielding a Strecker aldehyde. To discriminate between both (transition metal–assisted thermal decarboxylation and hydroxyl radical–initiated oxidative degradation of leucine) and, additionally, to elucidate the efficacy of hop α-acids in potentially suppressing these pathways, further experiments were conducted. Chelate complexes of EDTA and Fe^{2+} can be used to study the role and reactions of hydroxyl radicals in systems, without having complex formation of Fe^{2+} interfering, because it is firmly bound in the complex (34,79). The stoichiometry in this complex has a great influence on the effectiveness of radical generation, and high EDTA-to-Fe^{2+} ratios rapidly yield a depletion of radicals formed from this complex (34,81).

In individual trials, sample mixtures of leucine (0.762 mM) with 10 µM Fe^{2+}, 100 µM Fe^{2+}, or an EDTA-Fe^{2+} complex (EDTA/Fe^{2+}, 104:100 µM) were therefore heated from room temperature to 100°C and incubated at this temperature for 90 min. In the EDTA-Fe^{2+} complexes, a slight excess of EDTA as referred to Fe^{2+} was used (104 µM EDTA versus 100 µM Fe^{2+}) to ensure that all iron present in the solutions was complexed and no unbound iron was available. Samples containing leucine and Fe^{2+} or leucine and 100 µM EDTA served as references.

After heating and incubation for 90 min, 28.9 ± 2.8 µg/L of 3-methylbutanal was detected in the mixtures when 10 µM Fe^{2+} was present, whereas in samples with 100 µM Fe^{2+} added, 29.2 ± 0.3 µg/L of 3-methylbutanal was detected (Fig. 4). When no Fe^{2+} was added, only traces (1.2 ± 0.3 µg/L) of 3-methylbutanal were found, and in the sample for which solely 100 µM EDTA was added, no 3-methylbutanal was detected. As shown in Figure 4, when using the EDTA-Fe^{2+} complex instead of solely Fe^{2+}, and heating the model mixtures, 18.2 ± 0.9 µg/L of 3-methylbutanal was found in the model solution, which strongly indicates that hydroxyl radicals were involved in the oxidative degradation of leucine, eventually yielding 3-methylbutanal. Although it is likely that hydroxyl radicals as generated from EDTA-Fe^{2+} were involved in 3-methylbutanal formation, it is not clear whether the diminished formation of 3-methylbutanal when using EDTA-Fe^{2+} instead of Fe^{2+} was observed because fewer radicals were formed or because site-localized reactions of Fe^{2+} as proposed by Nashalian and Yaylayan (59) were inhibited.

Apart from that, the observation that no difference in 3-methylbutanal concentration was found when 10 or 100 µM Fe^{2+} was added implies that the amount of Fe^{2+} present in the system is not pivotal for 3-methylbutanal formation under these experimental conditions. This observation is in accordance with the aforementioned finding, in which addition of 500 µg/L of Fe^{2+} to samples containing 12 g/L of maltose and 25 mg/L of leucine also had no effect on aldehyde formation. Again, it can be anticipated that Fe^{2+}, once it is oxidized to Fe^{3+}, is rapidly reduced again to its lower valence state, and therefore, practically, only Fe^{2+} is present

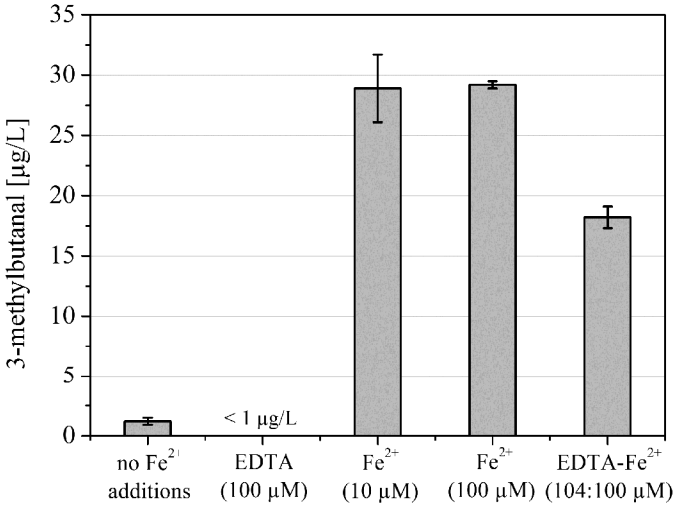

Fig. 4. Formation of 3-methylbutanal by transition metal–catalyzed decarboxylation of leucine. Leucine (0.762 mM) was incubated for 90 min at 100°C without Fe^{2+} additions, and with 100 µM ethylenediaminetetraacetic acid (EDTA), 10 µM Fe^{2+}, 100 µM Fe^{2+}, or the Fenton reagents EDTA and Fe^{2+} (100:104 µM) added. Mean values are presented. Error bars represent ±1 standard deviation of a triplicate experiment.

in the model systems. The comparatively low amounts of 3-methylbutanal found in the sample with only leucine added may be derived from traces of iron in the buffer system. Addition of excess EDTA to the model mixture containing only leucine can be assumed to have yielded a complete inactivation of all iron present, thus completely inhibiting aldehyde formation (Fig. 4).

Hop α-acids were reported to suppress hydroxyl radical formation by iron complexation but were shown to be ineffective in suppressing oxidative degradation of 2-deoxyribose via scavenging of hydroxyl radicals (79). Yet both possibilities were tested and applied to the model system used in this set of experiments. Because hop α-acids undergo isomerization to iso-α-acids under heat exposure (23), and solely hop α-acids were wanted to be assessed, reactions were carried out at 20°C.

The formation of H_2O_2 and hydroxyl radicals in beer or model systems is derived from complex oxidative chain reactions. Molecular oxygen is activated, thereby forming various oxygen radicals (e.g., $O_2^{\bullet-}$, $^{\bullet}OH$, $HO_2^{\bullet}$) and nonradical oxidizing agents (e.g., H_2O_2), all of which are termed reactive oxygen species (44). Fe^{2+} ions play a central role in these reaction cascades because they act as catalysts for H_2O_2 decay, thereby forming hydroxyl radicals. The initial activation of oxygen requires energy or a strong reducing agent. As opposed to the previous trial, in which oxygen activation occurred spontaneously in the model system owing to the high incubation temperature of 100°C (Fig. 4), when assessing the hop α-acids' efficacy at 20°C, reactions were started by adding H_2O_2 as the oxidizing agent and as the initiator for hydroxyl radical formation, and they were incubated for 24 h.

Adding H_2O_2 and incubating the model mixtures showed the highest formation of 3-methylbutanal when solely Fe^{2+} and H_2O_2 were present (Fig. 5). Adding hop α-acids to these mixtures and preincubating them for 1 h to allow the formation of hop α-acid–iron complexes caused a distinct reduction of 3-methylbutanal by 95.1%, which indicates that one possibility by which hop α-acids suppress leucine oxidative degradation is by complexing Fe^{2+} ions, thus rendering them harmless. Related to this, hop α-acids were reported to trigger autoxidation of Fe^{2+} to Fe^{3+}, which, in turn, is not capable of acting as a catalyst in the Fenton reaction system (79). Hydroxyl radical formation is consequently inhibited, as is leucine oxidative degradation that yields 3-methylbutanal.

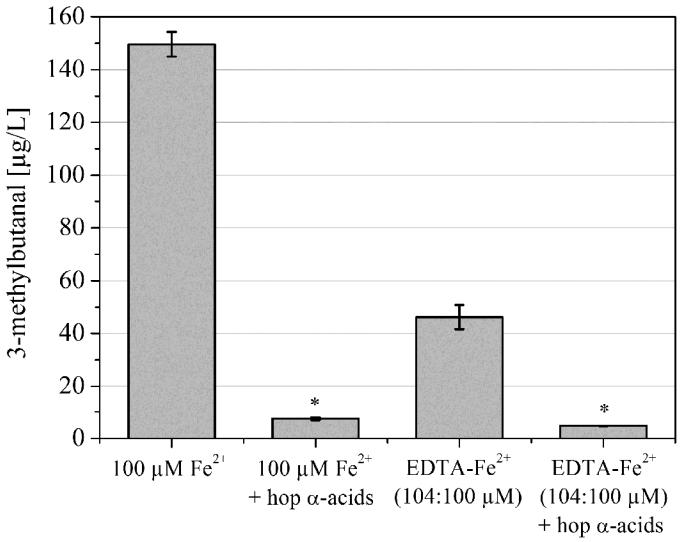

Fig. 5. Effectiveness of hop α-acids in suppressing the formation of 3-methylbutanal by iron complexation or hydroxyl radical scavenging. Reactions were started by the addition of 5 mM H_2O_2 and allowed to react for 24 h at room temperature. Mean values are presented. Error bars represent ±1 standard deviation of a triplicate experiment. Stars above bars indicate significant difference of mean percentages between the Fe^{2+} only sample and Fe^{2+} only sample with hop α-acids added, and between the EDTA-Fe^{2+} sample and EDTA-Fe^{2+} sample with hop α-acids added, by a Student's *t* test at the 99.9% confidence level. EDTA = ethylenediaminetetraacetic acid.

Testing the Fenton reagents EDTA and Fe^{2+} resulted in a distinctly lower formation of 3-methylbutanl compared with use of Fe^{2+} only. This observation may be explained by diminished radical formation from reactions of the EDTA-Fe^{2+} complex with H_2O_2 as opposed to when unbound Fe^{2+} reacts with H_2O_2. Furthermore, because Fe^{2+} is firmly bound to EDTA, site-localized reactions of Fe^{2+} with leucine that yield 3-methylbutanal may be blocked (15,59,73). The addition of hop α-acids to sample mixtures already containing EDTA-Fe^{2+} and leucine, and initializing hydroxyl radical formation by addition of H_2O_2, resulted in a clear reduction of 3-methylbutanal by 89.9%. In addition to the suppression of leucine oxidative degradation by Fe^{2+} complexation, these data clearly imply that hop α-acids are also capable of diminishing oxidative degradation of leucine by scavenging of hydroxyl radicals.

Summing up these data thus far further point to the significance of Fe^{2+} availability on Strecker-like reactions and degradation of amino acids to their corresponding aldehydes, because iron availability not only promotes certain pathways of the Maillard reaction but also triggers radical formation. Although independent of the pathway by which Fe^{2+} promotes the formation of 3-methylbutanal and 2-furfural, the outcome that hop α-acids, which were reported to be efficient in complexing ferrous (Fe^{2+}) or ferric (Fe^{3+}) ions (79), significantly diminished the formation of 2-furfural and 3-methylbutanal is a persuasive indication that the hop α-acids' mode of action may be in fact traced back to their iron complexation capability. The observation that iso-α-acids, which were reported to be only a little effective in complexing iron ions, were not able to abate aldehyde formation in these model systems supports this hypothesis. In addition to Fe^{2+} complexation, scavenging of hydroxyl radicals, which were also reported to trigger oxidative degradation of amino acids yielding Strecker aldehydes (80,81), may serve as a further explanation for the α-acids' mode of action. However, the α-acids' effectiveness may not be limited solely to their metal ion complexation or hydroxyl radical scavenging characteristics, and there are also other mechanisms imaginable by which hop constituents, and in particular hop α-acids, exert their capability to abate the formation of carbonyls. It is a well-known truth that Strecker degradation of amino acids in the presence of sugar-derived α-dicarbonyls yields the corresponding aldehydes (69). Because hop α-acids can form imines when reacting with *ortho*-phenylenediamine (77), Ting et al. (74) proposed that hop α-acids may also be capable of forming imines with amino compounds or amino acids in wort and beer, accordingly competing with reactive carbonyls for binding sites. Consequently, nonoxidative α-dicarbonyl reactions may be abated or blocked, thus diminishing the formation of odor-active aldehydes by this pathway. Observations from this study may point to the existence of such a mechanism; however, further research is needed to verify its relevance.

CONCLUSIONS

The current knowledge has been confirmed in the present study by additional information resulting from the application of previously unexplored experiments. New detailed views were presented by the elaboration of special methodologies, including RSM and CCRD. The functional principle by which hop α-acids and β-acids diminish the formation of 3-methylbutanal and 2-furfural may be ascribed to their unique molecular structure and chemical properties, which allow them to perturb oxidative or nonoxidative formation of aldehydes. As related to findings from this study, their antioxidative potential may be explained through, but not

necessarily limited to, two mechanisms. First, because of the hop α-acids' complexation functionality toward transition metal ions such as iron and copper, they can block or attenuate oxidative pathways of the Maillard reaction and suppress the generation of reactive oxygen species, thereby preventing oxidative degradation of leucine to 3-methylbutanal. Second, once hydroxyl radicals are formed, they are able to scavenge these radicals, thus directly acting as antioxidants and suppressing free radical reactions with leucine again, which can yield its corresponding aldehyde. Other aldehydes may show the same behavior and response to the presence to hop α-acids. Although working in model systems and with empirical models can only give an approximation of real conditions, outcomes from this study still expand the knowledge about factors affecting carbonyl formation during wort boiling. The hop acids' highly protective efficacy in suppressing the formation of odor-active aldehydes in the model systems used may also be applicable to real wort boiling conditions. Deductions from this study can therefore contribute to the clarification of processes that block or inhibit the formation of carbonyl compounds in beer.

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Publication F

As a consequence from the findings and associated deductions of the preliminary work, this study was designed to deliver brewers a simple and economic way to direct their in-house hop dosage towards a higher exploitation of the hops' full antioxidative potential. Because of the high efficacy and antiradical properties of hop α-acids, it was decided to limit the study to the exclusive use of hop extract from supercritical CO_2 extraction which is mostly free of hop phenolic material, also because hop CO_2 extract is a widely-used hop product in the brewing industry. The complete work was published in the *BrewingScience* journal under the title

"Influence of Hopping Technology on Oxidative Stability and Staling-Related Carbonyls in Pale Lager Beer"[281] *(Publication F).*

In accordance to the previous findings of this work[276, 282], outcomes from this study could clearly confirm and extend the knowledge around the hop dosage's high effectiveness on the ionic composition of wort and beer, particularly as related to prooxidative iron ions. As a main conclusion, it must be stated that the hop dosage clearly needs to be seen in relation to the constitution and composition of the wort matrix. In its industrial application, the hop dosage should therefore always be designed and adapted to the breweries' wort. Based on the findings, different hopping technologies were designed and their positive effects on beer quality could be confirmed analytically and sensorial. Findings and proposed technologies from this study can be directly transferred and applied to the brewing industry.

Wietstock, P. C., Kunz, T. and Methner, F.-J.

Influence of Hopping Technology on Oxidative Stability and Staling-Related Carbonyls in Pale Lager Beer

Storage-induced deterioration of beer flavor is a major issue for many breweries and technological measures to diminish staling are limited. In this study, the effect of different hopping technologies on content of pro-oxidative iron ions, concomitant oxidative beer stability and staling-related carbonyls was investigated. In lab-scale brewing trials, hop dosages during mashing-in or at the onset of boil caused a decrease of pro-oxidative iron ions in dependency of the amount of hop CO_2 extract dosed. Though, the effectiveness was clearly limited by the 'accessibility' of iron ions. The amount of 'free' iron was found to be dependent on the initial malt bill, and the hops' efficiency in terms of reducing the iron content was higher the more iron was present and the more hop CO_2 extract was dosed. Pilot-scale brewing trials (120 L) were carried out in duplicate using different hopping technologies: hops dosed only at the onset of boil (reference), mash hopping, divided hop dosage, first wort hopping, and continuous hop dosage. Hop CO_2 extract was the sole hop product used. While standard beer quality parameters were unaffected from the hopping, the bitter substance yields suffered from later hop dosages or in particular from hop dosages during mashing. All brews with modified hoppings showed reduced iron contents of up to ~30 % and improved oxidative stabilities as compared to the reference brews with the exception of the first wort hopping brews where the oxidative stability as measured by electron spin resonance spectroscopy was worse. After storage (12 weeks, 28 °C), the staled beers' carbonyl contents were noticeably distinguishable and were lowered up to 66.9 % when modified hopping was applied. Sensory analysis of the fresh and aged beers was in accordance with the analytical data and revealed improved sensory properties for the beers produced by applying modified hopping regimes with the exception of the mash hopping brew from brewing series 2, whose sensory properties were rated lower. This study provides new findings with regards to the anti-staling characteristic of hops and its application by simple modifications of the hopping technology.

Descriptors: Hop dosage, hops, beer ageing, mash hopping, first wort hopping, continuous hopping, aldehydes, iron

1 Introduction

Hops are next to water, malt and yeast one of the four ingredients used for beer production. They can be dosed at various stages during wort production; though, in Germany, the purity law limits the usage of extracts from hops to the hot part of beer production, while pelletized hops, hop powders, or cone hops can also be applied in the cold part, e.g. during fermentation or maturation [1]. Mostly, hops are added during early stages of wort production to allow sufficient time for the isomerisation of hop α-acids to iso α-acids via an acyloin ring contraction which contribute to the beer's final bitterness [2]. Yet, in brewing-related research history, also the effects of adding hops at earlier stages of the brewing process were tested. *Kolbach* and *Wilharm* [3] examined in 1943 the effects on dosing hops to the mash and found high bitter substance losses and no effect on the coagulation of nitrogen in the wort. *Schur* and *Pfenninger* [4] confirmed the high losses but reported a 'finer' and 'more distinct' hop aroma in beers when mash hopping was applied. *Gresser* [5] on the contrary found no hop aroma nor did he detect higher oil contents. *Preis* and *Mitter* [6] produced beers by adding certain amounts of the total hop bill to the first wort and found improved sensory properties and an improved bitter substance yield but milder bitterness when compared to beers where hops were not added to the first wort. But also the hop dosage during wort boiling as well as hop dosage modifications were shown to have a great effect on the oxidative stability of beer as investigations from *Wietstock* et al. [7], *Kunz* et al. [8], and *Mikyška* et al. [9] showed.

The high potential of hops and hop constituents in relation to not only providing bitterness and aroma but also featuring antioxidative and health beneficial properties was already discovered and studied intensively [7, 8, 10–18] making them also interesting for the pharmaceutical industry [19]. Particularly hop α-acids were shown to be capable of forming complexes with metal ions such as iron [8, 16, 20, 21], which, in turn, favors beer flavor stability because these metal ions are depleted as catalysts in the so-called Fenton reaction [16]. Hop α-acids were shown to possess a high antioxidative potential while iso-α-acids were less effective. The hop α-acids' metal chelation behavior was in fact reported to be

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very advantageous in terms of beer quality since metal ions which promote oxidative reactions such as Cu or Fe are complexed while other vital metal ions, such as e.g. essential yeast nutrients (Zn) remained unaffected [8, 22]. *Ting* et al. [14] additionally explained the antioxidative nature of hop α- and β-acids by their ability to form stable phenoxy radicals and/or block oxidative intermediates of the Maillard reaction.

Hop additions at the end of boiling or in the whirlpool were consequently demonstrated to be advantageous with regards to beer flavor stability as unisomerized α-acids are 'supplied' again which then do not isomerize but deploy their antioxidant activity during wort cooling [8]. This can be disadvantageous because the bitter substance yields become low when adding hops late on, and therefore, more hops need to be added when still aiming to achieve the same bitterness in the beers. Yet, even though hops possess considerable amounts of metal ions [23, 24], their addition during the process still yields a reduction of certain metal ions, in particular iron ions which, in turn, favors the beer's oxidative stability [8].

The effect of hop polyphenols is also discussed in literature and appears to be controversial. While there is great evidence that polyphenols possess the ability to attenuate oxidation reactions [13, 25, 26], the polyphenol's impact on oxidative beer flavor stability seems to be ambiguous [27] and is an ongoing debate. Mikyška et al. [9] performed brewing trials using hop extracts, sole hop pellets, or a combination of both, as single dosages or in divided dosages and found a good correlation between hop antioxidants and the appearance of carbonyl compounds during storage. This effect was mostly traced back to the antiradical activities of hop polyphenols which were imparted to the wort by the pellet dosages. Though, Wietstock et al. [7] reported no or only very little effect of hop polyphenols on the formation of harmful hydroxyl and ethoxy radicals in wort as measured by electron spin resonance spectroscopy, and the biggest antioxidative potential of hops was claimed to be derived from hop α-acids and hop β-acids. Other researchers see again hop polyphenols as the only source for the hops antioxidative capacity [13]. All these findings, however, appear to be a very much a matter of which method was used for determining the antioxidative capacity [28].

Ultimately, it seems therefore indispensable to verify analytical results from antioxidative measurements with storage trials in which ageing-related compounds are monitored and to verify analytical results with sensory analyses. Furthermore, asides from the debate which hop ingredient represents now the biggest antioxidative source, there is no doubt that dosing hops to beer reduces tremendously the formation of staling aldehydes as compared to an unhopped beer [7, 29, 30].

Various pathways have been suggested to be the origin of active off-flavor compounds in bottled beer. Amongst them, iso-α-acids were also identified as a potential source of flavor-active off-flavor compounds. *Hashimoto* and *Eshima* [31] reported the formation of staling aldehydes from the iso-α-acids' alkanoyl side chain by oxidative degradation. *De Clippeleer* et al. [32] anticipated the degradation of particularly *trans*-iso-α-acids as the source of carbonyls during beer storage first, but then found this pathway to be only of minor importance when performing brewing trials. This was confirmed by investigations from *Schmidt* et al. [33]. *Rakete* et al. [34] found evidence of the formation of flavor-active carboxylic acids from iso-α-acids by hydrolytic β-dicarbonyl cleavage under oxidative conditions and thus further clarified a mechanism originally proposed by *Williams* and *Wagner* [35]. Further reactions yielding beer deterioration comprise fatty-acid degradation, aldol condensation, oxidation of higher alcohols, and the Maillard reaction, all of which can occur at different reactions during beer production such as already during mashing, during wort boiling, or after bottling. For reviews of these pathways, see [36–38]. Additionally, free radicals such as the hydroxyl radical or hydroxyethyl radical were also shown to play a substantial role during ageing of beer [39–42]. They are formed via a mechanism involving activation of molecular oxygen as catalyzed by transition metal ions (Fe^{2+} and Cu^{+}) which can act as electron donors. Oxygen forms subsequently a series of active intermediates and reacts with transition metal ions again in the so-called Fenton and Haber-Weiss reaction thereby forming highly-reactive hydroxyl radicals. A direct formation of aldehydes by free radical attack was recently suggested by Wietstock and *Methner* [43]. Yet, independent from the substantial reactions yielding staling compounds, ongoing research imparts also the liberation of those compounds from a bound state during storage [44, 45]. Apart from that, it is evident that oxidative conditions trigger staling of beers whether it may be through a de novo formation or oxidation-promoted release of substances.

Every single step and event during beer production has undoubtedly a decisive influence on the final beer quality and its resistance against the appearance of ageing-related off-flavors. Oxidation and beer deterioration reactions occur already during early stages of beer production such as during mashing or during wort boiling. Diminishing oxidative reactions by complexing or removing pro-oxidative metal ions, e.g. Cu and Fe, at these early stages can therefore be advantageous for the final beer quality as antioxidants are preserved and/or oxidation reactions occur to a lower extent. One goal of this study was therefore to distinctively remove iron ions at the early stages of wort production, ultimately aiming at producing beers with an increased oxidative stability and diminished or delayed formation of staling off-flavors. The present study therefore aimed to scrutinize the hop dosage's influence at different points of addition during wort production, while taking the bitter substance yield into account. Hop CO_2 extract was used because it represents a hop product which is often applied in the industry. This study provides new insights in the hop acids' ability and mode of diminishing or delaying staling of beer during storage. Outcomes from this study therefore point to the importance to rethink the hopping technology applied in industry and adapt it with the goal of a higher efficiency, such as not only providing bitterness but also utilizing the hops antioxidant properties.

2 Materials and methods

2.1 Chemicals

Benzaldehyde, 2-furfural, iron II sulfate hepta hydrate, 2-methylbutanal, 3-methylbutanal, methional, pentanal, phenylacetalde-

hyde, and α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN) were purchased from Sigma Aldrich Inc., Steinheim, Germany. Sodium carbonate and sodium sulfate were obtained from Merck KGaA, Darmstadt, Germany. Diethylether and anhydrous ethanol were purchased from VWR international GmbH, Darmstadt, Germany. All chemicals were of analytical grade or higher. All aqueous solutions were made with double-distilled water and prepared freshly every day. The international calibration standards of iso-α-acids (ICS-I3) or a standardized calibration extract for α- and β-acids (ICE-3) were purchased from Labor Veritas AG, Switzerland. Purified α-acids (86.4 % purity), hop CO_2 extracts, or hop pellets were supplied courtesy from Hopsteiner.

2.2 Standard wort and beer analysis

Extract (2.9.2.3), alcohol (2.9.6.3), color (2.12.2), pH (2.13), foam stability (2.18.2), total nitrogen (2.6.1.1), free amino nitrogen (2.6.4.1.1), total polyphenols (2.16.1), bitter units (2.17.1), endogenous antioxidative potential (EAP) and radical levels (T_{600}) (2.15.3) were analyzed according to MEBAK [45]. The numbers in parentheses indicate the method used.

2.3 Determination of hop acid concentrations by HPLC

Hop acids were quantitated according to [47]. Chromatographic determination was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Böblingen, Germany) at a constant temperature of 40 °C and a flow rate of 1.2 mL/min with a 5 µL injection volume. Two mobile phases were used. Mobile phase A was 100 % methanol, mobile phase B was 55 % methanol, 44 % water and 1 % phosphoric acid. The elution began isocratically with 50 % of mobile phase B for the first 12 min followed by a gradient descent to 20 % of mobile phase B over the next 3 min, which was then held for 10 min. A Purosphere Star™ LC-18 5 µm C18 silica column was used for separation. Absorbance was measured at 270 nm and 314 nm. As reference for the iso-α-acids, an international calibration extract (ICS-I3) was used, whereas for α- and β-acids, a standardized hop extract (ICE-3) was deployed.

2.4 Determination of SO_2 levels in beers

Sulphur dioxide concentrations of beer were analyzed by a continuous flow analyzer (CFA -Skalar; Kat.-Nr.: 593-998) according to an optimized method using a new Teflon membrane [48]. Prior to measurement, beer samples were degassed by ultrasonic treatment for 5 minutes and filtered through a black band filter (Schleicher & Schuell, Dassel, Germany) during which the first 10 mL were discarded.

2.5 Determination of metal ion concentrations in worts and beers.

Metal ion 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 used for the determination of iron were 239.5 and 259.9 nm. A six-point calibration curve was used to quantify the test samples' concentrations. The calibration was done matrix-matched ranging from 0–1 mg/L or 0–250 µg/L in wort or beer, respectively, to deplete influences of the samples' organic matrices. All calibration curves showed good linearity ($R^2 > 0.99$).

2.6 Quantitation of aldehydes by solvent-assisted flavor evaporation (SAFE)-GC/MS

Solvent assisted flavour evaporation (SAFE) according to *Engel, Bahr* and *Schieberle* [49] and high resolution gas chromatography (HRGC) coupled to mass spectrometry (MS) analysis was used to measure staling aldehyde concentration in aged beers. An aliquot (100 mL) of beer was passed through a folded paper filter and spiked with 1 µg of pentanal as an internal standard. The sample was extracted twice with 150 mL diethyl ether. To remove the non-volatile material, the unified extracts were distilled under high vacuum by means of a SAFE apparatus. The distillate was washed twice with a 0.5 M Na_2CO_3 solution and water, dried over Na_2SO_4, and concentrated to 5 mL using a Vigreux column. A sample (1 µL) of this concentrated distillate was applied via a cold injection system (Gerstel, Mülheim, Germany) in 10:1 split mode to a gas chromatograph (6890, Agilent Technologies, Waldbronn, Germany) fitted with a capillary column (VF-5 MS, 60 m x 0.25 mm, 0.25 µm film, Varian, Darmstadt, Germany). The following temperature program was used for HRGC: after 12 min at 35 °C, the oven temperature was raised to 150 °C at a rate of 12 °C/min and then to 250 °C at 30 °C/min where it was held for 5 min. The flow rate of the helium carrier gas was 0.6 mL/min. The MS analysis was performed by an MSD 5973 mass spectrometer (Agilent Technologies, Waldbronn, Germany). Mass spectra in the electron impact mode (MS/EI) were generated at 70 eV using selected ion monitoring. Carbonyl concentrations were calculated by using a single point internal standard procedure. An internal response factor of the reference compounds in relation to the internal standard was determined, and was then used to calculate the amount of the compounds in the sample. Table 1 depicts the retention times and target ions of all compounds used for quantitation.

Table 1 **Retention Times and *m/z* Ratios Used for Quantitating Carbonyl Compounds by GC/MS. A Varian VF-5 MS column (60 m x 0.25 mm, 0.25 µm) was used for analyte separation. All aldehydes were quantitated by using commercially available reference compounds**

Substance	t_R [min][a]	m/z
3-methylbutanal	10.15	58
2-methylbutanal	10.63	58
pentanal (IS)	12.96	58
2-furfural	19.05	96
methional	21.50	104
benzaldehyd	23.03	106
phenylacetaldehyd	24.79	91
ethyl nicotinate	27.61	106
γ-nonalacton	29.55	85

[a] t_R = retention time

2.7 Sensory analysis

Sensory analysis of fresh and stored beers was conducted according to DLG (Deutsche Landwirtschafts-Gesellschaft e.V.) in multiple sessions with 10–12 trained panelists. The beers' attributes odor, taste, palate fullness, freshness and quality of bitterness are rated on a scale of 1 to 5, where 5 represents the highest rating and 1 the lowest rating. When ratings of 3 or lower are assigned to a sample, then it is declared 'not vendible' and the downgrading has to be justified with a detailed description of the off-notes. In order to get an improved comparability of the samples, a 'DLG score' was calculated based on the area that the final rating would make in a spider web diagram with these five attributes. Following the DLG standards, the ratings of the attributes 'purity of odor', 'purity of taste', and 'quality of bitterness' were included twice, and the attributes 'palate fullness' and 'freshness' were included single for the calculation.

2.8 Effects and hop bitter substance yields of hop dos ages during mashing-in

Worts were produced in lab-scale by mixing well 70 g of fine grist (100 % Pilsner malt) with 300 mL of double-distilled water. Mashing was conducted under continuous stirring using a laboratory masher (Bender & Hohbein, Bruchsal, Germany) and applying the following temperature program: mashing-in at 62 °C, heating up to 66 °C at 1 °C/min, 30 min rest, heating to 72 °C, 20 min rest, heating to 78 °C. After reaching 78 °C, the mash was immediately filtered using paper filters and the mashing-beakers and stirrers were rinsed with 100 mL of double-distilled water (T = 78 °C) which was added on top of the filters. The first 100 mL of wort were collected and were also poured back on top of the filters. Along with mashing-in the malt, 80 mg of hop CO_2 extract (Hallertauer Perle, c(α-acid) = 44.8 % wt./wt.) or hop pellets type 45 (Hallertauer Perle, c(α-acid) = 8.8 % wt./wt.) were added. 200 mL of collected wort were then boiled for 60 min under reflux and 200 mg/L of the same hop CO_2 extract were added at the start of boil. Sampling was done from the wort after filtration and from the boiled wort. Aliquots were taken and were immediately cooled using an ice bath. Hop acid concentrations from the worts were subsequently quantified by HPLC. The trials were conducted in triplicate.

2.9 Effects of hop CO_2 extract additions during mashing or during start of boil

The mashing and separation procedure in these trials was similar to the previous trial. First, the effects of adding hops during mashing-in were assessed and 0, 44.6, 89.3, 133.9, 223.2, 446.4 mg/L of hop CO_2 extract (Hallertauer Perle, c(α-acid) = 44.8 % wt./wt.) were added directly during mashing-in. In a separate trial, the mash's iron concentration was artificially increased by adding 200 µg/L of Fe^{2+} to the mashing-in liquor, and the hop dosage was limited to 0, 223.2, and 446.4 mg/L of hop CO_2 extract (Hallertauer Perle, c(α-acid) = 44.8 % wt./wt.) dosed during mashing-in. The sweet worts after filtration were collected and the iron concentrations from all worts were determined on the same day by ICP-OES.

In addition to dosing hops during the mashing-in step, the effects of adding hop CO_2 extract at the start of boil on iron concentration of sweet wort were assessed. In this trial, the same mashing procedure as described before was used again but this time in total, 3 L of unboiled wort were produced, divided into three 1 L aliquots and 0 µg/L Fe^{2+}, 100 µg/L Fe^{2+}, or 200 µg/L Fe^{2+} were added, respectively. The worts were then divided again in 100 mL aliquots and brought to a boil under reflux. Each aliquot was then mixed with 0, 44.6, 89.3, 133.9, 223.2, 446.4 mg/L of hop CO_2 extract (Hallertauer Perle, c(α-acid) = 44.8 % wt./wt.), respectively. After 5 minutes incubation at > 98 °C, the worts were immediately cooled in an ice bath and the iron concentration from all wort samples was determined on the same day by ICP-OES. All trials were done in triplicate.

2.10 Influence of malt type and hop dosage on iron ion concentration of wort

The effect of using 100 % Pilsner malt, 100 % Munich malt type I, or a mixture of both malts in equal amounts on iron concentration in wort was assessed in a lab-scale trial. Mashing and filtration was done again as described previously but this time, 1 L of each initial malt bill was produced in triplicate. After bringing the wort to a boil, 208.8 mg/L of hop CO_2 extract (Hallertauer Magnum, c(α-acid) = 47.9 % wt./wt.) were added and the worts were boiled under reflux for 60 minutes. Samples were taken from the worts after boiling and were immediately cooled prior to directly determining the iron concentrations using ICP-OES. All trials were done in triplicate.

2.11 Production of beers using different hop dosages in semi-technical scale

Five different beers were produced on a 1.2 hL scale in duplicate. The malt bill consisted of 90 % Pilsner malt and 10 % Munich malt type I and an infusion mash procedure similar to that of the previous trials was used for sweet wort production. The mash solids were separated from the sweet wort using a lauter tun. Table 2 depicts the different hop dosages, masses and points of addition. The amounts of hop α-acids added were designed to obtain an identical bitterness in the beers. The isomerization yield was assumed to be 33.33 % when dosing hops at the onset of boil. A yield of 5 % was expected from adding hops to the mash and the addition at the onset of boil was accordingly lowered. For the divided hop dosage, an additional amount of 10 % was given

Table 2 **Hop dosages used for beer production in semi-technical scale. All values are in mg/L. Please consult the materials and methods section for information about the design of the brews**

Brew	Abbrev.	Hop α-acid additions in [mg/L]					
		mash-in	first wort	start of boil	30 min boil	end of boil	whirlpool
Reference	REF	–	–	90	–	–	–
Mash hopping	MAH	60	–	81	–	–	–
Divided hop dosage	DIV	–	–	45	27	18	9
First wort hopping	FWH	–	45	–	27	18	–
Continuous hop dosage	CON	–	–	45	10 * 4,5 in 5 min intervals		

in the whirlpool to compensate a potential diminished isomerization yield caused by the later hop additions. For the first wort hopping and for the continuous hop dosage, no corrections as related to the quantity of hops dosed were made, and the total amounts added were similar to the reference (hops dosed only at the onset of boil). The hop product used was hop CO_2 extract (Hallertauer Magnum, c(α-acid) = 47.9 % wt./wt.). The wort was boiled under atmospheric pressure for 60 minutes, followed by a 15 min whirlpool rest. After subsequently cooling to pitching temperature of 14 °C using a plate heat exchanger, the worts were fermented in open vessels using the bottom-fermenting yeast strain W34/70 (Fermentis, Marcq en Baroeul, France) in cylindroconical tanks until a residual apparent extract of 3.5 % wt./wt. was reached. The green beers were subsequently stored at room temperature for 1 day, transferred to kegs and were maturated at 0–2 °C until filtered using membrane candle filters (5 µm/1 µm/0.45 µm; Donaldson, Haan, Germany). The beers were then bottled in 0.5 L bottles using a four organ filler (JS Maschinen GmbH, Bergen, Germany). The oxygen levels in the bottled beers were controlled regularly and did not exceed 50 µg/L as measured using a DIGOX 6.1 apparatus (Dr. Thiedig, Berlin, Germany). The pitching worts were sampled and kept frozen at –18 °C until analyzed. The beers were analyzed directly after filling and after 12 weeks storage at 28 °C in the dark.

3 Results and discussion

3.1 Effects and hop bitter substance yields of hop dosages during mashing-in

In general, the yield of hop bitter substances during wort boiling is considerably low and was shown to be dependent on many factors such as e.g. losses with trub during wort boiling, pH, temperature, etc. [50]. There is only little published of how the bitter substance yield is to be expected when applying a hop dosage during mashing. Therefore, as a preliminary trial, lab-bench trials were performed in which hops were added during mashing-in.

Table 3 depicts the results from lab-bench brewing trials. Hop iso-α-acid utilization was determined in both the filtered mashes and the worts after boiling. It is apparent from the data that the hop bitter substance yields were very low when adding hops during mashing-in. Adding pellets instead of hop CO_2 extract showed little higher yields for iso-α-acids which maybe traced back to the lower α-acid masses added at the onset of mashing. The hop

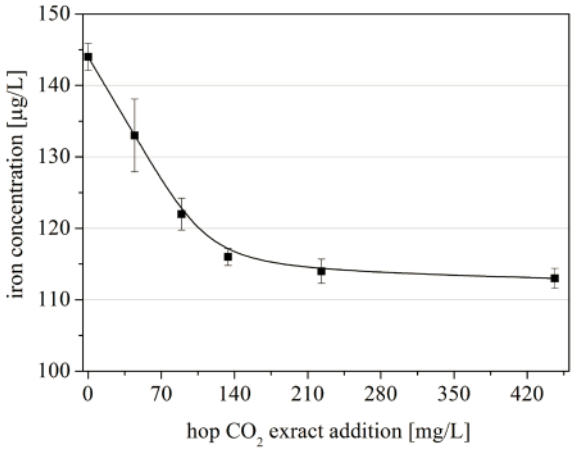

Fig. 1 Effect of hop CO_2 extract additions on iron concentration during mashing. Iron concentration was determined from filtered samples. Mean values are presented. Error bars represent ± 1 standard deviation. N = 3

α-acid utilization rates during wort boiling were in a normal range and showed no clear dependency as to what hop product was added during mashing-in. The little higher yields at end of boiling observed in the trials where hop CO_2 extract or hop pellets were added during mashing-in may also be derived from residual α-acids which 'survived' the filtration step and were therefore still present at the onset of boil.

Regarding the total yield, clearly, adding hops during mashing results in a worse yield of bitter substances as opposed to when adding hops only at the onset of boil. The higher total yield when adding pellets instead of hop CO_2 extract may also be explained by the difference of total α-acids added during mashing and boiling (hop pellets, 107.2 mg/L vs. hop CO_2-extract, 179.2 mg/L).

As shown in figure 1, adding hop CO_2 extract during mashing at amounts of 44.6 to 133.9 mg/L yielded a significant reduction of 144 µg/L of iron initially present in the mash until at maximum, a concentration of 114 µg/L (21 % reduction) was reached. This level was not further exceeded even when additions were increased up to 446.4 mg/L of hop CO_2 extract.

Recent studies [16] reported that hop α-acids are good iron chelators and the effect as seen in figure 1 can therefore most probably traced back to the α-acids' ability to bind iron thereby forming amorphous complexes which then precipitate and are then lost during the spent grain removal. It is apparent that still only ca. 21 % of the total iron was removed and it was therefore assumed that the residual 79 % of the iron was firmly bound in complexes

Table 3 Hop α-acids, iso-α-acids, and bitter acid yields of hop pellet or hop CO_2 extract additions during lab-scale mashing and wort boiling. Mean values ± 1 standard deviation are presented. N = 3

Hop product added during mashing-in	mashing				wort boiling				total yield[a] [%]
	α-acids added [mg/L]	iso-α-acids detected [mg/L]	α-acids detected [mg/L]	yield[a] [%]	α-acids added [mg/L]	iso-α-acids detected [mg/L]	α-acids detected [mg/L]	yield[a] [%]	
no hops added	–	–	–	–	89.6	33.9 ± 1.3	4.3 ± 0.3	37.8 ± 1.5	37.8
hop CO_2-extract	89.6	1.5 ± 0.2	0.3 ± 0.2	1.7 ± 0.2	89.6	36.8 ± 1.4	3.6 ± 0.1	41.1 ± 1.6	20.5
hop pellets (type 45)	17.6	1.3 ± 0.3	< 0.1	7.4 ± 1.7	89.6	37.6 ± 2.2	3.6 ± 0.2	42.0 ± 2.5	35.1

[a] Yields are calculated based on the sum of iso-α-acids at the end of boiling divided by the amount of α-acids added during mashing and/or wort boiling

Table 4 Effect of hop CO_2 extract additions and Fe^{2+} additions during mashing on free amino nitrogen content, bitter units and final iron concentration in sweet wort. Mean values ± 1 standard deviation are presented. N = 3

Hop CO_2 extract	Free amino nitrogen [mg/L]		Bitter units [BE]		Iron concentration [µg/L]	
	0 µg/L Fe^{2+}	200 µg/L Fe^{2+}	0 µg/L Fe^{2+}	200 µg/L Fe^{2+}	0 µg/L Fe^{2+}	200 µg/L Fe^{2+}
No addition	198.4 ± 7.2	197.0 ± 3.3	< 1	< 1	185.4 ± 8.2	179.8 ± 12.2
223.2 mg/L	196.5 ± 5.7	177.6 ± 5.8	6.0 ± 1.2	5.7 ± 0.6	124.9 ± 9.9	122.4 ± 5.8
446.4 mg/L	195.7 ± 1.7	177.2 ± 6.3	10.5 ± 0.4	10.0 ± 0.7	121.5 ± 11.2	119.8 ± 8.2

with other mash or wort constituents thus being not accessible for the α-acids' action.

In a separate trial, it was therefore assessed if an additional dosage of 'free' iron to the mash, added as iron(II) sulfate hepta hydrate, results in a higher relative depletion of iron. A supplementary addition of iron yielded no further increase in the iron concentration as measured by ICP-OES, and the addition of α-acids, added as hop CO_2 extract, showed no enhanced effectiveness (see Tab. 4). In accordance with the results from figure 1, it is likely that there are various constituents with free binding sites for iron present in the mash which immediately reacted with iron and made it non-accessible for the reaction with α-acids. *Svendsen* and *Lund* [51] reported that there are free binding sites for metal ions available in beer and it is most likely that there are even more binding sites in mash because there are more constituents in solution as opposed to beer. Accordingly, it can be anticipated that the additional iron was immediately bound and then removed with the spent grain.

From the free amino nitrogen data (Tab. 4), it can be observed that α-acid additions together with Fe^{2+} additions showed in fact an effect and provoked a distinct decrease of the mash FAN while no decline was seen when neither Fe^{2+} nor α-acids were added. Similar to α-acids, proteins or amino acids together with polyphenols form complexes with iron [52, 53] and it is therefore conceivable that α-acids together with mash nitrogen constituents and iron formed amorphous coagulates which subsequently precipitated. Hop α-acids appear to play a central role in these reactions as the effect was not observed when only Fe^{2+} was added. It is also interesting, though, that the effect was only seen when additional iron was brought into the mash. Schur and Pfenninger [4] reported no effect on the nitrogen composition in wort when adding hops to the mash; however, in their study also no additional iron was added.

The bitter units in trials were again very low and bitter substance yields ranged from 5–6 % which is in accordance with the previous findings (Tab. 3) and with literature data [3–5]. The extracts of the mashes were in the range of 13.95 to 14.04 % wt./wt. with deviations of at maximum 0.03 %, and differences in original gravity can thus not be accountable for the observations.

3.2 Influence of malt type and hop dosage on iron ion concentration of wort

Recent investigations demonstrated the hop dosage's strong influence on the iron concentration in wort and beer [8]. When aiming at a high efficiency of the hop dosage with regards to reaching a high precipitation of iron, it is crucial to know the maximum iron precipitation possible and at which amount of hops added this maximum is reached. An experiment was therefore designed in which a base wort's iron concentration was artificially increased by adding extra iron, and subsequently, various amounts of hop CO_2 extract were added to the wort at the start of boil.

In contrast to the trials where hops were dosed to the mash, in these trials, the iron additions yielded also a proportional increase of iron being detected. The recovery rates for iron were 55 % and 60 % for 100 µg/L and 200 µg/L of iron being added, respectively (Fig. 2).

The reduced iron concentrations in the worts after hop addition were clearly a function of the amount of hop CO_2 extract added at the onset of boil and the relative iron depletion was more pronounced, the more iron was present. At an addition of 200.9 mg/L of hop CO_2 extract, a maximum decrease of only 9 % was seen when no iron was added, and 33 % and 47 % when 100 and 200 µg/L iron ions were added, respectively. The response of the iron concentration plotted over the amount of hop CO_2 extract added followed an exponential descent and after an addition of 100.4 mg/L CO_2 extract (= 45.0 mg/L α-acids), > 90 % of the maximum iron depletion possible was already achieved in all trials while higher additions had only minor effects. Higher initial iron concentrations also resulted in little higher iron concentrations in the worts even at high hop additions which can be anticipated by a transition of 'free' iron ions into strongly bound forms which are not prone to be complexed by the hop constituents, in particular α-acids, anymore.

There is evidence that the malt type used has an influence on the wort's and beer's metal ion composition and concentrations [54,

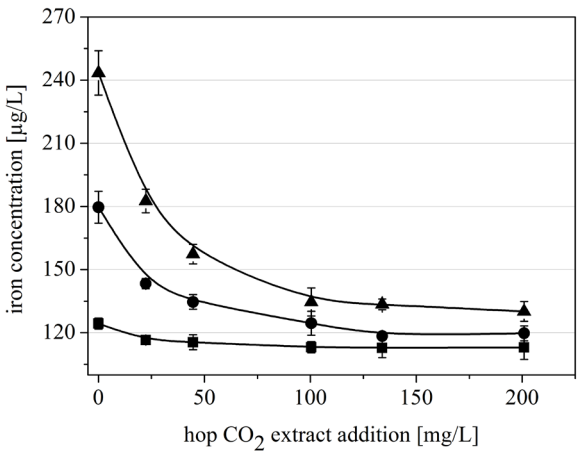

Fig. 2 Effect of hop CO_2 extract additions and Fe^{2+} additions on final iron concentration in wort. A base wort (■) was spiked with 100 µg/L Fe^{2+} (●) or 200 µg/L Fe^{2+} (▲) and brought to a boil. After 5 min, 0–200.4 mg/L hop CO_2 extract was added. Mean values are presented. Error bars represent ± 1 standard deviation. N = 3

Table 5 **Extract, pH value, and color of worts produced with 100 % Pilsner malt, an equal mixture of Pilsner malt and Munich malt type I, and 100 % Munich malt type I. Mean values ± 1 standard deviation are presented. N = 3**

		100 % Pilsner malt	50 % Pilsner malt 50 % Munich malt type I	100 % Munich malt type I
extract	[%-wt./wt.]	11.80 ± 0.06	11.87 ± 0.05	11.81 ± 0.03
pH-value	[–]	5.70 ± 0.02	5.41 ± 0.03	5.23 ± 0.02
color	[°EBC]	7.3 ± 0.3	18.6 ± 0.8	29.1 ± 0.4

55]. As a consequence from the previous trials' outcomes (cp. Fig. 2), it is conceivable that the hop dosage's influence and effectiveness also may change with the initial malt bill used and the wort composition. To test this hypothesis, trials were conducted in which worts were produced by using different initial malt bills: 100 % Pilsner malt; 50 % Pilsner malt, 50 % Munich malt type I; 100 % Munich malt type I. Hop CO_2 extract was then added at the onset of boil and the worts were incubated for 5 min prior to cooling them rapidly, and subsequent analysis.

Table 5 depicts the extracts, pH-values, and colors of the individual wort samples without hops added. While wort extracts were not influenced by initial malt bill used, the color increased when using higher proportions of Munich malt and the pH dropped which may be explained by the acidifying properties of certain Maillard reaction products [50].

As demonstrated in figure 3, the initial malt bill had a significant effect on the initial iron concentrations of the worts and increased with higher proportions of Munich malt (50 % Munich malt, 84 % increase; 100 % Munich malt, 202 % increase) (Fig. 3).

The hop dosage clearly caused a lowering of the iron concentration and its extent was strongly dependent on the malt bill used. The iron content decreased by 19 %, 29 %, and 59 % when 100 % Pilsner malt, an equal mixture of both malts, or 100 % Munich malt type I were used, respectively. It is not clear, though, if iron is present in a more 'vulnerable' form when using higher proportions of Munich malt type I, or if the iron reduction due to the hop dosage was more pronounced because a higher quantity of iron was present. Though, these data summed up imply that the direct binding capacity of iron in wort is much lower in comparison to mash. As a consequence, the proportional 'free' iron ion content with increasing munich malt additions is higher, and iron is then more 'vulnerable' to the hop acids' attack and complex formation.

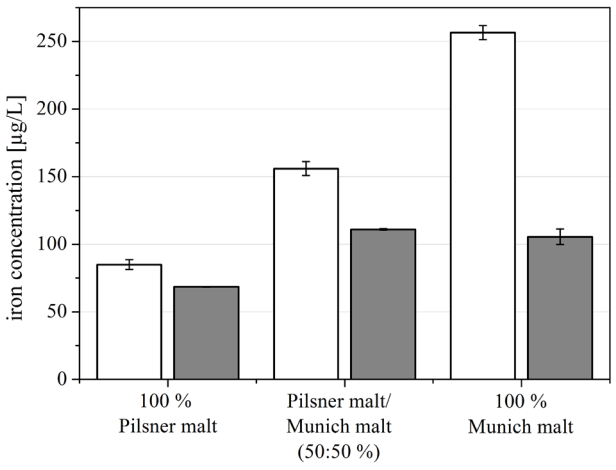

Fig. 3 **Effect of initial malt bill on iron concentration in sweet wort and iron depletion by hop CO_2 extract additions. Without hops added (□) and with 208.8 mg/L hop CO_2 extract added (■). Mean values are presented. Error bars represent ± 1 standard deviation. N = 3**

Taking all these data together thus far, the principal conclusions from these experiments are: first, an addition of ca. 45–60 mg/L hop α-acids can be considered as sufficient in terms of achieving a maximum iron precipitation during mashing or wort boiling at these experimental conditions such as e.g. the malt bill used, etc.; but secondly and more importantly, the hop dosage dependent iron removal is greatly dependent on the mash or wort matrix and the availability or 'vulnerability' of iron ions. The hop dosage should therefore be adapted to the individual wort matrix when aiming at a maximal iron precipitation at the onset of wort production.

3.3 Pilot-scale brewing trials

Based on these findings from the previous trials, brewing trials were designed and conducted in the institutes' pilot plant. The brews' hop dosages were chosen in a way to represent a high variety of points of addition and aiming at an increased oxidative stability of the final beers. In addition to the dosage points from the preliminary trials, first wort hopping and a continuous hop dosage were applied. Table 6 (see next page) depicts the beer analysis data from the duplicate brews.

The parameters original gravity, apparent extract, alcohol, apparent final degree of attenuation, color, pH, total nitrogen, free amino nitrogen, total polyphenols, and foam showed little variations within the treatments but no clear dependency on the type of hop dosage used. All values were in acceptable range for lager beers [56].

The hop bitter acid concentrations also displayed dissimilarities between the duplicate brews but an apparent influence of the hopping method used was observed. While α-acid and β-acid levens were both very low due to their limited solubility in beer [57], the total iso-α-acid concentration was generally lower than the reference when applying a divided hop dosage (DIV), first wort hopping (FWH), or a continuous hop dosage (CON). The iso-α-acid concentrations of the mash hopping (MAH) brews were dissimilar in both brews and was higher than the reference brew in the first brew and lower in the second brew. Concomitant to the iso-α-acid concentrations, the bitter units showed the same pattern and were 15–31 % lower as compared to the reference for the DIV, FWH, and CON brew in the first brews, and 4–17 % lower in the second brews. The brews where hops were dosed to the mash were either 4 % higher or 4 % lower than the reference. Though, when taking the total amount of hops used into consideration and looking at

Table 6 **Beer analytical data from beers as produced by applying different hopping technologies. REF, 100 % of hop CO_2 extract dosed at the onset of boil; MAH, mash hopping; DIV, divided hop dosage; FWH, first wort hopping; CON, continuous hop dosage**

		Brew series 1					Brew series 2				
		REF	MAH	DIV	FWH	CON	REF	MAH	DIV	FWH	CON
Original gravity	[%-wt./wt.]	11.27	11.41	10.94	11.36	11.12	11.14	10.89	11.11	11.21	11.08
Apparent extract	[%-wt./wt.]	1.83	1.90	2.01	2.06	1.96	2.31	2.03	1.99	1.97	1.95
Alcohol	[%-vol.]	4.99	5.03	4.71	4.92	4.84	4.67	4.68	4.82	4.88	4.82
Apparent degree of attenuation	[%]	83.7	83.3	81.6	81.8	82.4	79.2	81.4	82.1	82.4	82.4
Color	[EBC]	6.8	6.7	7.1	6.8	6.4	6.9	7.0	7.4	8.0	7.4
pH-value	-	4.40	4.37	4.31	4.41	4.35	4.30	4.33	4.35	4.37	4.33
Total N (12 %)	[mg/L]	738	667	671	631	694	717	733	770	808	724
FAN (12 %)	[mg/L]	79	82	58	81	72	73	75	89	90	72
NIBEM 30	[s]	231	229	226	213	215	243	239	222	215	220
Polyphenols (12 %)	[mg/L]	149	154	141	145	144	152	149	156	157	145
Hop iso-α-acids	[mg/L]	24.4	26.2	18.0	20.6	21.6	22.9	20.3	16.9	19.5	21.3
Hop α-acids	[mg/L]	< 1	1.0	< 1	2.3	< 1	< 1	< 1	< 1	1.1	< 1
Hop β-acids	[mg/L]	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Bitter units	[BU]	26	27	18	22	22	23	22	19	22	22
Bitter substance yield	[%]	29.9	19.1	18.2	24.4	24.4	25.6	15.6	19.2	24.4	24.4
SO_2	[mg/L]	4.2	3.9	3.5	3.8	3.5	4.7	3.2	4.1	3.4	3.2
Iron	[µg/L]	77	56	56	69	51	74	56	53	64	54
EAP-value	[min]	211	200	185	191	186	231	181	216	156	171
T_{600}-value [$*10^6$]	[–]	0.85	0.65	0.66	0.99	0.61	0.64	0.64	0.67	1.13	0.83

the overall bitter substance yield, the picture was different and the reference brews had the highest bitter substance yield from all trials. The mash hopping was the lowest followed by the divided hop dosage, while the first wort hop dosage and the continuous hop dosage displayed similar hop bitter substance yields of 24.4 % in both brewing series. Clearly, the total bitter substance yields suffered from later hop additions and in particular when adding hops during mashing.

The beers' SO_2 content ranged between 3.2 and 4.4 mg/L and were unaffected again by the hop dosages. These fluctuations are most likely dependent on the fermentation performance and yeast viability and not influenced by the hop dosage.

The beer's iron concentrations were clearly affected again by the hop dosage and were lowered by up to ~30 % when e.g. adding 50 % of the total hops at beginning of boiling and the remainder to be added continuously (CON). The mash hopping, the divided hop dosage, and the continuous hop dosage resulted in the lowest iron concentrations, followed by the first wort hop dosage. The reference brews were highest in iron throughout both brewing series. In consideration to the findings from the previous trials and literature data [8, 16], it is evident that the hop dosage's effect on the beers' iron concentrations can be traced back to the hop constituents' iron complexing properties.

The relation between beer constituents, the 'lag-time' or EAP-value and the radical concentration after a certain time measurement (T-value) is complex. While the EAP-value usually correlates well with substances that quench activated oxygen species such as e.g. the beer's SO_2 content, the T-value or radical concentration after a certain time is mostly influenced by substances that suppress or promote radical formation such as e.g. complexing agents or transition metals, respectively [39, 52, 58, 59].

In accordance with literature, the ESR measurements were affected strongly by the sample's SO_2 content, and it is therefore difficult to interpret the impact of the hop dosage as potential effects are most probably coped over by the samples' SO_2 content. To minimize the SO_2 influence on the measurement, the sample's SO_2 content can be artificially increased and the EAP-value is then divided by its SO_2 content giving the Beverage Antioxidative index (BAX) as an indicator for the sample's oxidative stability [60]. According to the method's functional principle, applied to this study, dividing the samples' EAP-value by their SO_2 contents gives an approximation of the BAX-values ranging from 50.2 to 53.1 $min*mg^{-1}*L$ in brewing series 1 and 45.9 to 56.6 $min*mg^{-1}*L$ in brewing series 2. With the exception of the FWH brew from brewing series 2, the BAX-value was always lowest for the reference brews, and the hop dosage modifications consequently yielded evidently an increase of the oxidative stability of the beers matrices. Thus, a lower 'SO_2-consumption rate' during storage can be expected by the brews produced using modified hop dosages in comparison to the reference brews.

The modified hop dosages also resulted in distinctly lower T_{600}-values (a measure for free radical levels) which can be ascribed to their effect on the beer's iron concentrations. The high T_{600}-values

Table 7 **Carbonyl contents of beers as produced by using different hopping technologies after prolonged storage of 12 weeks at 28 °C and from the fresh reference beers. REF, 100 % of hop CO_2 extract dosed at the onset of boil; MAH, mash hopping; DIV, divided hop dosage; FWH, first wort hopping; CON, continuous hop dosage**

	Carbonyl concentrations in µg/L[a]											
	Brew series 1						Brew series 2					
	REF (fresh)	REF	MAH	DIV	FWH	CON	REF (fresh)	REF	MAH	DIV	FWH	CON
3-methylbutanal	5.0	14.0	4.0	9.4	13.0	5.5	3.9	9.6	2.4	4.3	6.5	5.8
2-methylbutanal	2.9	9.3	3.4	5.7	8	4.7	2.4	6.2	1.9	3.4	5.1	4.8
2-furfural	1.7	53.0	18.0	12.0	18.0	14.0	2.0	44.0	14.9	12.8	18.7	16.0
methional	2.3	9.5	2.3	7.2	5.9	6.5	2.5	8.8	3.8	4.3	5.8	4.7
benzaldehyde	< 1.0	1.4	< 1.0	< 1.0	1.3	< 1.0	< 1.0	1.3	1.0	1.3	1.1	1.1
phenylethanal	2.6	5.3	2.4	3.1	4.8	3.3	1.9	3.9	3.3	4.1	4.0	3.5
ethyl nicotinate	3.2	7.4	6.2	3.6	9.3	3.7	2.8	5.6	9.1	4.9	6.7	4.5
γ-nonalacton	6.1	16.1	14.6	13.3	17.3	15.0	5.8	15.5	12.8	11.1	9.9	13.0
∑ aldehydes	23.8	116	50.9	54.3	77.6	52.7	21.3	94.3	49.2	46.2	57.8	53.4

[a] Carbonyl concentrations were calculated by using a single point internal standard procedure

of the brews where hops were dosed in the first wort (FWH) made an exception and there is no satisfactory explanation for this observation.

The described effects can be most probably traced back to the chelation and/or early removal of pro-oxidative metal ions due to the modified hop dosages. Kunz et al. [59] also found that higher iron values and higher contents of hop α-acids in beer promote deterioration reactions or improve the oxidative beer stability, respectively.

The beers' staling aldehyde concentrations were also determined and are depicted in table 7. From the fresh beers, only the reference brews were measured. After storage at 28 °C in the dark, the aldehyde concentrations of all beers were analyzed again. The reference brews exhibited the highest concentrations of all aldehydes measured after 12 weeks storage at 28 °C with the exception of benzaldehyde where concentrations were alike. The aldehyde levels as detected in the second brews were generally little lower than those in the first brews. Interestingly, the MAH beers from both brews displayed very low total aldehyde concentrations, and only the DIV beers from brewing series 2 were lower than the MAH beers' aldehyde concentrations from brewing series 2. This may be explained by the suppression of oxidative reactions already during the early process steps of wort production. Furfural, which is typically a good indicator for a sample's exposure to heat [61, 62], was also influenced by the hop dosage. However, recent findings from [63] also found a direct interaction between hop constituents and the formation of furfural in model solutions at wort boiling conditions thus confirming the observations from this study.

Summing up all aldehydes measured and comparing the brews within the two brewing series revealed a reduction of 43.9–66.9 % and 48.7–60.9 % as compared to the reference from brew series 1

Table 8 **Sensory analysis of fresh beers and of aged beers (28 °C, 12 weeks) as produced by using different hopping technologies. REF, 100 % of hop CO_2 extract dosed at the onset of boil; MAH, mash hopping; DIV, divided hop dosage; FWH, first wort hopping; CON, continuous hop dosage**

		Fresh beers					Aged beers (28 °C, 12 weeks)				
		REF	MAH	DIV	FWH	CON	REF	MAH	DIV	FWH	CON
Brew series 1	Purity of odor	4.6	5.0	4.8	5.0	5.0	3.2	4.3	4.2	4.1	4.2
	Purity of taste	4.5	5.0	4.0	4.7	4.6	3.2	4.4	4.5	4.3	4.3
	Palate fullness	4.6	4.6	4.0	5.0	4.8	4.4	4.5	4.2	4.5	3.6
	Freshness	4.6	5.0	4.4	4.4	5.0	4.3	4.7	4.5	4.6	4.4
	Quality of bitterness	4.0	4.4	4.0	4.4	4.7	3.2	4.5	4.3	4.0	4.6
	DLG scores[a]	39.5	46.5	36.8	44.8	46.5	24.2	39.6	37.6	35.9	36.6
Brew series 2	Purity of odor	4.3	4.5	4.2	4.6	4.8	3.7	3.3	4.4	4.3	4.3
	Purity of taste	4.5	4.6	4.5	4.6	4.5	3.8	3.1	4.1	4.1	4.4
	Palate fullness	4.4	4.5	4.5	4.7	4.6	4.0	4.0	4.0	4.3	4.0
	Freshness	4.6	4.4	4.6	4.4	4.6	4.3	4.1	4.4	4.6	4.6
	Quality of bitterness	4.4	4.7	4.8	4.4	4.7	4.0	4.0	3.9	3.9	4.0
	DLG scores[a]	39.2	41.7	40.4	41.4	43.6	30.5	25.9	34.8	35.3	36.3

[a] For calculation of DLG scores consult the materials and methods section

and 2, respectively. Even though the individual concentrations did not exceed their respective odor or flavor thresholds, the hop dosage's effectiveness in diminishing their concentrations was still apparent and the modified hopping technologies used were clearly superior in comparison to the brews where hops was solely dosed at the beginning of wort boiling.

Based on previous studies and literature data, the hops' strong reducing effect may be traced back to the following factors: Firstly, the hop constituents' reactions with iron consequently counteract the ferrous iron's catalytic actions in the Fenton and Haber-Weiss reaction systems thereby diminishing oxidative reactions during wort boiling and in beer. Wietstock and Methner [43] proposed a pathway for the formation of Strecker aldehydes from their parental amino acids by hydroxyl and hydroxyethyl radical attack, and this pathway is accordingly blocked or diminished when radical formation is lowered. This is supported by findings from Wietstock and *Shellhammer* [16] who demonstrated that particularly hop α-acids are capable of abating hydroxyl radical formation by complexing iron ions. Hop α-acids can also react with copper ions thus counteracting radical formation while other metal ions were shown to be unaffected [22] which may add additional effectiveness in counteracting the radical-provoked formation of staling aldehydes.

Secondly, but not less importantly, hop constituents may react with intermediates in the Maillard reaction or block or inhibit oxidative pathways of the Maillard reaction as recently suggested in [63]. This assumption is supported by the observation that the formation of furfural was also affected by the used hopping technology. Furfural can be formed in the Maillard reaction from pentose sugars such as xylose [64, 65] but was also shown by Rakete et al. [66] to be derived through an oxidation-mediated pathway from hexose sugars such as maltose via 3-desoxypentoson as reactive intermediate. Here again, the hop constituents may be capable of blocking or diminishing this oxidative pathway because of their reactions with transition metals.

Complementary to the aldehyde analysis, a sensory analysis of the fresh and aged beers was carried out. The data from the sensorial analysis is depicted in table 8. While no off-flavors were detected in all fresh beers, after storage, the reference beer from the brewing series 1 was clearly down-rated and off-flavor impressions were described as 'oxidized' as noticeably detected by all panelists. The beers as produced using the modified hopping technologies were also down-rated but all of them were still considered to be 'vendible' (rating > 3).

The fresh beers form brewing series 2 were rated between 4.2 and 4.8 implying that no defects were present. The reference beer was down-rated again after prolonged storage and was marked again with the descriptor 'oxidized' by all panelists. The beer which was produced using mash hopping got also ratings of < 3. This cannot be explained by the experimental data because the aldehydes measured were low, and also no other defects were observed. All other beers (DIV, FWH, CON) were rated lower than the fresh beers but again were still considered 'vendible'.

In terms of the 'DLG scores' and when comparing fresh and aged samples, it is obvious that REF beers from both brew series showed the most pronounced decrease of the overall sensory attributes (with the exception of MAH from brew series 2) upon ageing whereas the modified hoppings, and in particular the DIV beers, were more resistant against the appearance of staling characteristics.

4 Conclusions

Taking all these data together, clearly, the modified hop dosages were superior in suppressing staling as compared to a beer produced with hops dosed solely at the beginning of wort boiling. The hops effectiveness can be partly traced back but is not necessarily limited to the hop α- and β-acids' ability to suppress oxidative reactions during wort production which, in turn, abates the formation of staling aldehydes. The amount of hops dosed and the points of addition used clearly need to be adapted to the wort matrix to achieve a high efficiency. At this, the impact from the malt used and the availability or 'vulnerability' of metal ions for the interaction with hop α-acids appeared to be from great importance. *Lüers* [67] mentioned in 1950 that the addition of hops to the mash is a 'substantial waste'. This seems to be valid as related to the hop bitter acid utilization; yet, when taking the results from this study into consideration, this statement has to be qualified again as the positive effects on beer flavor stability were not known. Still, high losses have to be taken into account when adding hops to the mash. The continuous hop dosage was found to be best compromise between hop bitter substance yield and improved oxidative stability because only little lower bitter substance yields were found in comparison to the reference brews while the beers were clearly superior as related to their oxidative stability.

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4 Summarizing discussion

Freshness is a key attribute for beer and the brewing industry is therefore faced with the problem of beer flavor instability. There is the clear mission to deliver a product which tastes fresh and is resistant to flavor changes as long as possible. The present dissertation work was therefore devoted to oxidative reactions occurring during beer production and during ageing of beer, as well as to their suppression by target-oriented modifications of the hop dosage towards the goal to improve the resistance of beer against staling.

Substantially, the thesis was split into two parts which are conclusively closely related to each other, though their validity and significance are not necessarily limited to the findings, consequences as well as the connections of the content-wise separated parts, and both parts thus have their own *raison d'être*. The experimental work done within the scope of this thesis gave rise to six internationally accepted peer-reviewed publications. In the following chapter, the work is summarized and put into context. Critical points are addressed, the work is concluded, and the meaning of the findings for the food and brewing industry is clarified.

4.1 Oxygen radical-mediated formation of aroma-active aldehydes

The first part of the dissertation work comprises the publications A[283] and B.[281] Due to literature observations that oxygen promotes the formation of certain staling compounds and the evidently strong deteriorating character of oxygen-derived radicals during beer ageing, a direct connection between both, reactive oxygen species and the formation of staling compounds was suspected. Thorough literature research revealed a coherent pathway originally proposed by Stadtman[238] for the formation of Strecker aldehydes by transition metal-mediated oxidative degradation of their parent amino acids. It was to examine if this pathway is also valid for beer and wort environmental conditions as Stadtman and colleagues worked in human cell-related milieu.[238]

In the first publication of this dissertation work, the experimental design used from Stadtman and co-workers[238] was therefore transferred to beer environmental conditions. Experiments were started in a reduced beer matrix and a buffered model system was used in order to exclude potential side reactions of the complex beer constituents being irrelevant for the mechanism to be investigated. Solvent-assisted flavor evaporation technique (SAFE) was applied to isolate volatile aldehydes from buffered model solutions and was coupled with gas chromatography (GC) - mass spectrometry (MS) for the separation and quantitation of target molecules. This

procedure was a well-established analytical method at the TU Berlin's laboratories at the time when the thesis was carried out and it ensures a gentle sample treatment as well as a good recovery of analytes.[68] Electron spin resonance (ESR) spectroscopy with α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN) as spin trap was used to measure radical levels in solutions because it represents a contemporary and highly sophisticated method for the identification of hydroxyl and hydroxyethyl radicals in solution.[152, 154] In contrast to N-t-butyl-α-phenylnitrone (PBN), which is a wide-spread spin trap agent used in the brewing industry, POBN shows a higher affinity and stability in trapping hydroxyl and hydroxyethyl radicals. Lower concentrations of the spin trap are therefore needed than when using PBN to achieve similar ESR signal intensities, which has the concomitant advantage that only slight pH changes of the system are experienced in practice. Since the Fenton reaction rate is strongly pH dependent[182], an alkaline pH shift is anticipated to result in higher reaction rates and therefore in a faster depletion of the beer's endogenous antioxidative potential. Moreover, the mode of beer ageing appears to be significantly pH dependent[98, 137] and changes of system's pH may therefore lead to erroneously construed results. Even though using POBN requires longer ESR run times than when using PBN, the lack of a pH change produces more accurate results.[119]

Mixing leucine with the Fenton reagents H_2O_2 and Fe^{2+} at beer environmental conditions (pH 4.5, 5 % v/v ethanol) and incubating the mixture at 20 °C yielded 3-methylbutanal. In accordance with this finding, 2-methylbutanal was derived from isoleucine, and phenylacetaldehyde and benzaldehyde were derived from oxidative degradation of phenylalanine (cp. Figure 1, publication A[283]). This observation was explained by the consequences of hydroxyl radical formation in course of the Fenton reaction, which were anticipated to react directly with amino acids thereby yielding Strecker aldehydes with one carbon atom less than the corresponding amino acid, or which react first with ethanol giving rise of hydroxyethyl radicals that subsequently also yield Strecker aldehydes after oxidizing amino acids. Further evidence of the involvement of free radicals in the reactions yielding aldehydes was demonstrated in subsequent trials where a linear relationship between the Strecker aldehydes formed and radical concentration was identified (cp. Figure 3, publication A[283]). Reactions were started by the addition of H_2O_2 and a complex of ethylenediaminetetraacetic acid (EDTA) with Fe^{2+} in an equimolar ratio was used to catalyze radical generation instead of using solely Fe^{2+}. By using this procedure, side reaction and precipitation of iron are diminished.[28, 97] When Fe^{3+} instead of Fe^{2+} was used, no or only little amounts of aldehydes were found and no radicals were detected by ESR spectroscopy. This

observation clearly supports the theory of the involvement of radicals in the formation of Strecker aldehydes from amino acids as Fe^{3+} is incapable of acting as a catalyst in the Fenton reaction.[131]

In sum, the results were well in accordance with published claims that aromatic amino acids, such as phenylalanine are better `sinks' for radical attack[87, 265], though, also leucine and isoleucine were shown to be clearly susceptible to free radical attack. Further experiments even strongly suggested the involvement of hydroxyethyl radicals in the reaction as higher ethanol concentrations in the solutions also yielded a linear response and increase of Strecker aldehydes being formed (cp. Figure 4, publication A[283]). A possible pH effect as provoked by increasing ethanol concentrations could be excluded.

Taking all these data together, a pathway initially suggested by Stadtman[238] could be adapted to beer environmental conditions, and furthermore, was expanded to the involvement of hydroxyethyl radicals in the reaction cascade. A reaction mechanism valid for beer systems was therefore proposed (cp. Figure 5, publication A[283]). The existence of this pathway could be verified in a beer-like model system, and preliminary assumptions, that reactions of ROS with amino acids can yield aroma-active Strecker aldehydes, could be confirmed.

As a consequential step, knowledge as gained from this first publication was transferred to actual beer production and storage. Numerous publications propose reactions yielding carbonyl compounds and the appearance of off-flavors during beer ageing, though only a small number of pathways may serve as an explanation for the detrimental role of oxygen in promoting beer staling. The benighted state of the actual relevance of these pathways and the complex beer matrix make it difficult to study and clarify the relevance of the proposed pathway from publication A[283] for beer staling, also because e.g. detectable intermediates in the Strecker or Strecker-like reaction are similar whether they are derived from ‘ordinary’ Strecker degradation as initialized by α-dicarbonyl compounds[61, 224], or by Strecker-like reactions, as initialized e.g. by *ortho*-quinones.[215, 222]

In coherent consequence of these premises, it was thus decided to approach the proof or disproof of the proposed pathway by application of statistical methods in order to narrow down the probability of this pathway to be relevant during beer ageing. Response surface methodology (RSM) and central-composite rotatable designs (CCRD) were the statistical methods of choice. RSM is a very powerful tool for which a response of interest is influenced by several factors. An empirical model can then be established and mathematical functions are applied to describe

the system which is investigated. The significant factors are then identified and pathways can be anticipated with a calculated probability. Finally, as an add-on feature, optimization of the response variable in dependency of the factors involved is possible.[187]

Results and deductions of the findings were published in the *Journal of Agricultural of Food Chemistry* (publication B[281]). The experiments were planned and conducted over a long period in a step-to-step approach. Consecutive storage trials were carried out at 'gentle' storage temperatures of 20 °C or 28 °C and in the dark to exclude the potential effects of elevated temperatures and/or light exposure, respectively.

In a first storage experiment, which was conducted over prolonged storage of 18 months at 20 °C in the dark, the potential role of the Maillard reaction and particularly α-dicarbonyl compounds in terms of promoting the formation of Strecker aldehydes was investigated. A mixture of xylose, a pentose sugar, and glucose, a hexose sugar, were thus varied along with amino acids (a mixture of leucine, isoleucine, and phenylalanine) and Fe^{2+}. If α-dicarbonyl compounds as generated from xylose and glucose were the initiators of Strecker degradation and thus origin of Strecker aldehyde formation during beer storage, a positive factorial interaction between the independent factors sugar concentration and amino acids was to be expected. Fe^{2+} was also implemented in the study because it was reported to promote the Maillard reaction[70, 89, 111, 194, 212, 264] and beer ageing[131, 134, 147, 155, 296], and may therefore also play a promoting role in α-dicarbonyl-mediated beer staling reactions.

The only significant factor for Strecker aldehyde formation found in this storage trial was the factor amino acid concentration, which was a first indication that a *de novo* formation in the bottles occurred, and that this reaction was affected by the availability of its precursors (cp. Table 4, publication B[281]). Beer can be exposed to high temperatures e.g. during oversea transport[200], and the Maillard reaction may have more importance then. Also, carbohydrates other than glucose and xylose may be more reactive in promoting Strecker degradation, and a potential role of the Maillard reaction for Strecker aldehyde emergence during beer ageing can therefore clearly not be ruled out from this experiment. Yet, glucose and xylose concentration was in fact found to be a significant factor ($p = 0.046$) for 2-furfural formation thus demonstrating that the Maillard reaction occurred to some degree in this storage experiment, though it was clearly not responsible for the formation of Strecker aldehydes. Two pathways may explain the dependency between 2-furfural formation and xylose/glucose levels in these experiments, non-oxidative xylose degradation[13, 51] and oxidative hexose degradation.[211]

However, this result was not further investigated and it was not broken down to which pathway or carbohydrate was responsible.

Because carbohydrate concentration was not a significant factor for Strecker aldehyde formation, the approach was now shifted towards a free radical-mediated pathway as previously suggested in publication A.[283] Findings from a subsequent storage trial demonstrated that oxygen presence in beer indeed only affected Strecker aldehyde formation and none of the other carbonyl compounds measured. Exposing beers to oxygen during bottling and storing the beers for 30 weeks at 28 °C resulted in increasing Strecker aldehyde levels from 44 % (methional) of up to 583 % (benzaldehyde) as compared to a reference where oxygen was omitted (cp. Figure 1, publication B[281]). Interestingly, in accordance to the previous trial (cp. Table 4, publication B[281]), the addition of Fe^{2+} had no effect on the content of carbonyl compounds after prolonged storage. Aside from this observation, data implied that the effects were derived from *de novo* formation of Strecker aldehydes from their parent amino acids during storage, and there was additionally an indication that *de novo* formation started once reactive oxygen species were present (when SO_2 was < 0.1 mg/L). Yet, clear evidence of a *de novo* formation of Strecker aldehydes was still outstanding, and was therefore further investigated by working with labeled amino acids. D^5-labeled phenylalanine was the amino acid of choice because of its deuterated benzene ring which is very stable and thus not very likely to be degraded during beer storage, and also because phenylalanine was shown before to be a good 'sink' of oxygen radical attack.[87, 283] Exactly adjusting different total packaged oxygen concentrations in the bottles during spiking was vital in order to demonstrate the involvement of oxygen in the reaction. This premise was successfully accomplished by working in an inert gas glove box, adjust it to the desired atmospheres, and package beers then under defined oxygen concentrations (see supplementary material, publication B[281]).

One possibility for the emergence of aldehydes during beer storage is the liberation from a bound state. In fact, many studies point to the significance and relevance of a pool of intrinsic aldehydes.[19-21, 27, 67, 136, 160, 266] While an imine formation between amino acids and aldehydes was disproved by Baert and co-workers[20], a release of aldehydes from bisulfite complexes as initially suggested by Barker et al.[27], and confirmed by others[20, 21, 27, 136], is strongly evident to play a role during beer staling. Because bisulfite can protect beer from staling in two ways, by blocking oxidative chain reactions through quenching of ROS, and by the aforementioned complex formation between bisulfite and aldehydes, it was decided to wait until the base beer's

bisulfite concentration was < 0.1 mg/L (the method's LOQ) before preparing the samples, and starting the storage trial.

As a clear emphasis of this study, total packaged oxygen concentration could be identified as a major significant factor for *de novo* formation of Strecker aldehydes during these experimental conditions as verified by measuring the labeled derivate aldehydes phenylacetaldehyde-D^5 and benzaldehyde-D^5 from D^5-labeled phenylalanine (cp. Figure 2, publication B^{281}). Strecker aldehyde levels after storage and total packaged oxygen even featured linear relationships with model R^2 ranging from 0.82 to 0.93. All Strecker aldehydes measured were clearly significantly ($p < 0.05$) affected by the factor total packaged oxygen with the exception of labeled benzaldehyde-D^5, most likely because this Strecker aldehyde was only found at concentrations < 1.0 µg/L. Fe^{2+} concentration was insignificant for all responses again with the exception of 3-methylbutanal where it was kept in the model at $p = 0.0879$ (cp. Table 5, publication B^{281}). All other carbonyl compounds measured were not affected from both, the factor total packaged oxygen and the factor Fe^{2+} concentration.

The insignificance of Fe^{2+} in all trials in fact queried to some degree the proposed pathway in terms of being relevant for beer ageing as Fe^{2+} is clearly promoting free radical generation. This behavior was finally hypothesized to be originated from the assumption that Fe^{2+}, once it's oxidized to its higher valence state, Fe^{3+}, is rapidly reduced again by reducing substances present in beer, thus being available again for its catalytic action in the formation of ROS. Fe^{2+} can therefore be anticipated to be the predominant iron species in beer which is also in accordance with literature data.[131, 155, 166] In terms of reducing substances, numerous compounds are conceivable and comprise e.g. intermediates of the Maillard reaction[45, 113, 138, 147, 155, 191] or polyphenols with prooxidative character[2, 50, 124, 221, 285] which is anticipated to not only affect radical formation in the Fenton reaction system but also in the activation of atmospheric activation of oxygen (see also chapter 1.3). An additional explanation for the insignificance of Fe^{2+} may be that oxygen and particularly activated oxygen can be assumed to always being the rate limiting factor in bottled beer, only little amounts of Fe^{2+} are needed to catalyze radical formation; or in other words, Fe^{2+} is anticipated to always being in excess as compared to activated oxygen, and oxygen is thus the Fenton reaction's limiting factor in bottled beer. As a consequence of these hypotheses, additional Fe^{2+} additions have no effect which adequately explains the observations from the RSM experiments.

Yet, for the proposed reaction to be valid, Fe^{2+} must still be crucial because it is required as an electron carrier for oxygen activation and as a catalyst in radical generation. Excess EDTA was

therefore added to beers in a supplementary trial in order to withdraw Fe^{2+} and Fe^{3+} ions completely from the Fenton reaction system. In fact, significantly diminished Strecker aldehyde formation was detected and *de novo* formation of D^5-labeled aldehydes was completely suppressed when EDTA was added (see Table 6, publication B[281]). Concomitantly, capping beers with oxygen barrier crown corks diminished also *de novo* Strecker aldehyde formation thus demonstrating the consequences of oxygen which diffuses through the crown cork liner materials when using standard crown corks without oxygen barrier functionality. Oxygen barrier crown corks can therefore be considered a good measure to diminish oxygen-mediated *de novo* formation of Strecker aldehydes during beer storage.

In coherent consequence of these findings, oxygen exposure or minimization was shown to also having a clear effect on Strecker aldehyde formation during wort production (cp. Table 7 and 8, publication B[281]), however it must be considered that particularly during mashing and partly also during heating of wort to the onset of boil, also enzymatic reactions may play a role.[241] The results are still in reasonable agreement with studies from Narziß, Reicheneder, and Bauer[189] who found that beers which were produced under oxygen avoidance were superior as related to their analytical and sensory quality. Even though, diminishing oxygen during wort production is not a new technological measure given, the only industrial publication published as related to the influence of oxygen during mashing and wort production and suggestions for practical measures to decrease oxygen was published by Zuercher and Gruss.[294]

Taken all these data together, further evidence for the validity of the proposed reaction was delivered and a complete reaction mechanism could be drafted (cp. Figure 3, publication B[281]). These findings expand the knowledge of previously reported beer storage mechanisms by of yet unexplored effects of beer constituents and furthermore complement proposed pathways from literature where oxygen or ROS are involved. Yet, the existence of the pathway remains hypothetical, not at least because radical intermediates were not possible to be identified and an adduct- or product-targeted characterization was also impossible because products from the proposed reaction are similar to those from ‘ordinary’ Strecker degradation.

Deductions from these results certainly do not arrogate to be exclusively responsible for the storage-induced appearance of Strecker aldehydes during beer storage. In fact, because of the relatively little formation detected (6.3 µg/L phenylacetaldehyde-D^5 at 20.99 % oxygen in atmosphere during spiking), *de novo* formation during storage may only play a subordinate role for the appearance and emergence of Strecker aldehydes during storage. Modern bottling machines can achieve total packaged oxygen concentrations of < 10 µg/L, and the relevance of

an oxygen-induced *de novo* formation of aldehydes is therefore debatable. Still, when bottles were prepared at minimized oxygen concentrations, an amount of 1.6 µg/L phenylacetaldehyde-D^5 was formed *de novo* during a period of 12 weeks of storage. Because storage experiments were carried out at moderate storage temperatures of 28 °C, and in a limited storage time of 12 weeks, it can be anticipated that the relevance of a *de novo* formation is emphasized when there is faulty oxygen-pickup during bottling, when beer is exposed to higher storage temperatures, e.g. during oversea transport, or when it is stored for longer periods of time. Still, other mechanisms, such as the liberation from bisulfite[20, 27, 67, 136] or 2-substituted 1,3-thiazolidine-4-carboxylic acid complexes[21], may be of more importance which points to the well-known truth that measures against staling reactions should be taken early on during the brewing process and by the choice of the raw materials. Despite that, it is unambiguous that ROS play a detrimental role during beer ageing, wort production, or already during malt production. The fact that oxygen is known to have a deteriorating character during beer storage together with the detection and characterization of the role ROS, and eventually, the proposed reaction mechanism unmistakably point to the need of the brewing industry to minimize oxygen pickup in every step during beer production where oxygen is not a necessity.

Measure to avoid oxygen-pickup comprise e.g. the use of deoxygenated water, the flushing of product-sided pipes, tanks, and equipment with inert gases as well as the avoidance of oxygen-pickup during filling, and eventually the use of packaging with very low or no gas permeability. Furthermore, as a reasonable deduction of this work, amino acid concentrations in finished beer and during wort production should be as low as possible. Processing of barley with low protein content, partial suppression of proteolysis during malting by e.g. steeping at low temperatures, enriching CO_2 at the end of germination, and withering at low temperatures are potential steps during malting.[186] Mashing-in at high temperatures, e.g. > 60 °C, thus skipping proteolysis during mashing is an advisable technological measure in the brewhouse.[39] However, attention should be paid that the free amino nitrogen content of wort is not too low as the amino acid pool is certainly vital for the yeast and for the fermentation performance. A free amino nitrogen content of 120 mg/L was found to be the minimum level possible.[176]

It is clear that the results from the buffer system (cp. results from publication A[283], and Figure 4, publication B[281]) were different than those from actual beer storage (see beer storage data from publication B[281]) as the products from the pertinent reactions are eventually a result of the availability, abundance, and liability of reaction partners. Beer contains numerous substances other than amino acids which can potentially react with hydroxyl and hydroxyethyl radicals,

and above all, also a numerous antioxidants that are capable of quenching those radicals.[11] As compared to actual beer, the buffer system used in publication A[283] contained only amino acids and the Fenton reagents H_2O_2 and Fe^{2+}. All trials in publication A[283] were done with high concentrations of amino acids and were started by the addition of H_2O_2, which is, in an actual beer environment, formed in course of a complex reaction cascade that is still far from being completely understood. Future research on oxidative beer flavor stability should therefore also attempt monitoring and examining the activation and consumption of oxygen therefore approaching the problem of oxidative flavor instability from the oxygen activation, so to say from the 'front-end' of oxidative chain reactions. It is evident that the activation of oxygen needs the presence of a complex electron transport system and there is an indication that amongst other substances, polyphenols may play a crucial role.[2] The presence of transition metals functioning as electron carriers may be also essential.[50, 205, 296]

Additionally, as aforementioned, oxygen is mostly present in very low concentrations in bottled beer and it is therefore clear that other factors can be limiting in beer during storage than in buffered model solutions where all reagents are added in desired quantities and oxygen or oxidizing agents are abundant. This is also a reasonable explanation why e.g. the concentration of the Fenton reagent, in this case ETDA-Fe^{2+}, had an effect on the amount of aldehydes being formed in the model systems (cp. Figure 3 in publication A[283]) while Fe^{2+} concentration was an insignificant factor in RSM experiments during actual beer storage (cp. publication B[281]). At this, it could be of major interest to study the Janus-faced role of reducing agents in terms of exerting pro- and antioxidative character by interaction with metal ions in beer in greater detail.[2, 42, 44, 45, 113, 155, 204, 208, 226, 292]

Knowledge from these studies may also be transferred to other food systems. For instance, in wine, environmental conditions are comparable to those in beer, and observations from published studies are very similar to those found in beer.[40, 69, 231, 255] Similar mechanisms can be anticipated and similar measures should be taken to improve product quality.

4.2 Anti-staling effects of the hop dosage and optimization of the hopping technology

As a lucid outcome of the findings from publication A[283] and B[281] but not essentially limited to the damaging role of ROS, the purposeful utilization of constituents with antiradical character come into effect. **The second part** of this dissertation work therefore focused on the hop dosage and in particular hop acids in terms of their capability and mode of exerting their antioxidative properties and particularly as related to their function to diminish ROS-related reactions.

The work on hop antioxidants and their application in the brewing process comprises four publications which are listed in the chapter 3.2 of this dissertation work. The hops fundamental mode of action was characterized analytically in publication C[276], D[282], and E.[273] The objective evidence of the hops' impact on oxidative beer flavor stability, the practical applications and consecutive effects were investigated in publication F.[280] The publications' content, context, and relation with findings from chapter 3.1 are now summarized in the following section.

In publication C[276], the hop α-acids and *iso*-α-acids' mode of action were further characterized. Quenching of ROS (Figure 2, pathway a) from hop constituents could be excluded because the addition of hop acids did not result in an EAP-value or 'lag-time' during ESR measurements[250, 274], which is an indication for scavenging of activated oxygen forms.[119] This work therefore focused on iron complexation and hydroxyl radical scavenging (Figure 2, pathway b and c). A Ferrozine assay was applied to test the hop acids' capability to complex Fe^{2+} ions. The hop acids' effectiveness against hydroxyl radicals was assessed by quantitating the extent of oxidative degradation of 2-desoxyribose (2-DR), a marker substance for hydroxyl radical attack. Both assays are simple and when adequately combined, allow gaining valuable information for a test compounds' antioxidative mode of action.

At a glance, both hop α- and *iso*-α-acids were shown to be able to diminish 2-DR oxidative degradation, but α-acids were clearly more efficient. Because an inverse relationship between their protective effect and Fe^{2+} concentration was found (cp. Figure 4, publication C[276]), it was anticipated that their effectiveness was related to their Fe^{2+} complexation functionality. This was further verified by using a Ferrozine assay[43] and by observing the hop acids' spectral changes when mixing them with Fe^{2+}. These data additionally suggested that α- and *iso*-α-acids can trigger autoxidation of Fe^{2+} to Fe^{3+}, and furthermore, stabilize iron in its higher valence state.

By using an EDTA-Fe complex to generate $^{\bullet}OH$, instead of using Fe^{2+} only, $^{\bullet}OH$ radical scavenging (Figure 2, pathway c) could be tested without having complexation of Fe^{2+} (Figure 2, pathway b) interfering. The use of such EDTA-Fe complexes provides useful properties for studying radical-initiated decomposition reaction including e.g. the ability to keep iron soluble and reactive in solution[72] and allowing the complex to participate in both the oxidative and reductive steps of Fenton-related reactions.[93] Though, one disadvantage of using the EDTA-Fe complex is that it may be idiosyncratic and thus may not be applicable to biologically occurring iron complexes.[96] Also, this assay is inapplicable to certain compounds such as e.g. strong iron chelators that can withdraw iron from EDTA.[103] Because binding and withdrawal of iron from

EDTA by hop α- and *iso*-α-acids was unlikely to happen, also because EDTA appeared to be the stronger iron chelator (cp. Figure 7, publication C[276]), this assay was decided to be applicable to be used to study •OH scavenging. Furthermore, typical •OH scavengers that were also tested reacted as expected and diminished 2-DR oxidative degradation (cp. Figure 5, publication C[276]). Yet, both hop α- and *iso*-α-acids did not exert any capability to abate 2-DR oxidative degradation and it was therefore followed that they do not possess •OH scavenging functionality at these experimental conditions and concentrations tested. This contradicts claims from Liu et al.[164] who indeed reported •OH scavenging activity of hop α-acids. Though, in their study, a phenanthroline assay was used, which also utilizes the Fenton reagents H_2O_2 and Fe^{2+}, and Liu et al.[164] may not have considered the hop acids' Fe^{2+} complexation properties.

Interestingly, *iso*-α-acids showed also antiradical characteristics and prevention of 2-DR oxidative degradation even though they were not showing effectiveness in studies using ESR spectroscopy.[250, 274] It may be hypothesized that the *iso*-α-acids' iron chelating functionality towards iron ions is not active in suppressing •OH generation in 'real' systems. This may be traced back to the nature of the complex. Open structure complexes such as e.g. equimolar EDTA-Fe^{2+} complexes allow reactions with H_2O_2 and release of •OH into the solution.[95] As opposed to α-acids who were claimed to form coordinative bonds with iron ions[282], *iso*-α-acids were reported to form sandwich complexes with ferrous iron[77], which may, because of its open structure, still be accessible to H_2O_2 and therefore not sufficiently prevent oxidative damage to •OH acceptor molecules. The *iso*-α-acid-Fe-complex may also be not as stable as the α-acid-Fe-complex and may therefore lose effectivity when e.g. exposed to higher temperatures such as during wort boiling or during ESR measurements. Additionally, *iso*-α-acids do not possess the same functional group as α-acids such as the six-member ring structure, and formation of antiradical, resonance-stabilized phenoxy radicals as suggested by Ting et al.[250] is therefore not possible. This is of interest as the oxidation of Fe^{2+} to Fe^{3+} was reported to also yield a concomitant oxidation of the ligand possibly due to site-localized oxidation reactions.[102] Hop α-acids can thereby form resonance-stabilized phenoxy radicals when oxidized. Hop *iso*-α-acids do not possess this functionality and even though they trigger oxidation of Fe^{2+} to Fe^{3+}, they cannot stabilize the loss of an electron, become a reactive radical species themselves, and may therefore allow or trigger further radical reactions. The fact that they promote autoxidation of Fe^{2+} and formation of a radical species would even imply prooxidative behavior as observed in ref. 274.

Taken these data together, clearly, one of the main features of hop α-acids appeared to be their iron ion complexation functionality, though reactions of hop acids with other metal ions were also imaginable which potentially can have also negative consequences. Metal ions and the beer's ionic composition play a vital role for the brewing process, the fermentation, and eventually for beer quality. The experimental work load performed within the scope of publication D[282] was done to narrow down the hop acids complexation functionality towards different metal ions. UV-VIS spectroscopy was the method of choice to study the hop acids complexation characteristic because hop α-acid-complexes with iron or copper show absorbance at 550 nm while sole hop α-acids do not. This wavelength can therefore be used to study the complex's chemistry. A further feature was that hop α-acid-Fe-complexes could be removed by syringe membrane filtration using a pore size of 0.45 µm. This characteristic was used to test again the hop acids' ability to remove metal ions from solution by mixing them with metal ions of interest, and after incubation and filtration, measure the metal ion concentrations at different pH values in the retantate by using ICP-OES. The results and the pH effect are discussed in detail in consideration of the metal-interactions in publication D.[282] Based on the data, a structure of the hop α-acid-Fe-complex could be proposed (cp. Figure 4, publication D[282]). It was anticipated that the delocalized electron from the α-acid's cyclic *β,β'*-triketone skeleton forms a coordinative bond with the metal ion thus resulting in an uncharged complex. By observing the structure of the complex, it becomes clear that Fe^{2+} must autoxidize to Fe^{3+} when reacting with α-acids thus allowing the formation of three coordinative bonds, which is in good agreement with results from publication C.[276] Steric hindrance may suppress further ligands to bind to the metal ion.

Outcomes from publications C[276] and D[282] provide substantial information that one of the main characteristics of in particular hop α-acids lies in their principle complexation functionalities towards prooxidative transition metal ions which, in turn, results in abating or suppressing the formation of $^{\bullet}OH$ and their consecutive reactions. At this, particularly hop α-acids were shown to have the tendency to render and stabilize iron in a state which is incapable of participating in redox reactions. Moreover, the hop acids' complexation functionality was clearly demonstrated to being oriented towards the transition metal ions iron and copper. With the exception of manganese that was also not affected by hop acids and is claimed to be prooxidative[33, 296], other metal ions were unaffected which can be considered as very advantageous for beer quality.

Yet, there were also inconsistencies as related to the findings. In publication C[276], an effective concentration in terms of completely diminishing 2-DR oxidative degradation was found to be

achieved at a hop α-acid to Fe^{2+} ratio of 15:1. This partly contradicts findings from publication D[282] where a 3:1 ratio was detected and an associated structure of the complex was proposed. It must therefore be assumed that a 3:1 α-acid-Fe complex can still release 2-DR deteriorating material. Even though no more α-acids can bind to iron, it may be anticipated that this complex is still partly accessible to H_2O_2 thus releasing hydroxyl radicals. It may be further hypothesized that albeit no more α-acids can bind covalently to iron, because of the unpolar character of the complex, still more α-acids are attracted by it thereby undergoing a weak interaction with it. Consequently, when the α-acid to Fe^{2+} ratio is increased, the complex is shielded more effectively, becomes less accessible to H_2O_2, and 2-DR oxidative damage is further diminished. Additionally, because the hop α-acids' antiradical character in terms of iron complexation is related to their tendency to render iron in a state that is redox inactive, this functionality is a question of how strong this electrochemical effect is. It is likely that the potential of Fe^{2+} to autoxidize to Fe^{3+} is increased when more α-acids become attached to it. EDTA has a high formation constant of Fe^{3+} and high oxidation potential of ferrous iron[214] and was very efficient in suppressing 2-DR oxidative degradation when used in supermolar concentrations (cp. Figure 3, publication A[276]), which supports the aforementioned assumption. The formation constants and redox potential difference of the redox pairs α-acid/Fe^{2+} or α-acid/Fe^{3+} are unknown and may be investigated in further studies.

With regards to the focus and goal of this dissertation work, the hop acids' complexation activities were demonstrated to be advantageous for wort and beer quality. This feature of hop acids allows target-oriented application of hop additions which may also be applied to other food products or industries where certain metal ions are unwanted. An additional future field for the hop acids' mode of action may be to investigate their complexation functionality towards different heavy metal ions which are not found in high quantitities in beer; yet, they are still a risk for the consumers' health.

Towards the goal of this dissertation work to improve beer oxidative stability, the focus was shifted towards the antioxidative and anti-staling effects of hops. Because the hops' positive impact as related to the suppression or delaying of staling was anticipated to come into effect already during wort boiling, and with regards to the clear role of the intrinsic aldehyde pool of beer, the effects of the hop dosage along with the effectiveness of different hop constituents was now further characterized by means of the experiments and work done in publication E.[273] In order to examine the hops' mode of diminishing staling compounds during wort production, the complex wort matrix was reduced to the presence of maltose, a carbohydrate source,

leucine, an amino source, and Fe^{2+}, as a potential catalyst and electron carrier of redox reactions. Mixtures of these constituents were heated together with hops and certain hop products, and carbonyl compounds were analyzed subsequently using SAFE-GC/MS. The thiobarbituric acid index (TBI) of the heated samples was additionally analyzed because thiobarbituric acid reacts nonspecifically with carbonyl compounds and is usually used as an indicator for heat load.

As a first and central step, the simplified model system used in the study was validated on its applicability to mimic the relevant reactions during wort boiling. Addition of hop pellets type 90 to a model system containing maltose only, or maltose and leucine was found to abate the mixtures TBI by 14.5-16.6 % after incubation at 100 °C (cp. Table IV, publication E^{273}). This result was confirmed by the SAFE-GC/MS measurements where reduced 2-furfural formation and both, reduced 3-methylbutanal and 2-furfural formation, in test mixtures containing maltose, or maltose + leucine, respectively, were observed.

Dissimilar reactivity in abating carbonyl compounds by different hop constituents (hop α-acids, *iso*-α-acids, β-acids, tetrahydro-*iso*-α-acids, or spent hops residues from the CO_2 extraction process) was furthermore detected in separate experiments. While all hop constituents diminished 2-furfural formation, though to a varying degree, only α-acids and β-acids significantly diminished 3-methylbutanal formation (cp. Figure 1, publication E^{273}). Spent hops residues from the CO_2 extract production process were most effective in suppressing 2-furfural formation, yet, also hop α-acids and β-acids diminished remarkably 2-furfural formation by 38.9 % and 38.0 %, respectively.

With the focus to thoroughly study the hops' effects and potential interactions with compounds influencing carbonyl formation during heat exposure, in subsequent trials, RSM and CCRD was applied. Purified hop α-acids were used because they were very effective in diminishing aldehyde formation as found in preliminary trials, and combined with the other independent factors maltose, leucine, and Fe^{2+}. 2-Furfural and 3-methylbutanal were put in as responses of the CCRD. Linear models were found to fit best the experimental data indicating that none of the significant factors was limiting (cp. Table VI, publication E^{273}). Fe^{2+} concentration was eliminated from the models by hierarchic backward elimination because it was insignificant at $\alpha = 0.1$ for the both responses 2-furfural and 3-methylbutanal. For 3-methylbutanal, maltose and leucine concentration were highly significant, while α-acids also showed effectiveness but at a lower confidence level. For the response 2-furfural, maltose and α-acids were both significant model terms at $p < 0.05$.

In similarity to previous investigations (cp. publication B[281]), Fe^{2+} concentration was again an insignificant factor for aldehyde formation. Though, as found in a separate trial, EDTA in fact diminished aldehyde formation, and varying the Fe^{2+} concentration had virtually no impact (cp. Figure 4, publication E[273]) it was concluded that aforementioned mechanisms (Fe^{3+} recycling by reducing agents) were again responsible for the insignificance of Fe^{2+} in the RSM experiments. At this, it is important to note that hop α-acids promote Fe^{2+} autoxidation to Fe^{3+} and stabilize iron in its higher valence state (cp. publication C[276]) which can serve as an explanation of their effectiveness in these experiments. This behavior will be discussed in greater detail later in this section. The hop α-acids' complexation functionality may further also suppress iron-assisted Strecker-like reactions yielding 3-methylbutanal as proposed by Nashalian and Yaylayan.[190] However, the relevance of this pathway was not further investigated. In addition, it may be stated that because of the reactivity of α-acids towards *ortho*-phenylenediamine[267], they may also react with amino compounds thereby forming imines and thus competing with α-dicarbonyls for binding sites. Evidence of this non-oxidative pathway could also not be delivered within the scope of this study, though.

A formation of 3-methylbutanal by oxygen radical-initiated oxidative degradation of leucine was also tested and found at these experimental conditions. This is in agreement with previous findings from publication B[281] and points to the importance and relevance of the postulated mechanism for the oxidative degradation of amino acids at wort production conditions. Interestingly, in opposite to the previous publication C[276], hop α-acids clearly also showed antioxidant activity and suppression of 3-methylbutanal formation by scavenging of radicals. As hop α-acids where shown to be incapable of scavenging $^{\bullet}OH$ (cp. publication C[276]), this activity may be explained by scavenging of organic radicals ($R^{\bullet}$) such as e.g. the carbon-centered radical, which is formed as the first intermediate reaction product when amino acids are attacked by $^{\bullet}OH$.[283] In fact, hop α-acids were reported to be capable of scavenging of organic radicals such as the DPPH-radical which supports this assumption.[250, 274] The hop α-acids' conjugated electron system thereby allows them to form resonance-stabilized phenoxy radicals which are less harmful than the original $^{\bullet}OH$ or $R^{\bullet}$ and further oxidation reactions are therefore diminished. With regards to the postulated oxidative pathway[283], two ways of the α-acids' mode of action can therefore be postulated: effective complexation of transition metal ions rendering them redox inactive thus preventing $^{\bullet}OH$ formation, and scavenging of organic radicals, both of which diminish the consecutive formation of 3-methylbutanal. As related to the hop α-acids' radical scavenging activity, the different outcomes from both studies, publication C[276] and

publication E[273], may be traced back to the nature of the radical and to the α-acids' reactivity towards these radicals.

It is noteworthy mentioning that in addition to suppressing the oxidative degradation of amino acids, hop α-acids were also clearly capable of significantly suppressing 2-furfural formation. The α-acids' effectiveness in this regard may be traced back to the suppression of the formation of 2-furfural via an oxidative mechanism as proposed by Rakete et al.[211] 2-Furfural formation from xylose was also found to be greatly enhanced by iron presence in biobased systems[264], and this may be also of relevance for 2-furfural formation during wort production. Hop α-acids may again affect this pathway because of their capability to complex iron ions.

In sum, these data bring further substantiation of the strong effectiveness of hop α-acids in diminishing carbonyl formation and allow deductions to their importance during wort boiling. An obvious connection between the antiradical properties of hops and their protective effects could be demonstrated. Against claims from ref. 157, clearly, hop acids, and particularly α- and β-acids represent evidently a very powerful fraction of hops in terms of their antioxidative potential. It is therefore unambiguous from all these data taken together and from published literature[250, 274, 276, 282] that α-acids and also the hop dosage are very effective tools for suppressing staling reactions. A practical transfer of this knowledge was still outstanding and was therefore realized in the last publication of this dissertation work.

In publication F[280], the hop dosage was optimized and directed towards an improved utilization of the hops' antioxidative properties. Concomitantly, it was monitored if changes of the hopping technology also negatively impact other important beer quality parameters such as e.g. the foam, bitterness yield, etc. Exclusively hop extract as received from supercritical CO_2 extraction was used, which contains very little phenolic material.[32] It was the intention of this work to deliver brewers a simple and economic way of optimizing their hopping technologies and direct them towards a higher utilization of all the properties of hops. Along with standard wort and beer analysis according to MEBAK[176], ESR spectroscopy to measure free radical levels, quantitation of iron using ICP-OES, staling aldehyde analysis by SAFE-GC/MS, and evaluation of the organoleptic deterioration of the beers during storage by means of the DLG were the methods of choice to obtain a holistic picture of the effects of the hopping technologies used.

Because of the limited knowledge about hop dosages during mashing, in the first part of this work, trials were conducted in which the application of hops during this process step was investigated. Two outcomes can be highlighted from these initial experiments: firstly, the

bitterness yield of hop dosages during mashing is very low (< 2 % when working with hop CO_2 extract), and secondly, adding hops during mashing-in yields a reduction of iron levels in wort, though there was a maximum of iron being removed of 21 % while 79 % remained in solution even when high amounts of hop CO_2 extract/α-acids were added (cp. Table 3 and Figure 1, publication F[280]). It was thus anticipated that the major part of iron was firmly bound in complexes with other organic molecules and for that reason, was not vulnerable to the hop acids' mode of action.

As opposed to hop additions during mashing, iron depletion from wort was clearly more pronounced when the hop dosage was done at the onset of boil (cp. Figure 2, publication F[280]). The wort's iron concentration was clearly a function of the added hop CO_2 extract, and was lowered percentage-wise more, the more hop CO_2 extract were added, and the more iron was initially present in the wort. At the experimental conditions used, a maximum iron reduction was found at an addition of ~45 mg hop α-acids per liter of brewing liquor. The wort's iron content but even more importantly, the availability or vulnerability of iron to being complexed by hop acids was additionally shown to be clearly dependent on the initial malt bill used (cp. Figure 3, publication F[280]). These findings together with available literature data[113, 156] strongly suggest that iron is bound firmly to organic matter in barley or barley malt. When exposed to high temperatures (> 80 °C), e.g. during kilning or roasting of the malt, the iron's chemical condition is anticipated to be altered and more iron is thus released into the wort. In lab-scale brewing trials using 100 % Pilsner malt, 95.2 % wt/wt of the initial iron present in the wort was shown to be removed with the spent grains.[275] There is indication that this balance is altered when other malt types are used.[156] Accordingly, the percentage share of iron which is 'leached out' of the spent grains and released into the sweet wort also changes. It must therefore be noted that the effectiveness of the hop dosage in terms of iron removal is greatly dependent on the constitution and composition of the wort matrix and initial malt bill. In its practical application, this implies that the hop dosage should always be adapted to the breweries' recipes and malt bill when aiming at a high efficacy.

Based on the initial findings, brewing trials with varied hopping were designed: hops dosed only at the onset of boil (reference), mash hopping, divided hop dosage, first wort hopping, and continuous hop dosage. For the detailed design of the brews, see Table 2, publication F.[280] The grist comprised in addition to 90 % Pilsner malt, 10 % Munich malt type I. This malt bill was chosen to account for the effect of the malt composition on the concentration and condition of 'free' iron, which, in turn, was also anticipated to affect the effectiveness of the hop dosages.

Important beer quality parameters such as original gravity, apparent extract, alcohol content, final degree of attenuation, color, pH, total nitrogen, free amino nitrogen, total polyphenols, and foam showed little variations within the treatments but no clear dependency on the type of hop dosage used. The bitter substance yield clearly suffered from later hop additions as compared to the reference, and was worst when applying mash hopping. This has important economic consequences as higher hopping rates and thus raw material costs have to be taken into account when the same bitterness is to be achieved in the beers.

Iron concentration was evidently lowered by the modified hopping technologies as compared to the reference which concomitantly also resulted in an improved oxidative stability as determined using ESR spectroscopy. The brews where hops were dosed in the first wort formed an exception and higher radical levels were measured although iron levels were lower. No satisfactory explanation for this observation could be found. Staling aldehyde of the fresh and stored beers (12 weeks at 28 °C) confirmed the ESR measurements and clear improvements of the oxidative stability by means of lower aldehyde levels were found. Staling aldehydes were remarkably diminished by up to 66.9 % as compared to the reference when applying mash hopping. But also the other treatments yielded a reduction of at minimum 43.9 % of total aldehydes measured as compared to a reference where hops were dosed solely at the beginning of boiling. Interestingly, in analogy to the previous study[273], 2-furfural was also clearly affected by the hopping technologies. This is not only confirmation of findings from publication E[273] and proof of the hops' effectiveness in abating oxidative or non-oxidative pathways of the Maillard reaction, but also has important consequences for the usage of 2-furfural as an analytical marker substance for heat exposure[59] as its concentration is clearly affected by the hopping technology used and not only affected by heat treatment. Similar assumptions may be valid for the TBI. Further investigations as related to this issue should also revise other marker substances such as e.g. 5-hydroxymethylfurfural (HMF). The instrumental evidence of an increased oxidative stability was eventually reflected by the sensory analysis of the beers where the modified hop treatments were clearly found superior as compared to the reference (cp. Table 8, publication F[280]). As opposed to the modified hopping and in accordance with the instrumental data, the reference beers where hops were dosed only at the onset of boil showed a faster deterioration of the overall organoleptic properties of the beers during storage. In accordance with the objective of this dissertation work, the resistance of beers against staling could clearly be improved by modifying the hop dosage.

In consideration of all data from the publications A-F taken together, there are still ambiguities. Outcomes from publication C[276] and F[280] clearly point to the assumption that the hop dosage's effects can be traced back to their interaction with transition metal ions. With regards to this, results from publication C[276] ascribe the α-acids' efficacy to their complexation of prooxidative Fe^{2+} ions thereby suppressing radical reactions, while publication F[280] even implies an efficacy by iron removal. One may assume that these findings partly interfere with results from publication B[281] and publication E[273] where Fe^{2+} concentration was evidently shown to be insignificant for carbonyl formation during beer production or wort boiling conditions. However, the state in which iron (or other transition metal ions) is present or rendered by interplay of all reducing or oxidative substances in the wort matrix can be anticipated to have a higher significance for the oxidative wort or beer stability than the 'crude' concentration of iron. In the RSM experimental designs from publications B[281] and E[273], solely the Fe^{2+} concentration was altered while iron was anticipated to be primarily present as Fe^{2+} because of its subsequent fast reduction, once oxidized. Hop α-acids were shown in publication C[283] being effective in terms of rendering Fe^{2+} less harmful by promoting its autoxidation to Fe^{3+}. As related to the hop α-acids' efficacy, it is therefore conceivable that they render or stabilize iron in a less harmful valence state thereby counteracting Fe^{3+} reduction to Fe^{2+} and thus considerably improving the oxidative wort or beer stability. In similarity to the effectiveness of EDTA (cp. Table 6, publication B[281] & Figure 4, publication E[273]), hops withdraw iron from its reaction partners without necessarily having an effect on the system's iron concentration. Iron removal as detected in publication D[282] or publication F[280] may represent a supplementary effect.

As an ancillary deduction from the studies' findings, it must therefore be accepted that analyzing solely iron concentration is not a good measure for determining the oxidative stability of wort or beer. By contrast, a holistic assay for exploiting the 'true' oxidative state of a food system, must acquire and comprise the interplay of all reducing and oxidative substances and concomitant state of all transition metal ions in the system. Even though ESR measurements may not serve as a good predictor for aldehyde levels in beer during storage because those levels are mostly dependent on oxygen presence (and ESR measurements are independent of the initial oxygen concentration, cp. publication B[281]), ESR spectroscopy is still an adequate tool to assess precisely the oxidative state and interplay of transition metal ions in interplay with all substances present in beer.

With regards again to the hop acid's efficacy, one must embrace the fact that also mechanisms other than interaction with iron or free radical scavenging may be responsible for the hop acids' activity which could not be identified within the scope of this dissertation work. With regards to the high effectiveness of hop α-acids on staling aldehydes suppression during wort boiling, it may be presumed that hop α-acids are capable of interacting with certain intermediates or pathways of the Maillard reaction thus blocking or abating chain reactions which can eventually yield off-flavor compounds. Evidence pointing to the existence of such functionality was not found within this work.

5 Conclusions

In accordance with the objectives of this study, this dissertation work accomplished to identify and expand the knowledge of beer staling mechanisms and the protective role of hops. Outcomes from this work therefore point to numerous starting points for practicable minimization strategies to diminish and delay staling of beer.

Oxygen, which is unavoidably always encountering wort or beer at some point during the brewing process, could be demonstrated to have a detrimental role and deteriorating character for beer flavor. Despite this fact is not a new given, a direct connection between oxygen-derived radicals and the formation of aldehydes could be established and a pathway could be postulated thus conveying the fundamentals about the well-known truth that particularly amino acid-derived aldehydes respond to oxygen exposure of beer while other staling aldehydes are mostly unaffected. For the first time, *de novo* formation of Strecker aldehydes during storage of beer was significantly correlated with total packaged oxygen using labeling experiments. Next to minimizing oxygen during brewing, packaging, and oxygen diffusion through the packaging material, future research should, on one thing, investigate reactions and substances that render oxygen from its relatively stable ground state to reactive oxygen species, and on the other, attempt to find strategies to lower amino acid concentrations in wort and beer because of their crucial role as off-flavor precursor. Though, considering the limited extent of *de novo* aldehyde formation relative to the total amounts of aldehydes found, and taking into account the levels of oxygen on average present in beer after bottling on industrial-scale, it is with certainty that the functional interaction of a multitude of reactions and mechanisms is responsible for off-flavor emergence during beer storage. The intricate composition of the wort and beer matrix, along with the complexity of the technological factors, makes it difficult investigating these mechanisms detached from each other.

In addition to the gained knowledge about the role of reactive oxygen species and origin of Strecker aldehydes, yet unexplored findings about the central role of iron in beer flavor deterioration reactions could be delivered. It is very likely that iron concentration per se is not stringently affecting Strecker aldehyde concentrations but that finally, the interplay of all reducing and oxidative substances in wort or beer, the wort's or beer's oxidative condition, and the concomitant balance or imbalance of the iron's valence state must be considered more important. These findings are not limited to iron ions and may also be assigned to other transition metal ions such as copper or manganese, which not only brings up important

conclusions for the analytical determination of beer flavor stability, but also allows deductions and implications for future research as to which and how wort or beer constituents interact with iron or other transition metal ions.

With regards to staling reaction, the high potential of the hop dosage in terms of improving oxidative beer stability could be successfully elucidated, utilized and demonstrated within this work. Knowledge regarding the preserving effects of hops was expanded by novel findings and yet unknown functionalities. Clearly, one of the main characteristic of hop α-acids is that they are capable of rendering iron ions in a state which is inert to participating in radical-driven flavor deteriorating reactions. Hop additions therefore shift the oxidative balance or imbalance of beer to a more favorable state, eventually improving its resistance against oxidation and staling. As a beneficial side effect, other metal ions which are considered vital for the brewing process are not affected from the hop acid's actions. Albeit hop α-acids were found incapable of scavenging $^{\bullet}OH$, a further antioxidant mode of hop α-acids comprises blockage or inhibition of oxidative chain reactions by quenching of organic radicals and concomitant formation of stable resonance-stabilized phenoxy-radicals. Early blockage of oxidation reactions by e.g. adding hops during mashing is very effective in improving beer flavor stability but also has the concomitant downside of high losses of hop bitter acids and is thus economically doubtful. During wort boiling, because of the high effectiveness of hop α-acids, and the affiliated loss of their reactivity when isomerized, constantly allocating fresh α-acids is a key strategy which can be realized in practice e.g. by a divided hop dosage or by implementing a continuous hop dosage. Adding hops continuously during wort production was in fact found to be the best compromise between losses of hop bitter substances and an improved oxidative stability. As hop polyphenols are suggested to also contributing to beer flavor resistance against staling, it is of interest to adapt findings from this research to hop products which contain hop phenolic material and look for further optimization potentials. The hop industry can provide products for breweries which can be applied purposely in terms of increased oxidative beer stabilities but may also use the knowledge gained in this study for industries other than the brewing industry and design pertinent products.

By means of this work, another piece to the intricate puzzle of reactions and factors affecting beer flavor instability could be added eventually helping to improve and preserve beer organoleptic properties over a longer period. Continued research is certainly still needed to further identify and optimize critical factors during beer production.

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Appendix

List of publications

Publications in international peer-reviewed journals related to this thesis

Publication A

Wietstock, P. C.; Methner, F.-J. (2013) Formation of aldehydes by direct oxidative degradation of amino acids via hydroxyl and ethoxy radical attack in buffered model solutions. BrewingScience 66 (7/8), 104-113.

Publication B

Wietstock, P. C.; Kunz, T.; Methner, F.-J. (2016) Relevance of oxygen for the formation of Strecker aldehydes during beer production and storage. *Journal of Agricultural and Food Chemistry* **64** (42), 8035-8044. DOI: 10.1021/acs.jafc.6b03502

Publication C

Wietstock, P. C.; Shellhammer, T. H. (2011) Chelating properties and hydroxyl-scavenging activities of hop-α and iso-α-acids. Journal of the American Society of Brewing Chemists **69** (3), 133-138. DOI: 10.1094/ASBCJ-2011-0718-01

Publication D

Wietstock, P. C.; Kunz, T.; Perreira, F.; Methner, F.-J. (2016) Metal chelation behavior of hop acids in buffered model solutions. BrewingScience 69 (9/10), 56-63.

Publication E

Wietstock, P. C.; Baldus, M.; Öhlschläger, M.; Methner, F.-J. (2017) 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** (1), 41-51. DOI: 10.1094/ASBCJ-2017-2001-01

Publication F

Wietstock, P. C.; Kunz, T.; Methner, F.-J. (2016) Influence of hopping technology on oxidative stability and staling-related carbonyls in pale lager beer. BrewingScience 69 (11/12), 73-84.

Further publications in other international peer-reviewed journals

Wietstock, P. C.; Glattfelder, R.; Garbe, L.-A.; Methner, F.-J. (2016) Characterization of the Migration of Hop Volatiles into Different Crown Cork Liner Polymers and Can Coatings. *Journal of Agricultural and Food Chemistry* **64** (13), 2737-2745.

Wietstock, P. C.; Götz, F.; Klie, R.; Methner, F.-J.; Scheuren, H. (2015) Feasibility Study using a Novel Desorption/Absorption Process for Transferring Hop Volatiles to Beer. *BrewingScience* **68** (1/2), 1-7.

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Karabín, M.; Rýparová, A.; Jelínek, L.; Kunz, T.; **Wietstock, P. C.**; Methner, F.-J. (2014) Dostálek, P., Relationship of iso-α-acid content and endogenous antioxidative potential during storage of lager beer. *Journal of the Institute of Brewing* **120**, 212-219.

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Wietstock, P. C.; Kunz, T.; Shellhammer, T. H.; Schön, T.; Methner, F.-J. (2010) Behaviour of antioxidants derived from hops during wort boiling. *Journal of the Institute of Brewing* **16**, 157-166.

Publications in non-peer reviewed journals with academic editorial board

Wietstock, P. C.; Götz, F.; Methner, F.-J.; Scheuren, H., Verbesserte Aromahopfennutzung - Machbarkeitsprüfung. *BRAUWELT* **2014**, *46*, 1390-1392.

Wietstock, P. C.; Götz, F.; Methner, F.-J.; Scheuren, H., Improved utilization of aroma hops. *Brauwelt International* **2014**, *34*, 80-81.

Conference contributions

Wietstock, P. C.; Glattfelder, R.; Garbe, L.-A.; Methner, F.-J. *Scalping of Hop Volatiles from Beer into Crown Cork Liner Polymers and Can Coatings* (Poster), World Brewing Congress, Denver, USA, 2016.

Öhlschläger, M.; **Wietstock, P. C.**, Baldus, M.; Methner, F.-J. *Impact of Hop Constituents on the Formation of Staling Aldehydes in Wort-Like Model Solutions* (Poster), 12th International Trends in Brewing, Gent, Belgium, 2016.

Glattfelder, R.; **Wietstock, P. C.**; Garbe, L.-A.; Methner, F.-J. *Comparison of Hop Volatile Scalping into Can Linings and Crown Liner Polymers* (Poster), 12th International Trends in Brewing, Gent, Belgium, 2016.

McIlmoyle, D.; **Wietstock, P. C.**, Methner, F.-J. *Effects of Process Parameters During Dry Hopping on Hop Oil Transfer Rates and Hop Aroma in Beer* (Poster), 12th International Trends in Brewing, Gent, Belgium, 2016.

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt durch meine Unterschrift, dass ich die vorliegende Dissertation "Free Radical-Mediated Formation of Aroma-Active Aldehydes During Beer Production and Storage and Anti-Staling Effects of the Hop Dosage" in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben sind.

Vorveröffentlichungen, die in der vorliegenden kumulativen Dissertation verwendet wurden, können dem Anhang entnommen werden und sind in Kapitel 3 aufgelistet. Alle Stellen, die wörtlich aus Veröffentlichungen entnommen wurden, sind als solche kenntlich gemacht und es wurde keine andere als die angegebene Literatur oder sonstige Hilfsmittel verwendet. Nach § 5, Abs. 1, Satz 3, Nr. 5 und 6 PromO der Technischen Universität Berlin versichere ich an Eides statt, dass die Darstellung des Eigenanteils in Kapitel 3 der Wahrheit entspricht. Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Berlin, den 29.03.2017